THE UNIVERSITY OF HULL

RADIORESISTANCE IN ORAL SQUAMOUS CELL CARCINOMA.

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By

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LIST OF ABBREVIATIONS:

AJCC	American Joint Committee on Cancer
BECN1	Beclin 1
BMP5	Bone Morphogenic Protein 5
BSA	Bovine Serum Albumin
CEA	Carcinoembryonic Antigen
CCNE1	Cyclin E
CLCN6	Chloride Channel 6
CLU	Clusterin
COX	Cyclo-oxygenase
cDNA	Complementary
DAB	Diaminobenzidine Tetrahydrochloride
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribose Nucleic Acid
DNA-PK	Deoxyribose Nucleic Acid – Protein Kinase
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EGR1	Early Growth Response 1
FAP	Familial Adenomatous Polyposis
GMEB1	Glucocorticoid Modulatory Element Binding protein 1
HCl	Hydrochloric acid
HEPA	High Efficiency Particulate Air filter

HER2	Human Epidermal Growth Factor Receptor 2
HNSCC	Head and Neck Squamous Cell Carcinoma
HRMT1L2	Heterogeneous Nuclear Ribonucleotein Methyltransferase 1-Like 2
ICAM3	Intracellular Adhesion Molecule 3
IGHMBP2	**I**mmunoglobulin Mu Binding Protein 2
KLK8	Kallikrein 8
LRR	Loco-regional Recurrence Rate
LRRFIP1	Leucine Rich Repeat Interacting Protein 1
MeSH	Medical Subject Heading
MNC	Multi Nucleated Cell
mRNA	messenger Ribose Nucleic Acid
MHC-II	Major Histocompatability Complex - II
MVD	Micro Vessel Density
PBS	Phosphate Buffered Saline
PITX1	Pituitary Homeobox 1
PTK7	Protein Tyrosine Kinase 7
RNA	Ribose Nucleic Acid
RREB1	Ras Responsive Element Binding protein 1
SCC	Squamous Cell Carcinoma
SDS	Sodium Dodecyl Sulphate
SSC	Sodium Salt Citrate
TEAD4	Transcriptional Enhancer Factor 3
TGF-α	Transforming Growth Factor Alpha

TGFB	Transforming Growth Factor Beta
TBS	Tris Buffered Saline
TNM	Tumour Node Metastasis
VEGF	Vascular Endothelial Growth Factor
UICC	Union Internationale Contre le Cancer
WT	Wild Type

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Abstract.

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common cancer accounting for approximately 6% of all cancers worldwide. However the distribution across the globe varies considerably. The majority of small tumours of the oral cavity and upper aerodigestive tract, in the absence of metastatic disease, can be successfully treated with surgery or radiotherapy. Despite this most small tumours of the oral cavity are now treated with surgery as the primary treatment modality with radiotherapy being reserved for adjuvant therapy, palliation or in patients unfit for surgery. Radiotherapy is also used in cases where there is doubt about the completeness of resection and where adverse histological characteristics are present. Unfortunately, on average about 10% of tumours treated in this way are resistant to radiotherapy, developing tumour recurrence within the original radiotherapy field during the ensuing 12 months. Patients with radioresistant tumours are not only receiving a therapy that is unnecessary but are also being put at risk of potentially serious complications, e.g. osteoradionecrosis of the cervical spine. The primary aim of this thesis was to investigate the mechanism of radioresistance and create an in vitro model of a radioresistant oral squamous cell carcinoma. The methods of cell culture, microarray analysis and immunohistochemistry were employed to this end. Two novel radioresistant cell lines, PE-CAPJ41**RR** and PE-CAPJ49**RR**, were created and a number of targets identified using microarray analysis. Immunohistochemistry was used to investigate the relationship EGFR, Bcl-2, BAX and COX-2 had with radiotherapy response.

1.1. Introduction

Head and Neck Squamous Cell Carcinoma is the sixth most prevalent cancer worldwide (Parkin et al., 2005). Oral cancer is the most common form of head and neck cancer with an estimated 275,000 new cases in 2002 far exceeding laryngeal caner which has an estimated 159,000 new cases per year (Parkin et al., 2005). Oral cancer makes up between 2-6% of all cancers diagnosed in Europe and the United States (Moore et al., 2000b). In the UK, Europe and the USA, the incidence of oral cancer increased in the last three decades (Hindle et al., 1996). Truncated age standardised rates for mortality have risen from 1.67 per 100 000 in 1970 to a rate of 2.91 per 100 000 in 1990, with the incidence rates rising from 3.61 per 100 000 to 5.52 per 100 000 in a similar time period (Moore et al., 2000a). A number of factors have been postulated for this increase including the large rise in the consumption of alcohol in these countries in the latter part of this century (Hindle et al., 1996). The cohort of adults in whom this increase is occurring is now entering the older age group were there is traditionally a higher disease frequency. Thus in the next decade the incidence of oral cancer may rise steeply with the subsequent extra burden on the NHS. Therefore research needs to be targeted on optimising current treatment regimens and developing new strategies.

Currently the majority of oral cancers are treated with surgery, radiotherapy chemotherapy or a combination of these (Davidson et al., 2001). Small tumours are usually treated surgically with radiotherapy being most commonly employed for those cases with adverse histological findings such as involved resection margins. A group of patients exists in whom radiotherapy may be of benefit. Where resection margins are close, but still considered clear, radiotherapy may improve the long term survival

(Brown et al., 2007). Predicting the response this group of tumours has to radiotherapy will enhance the decision making process. Each treatment modality is associated with co morbidities and failure rates ranging from 20-40% depending on stage of disease and the treatment chosen (Carvalho et al., 2003). Different tumour types are already known to have a varied response to radiotherapy, with tumours such as mesotheliomas having an "intrinsic" resistance to radiotherapy (Zucali and Giaccone, 2006) and tumours such as seminomas having an "intrinsic" sensitivity (Christoph et al., 2005). A tumours' intrinsic response to radiotherapy along with other factors such as the primary site of a tumour and its' size (Corvo et al., 2001; Fukuda et al., 2004; Raybaud-Diogene et al., 1997), already exist which enable the clinician to make an informed decision about the efficacy of radiotherapy. However, they are of limited value (Corvo et al., 2001). The ability to predict likely treatment failures before the commencement of radiotherapy would increase the efficacy of the treatment and spare the patient the potentially unpleasant side effects such as xerostomia, dysgeusia, fibrosis (Lapeyre et al., 2004) and more wide reaching effects such as immunosuppression (Fukuda et al., 2004).

During this thesis when the term "oral cancer" is used it will mean any malignant neoplasm affecting the oral cavity (consisting of the vestibule and the oral cavity proper). Thus cancers of the lateral border of tongue, floor of mouth, buccal mucosa or a combination of these will be considered, and those of the lip and posterior third, or fixed portion, of the tongue will not be discussed.

1.2. Anatomy

The oral cavity can be divided up into two main parts; an outer, smaller, slit like portion, the vestibule; and a larger, oval-shaped portion referred to as the mouth cavity proper. The vestibule is a slit like space which communicates with the mouth cavity behind the third molar tooth on each side. With the mouth closed the roof and floor of the vestibule are bounded by the reflection of the mucosa from the lips and cheek onto the lateral gingival margin. The lateral wall of the vestibule is made up primarily of the buccinator muscle and its overlying mucous membranes and medially it is bound by the teeth and lateral wall of the alveolar process. See figure 1.1.

The boundaries of the oral cavity are made up anteriorly of the lips, posteriorly by the anterior tonsillar pillar and laterally of the buccal mucosa. The roof consists of the hard palate and the floor is formed primarily by the anterior two thirds of the tongue and by the reflection of the mucous membranes from the sides of the tongue to the gums on the mandible (Snell, 1992). That portion of the tongue lying posterior to the foramen caecum and the soft palate are considered to be part of the oropharynx. The oral cavity contains the alveolar processes and attached teeth, the hard palate, that portion of the tongue which lies anterior to the circumvallate papilla, the orifice of the parotid duct opposite the upper second molar and the orifices of the submandibular and sublingual ducts.

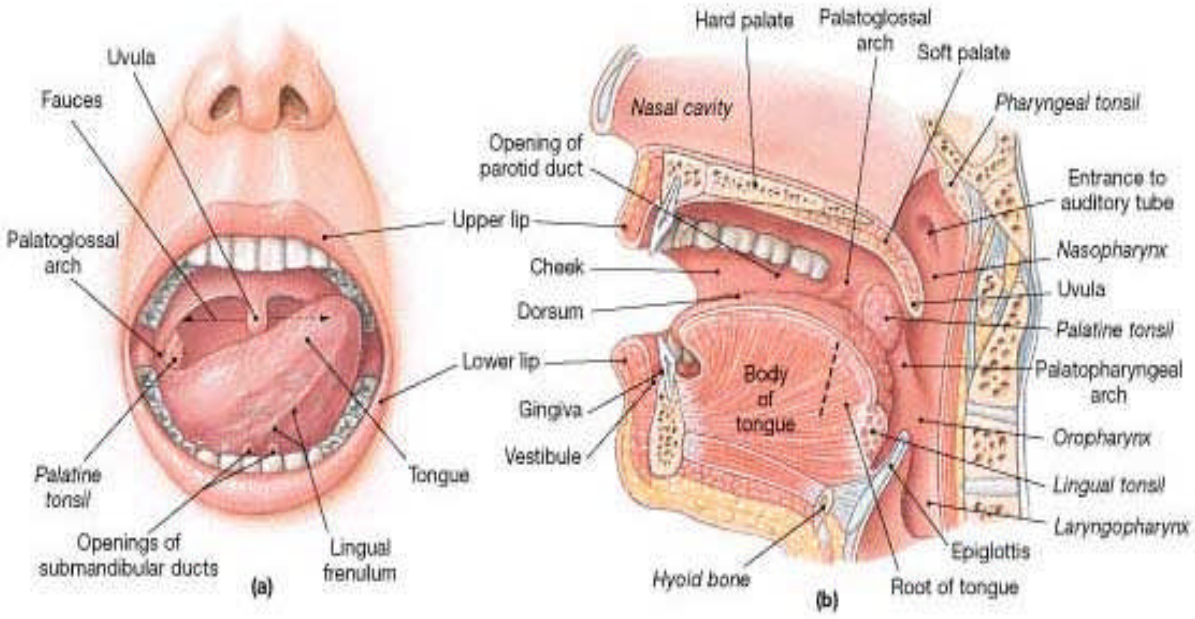


Figure 1.1 From www.mededucation.bhmu.edu.cu showing anatomy of oral cavity from an anterior view in (a) and a cross section in (b).

The anterior two thirds of the tongue is referred to as the oral or mobile tongue and is bounded posteriorly by the "V" shaped line of circumvallate papillae (figure 1.2). The mobile tongue has a dorsal and ventral surface which is covered by a mucosa of simple stratified squamous epithelium with interspersed papillae or taste buds. The tongue is comprised of both intrinsic and extrinsic muscles. The intrinsic muscles of the tongue are responsible for changing its shape and consistency whilst the extrinsic muscles provide the tongues mobility. There are three pairs of extrinsic muscles: genioglossus, hyoglossus and styloglossus (figure 1.3). These muscles receive their motor supply directly from the hypoglossal nerve (Boyle and Strong, 2001). The sensory innervation of the anterior two thirds of the tongue is supplied by fibres from the lingual nerve, a branch of the mandibular division of the trigeminal nerve, which also supplies the mucous membranes lining the floor. Taste sensation is supplied via the chorda tympani which is a branch of the facial nerve. The blood supply to the

tongue is derived from the lingual artery, the tonsillar branch of the facial artery and the ascending pharyngeal artery, with the corresponding veins draining into the ipsilateral internal jugular vein (Snell, 1992).

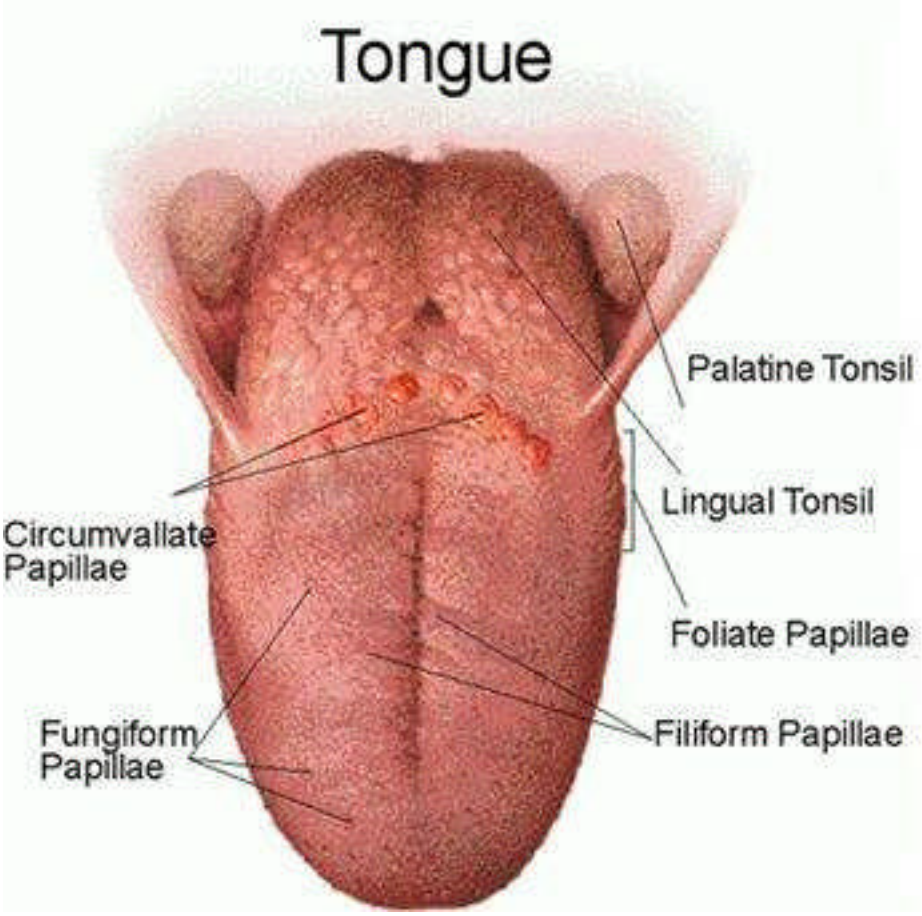


Figure 1.2. From www.doctorspiller.com showing the tongue and the circumvallate papillae dividing it into its anterior and posterior portions.

The hard palate lies within the horseshoe shape of the maxillary alveolar process and can be divided into the primary and secondary bony palate. The palatal processes of the maxillary bones make up the primary bony palate whilst the secondary bony palate is made up of the horizontal processes of the “L” shaped palatine bones. The greater and lesser palatine foramina are situated adjacent to the second and third molar on the hard palate and transmit the terminal branches of the sphenopalatine

artery (posterior superior alveolar artery) and greater palatine nerve, which are braches of the maxillary artery and maxillary division of the trigeminal nerve respectively. The vessels supplying the primary palate region are terminal branches of the nasocilliary nerve and vessels and reach the palate through the incisive foramen (Boyle and Strong, 2001).

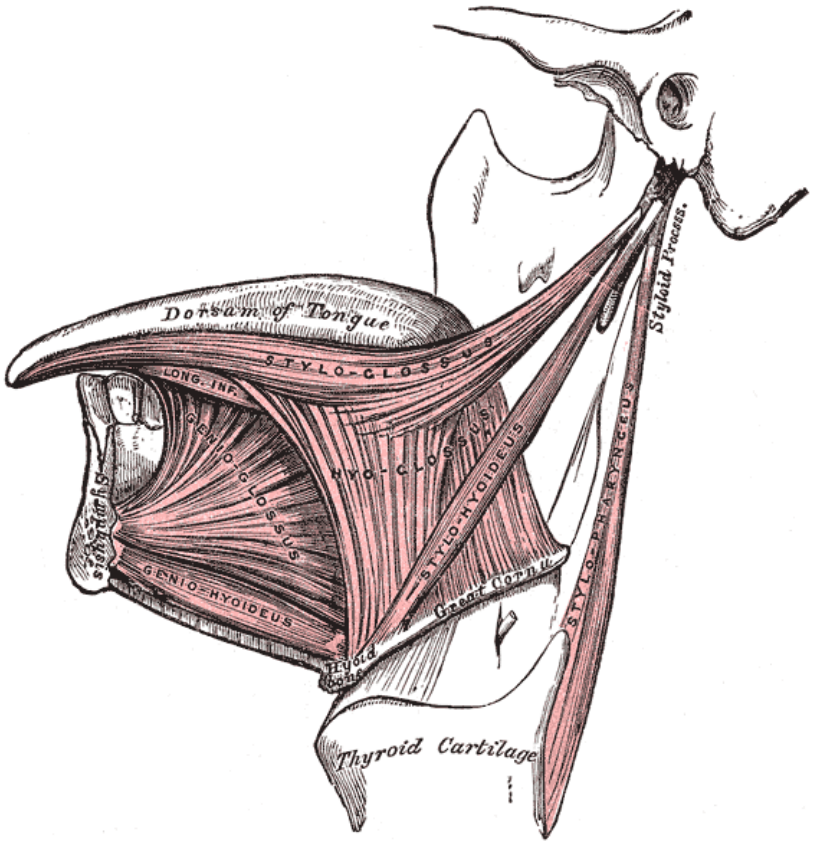


Figure 1.3 from Greys Anatomy showing the muscles of the tongue.

A soft, thin “U” shaped layer of mucosa makes up the floor of the mouth. This overlies the insertion of the mylohyoid muscle laterally, the hyoglossus medially and the genioglossus anteriorly. It overlies the submandibular gland and duct and the lingual nerve. The blood supply is derived from the lingual artery (Boyle and Strong, 2001).

The mouth is lined by stratified squamous epithelium which overlies richly vascular connective tissue. The epithelium varies in thickness throughout the oral cavity being thickest over the tongue where there are also papillary projections. Most of the epithelium is non-keratinising except over the lips, gums and hard palate where a degree of keratinisation occurs. When keratinisation occurs elsewhere in the oral cavity this can lead to the formation of white plaques on the mucosa termed leucoplakia (Dixon, 1996).

1.2.1. Lymph drainage

The oral cavity contains a rich supply of lymph channels which ultimately drain into the lymph nodes of the neck as can be seen in figure 1.4. Upto seven different lymph node levels have been described in the literature (see figure 1.5), with each head and neck region preferentially draining to one of these levels.

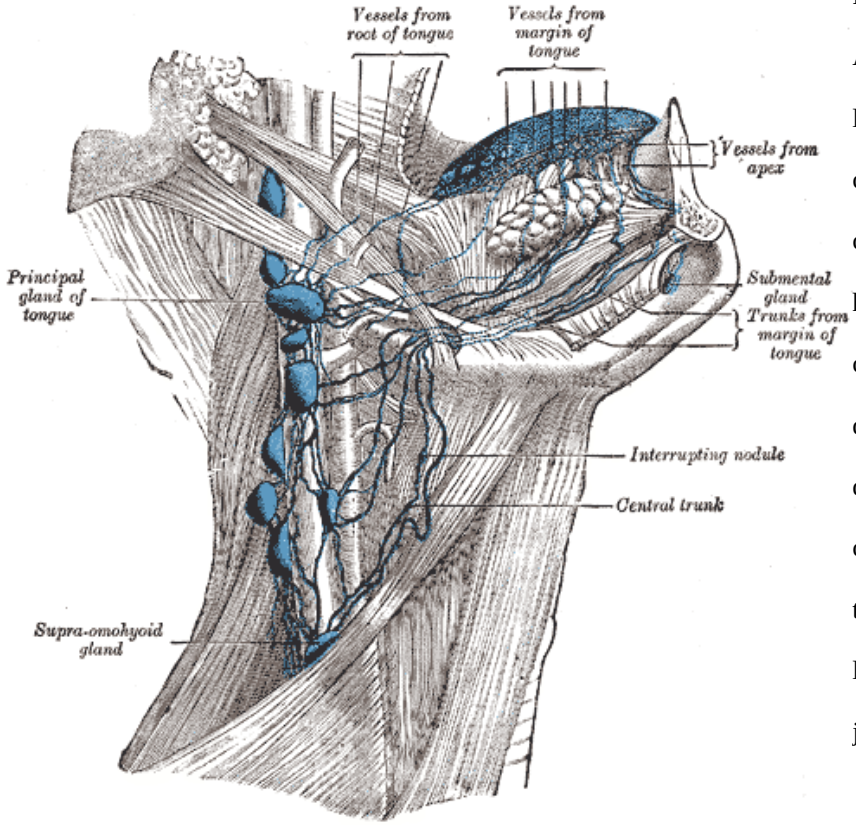


Fig 1.4. From Greys Anatomy showing lymph drainage of oral cavity, lymph drainage channels can be seen highlighted in blue. As can be seen in the diagram the lymph drainage of the oral cavity ultimately leads to the lymph nodes lying along the internal jugular vein.

The lymph vessels of the oral cavity drain into level one lymph nodes via a number of routes. The vessels draining the alveolae initially pass to the submaxillary glands, those of the palate pass backwards to either drain into the subparotid or directly into level 2 nodes and those of the floor of the mouth either pass through the submental/level 1 nodes to level 2 or drain directly into level 2 nodes. The tonsil usually has three to five lymph vessels which drain into a single level 2 node lying immediately inferior to the posterior belly of the digastric muscle where it crosses the internal jugular vein. This lymph node is often referred to as the jugulodigastric node.

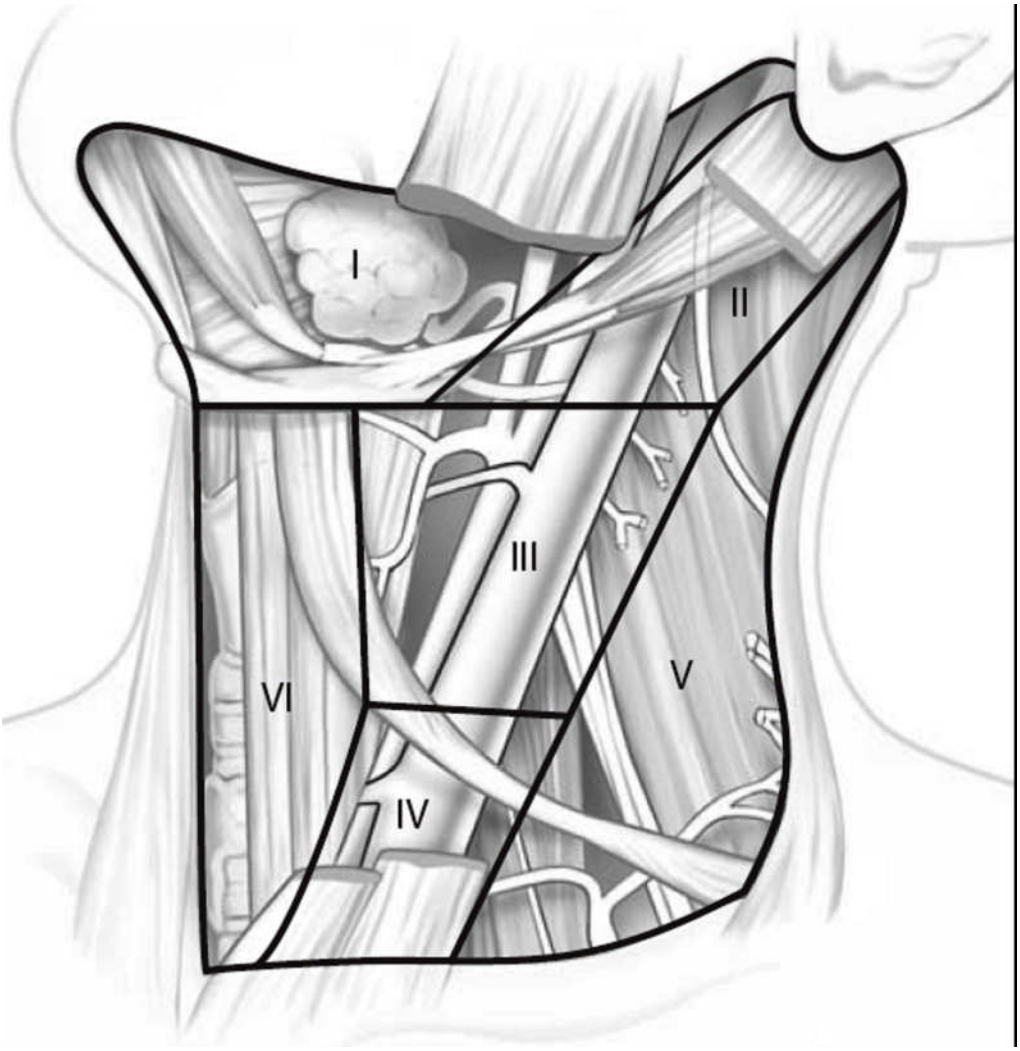


Figure 1.5. From (Cummings et al., 2005) showing the different levels of the neck in which lymph nodes are contained.

The lymph drainage of the tongue is a little more complex than the rest of the oral cavity. The lymph vessels which drain the tongue can be divided up into four main groups, the apical, lateral, basal and median. The apical vessels drain the tip of the tongue to the submental/suprahyoid nodes. The lateral vessels, as the name suggests, drain the margin of the tongue either to the submaxillary nodes or directly to level 2 nodes. The basal vessels drain from the region of the circumvallate papillae and pass directly to level 2 and the median vessels also pass directly to level 2 lymph nodes (Grey's anatomy). Despite this, lesions which approach the midline can drain bilaterally and thus present with metastasis in the contra-lateral neck. Cases have also been reported where lesions of the anterior tongue metastasis directly to nodes low down in the neck bypassing the "front line" lymph drainage (Boyle and Strong, 2001).

1.2.2. Function

The oral cavity and oropharynx are that part of the aero-digestive tract concerned with mastication of food, initiation of swallowing, articulation and the provision of an alternative airway should the nasal cavity become compromised. It is also where digestion of food begins and, via the tongue, where food is tasted and deemed safe to swallow.

Once solid food enters the mouth it is usually broken down by the grinding action of the teeth which also facilitates the mixing of food with saliva. The functions of saliva are mainly mechanical, lubricating the mouth during chewing and aiding swallowing. It has a higher concentration of potassium than plasma, making it relatively alkaline, and contains an amylase like enzyme, ptyalin, which initiates the digestion of starch (Keele et al., 1992).

The action of the tongue and the cheek muscles (especially buccinator) cause the food to pass repeatedly between the teeth to allow thorough mixing. Swallowing begins when the mouth is closed and there is voluntary contraction of mylohyoid. There are three phases of swallowing: the oral, pharyngeal and oesophageal phase. The first is under voluntary control and the rest are under reflex control. Once the oral phase has been initiated the food is formed into a bolus which eventually comes to lie on the dorsum of the tongue. This food bolus is then pushed upward and backwards against the under surface of the hard palate by the tongue and propelled into the oropharynx which triggers the rest of the swallowing process (see figure 1.6). The oropharynx has a rich sensory innervation from the glossopharyngeal nerve. Afferent impulses are sent up to the so called "deglutination centre" in the medulla to produce the complex co-ordinated movements which occur in the involuntary phase of swallowing (Keele et al., 1992).

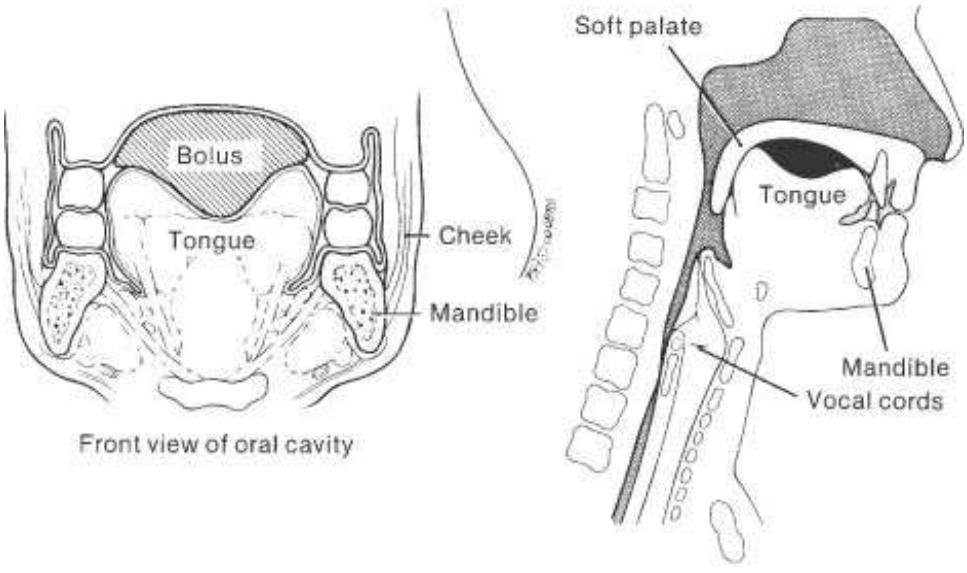


Figure 1.6 From (Cummings et al., 2005). Showing food bolus lying on the dorsum of the tongue as it is passed back through the oral cavity towards the oropharynx.

The pharyngeal phase of swallowing is the most complex yet only lasts half a second and occurs during a period of apnoea. Once the bolus has entered the oropharynx the soft palate is elevated to close of the nasopharynx, the larynx also elevates, causing the epiglottis to flap backwards, the cricoid tilts forward, the vocal cords are approximated and the airway is thus protected from aspiration. The oesophageal phase of swallowing consists of a wave of peristalsis that propagates downwards leading to the entry of the food bolus into the stomach (Narcy et al., 2005).

The production of speech is a complex process involving the lungs, larynx, oropharnyx and oral cavity, the later two are often referred to as the so called articulators or voice tract. Once sound is initiated by air passing through the larynx (so called phonation) the vocal tract modifies this to turn the rudimentary sound into intelligible speech. Various parts of the oral cavity and oropharynx are concerned with producing different sounds and work together to produce words and sentences. The size and acoustics of the oral cavity can be changed by the movements of the palate, tongue, lips, cheeks and jaw.

The soft palate (also known as the velum) is the mobile part of the roof of the oropharynx and is normally in a position which allows air to pass through the nose and mouth. During speech it is often raised so air cannot escape into the nose. Whilst speaking the soft palate articulates with the tongue to produce the so called velar consonants “k” and “g”. When the tongue articulates with the upper alveolar ridge, just above the top front teeth, the sounds such as “t” and “d” are produced and are sometimes referred to as alveolar sounds.

The tongue is probably the most important articulator as it interacts with so many of the other articulators and can deform into many shapes. It therefore does not have any particular sounds attributable to it. Lips also play a major role and have two main methods of producing sound, pressing against each other or pressing against the teeth. When the lips are pressed together bilabial sounds such as p and b are produced and when they articulate with the teeth labiodental sounds such as f and v are formed. (www.personal.rdg.ac.uk)

1.3 Incidence of Oral Cancer.

During the early part of the 20th century the incidence rate of oral cancer was declining. This decline has been replaced by the subsequent increase in its incidence over the past thirty years. The age-adjusted incidence rate for oral cancers was 8.3 per 100 000 in 1994 – 1998 but varied greatly between the sexes and across the globe (Canto and Devesa, 2002) as illustrated in figure 1.7. Current figures show that the area with the highest incidence is Melanesia, with an incidence rate of 31.5 per 100 000 (Parkin et al., 2005). There are also a high incidences reported in certain parts of the developed world such as eastern, western and southern Europe and Australia (Wunsch-Filho, 2002), with oral cancer being 45 times more common in parts of France than in the Gambia (Davidson, 2001). Australia's' high rate of oral cancer, 10.2 per 100 000, is attributed to the high rate of lip cancer secondary to sun exposure in the Caucasian population (Parkin et al., 2005). In India oral cancer is the most common cancer affecting males, the third most common affecting females (Wunsch-Filho, 2002) and accounts for over 50% of all newly diagnosed cancers (Ford and Grandis, 2002). The notably high rate, 12.7 per 100 000, is most often attributed to the

high incidence of betel nut chewing, which is known to be linked to the pathogenesis of oral cancer (Chen et al., 2003), and will be discussed further in section 1.4.

Latin America and the Caribbean have intermediate rates with some countries actually reporting a decline. Uruguay has one of the highest incidence rates among the South American countries with an incidence rate of 15.8 per 100 000 in the male population, possibly due to the high consumption of spirits as compared to wine and beer (De Stefani et al., 1998). The incidence rates across eastern Asia vary considerably. The overall incidence rate for males in 2000 was 2.31 per 100 000 but in Taiwan in 1999 this was at 25.31 per 100 000 (Chen et al., 2004).

In the UK there has been a change in the incidence rate over the last century. The elderly have seen a fall in the incidence rate over the last twenty years but successive cohorts of younger age groups have seen a dramatic rise in incidence. Those aged 45-49 have seen doubling of the incidence rate from 2.10 to 4.3 per 100 000 and in the age group 50-54 there has been a rise from 3.9 to 6.9 (Hindle et al., 1996). It is estimated that 6% of all oral cancers in the UK are diagnosed in patients under the age of 45 years (Llewellyn et al., 2004) and 3% under the age of 40 years (Pitman et al., 1999). The male:female ratio has also changed substantially over this time. In 1916 the male:female ratio was 10.3:1 but has subsequently fallen to 2.5:1 (Hindle et al., 1996) possibly due to the increased rates of smoking and drinking among the female population.

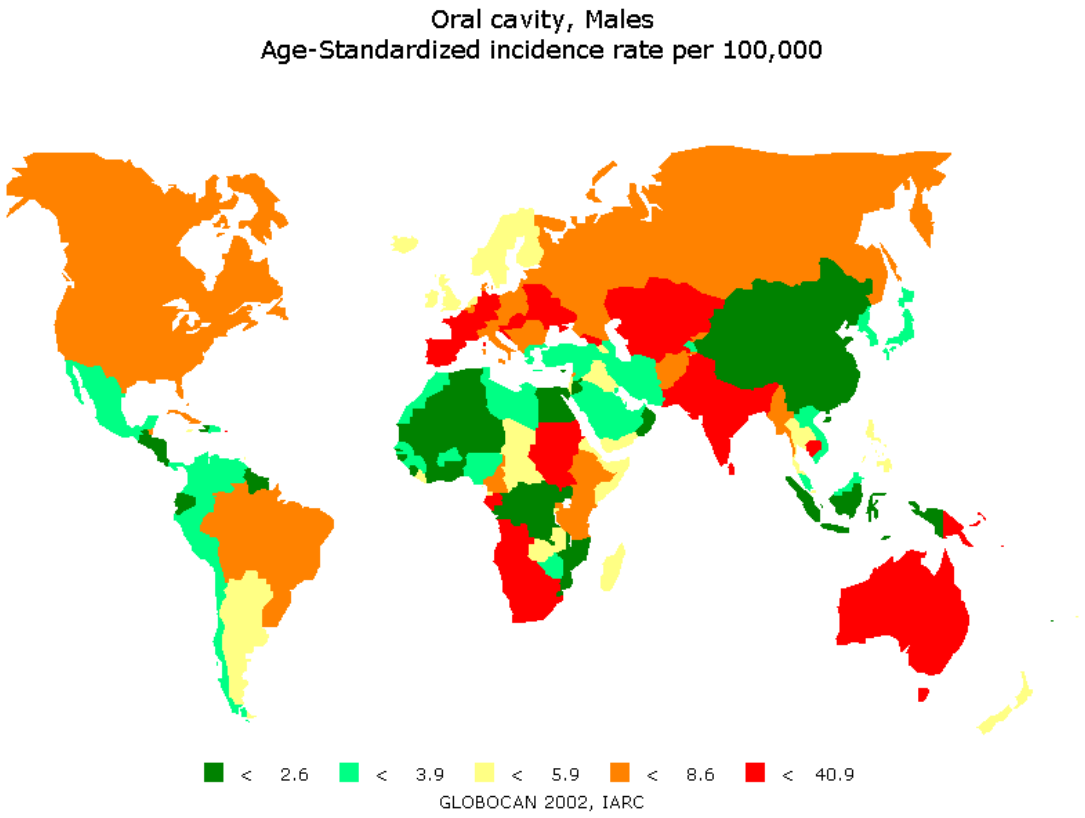


Figure 1.7 from (Parkin et al., 2005) showing incidence rates for oral cancer across the globe. The highest incidence is shown in red.

1.4 Aetiology.

The link between alcohol, smoking and head and neck cancers has now been long established (Elwood et al., 1984b). When alcohol and smoking habits were examined with specific regard to oral cavity malignancy a number of studies have suggested that alcohol may play a more important role than smoking (Choi and Kahyo, 1991; Elwood et al., 1984a) and can act as an independent risk factor for the development of oral cancer (Schlecht et al., 1999; Rothman, 1978). The risk of developing head and neck SCC is between 5-25x higher for those who smoke (Licitra et al., 2002). However these two factors alone cannot explain every case as cancers occur in those who have neither smoked tobacco nor drunk alcohol.

Epidemiological data strongly link cigarette smoking to the development of cancer (Brennan et al., 1999). Repeated exposure to specific carcinogens in cigarette smoke are thought to cause multiple dysplastic lesions in the mucosa of the aerodigestive tract, the so called phenomenon of field carcinogenesis (El-Gazzar et al., 2005). A study by Brennan et al., (1999) found a link between cigarette smoking and mutations in the p53 gene suggesting a possible mechanism of action. There are two main types of tobacco on the market, black or dark tobacco which is air cured and mainly used in cigars and pipes, and blond or light tobacco, which is flu cured and used mainly in the manufacture of cigarettes. The two types of tobacco have been studied in the past to see if they carry the same cancer risk (Castellsague et al., 2004). Castellsague et al., (2004) studied data on 375 patients newly diagnosed with oral cancer and matched these with 375 controls. They found that black tobacco was associated with a 2 to 4 fold increase in the risk of developing cancer when compared to blond tobacco.

“Areca cattechu Linn” is more commonly known as the betel or arcacia nut and is a widely cultivated plant in places such as India, Bangladesh, China and Malaysia (Arjungi, 1976). In its most basic form betel quid is a combination of betel leaf, areca nut and slaked lime. In many countries tobacco is added to this mixture along with various other ingredients such as lime juice and spices. Areca nut has psychoactive properties due to the presence of the alkaloid arecoline (Nair et al., 2004). The practice of betel quid chewing has been found to have a causal relationship with oral cancer, either through mutations caused from the alkaloid contained in the areca nut, the tobacco chewed or as a combination of both (Gupta and Ray, 2004).

Viruses have been found to play a role in other cancers with the most notable example being Human Papilloma Virus (HPV) and its' link with cervical cancer (Mckaig et al., 1998). HPV is also found in around 15% of all squamous cell cancers of the head and neck region (Canto and Devesa, 2002), with tumours of the tonsil being the most commonly infected (Canto and Devesa, 2002). A study in Sao Paulo found that 25% of tonsil tumours were infected with HPV-16 (Wunsch-Filho, 2002). HPV is thought to induce cancer by interfering with the genes or gene products of the cell cycle (Mckaig et al., 1998). A systematic review of HPV and its links with head and neck squamous cell carcinoma found that its link was strongest for those cancers affecting the oropharynx and weakest for oral cavity tumours (Hobbs et al., 2006).

There is a higher incidence of oral cancer in those individuals from a lower social status (Wunsch-Filho, 2002). A link between poor diet, poor dentition and malnourishment may help to explain this (Wunsch-Filho, 2002). Other factors reported to increase the risk of oral cancer include infrequent tooth brushing, low intake of fresh fruit and vegetables, poorly fitting dental prosthesis, riboflavin deficiency and charcoal grilled red meat (Wunsch-Filho, 2002).

Tea and coffee have not been found to be linked to oral cancer and it has been hypothesised that green tea may have a protective effect (Wunsch-Filho, 2002). There is a long list of occupations which have an association with oral cancer including plumbers, carpet fitters, painters and decorators, blacksmiths and electricians. Whilst no study has proved a direct link with occupation, it has been postulated that long term exposure to burning fossil fuels may have a role to play (Huebner et al., 1992; Merletti et al., 1991; Oreggia et al., 1989). Environmental exposures to radiation and

nitrosamines have also been linked with oral cancer (Canto and Devesa, 2002). The risk marijuana smoking poses to the development of oral cancer is yet to be properly understood (La Vecchia et al., 1997).

1.5 Pathology of Oral Cancer.

1.5.1 Pre-malignant conditions.

There are a number of pre-malignant conditions which are known to affect the oral mucosa each with a different pre-disposition to develop into cancer. Lichen planus is a keratin producing disease that usually affects the buccal mucosa. It is characterised by the presence of "Wickham's striae" which are multiple, grey, raised lines converging towards each other to form a mesh with normal mucosa in between. It is a relatively benign lesion with a low propensity to transform into SCC. Conversion rates in the published literature range from 0.4% to 6.5% (Murti et al., 1986; Holmstrup et al., 1988; Lanfranchi-Tizeira et al., 2003; Bornstein et al., 2006; Markopoulos et al., 1997; Hsue et al., 2007).

Leucoplakia is commonly referred to as a pre-malignant lesion, but in actual fact it literally means "a white plaque". Oral leucoplakia is a predominantly white area of the oral mucosa that cannot be characterised as any other definable lesion (Axell et al., 1996). Histologically it is represented by hyperkeratosis with varying degrees of dysplasia (Lodi et al., 2006). A proportion of these lesions undergo malignant transformation with reported rates in the literature ranging from almost 0% to 20% (Lind, 1987; Schepman et al., 1998; Silverman S Jr et al., 1984). Erythroplakia is a variant of leucoplakia which appears as a red, velvety, mucosal patch. It represents

epithelial atrophy, inflammation and subepithelial telangectasia. This type of lesion confers a much greater risk of being associated with cancer with upto 91% representing either carcinoma in situ or invasive SCC (Boyle and Strong, 2001).

Histopathological analysis of lesions biopsied from the oral mucosa are often referred to as dysplastic. The term “dysplasia” was originally used to describe an abnormality of development, such as renal dysplasia and bronchopulmonary dysplasia, which have no pre-cancerous association. In pathology, dysplasia refers to a combination of abnormal cytological appearances and abnormal tissue architecture which occurs principally in the epithelia. Its importance lies in its pre-cancerous association (Lakhani et al., 1993). A dysplastic cell is one which displays considerable pleomorphism (variation in size and shape) and has deeply stained (hyperchromic) nuclei which are abnormally large for the cell. There is also an increase in mitotic activity which is not confined to the basal layer of the epithelium where this usually occurs. The cells lose their usual ordered architecture and exhibit a haphazard arrangement (Kumar et al., 1997).

Dysplasia is often categorised as mild, moderate or severe in nature. The distinction between the three is made on the extent of the tissues involved. Mild dysplasia usually refers to changes affecting the lower third of the epithelium and can be difficult to distinguish from an inflammatory response. Moderate dysplasia refers to changes reaching the middle third of the epithelium and severe dysplasia occurs when the full thickness of the epithelium is involved and is often referred to as carcinoma in situ (Lakhani et al., 1993). Attempts to replace this traditional subdivision by other grading systems, such as the squamous intraepithelial neoplasia grading system or the

Ljubljana classification, have not been successful and are not in widespread use (Brennan et al., 2007).

1.5.2 Malignant Conditions.

The vast majority of malignant tumours found in the oral cavity are of the squamous cell carcinoma type, so much so that oral cancer and oral squamous cell carcinoma are often used synonymously. Oral squamous cell carcinoma makes up almost 90% of all tumours found in the oral cavity (Brown et al., 2001). Salivary gland tumours are the second largest group of tumours affecting the oral cavity making up around 7% of the total. Melanomas, sarcomas, lymphomas and metastases from other sites make up the remaining (Watkinson et al., 2004). The majority of oral cancers (35%) occur in the tongue making this the most common site followed by carcinomas of the floor of mouth (30%), lower alveolus (15%), buccal mucosa (10%), upper alveolus/hard palate (8%) and the retromolar region (2%) (Shiboski et al., 2000; Barasch et al., 1995). Oral squamous cell carcinoma primarily spreads to the regional nodes in the head and neck region. Tumours of advanced T stage are most often associated with distant spread. The lung is the most common site for distant spread (66% of lesions), with bone (22%), liver (10%), skin and mediastinum being other sites for distant metastasis (Ferlito et al., 2001).

1.5.2.1 Squamous cell carcinoma(SCC).

As previously stated this type of cancer makes up the vast majority of oral cancers. The average age at diagnosis is 60 years with 95% of all SCCs of the oral cavity being diagnosed in patients aged >40 years. The male:female ratio was around 6:1 in the 1950s, but more recently there has been an increase in the female incidence and the male:female ratio was estimated at 2:1 in 1997 (Broom et al., 1998).

SCCs of the oral cavity can be further divided up into three distinct macroscopic sub-groups those being exophytic, ulcerative or infiltrative. These types often coexist but the most commonly occurring types are the ulcerative and infiltrative cancers with exophytic ones being much rarer. SCC consists of malignant cells of squamous differentiation as evidenced by keratin formation and the presence of intracellular bridges. It is graded on a three point scale from well differentiated through moderately differentiated to poorly differentiated depending on the presence of intra or extra cellular keratin. Despite the widespread use of this grading system it has little bearing on the prognosis of the patient (Prasad and Huvos, 2001). Various subtypes of SCC exist including keratinizing, non-keratinising, verrucous, basaloid, spindle cell and adenosquamous carcinoma.

Keratinising SCC tends to be both ulcerating and fungating with infiltrating margins. They produce variable amounts of intra and extra-cellular keratin. Microscopically the cells are large with prominent intracellular bridges. Non-keratinising SCCs are though to be derived from respiratory tract mucosa and are the less common type. They grow in a plexiform (weblike) pattern of connecting broad bands (Gassner et al., 2005).

Verrucous carcinomas are a variant of squamous cell carcinomas and represent a very well differentiated type. They present in the oral cavity as a warty tumour which grows slowly. Although undoubtedly malignant, they rarely if ever metastasise. They are difficult to diagnose histologically unless the specimen includes the deep margin of the tumour which demonstrates keratinising epithelium in long papillomatous folds growing into the subepithelial tissue.

Basaloid SCCs are usually found in the posterior part of the oral cavity and are much more aggressive than other forms of SCC. At presentation around two thirds of patients with basaloid SCC will have nodal disease thus the prognosis for this type of SCC is relatively poor with median survival being around 18 months. This tumour grows submucosally around a central ulceration. It is composed of closely packed, pleomorphic cells that form nests and cords.

Spindle cell SCC, also known as sarcomatoid SCC, is again exophytic in nature with a histological profile resembling malignant fibrous histiocytoma. It has a squamous cell component and a spindle cell component. The spindle cell component usually makes up the bulk of the tumour. Most of these lesions are polypoid, firm and pink with a mucosal attachment that varies from a thin stalk to a broad base. Adenosquamous carcinoma and adenocarcinoma are thought to arise from the minor salivary glands found throughout the oral mucosa (Prasad and Huvos, 2001; Gassner et al., 2005).

1.5.2.2 Salivary gland tumours.

Salivary gland tumours can occur within the minor salivary glands of the oral cavity with the most common malignant tumours being mucoepidermoid carcinoma, adenoid cystic carcinoma and adenocarcinomas (Spiro, 1986; Spiro et al., 1973; Waldron et al., 1988). The incidence of malignancy in minor salivary gland tumours has been reported to be as high as 88%, but more commonly reported to be in the region of 50% (Yih et al., 2005; Satko et al., 2000). These tumours tend to present as slow growing, non-ulcerative masses in patients between the ages of 30 and 60 years, with the peak age of incidence around 50 years (Ito et al., 2005; Watkinson et al., 2004;

Yih et al., 2005). Other rarer malignant tumours affecting the minor salivary glands include acinic cell carcinoma, primary squamous cell carcinoma and carcinoma ex-pleomorphic adenoma.

Adenoid cystic carcinoma is one of the most common tumours affecting the minor salivary glands and represents between 27% and 55% of this type of tumour (Satko et al., 2000; Gassner et al., 2005). It usually affects patients in their fifth decade with a male:female ratio of 1:1.2 (Yih et al., 2005). It has a characteristic cribriform appearance on microscopy with the interruption of sheets of tumour cells by cylindrical pseudospaces. Adenoid cystics exhibit perineural invasion which is one of the hallmarks of this type of tumour. Distant metastasis occur in about one third of patients and they tend to exhibit haematogenous spread to lungs and bone in preference to lymphatics (Gassner et al., 2005).

Mucoepidermoid carcinoma is also a commonly found tumour affecting the minor salivary glands. Reports in the literature state that this type of tumour accounts for between 22% and 51% of all minor salivary gland malignancy (Pinkston and Cole, 1999; Satko et al., 2000). It can affect patients from 25 years to 90 years with the average age at diagnosis being 55 years. It affects women more commonly than men with the male:female ratio being 1:2.5 (Yih et al., 2005). Microscopically this tumour is composed of mucous secreting and epidermoid type cells. It primarily spreads to regional lymph nodes with cervical node metastasis reported in upto 24% of patients at diagnosis (Gassner et al., 2005).

Adenocarcinoma is the third most common malignant tumour affecting the minor salivary glands making up between 12% and 26% of this type of tumour (Pinkston and Cole, 1999; Waldron et al., 1988; Spiro, 1986). There are two main types of adenocarcinoma, polymorphous low-grade adenocarcinoma and adenocarcinomas which lack any characteristic features enabling them to be classified as any other specific types, hence this is a diagnosis of exclusion (Prasad and Huvos, 2001). Polymorphous low-grade adenocarcinoma almost exclusively affects the minor salivary glands. The average age at diagnosis is approximately 60 years but it has been reported in patients from as young as 20 years to as old as 94 years (Yih et al., 2005). Microscopically cells are usually small to medium sized, regular in shape and lack nuclear atypia.

1.6 Symptoms.

Cancers of the oral cavity are often picked up on routine dental examination or brought to the attention of a family doctor as they are usually felt or seen quite easily by the patient. They can present as roughness or ulceration on the mucosa of the oral cavity. Oral cancer can also present in an early form as painless white or red patches (Zakrzewska, 1999). Due to the complex sensory innervation of the ear provided by a large number of nerves it is often a site of referred pain. The auriculotemporal branch of the trigeminal nerve supplies sensation to the tragus, anterior pinna, anterolateral surface of the tympanic membrane and part of the external auditory canal (Bauer and Jenkins, 2005). The trigeminal nerve also supplies sensation to parts of the oral cavity and when oral cancers start to involve these braches patients can present with referred pain to the ear, which can be difficult to control.

1.7 Staging.

The natural history and response to treatment of oral cancer can be difficult to predict in the clinical setting. The process by which cases of oral cancer are sub-divided into groups in which the behaviour may be similar is called staging. The process of staging not only allows an attempt to predict the behaviour of a cancer but also allows for a meaningful comparison of clinical and biological data both nationally and internationally. An idea of the patient prognosis for each stage of the disease can be formed and, most importantly, a guide to suitable treatment is provided by the stage of the disease.

In order to accurately stage a cancer a number of factors need consideration. Firstly the primary site of the tumour e.g. whether this is oral cavity or lung, as the primary site alone has a bearing on outcome. Once the primary site has been determined the size of the tumour at this site must then be considered, with larger tumours and those which involve surrounding tissues being associated with a less favourable outcome. Once this has been established other factors to consider are the presence or absence of lymphatic or blood borne metastasis. Together these make up the so-called tumour (T), node (N), metastasis (M) or TNM system of staging cancer. There are traditionally two staging classifications for head and neck cancer: those of the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC), which over the years have converged to an almost identical classification system. The TNM classification for oral cavity cancer is shown below.

Tumour :

T_1 tumour 2cm or less in greatest diameter

T_2 tumour more than 2cm but less than 4cm in greatest diameter

T_3 tumour more than 4cm in greatest diameter

T_4 tumour invades adjacent structures

Node :

N_x regional nodes cannot be assessed

N_0 no regional lymph node metastasis

N_1 metastasis in a single ipsilateral lymph node 3cm or less in greatest diameter

N_{2a} metastasis in a single ipsilateral lymph node greater than 3cm but less than 6cm in greatest diameter

N_{2b} metastasis in multiple ipsilateral lymph nodes all less than 6cm in greatest diameter

N_{2c} metastasis in bilateral / contralateral lymph nodes all less than 6cm in greatest diameter

N_3 metastasis in any regional lymph node of greater than 6cm in maximum diameter

Metastasis :

M_x distant metastasis not assessed

M_0 no distant metastasis

M_1 presence of distant metastasis

(reproduced from UICC handbook 1997)

1.8 Treatment.

There are a number of standard treatment options for oral cancer. These include surgery, radiotherapy, chemotherapy or a combination of all three. The selection of the treatment regime depends mainly on the extent of the primary tumour, the T-stage, but other factors such as tumour infiltration, anatomical sub-site and lymph node status also play a role (Stambuk et al., 2007). The histological grade of the tumour, whilst needing consideration, is overridden by the T stage and anatomical subsite. No large, well designed prospective randomised controlled trial yet exists comparing each of the above three modalities (Licitra et al., 2002; Oliver et al., 2007b).

The management of the primary disease in oral squamous cell carcinoma has changed considerably over the last twenty years. The treatment of choice in many countries is now surgery with radiotherapy being used as an adjunct for those patients at risk of recurrence (Day et al., 2003; Woolgar et al., 1999). The impact of surgical treatment for oral cancer can have a profound effect on a patients quality of life, compromising speech, mastication and appearance (Rogers et al., 1998). In some anatomical subsites, such as the mobile tongue, surgery is preferred to radiotherapy due to its proximity to adjacent radiosensitive structures of the teeth and mandible (Oliver et al., 2007b). Although post operative radiotherapy is used to achieve improved local control rates. The decision to follow surgery with radiotherapy is based on a pathological assessment including pattern of invasion, resection margins and nodal status (Langendijk et al., 2005). There is little doubt as to the usefulness of radiotherapy in those cases were patients are at high risk of tumour recurrence with involved resection margins and extra-capsular spread in the nodal disease (Brown et al., 2007). Doubt still exists as to the role radiotherapy plays in those patients at

intermediate risk of recurrence were resection margins are clear but within 5mm and were there is positive nodal disease in the neck without any extra-capsular spread (Oliver et al., 2007b).

For more advanced tumours staged T3 or T4, where failure rates following radiotherapy are relatively high (Licitra et al., 2002), or for those cases involving cervical lymph node metastasis, surgery is the preferred option in combination with radiotherapy ,to gain disease control. Inagi et al.,(2002) showed a five year survival rate for stage three and four disease of 74% for those cases of oral cancer treated with surgery alone and 47% for those treated with radiotherapy alone. When considering the survival of patients with stage four disease the five year survival is much worse. One study showed a five year survival of 43% when patients were treated with surgery alone and 58% when surgery was combined with post operative radiotherapy (Hicks, Jr. et al., 1997). Surgical excision often needs to be quite extensive but, with the improvement in reconstructive surgical techniques, functions such as swallowing are more often maintained now than was previously possible. In many cases radiotherapy is needed after the surgical resection. For example if the original tumour was considered bulky, resection margins were positive or if there was evidence of extracapsular spread of an involved lymph node on histological examination (Zelefsky et al., 1992). When radiotherapy is to be given post operatively it is best commenced three weeks after the initial surgery and certainly not delayed longer than six weeks (Lapeyre et al., 2004). This allows some healing to take place at the site of surgery without allowing the tumour to repopulate. Improved outcome has been shown with the addition of post operative radiotherapy for those tumours staged at T4

on initial diagnosis (Zelefsky et al., 1992; Gehanno et al., 1993; Spiro and Spiro, 1989; Pinsolle et al., 1992).

Treatment of the N0 neck has remained an area of some debate for those cancers affecting the oral cavity. Whilst some papers have shown no increase in survival if either elective neck dissection is carried out at the time of the initial surgery or when neck metastasis are identified during follow-up, they still advocate a policy of elective neck dissection (Hicks, Jr. et al., 1997; Inagi et al., 2002). A retrospective study with nearly 1000 cases of oral cancer over a 40 year period again showed no significant improvement in survival when treating the N0 neck. They recommend a policy of observation with treatment reserved for positive neck nodes found during follow-up (Sessions et al., 2000).

Where metastatic disease i.e. M_1 disease, is encountered, palliation is usually the aim of any treatment. Although the risk of distant metastasis is low at presentation it can be as much as 40% during the follow-up period (Lee et al., 1993; Leemans et al., 1994; Calhourn et al., 1994). The most common site for metastatic deposition is the lung followed by the liver and then bone (Calhourn et al., 1994; Leemans et al., 1994; Massard et al., 1993). Chemotherapy is the usual modality of treatment chosen but there is only a 40-70% expected response rate (Licitra et al., 2002). Surgery can play a role for some types of lung metastasis. When it is a solitary lesion presenting more than one year from the initial head and neck primary, it is very difficult to differentiate between this being a metastasis or a primary lung tumour. Therefore an aggressive surgical approach should be taken. Five year survival rates for this type of situation range from 20-30% (Mazer et al., 1988; Adelstein, 1994).

1.9 Prognosis.

Prognosis depends on a number of factors. Some are related to the tumour site, stage and histological grade and some are related to the patient. Absolute survival of the patient is usually lower than the disease specific survival due to deaths from other related diseases or second primaries (Bataini et al., 1990).

Five year survival rates for T_1 and T_2 oral cancer are reported between the 70-90% range, but as with other head and neck cancer subsites the prognosis decreases by 50% in the presence of metastatic neck nodes (Boyle and Strong, 2001). The five year survival for patients with much more advanced stage, especially those with bulky or bilateral lymph node involvement, is less than 20%. By the addition of post operative radiotherapy the survival of later stage disease has been increased to between 50-60% (Vikram et al., 1984). Factors which predict improved survival in oral cancer patients are low T-stage, low N-stage and absence of significant co-morbidities (Boyle and Strong, 2001). The histological grade of the tumour probably has some bearing on the outcome, but this is by no means as significant as the T and N stage (Bataini et al., 1990). Many patient factors have been shown to adversely affect the overall outcome. Daily alcohol intake, smoking, gender, earache, pain, dysphagia, weight loss, oral bleeding, odynophagia, the American Society of Anaesthesiologists surgical risk score, haematocrit level and lymphocyte count all have a bearing on the prognosis (Lee et al., 1993; Massano et al., 2006).

The recurrence rates quoted in the literature for all head and neck squamous cell carcinoma (HNSCC) range from 18-76% (Carvalho et al., 2003). Fewer studies exist which focus exclusively on oral cancer but in those which have been published

recurrence rates range from 19-53% (Shikama et al., 2003; Eckardt et al., 2004; Carvalho et al., 2003). Obviously this is quite a large range, and a number of factors may contribute. Each team seems to use a different definition of recurrence and many of the papers in the literature do not concentrate on any particular head and neck sub site.

Shikama et al., (2003) studied a population if 161 patients with HNSCC who were treated with a combination of radiotherapy and surgery. Of this group 82 patients had tumours of the oral cavity. In patients with oral cavity tumours the 5-year overall survival rate was 56% and the 5-year loco-regional recurrence rate (LRR), was found to be 47% compared to 23% for other sites (p=0.016). A recurrence was defined as a local recurrence, regional nodal recurrence or both. Further analysis was performed on the individual oral sites and the LRR for tongue, oral floor and other oral cavity cancer was 42%, 47% and 52% respectively.

(Eckardt et al., 2004)Eckardt et al., (2004) looked at a sample of 1000 HNSCC of whom 883 patients had oral cavity or oropharyngeal tumours. The patients in this study were treated with a range of modalities including radiotherapy, surgery and a combination of both. The recurrence rate was defined as any recurrence of the primary tumour over the length of the study (20 years). An overall recurrence rate of 19.8% was found with 36.4% of these reported between 6 months and 1 year after the initial treatment.

Carvalho et al., (2003), between 1954 and 1998, studied a total of 2067 patients attending their department with oral or oropharyngeal tumours during 1954 and 1998.

The patients were treated with a variety of modalities reflecting the change in regimens over the decades. When the data was analysed an overall recurrence rate of 52.2% was found. This was further classified according to treatment: of 729 (35.3%) treated with radiotherapy alone a recurrence rate of 22.5% was noted.

The use of radiotherapy has been shown to improve the prognosis of certain groups of oral cancer (Vikram et al., 1984). When it is used as the primary treatment modality it not only treats the cancer in question but also treats the surrounding mucosal field in the oral cavity. If however, the tumour does not respond to radiotherapy, then not only is the prognosis worsened but the patient is left with unpleasant side effects from the radiotherapy itself, with no benefit. Being able to predict this response to radiotherapy before treatment would improve prognosis and avoid unwanted side effects for these patients.

1.10 Potential Markers of Radioresistance.

With the advance in modern molecular research techniques the mechanisms by which radiotherapy causes cell death have become clearer. Briefly, the ionising radiation causes damage to a cells' double stranded DNA. This damage is then detected via various proteins and the cell cycle is paused whilst an attempt is made to repair the DNA. In cancers which are radiosensitive the mechanisms of detection and repair are inappropriate and less effective than in normal tissue or the damage is so great that the apoptosis pathway is activated and the cell starts the process of programmed cell death (Rosen et al., 2000). DNA is more susceptible to damage in rapidly dividing cells which means that although cancer cells are primarily targeted, rapidly dividing cells in normal tissue i.e. mucous membranes and salivary tissue, are also affected (Oliver et al., 2007a). The mechanisms by which tumour cells become radioresistant

are complex and the exact molecular pathways remains to be elucidated. Many factors exist which act at different points of the cell cycle changing the susceptibility to radiotherapy. After exposure to ionising radiation the cell cycle is usually paused via various check points, if these are defective through mutation or carcinogenesis then a cell may avoid apoptosis (Bristow et al., 1996).

Early studies into radioresistant oral cancer employed techniques available at the time such as cytological analysis (Silverman and Sheline, 1961; Gupta et al., 1987; Memon and Jafarey, 1970). As things have moved on various genes and gene products have been investigated using techniques such as polymerase chain reaction (PCR) and immunohistochemistry which are good at studying single factors. Further advancements in molecular techniques have opened up a new field of study, namely microarrays, which enable thousands of genes/ gene products to be analysed simultaneously. Data from each of these techniques will be discussed in turn.

1.10.1 Cytological Predictors.

Cytological analysis of the nuclei of cancer cells has been used as an attempt to predict radiotherapy response in oral cavity SCC in the past by examining the histomorphological changes of the nucleus such as micronucleus formation (Silverman and Sheline, 1961; Gupta et al., 1987; Memon and Jafarey, 1970). During mitosis any chromosomes or chromosome fragments which lag behind and are not incorporated into the daughter nucleus are known as micronuclei. The presence of micronuclei suggests that the cell suffered some form of chromosomal damage and micronucleated cells are considered to be dying (Midander and Revesz, 1980). There have also been a number of recent studies using serial cytological changes induced by a course of radiotherapy to aid in the prediction of response to radiotherapy

(Narayanan et al., 1998; Bhattathiri et al., 1998; Bhattathiri et al., 1996; Bindu et al., 2003).

Bhattathiri et al., (1996) analysed tumour cytology smears from 49 patients with oral SCC whilst they underwent a course of fractionated radiotherapy to a total of 60Gy. During the follow up (median time of follow-up being 14 months) 57% of the patients developed a recurrence and were classified as radioresistant. Light microscopy was used to identify micronucleated cells (MNC) in the smears. In this study no smears were taken after 12Gy as it was deemed too difficult to distinguish tumour from normal tissue. It was found that radioresistant and radiosensitive tumours demonstrated the same proportion of MNC before and after treatment, and they both showed an increase in MNC during treatment. Although the increase in MNC occurred earlier in the radioresistant group, and reached a plateau which did not occur in the radiosensitive group, these differences were not found to be statistically significant.

Narayanan et al., (1998) performed a similar study on 44 patients with oral SCC. During this study 59% of patients developed recurrence and the smears were taken up to a dose of 28Gy i.e. half the total treatment dose. The tumours classed as radioresistant were defined as those which developed a recurrence at any time during follow up. Patients were followed up for a minimum of 30 months or until they developed a recurrence. When the data were analysed a significant difference was found in the MNC count at 28Gy (p-value=0.05). This group concluded that the method could help detect treatment response during the course of radiotherapy but not before commencement. This second study (Narayanan et al., 1998) found a significant

difference after extending the dose at which smears were obtained, as compared with Bhattathiri et al., (1996). However, these results require further verification due to the difficulty in identifying tumour during the course of radiotherapy and it must be remembered that the technique does not appear to have any pre-treatment predictive value in oral SCC despite having proved useful in other tumour types such as cervical (Zolzer et al., 1995).

Bhattathiri et al., (1998) performed a study on 69 patients with OSCC undergoing radiotherapy to a mean dose of 52.5Gy. They again took smears from the tumour both before and during the course of radiotherapy to a dose of 38Gy. The patients were followed up for 18 months or until they developed a recurrence at the primary site. During this time 54% of the patients developed a recurrence and were termed radioresistant. By light microscopy multiple changes in the morphology of the cell, including multinucleation, micronucleation and nuclear budding were observed. It was concluded that the radioresistant group of tumours exhibited a lesser degree of change when compared to the radiosensitive group and although the change in the characteristics was noted to be statistically significant ($p<0.05$) during the treatment period no mention of the statistical significance between the two groups was mentioned after the final analysis. The highly subjective nature of the assessment also raises questions regarding widespread applicability.

Whilst a number of reports seem to offer a method by which response to radiotherapy can be monitored and predicted during the treatment, none offer a reliable way of predicting response to radiotherapy prior to treatment. As the two studies with statistically significant results took smears upto 28Gy (Narayanan et al., 1998) and

38Gy (Bhattathiri et al., 1998) respectively there will be little benefit gained in terms of avoiding the unpleasant side effects of radiotherapy.

1.10.2 Microvessel Density (MVD)

For a tumour to grow and survive past approximately $1mm^3$ it must induce a blood supply to gain oxygen and nutrients, thus angiogenesis plays an important role in the development of a tumour. Conversely hypoxia is thought to be one of the major causes of radiotherapy failure as the formation of oxygen free radicals is how the DNA is damaged (Littbrand and Revesz, 1969). So, increased angiogenesis can be both a poor prognostic factor as well as potentially being responsible for improving the chances for successful radiotherapy. One of the simplest and most widely accepted ways of assessing angiogenesis is to look at microvessel density (MVD) i.e. to examine how many new vessels have been formed. An alternative way is to examine the expression of vascular endothelial growth factor (VEGF) which will be considered later in this review.

Only three studies exist which have focussed exclusively on oral SCC, MVD and response to radiotherapy (Brun et al., 2001; Shintani et al., 2000; Aebersold et al., 2000). Brun et al., (2001) looked at 39 cases of oral SCC which all underwent pre-operative radiotherapy (50Gy) and assessed the MVD both before and after radiotherapy by staining with antibodies to von Willebrand factor. Although a high degree of MVD correlated with a poor prognosis, it had no relation with response to radiotherapy. Shintani et al., (2000) studied 41 cases of OSCC again with regards to MVD before and after radiotherapy, and found that MVD decreased after radiotherapy but once again established that there was no correlation between MVD

and radiotherapy response. In the largest study of its type Aerbersold et al., (2000) studied a population of 100 patients with OSCC all of whom received radical radiotherapy with a median dose of 74Gy. Intratumoral MVD was identified by staining with antibodies to CD31. This study found that MVD did predict for response to radiotherapy (p=0.01).

These three studies have given disparate results in terms of predicting the response of a tumour to radiotherapy. A variety of reasons may be responsible for this including the number of subjects in each study, amount of radiotherapy, stage of the tumours in the study and the combined use of other treatment modalities (chemotherapy and surgery) all are likely to confuse the picture. Aerersold et al., (2000) studied a population of patients of whom 88% had T-stage 3 or 4, whilst the other two studies had only approximately 50% of their study population falling into this group. Whilst this will have an effect on the outcomes of the studies the most important factor is the definition used for a radioresistant tumour. Whilst Brun et al., (2001) and Shintani et al., (2000) used a definition which effectively excluded any late recurrences by only looking at the initial response to radiotherapy Aerbersold et al., (2000) used a more lenient definition which allowed for any recurrence be it early, late or at a distant site. Thus the role MVD has in predicting radioresistance is not currently clear.

1.10.3 Molecular Determinants of Response to Radiotherapy.

1.10.3.1 The role of p53.

The p53 gene is one of the most commonly occurring mutated genes in cancer. The p53 gene encodes a nuclear protein which is involved in many important cellular processes such as apoptosis, DNA synthesis and gene transcription. When a cells' DNA is damaged by ionising radiation this damage is detected and the level of p53

protein is stabilised. The cell cycle is paused and the resultant response determines whether the cell enters into apoptosis or a DNA repair/survival pathway (Rosen et al., 2000). A cell may however undergo p53 independent apoptosis if the dose of radiotherapy is high enough and DNA is sufficiently damaged (Jayasurya et al., 2004). Many studies utilise immunohistochemistry as a method to detect the presence of mutated p53. This method assumes that very occasional positive staining of tumour cells correlates with an accumulation of wild type (WT) p53 in response to DNA damage while intense staining of most cells is due to mutation of the gene itself as p53 has a much longer half life than the normal counterpart which is usually undetectable (Aebersold et al., 2000).

The current literature was searched using the search terms radiation tolerance, radioresistance, radiotherapy, radiosensitivity, mouth neoplasm and oral cancer and a total of six studies which looked exclusively at the relationship between p53, oral cancer and radiotherapy were identified (Grabenbauer et al., 2000; Jayasurya et al., 2004; Girod et al., 1998; Yamazaki et al., 2003; Alsner et al., 2001; Koelbl et al., 2001) (Table 1.1). The results obtained from these papers are both confusing and, in some cases, contradictory. Whilst Grabenbauer et al., (2000) found that increased staining for p53 protein correlated with tumour radiosensitivity ($p=0.002$) Jayasurya et al., (2004) concluded the exact opposite ($p=0.002$) and Koelbl et al., (2001) found no relationship between the two factors. Why the results should be so varied has a number of likely explanations.

Author	Year	Country of Study	Number	Predictor for radioresistance	P-value	F/U	Method
Girod et al., (1998)	1998	Germany	90	Decreased p53 staining	-	Immediate response	Immuno
Grabenbauer et al., (2000)	2000	Germany	102	Decreased p53 staining	0.002	43 months	Immuno
Alsner et al., (2001)	2001	Denmark	58	Mutated P53	0.003	74 months	DGGE
Jayasurya et al., (2004)	2004	India	125	Increased p53 staining	0.002	48 months	Immuno
Yamazaki et al., (2003)	2003	Japan	49	Mutated p53	-	Immediate response	PCR
Koelbl et al., (2001)	2001	Germany	88	No relationship	-	53 months	Immuno

Table 1.1 Studies showing relationship between p53 and response to radiotherapy. Immuno – immunohistochemistry, DGGE – denaturing gradient gel electrophoresis, PCR – polymerase chain reaction.

Firstly, different parts of the world have different habits and cultures. Whilst chewing tobacco and related products is popular in India it is relatively uncommon in Western Europe. This may lead to different tumour characteristics in different populations and effect study results. Jayasurya et al., (2004) concluded that an increased staining for p53 predicted for radioresistance, studied a group of 125 patients with oral SCC all from the Indian sub-continent out of which just over 80% chewed tobacco but the group studied by Koelbe et al., (2001), whose study showed no relationship, came from Germany where chewing tobacco is relatively uncommon.

The choice of treatment regime may also have an effect on the study results. Two of the studies, Jayasurya et al., (2004) and Alsner et al., (2001) examined cases that were treated with radiotherapy alone and concluded similar results, that increased

expression of p53 correlates with a radioresistant tumour. However, the group studied by Grabenbauer et al., (2001) received post operative radiotherapy and concluded that increased expression of p53 correlates with a radiosensitive tumour and the group studied by Koelbl et al., (2001) received pre-operative radiochemotherapy and could find no correlation between the expression of p53 and radiotherapy response. So it seems that the timing of radiotherapy as well as the other choice of treatments has a direct effect on the predictive value of p53.

When looking at the studies that utilised immunohistochemistry to detect over or under expression of p53 (Girod et al., 1998; Jayasurya et al., 2004; Grabenbauer et al., 2000; Koelbl et al., 2001) a number of different scoring methods have been used. Grabenbauer et al., (2000) used a densitometer to score the slides, Jayasurya et al., (2004) used two people blinded to the pathological information who scored the slides by examining the invading tumour fronts and, although Koelbl et al., (2001) again utilised two independent scorers, they evaluated 400 tumour cells in four randomly chosen high power fields of the tumour mass.

The definition used by each of the groups as to what constitutes a radioresistant tumour varies widely and hence will also have an effect on the results. Whilst Grabenbauer et al., (2000) and Alsner et al., (2001) included any recurrence at local or distant sites during the follow up period (43 and 74 months respectively), Jayasurya et al., (2004) and Koelbl et al., (2001) included only local recurrence during the follow up period (48 and 52 months respectively) and (Girod et al., 1998)Girod et al., (1998) simply looked at the initial effect radiotherapy had on the tumour thus excluding any later local or distant recurrences.

From the published studies it is not possible to gain a consensus at the current time over the role p53 has in predicting response to radiotherapy. The conflicting results are almost certainly in part due to the different characteristics of the individual studies but the complex role p53 has on the function of both normal and cancerous cells will undoubtedly further confuse the issue.

1.10.3.2 DNA Protein Kinases.

The DNA protein kinase complex (DNA-PK) is involved in one of the major pathways by which a cell responds to DNA double strand breaks induced by ionising radiation (Jeggo et al., 1995). It consists of a heterodimer comprising 70- and 80 kDa proteins termed Ku and a 465-kDa serine/threonine protein kinase catalytic subunit termed DNA-PKca. The Ku component functions as an activator of the catalytic subunit, and also represents the major double-stranded DNA-binding protein (Gottlieb and Jackson, 1993). As such it would be expected that the over or under expression of proteins which make up this complex could have some predictive value with regards to the response to radiotherapy. When the PubMed database was searched using the MeSH terms mouth neoplasms, radiation tolerance and DNA protein kinases only one paper was found (Shintani et al., 2003).

Shintani et al., (2003) performed a study on 42 human oral SCC specimens, all of whom had received pre-operative radiotherapy. An immunohistochemical analysis was performed with antibodies against DNA-PK complex proteins. Although the study found an increase in the expression of DNA-PK complex proteins after radiotherapy there was no relationship between response to radiotherapy and DNA-PK complex proteins.

1.10.3.3 Vascular Endothelial Growth Factor (VEGF).

Angiogenesis plays an important role in the development of radioresistance in many types of cancer and a link has already been established in cervical and breast cancer (Toi et al., 2001). VEGF encompasses a family of proteins which strongly promote new vessel growth and formation. They act via receptors principally found on the vascular endothelium (Ferrara et al., 1992). VEGF causes an up regulation of the antiapoptotic protein Bcl-2 thus protecting cells from the effects of radiotherapy (Ferrara, 2004). There have only been four studies on oral cancer exclusively, which have attempted to correlate the levels of VEGF with the outcome of radiotherapy (Harari and Huang, 2001; Tamatani et al., 2004; Shintani et al., 2000; Smith et al., 2000). Shintani et al., (2000) analysed 41 oral SCCs using immunohistochemistry to detect the presence of VEGF both before and after the tumour had been exposed to fractionated radiotherapy. The polyclonal antibody used in this study detected the precursor to VEGF and the VEGF-A isoform. In this study radioresistance was defined as tumour regression of <50% of the initial tumour size on macroscopic examination. Detection of VEGF could not be done on all of the specimens both before and after radiotherapy as 12 of the cases showed a complete response and no tumour tissue remained. This study concluded that VEGF was up-regulated in all of the tumours surviving radiotherapy as the levels of VEGF in the post-radiotherapy specimens were increased. The authors hypothesised that the radioresistant tumour cells had the potential to up-regulate VEGF in response to DNA damage. (Smith et al., 2000)Smith et al., (2000) studied a total of 56 patients treated with a combination of surgery and post operative radiotherapy (to a median dose of 60Gy). Immunohistochemistry was performed on tissue sections from the primary tumour before therapy began. Any tumour at local, regional or distant sites was taken to be a

recurrence and thus indicate a resistance to radiotherapy, however they did analyse each site of recurrence separately. They found that increased staining for VEGF predicted any recurrence (p=0.007) and also predicted for local recurrence (p=0.04). Harari and Huang, (2001) and Tamatani et al., (2004) both performed in vitro studies on Oral SCC and whilst neither found any predictive value to VEGF they both found VEGF to be of key importance to the mechanism by which tumours respond to radiotherapy.

These studies suggest both a possible mechanism for the development of radioresistance and a way of predicting a response to radiotherapy before treatment begins. More clinical studies with a better standardised definition of radioresistance are needed before any firm conclusions can be drawn.

1.10.3.4. Cyclooxygenase-2 (COX-2).

Prostaglandins are synthesised from arachidonic acid and the cyclooxygenase (COX) enzymes are essential for this process. There are two isoforms of this enzyme conveniently named COX-1 and COX-2. Of the two isoforms COX-2 is induced during pathological processes such as inflammation and cancer (Lin et al., 2002) including that of the head and neck region. One study exists that investigated COX-2 expression in histological biopsy specimens of oral SCC (Terakado et al., 2004). In this study biopsy samples were taken from 41 patients with oral SCC both before radiotherapy and after surgery, and immunohistochemistry was used to evaluate the expression of COX-2. Radioresistance was determined from the surgical specimens by analysing tumour mass remaining after the course of radiotherapy i.e. if two thirds or less of the tumour mass did not show a response to radiotherapy the tumour was

deemed radioresistant. Of the biopsy specimens 26 were scored as having a poor response to radiotherapy. When this was combined with the expression of COX-2, 17 of these specimens had a high level of COX-2 expression and statistical analysis showed a significant relationship between the over expression of COX-2 and radioresistance (P=0.047). Although this study is encouraging in its positive findings, and correlates well with the findings from a study on laryngeal biopsy specimens (Nix et al., 2004), the definition of radioresistance used in this instance does not take into account any tumours which may recur after the immediate treatment, thus some radioresistant and poor prognosis tumours will have been excluded from this group.

1.10.3.5 Bcl-2 Family.

These are a group of proteins which are intimately involved in programmed cell death and are best described as being in two groups, those which promote and those which inhibit cell death. Promoters include bax, bak, bcl-xS, bad and bid and inhibitors include bcl-2, bcl-xL and bcl-w (Nikitakis et al., 2004). A number of radioresistance factors discussed e.g. EGFR, p53, DNA-PK complex and COX-2 exert their effects by modulating the apoptotic process. Whilst studies exist on the prevalence and prognostic value of the Bcl-2 family in oral SCC (Loro et al., 1999; Xie et al., 2003; Xie et al., 1999; Vora et al., 2003) studies relating the response to radiotherapy, Bcl-2 and oral SCC are awaited .

1.10.3.6 Epidermal Growth Factor Receptor (EGFR).

The family of epidermal growth factor receptors (EGFR) consists of four tyrosine kinase transmembrane receptors which lie at the heart of a multitude of cell processes including cell proliferation, angiogenesis, migration and apoptosis (Harari, 2004). The

EGFR signalling network is highly complex and consists of many layers. At present there are ten different ligands known to activate this pathway in mammals, epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) being two of the more well known. When the pathway is activated in the majority of cases the result is stimulation of cell growth (Yarden, 2001). However, this network has many other effects on the cell and when the receptor is activated by its' ligands e.g. TGF-α, it can cause activation of the signal transduction molecule stat3 which in turn promotes a rise in the antiapoptotic gene products such as bcl-xL (Nikitakis et al., 2004; Song and Grandis, 2000). The theory that blocking this receptor would enhance the effects of radiotherapy, by decreasing the anti-apoptotic proteins, has been tested on a number of occasions (Gee and Nicholson, 2003; Huang et al., 1999; Harari and Huang, 2001; Huang et al., 2002). Huang et al., (1999) performed a study on cell lines derived from human HNSCC. The cell lines used in the study were from the floor of mouth, tonsil and facial epidermis. An antibody, C225, was used to inhibit the effects of EGFR, and the growth of all cancer cell lines was inhibited. The cells were then exposed to a standard regime of radiotherapy in combination with the C225 antibody. It was found that C225 significantly enhanced the effect of radiotherapy (P=0.001). Harari and Huang, (2001) took this theory one step further and using the same cell lines they induced tumour growth in athymic mice, exposed them to C225 and then to fractionated radiotherapy. They also found a significant increase in the response to radiotherapy in those tumours exposed to C225. Huang et al., (2002) used a different anti-EGFR agent, Iressa/ZD1839 a tyrosine kinase inhibitor, on tumours induced in athymic mice and once again showed a markedly improved response to radiotherapy.

Harari and Huang, (2001) performed a study on cell lines developed from oral SCC which induced tumours in mice. They used C225 combined with radiotherapy and assessed levels of VEGF using immunohistochemistry both before and after treatment. They found that the expression of VEGF was reduced after radiotherapy alone and was reduced further when combined with the effect of C225. A further study performed by a different research group (Tamatani et al., 2004) using tumour bearing nude mice concluded that the production of angiogenic factors such as VEGF in response to radiotherapy, was the major method of inducible radioresistance.

The C225 antibody has been used in a number of phase one, two and three trials, under the trade name "Cetuximab", treating a broad group of patients with all types of head and neck cancer (Baselga, 2001). The trials also involved many other forms of cancer including lung and colorectal cancer. The results of a large randomised controlled trial involving 424 patients with advanced head and neck cancer were published in June 2004. In the two arms of the study the patients were either given high dose fractionated radiotherapy alone or this in combination with cetuximab. It was found that the median survival of patients in the cetuximab arm had a near doubling of their median survival time from 28 months to 54 months (Harari, 2004).

1.10.4 Future Techniques.

All of the studies quoted in this review so far have studied expression of a single gene or protein with varying degrees of success. The process of studying gene expression has been a time consuming process in the past with individual genes having to be studied separately. In the last few years the advent of microarray technology has revolutionised this field, allowing a large number of genes to be studied

simultaneously and comparisons to be made between two distinct groups of tumours or between normal and tumour tissue (Varley, 2001).

1.10.4.1 Microarray Techniques.

Hanna et al., (2001) obtained biopsies from patients undergoing radical resections for head and neck SCC but do not specify in their paper from which site these were taken. They performed gene expression analysis using the Atlas human cancer 1.2 cDNA microarray on two biopsies from radioresistant tumours and two from radiosensitive tumours. The aim of the study was to try and identify a cluster of genes which would more accurately predict the radiotherapy response than a single marker.

Out of the 1187 genes on the microarray, they selected 60 for further study. Using bioinformatics they were able to predict the response to radiotherapy. Many of the genes that were in the cluster have not been previously studied in the literature e.g. JAG-2, STAT-3 (Hanna et al., 2001). However, this approach was undertaken knowing the response to the radiotherapy, has very low numbers and, as stated by the authors, does not take into consideration the expression of radio-inducible genes that may also affect the response to radiotherapy. A larger, more controlled study is required to validate this approach in the hunt for radioresistance genes, but this study provides encouragement that the approach may prove useful as a profile for radioresistance was obtained.

1.10.5 Summary of markers of radioresistance.

Out of the potential markers of radioresistance which have been identified so far in this thesis only a few have demonstrated any real predictive value. Whilst cytological

analysis seems able to aid in prediction once a course of therapy has begun, they have little value beforehand. Intratumoral microvessel density seems to be a more promising area of study but only one of the three studies found a statistical significance to their results. VEGF, VEGF-Receptor and COX-2 again look like possible targets for further research with larger studies and a more standard definition of radioresistance being essential. Of all the markers referred to in the literature EGFR is undoubtedly the most promising as a therapeutic target, although as it is over expressed by 80% of all HNSCC (Harari, 2004), its value as a predictive marker is limited. With the advent of microarray technology the opportunity has arisen to profile many genes from each patient with cancer. If a profile, such as the one developed by Hanna et al., (2001) could be correlated with the outcome of different treatment regimes this would be an invaluable tool in deciding the treatment regime of cancer patients in the future. Ideally from the increasing body of microarray data will come a small panel of key genes that can be tested in a routine screen, and on the basis of this profile the most appropriate treatment strategy can be adopted.

1.11 Microarray Technology.

A microarray is a small analytical device that allows genomic exploration with greater speed and precision than previously possible. Glass chips containing tens of thousands of genes are used to analyse labelled nucleic acids extracted from cells, tissues and other biological sources (Schena et al., 1998). Molecules in the fluorescent sample react with the corresponding sequence on the chip which causes each spot to glow with an intensity proportional to the activity of the expressed gene.

This technology is a relatively new entrant to the world of research. It was developed and in use during the 1980s, but did not come into prominence until the mid 1990s (Sasik et al., 2004). When it came onto the market in the late 1990s the high price pushed the technology beyond many labs, and researchers were forced to build arrayers themselves. With methods of manufacture changing and greater demand, the price has come down over recent years and the technology has become available to a growing number of research centres (Gershon, 2002).

Until the conception of microarrays studying gene expression was time consuming and involved techniques such as Northern and dot blotting. Both of these techniques involve hybridisation of a labelled gene probe to the RNA from the tissue of interest in order to assess relative expression levels. They each have their own draw backs primarily that only one gene at a time can be examined and in the case of Northern blotting relatively large quantities of RNA are required (Varley, 2001). Microarrays allow parallelism, miniaturisation, multiplexing (the process by which multiple genes are analysed in a single assay) and automation which cannot be achieved with the earlier technologies (Schena et al., 1998).

A simple idea was conceived to improve this process and overcome the disadvantages, instead of immobilising the RNA from tissues of interest and probe one at a time for each gene, the process was essentially reversed. The genes were immobilised on a matrix and the RNA was labelled as a probe. As the Human Genome Project identified more and more genes so the “array” of genes available to attach to a matrix became larger and larger (Varley, 2001). Initial estimates on the number of genes in the human genome were around 150 000 but have steadily

dropped to a figure of 30 000 (Varley, 2001). This led researchers to miniaturise the matrix on which these can be immobilised and the resulting creation was termed a gene chip. They are also referred to as DNA chips and microarrays.

1.11.1 Types of microarrays.

The first microarray experiments were performed using complementary DNA (cDNA). A cDNA molecule is a nucleic acid derived from messenger RNA (mRNA). It is a double stranded molecule that is an exact "complement" of the corresponding mRNA molecule. As the mRNA only contains the nucleotides corresponding to the original base pairs contained in the exon portion of the original gene, the sequence representing the introns is not present in the cDNA. The length of cDNA used in microarray analysis is typically between 500 – 2500 base pairs. Oligonucleotide microarrays have now also become commonly used. Oligonucleotides are single stranded 15 to 70 nucleotide molecules made by chemical synthesis. They are constructed from genes where the exact sequence is known which means the investigator can be quite specific about which target is printed onto the microarray.

1.11.2 Manufacturing a gene chip.

Many ways of manufacturing a gene chip have been tried in the past, and these different methods can be categorised into the method of synthesis or delivery. When a synthesis approach is used to manufacture chips, they are prepared in a step wise fashion by the in situ synthesis of nucleic acids and other biopolymers. Oligonucleotides are built up with each round of synthesis adding more and more nucleotides until the desired length has been achieved. On the other side, delivery techniques use exogenous preparations of molecules, such as complementary DNA, and drop these onto known locations on the "chip" in an alternative approach to using

oligonucletides (Khan et al., 1999). There are three main methods of manufacturing DNA chips photolithography, mechanical microspotting and ink jet technology (Schena et al., 1998).

1.11.2.1 Mechanical Microspotting.

This was the first widely used method of producing microarrays (Sasik et al., 2004). Essentially it involves the printing of a small quantity of premade biochemical substance onto a solid surface i.e. a microscope slide. There is direct surface contact between the printing substrate and a delivery mechanism containing an array of tweezers, pins or capillaries. The largest manufacturer of this type of chip is Synteni USA, and their chips measure approximately 3.6cm^2 containing as many as 10 000 groups of cDNA. This method is cheaper than that of photolithography and by combining chips, upto 40 000 genes can be analysed at any one time (Sasik et al., 2004; Schena et al., 1998).

1.11.2.2 Photolithography.

The next type of array to emerge onto the market involved in situ synthesised oligonucleotide arrays using photolithography (Sasik et al., 2004). This is a synthetic technology which combines methods used in the semiconductor industry with DNA-synthetic chemistry (Fodor et al., 1991). Unlike the microspotting method, this type of chip allows hybridisation with cRNA from the test tissues (Sasik et al., 2004). These chips are manufactured by Affymetrix in the USA. They are about 1.6cm^2 in size and contain 400 000 oligonucleotide groups. As photoprotected versions of the four DNA building blocks are used in this method, chips can be manufactured from a sequence database, and the use of synthetic agents minimises any chip to chip variation (Schena

et al., 1998). This method is cartridge based and requires the use of specific equipment that comes as a package. It is much more expensive to use than the other methods.

1.11.2.3 Ink Jet Technology.

This is the newest method of manufacturing DNA chips. It is similar to the microspotting approach and there is no direct surface contact instead piezoelectric forms of propulsion are utilised to transfer biochemical substances from miniature nozzles to solid surfaces. This has the added benefit of having a theoretically much higher throughput than the microspotting method (Schena et al., 1998).

1.11.3 Methodology.

Performing a microarray experiment involves a number of steps. Throughout the experiment the DNA of the test is compared to that of a standard and any differences in expression are analysed. The first part of the experiment involves extracting mRNA from the pair of tissues under investigation e.g. RNA from an oral cancer cell line and RNA from a normal oral cell line. This mRNA is then converted into cDNA using the method of reverse transcription. The cDNA for the test and standard are then labelled with different fluorescent dyes, typically one that fluoresces red and one green, see fig 1.8.

Once this has been done, the test samples are mixed and placed onto the microarray slide where they are left to incubate. At this stage some of the cDNA labelled red or green will bind to the corresponding oligonucleotide on the slide. Genes which have been over expressed by one or the other of the test tissues will have relatively more cDNA in the solution on the array slide. Thus, each spot on the array slide will bind

relatively more of that cDNA. Once the slide has been left to incubate the excess, unbound cDNA is washed off along with the solution in which they were contained. The slide is then scanned by a laser, first in the red spectrum and then in the green. Two images are created which are then combined to form a final microarray image. Each spot can fluoresce red, green, yellow or white. Red or green spots represent those genes which have been over expressed by that tissue whose cDNA was labelled with that florescent dye, shades of yellow arise when there has been an almost equal number of cDNAs' binding and white spots arise when the spot has been saturated with cDNA of either type.

1.11.4 Applications.

The most widely used application of microarray technology is in the comparison of gene expression in two or more populations of cells (Schena et al., 1998; Varley, 2001). All expression studies involve the hybridisation of labelled RNA to either the cDNA spot or the oligonucleotide attached to the solid support (Varley, 2001). Gene chips are well suited to this kind of analysis as they focus on the functional (expressed) parts of the genome. This is an important consideration when working with the human genome as the ratio of coding to non coding DNA is low (Schena et al., 1998). Tumour profiling using gene chips has provided the most dramatic example of the power of the microarray system. The profiling of a tumour can produce huge amounts of information, but sometimes it is possible to identify groups of genes whose expression pattern is closely related to specific clinical features such as drug response, metastatic potential and response to radiotherapy.

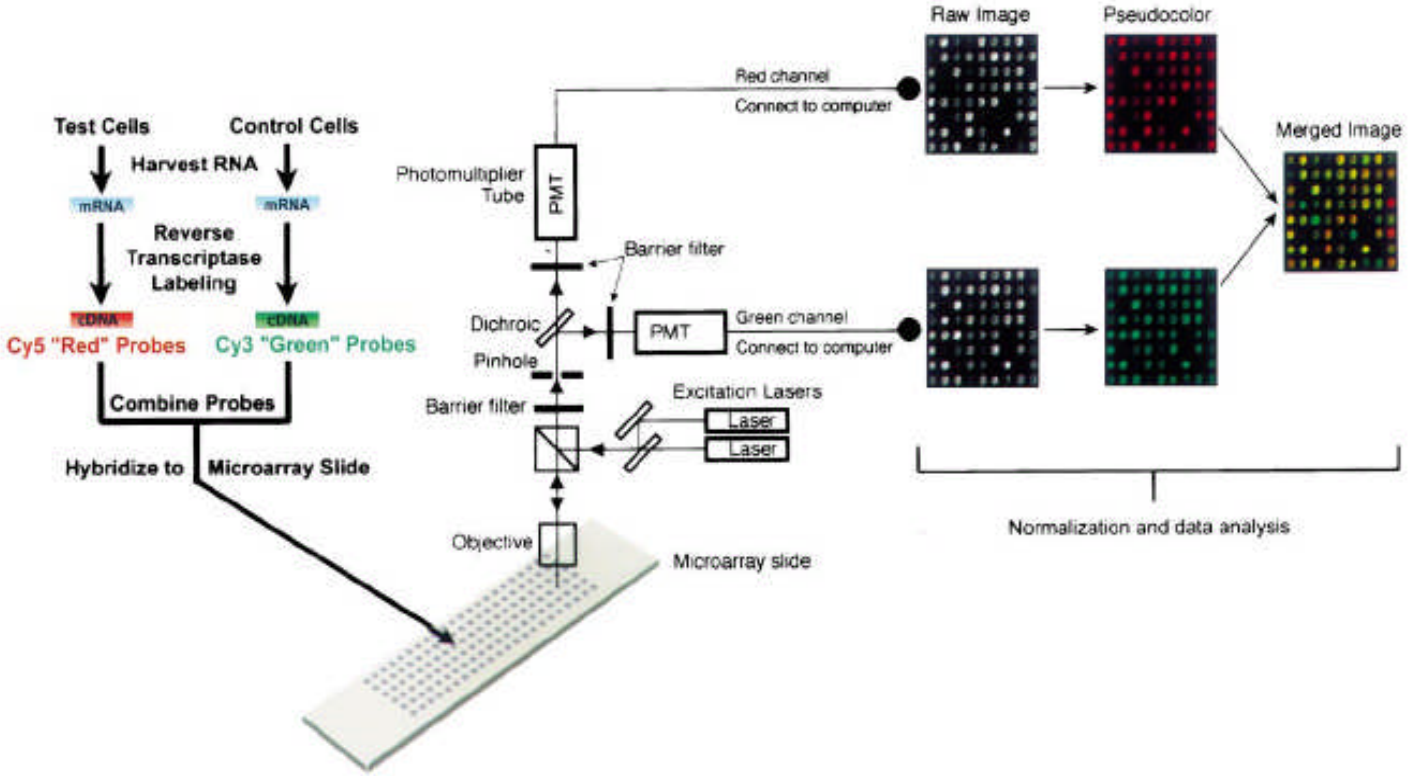


Figure 1.8 Schematic diagram to show the methodology behind performing a microarray experiment. cDNA is created from mRNA, labelled with each dye, then hybridised to the array slide. The slide is then read by the scanner and the data is then ready to be interpreted.

Knowing which genes are associated with these characteristics will give clues to the biological mechanism behind them and may provide novel targets for therapies (Varley, 2001).

Compounds and therapies can be applied to tumour cell lines and the resultant change in gene expression determined. This can be used for screening new anti cancer agents, investigating their mechanism of action and help in establishing how a tumour resists a particular therapy. The mechanism of key cellular processes can also be investigated using gene chips by comparing one group of genes to another e.g. comparing the expression profiles of young, senescent and immortal cells to identify genes involved in cellular ageing (Varley, 2001).

1.11.5 Advantages of Microarrays.

Microarray assays possess a set of performance specifications that older technologies are unable to achieve. They allow for massive parallel data acquisition and analysis thus increasing the speed of experimental progress and allowing meaningful comparisons of the genes or gene products in the array. In the future it may be possible to allow the analysis of the whole of the human genome in one experiment (Schena et al., 1998).

Gene chip technology allows for the miniaturisation of conventional assays thus reducing reagent consumption, minimising reaction volumes, increasing sample concentration and accelerating the reaction kinetics. They allow sensitive and rapid data detection with scanners or cameras equipped with charged-couple devices. The ability to process multiple samples at the same time “multiplexing” is also possible with microarrays. In this case, instead of two florescent dyes being used to label two

samples, multiple dyes are used for multiple samples which are then analysed on the same chip. This increases the accuracy of comparative analysis by eliminating factors such as chip to chip variation, different reaction conditions and other problems in conducting two separate experiments (Schena et al., 1998).

1.11.6 Disadvantages of Microarrays.

Despite the falling cost of running microarray experiments over the past few years, the expensive nature of the equipment is still one of the major limiting factors of this technology (Hafian et al., 2004). This leads to the problem of few researchers being in a position to repeat their experiments to confirm their results or to compare results between different microarray technologies (Tilstone and Aldhous, 2003). Commercial DNA chips were retailing for around $1000 each in 2003, this meant that some researchers were forced to pool several different samples and apply them to one array. However, in this type of experiment valuable information on sample to sample variation maybe lost (Tilstone and Aldhous, 2003).

One of the benefits of microarrays can conversely also be one of its downfalls. The amount of data that the analysis generates can sometimes be overwhelming and meaningless. Statistical analysis of the results is often difficult, time consuming and often requires powerful computer programmes. The relatively high cost of the gene chips means that the experiments are often not repeated to allow for variations. It is also difficult to determine whether an apparent two fold change in gene expression is significant or not (Tilstone and Aldhous, 2003). There is no consensus on the number of experimental replicates to perform and researchers often use computer software incorrectly to perform the analysis, thus resulting in spurious results (Tilstone and Aldhous, 2003).

When using microarray technology it is very difficult to know how to correct for false positive results, for example when studying a single gene there maybe a 5% chance that the recorded difference in its activity are the result of a chance fluctuation, but when this is repeated over 10 000 genes it becomes 500 apparently significant differences in gene expression when in fact there may be none! For simple data sets statistical models exist to combat this but as yet none exist for gene chips (Tilstone and Aldhous, 2003).

At every stage of the microarray process errors can creep in, from the preparation of tissue samples to the data extraction. The source of mRNA should be isolated from a homogenous population of cells and collected in a way that preserves it as RNA in solution is an unstable substance which degrades easily, thus apparent differences in expression may just be due to the RNA not being preserved properly when collected (Tilstone and Aldhous, 2003). Whole samples of tissues can be difficult to study as they have multiple cell types. Techniques and timing of collection of the samples vary as does the quality and quantity of the RNA (Tilstone and Aldhous, 2003). The use of different dyes can influence the results recorded by the lasers that measure the fluorescent signals as can the location of the spots on the chip. Unevenness of the glass slide or even dust on the slide can also affect the readings of fluorescence (Tilstone and Aldhous, 2003).

1.12 Immunohistochemistry.

1.12.1 Introduction.

Over many years immunohistochemistry has played an important role in the fields of cell and tissue biology, embryology and diagnostic pathology. It has revolutionalised the field of tumour diagnosis and provided pathologists with a way of better

characterising difficult or unusual tumours. As a result it has led to the reclassification of many neoplasms and the creation of new categories previously unknown.

Immunohistochemistry works by localising and identifying a specific antigen in a cell or tissue specimen. The specimens are usually first fixed in formalin, embedded in paraffin and then cut into sections of approximately 4 – 5 μm thick. These are then deparaffinised with a xylene solution and rehydrated with a series of ethanol solutions of decreasing concentrations. The sections are then dried and treated with 3% hydrogen peroxide solution to block any endogenous peroxidase. The final staining chromogen reaction utilises an enzyme isolated from the root of the horseradish plant, horseradish peroxidase. Its active site is iron containing and is able to form complexes with hydrogen peroxide. This reaction breaks down the hydrogen peroxide into water and atomic oxygen, which is then able to go on and oxidise any available substrate. In this thesis the substrate used for the staining reaction is 3-diaminobenzidine tetrahydrochloride (DAB) which forms a brown precipitate when oxidised. If the endogenous peroxidase contained in cells types such as erythrocytes, monocytes and myocytes is not blocked then increased background staining will occur.

Once the endogenous peroxidase has been blocked the sections may need to be subjected to an antigen retrieval method, described in section 1.12.5. The sections are then treated with caesin to block any non-specific staining and the primary antibody, either monoclonal or polyclonal, is then applied. A second antibody linked with a visible marker, is then applied to enable detection of the primary (Hayat, 2002b).

1.12.2 Antigens.

An antigen is a substance which reacts specifically with receptors on the surface of lymphocytes and with their soluble products such as antibodies. They are usually large complex proteins or polysaccharides. Most antigens have a variety of antigenic determinants on their surface, so called “epitopes”. When an antibody detects an antigen it does not recognise the whole of the antigen, instead it just homes in on a particular epitope on the antigens’ surface. Epitopes can be defined as immunologically active regions of an antigen which bind to specific membrane receptors on secreted antibodies (Hayat, 2002b).

1.12.3 Antibodies.

This term refers to a group of proteins that belong to the immunoglobulin family. There are five types of human antibodies with IgG being the most abundant and IgA, IgM, IgD and IgE being the rest in order of decreasing abundance. IgG and IgM are by far the most utilised antibodies in the field of immunohistochemistry.

Each antibody is essentially the same in structure consisting of two heavy and two light chains which are linked together by a varying number of disulfide bonds. Each light chain can consist of either a kappa or lambda portion. An antibody can be split up into various “domains” using either papain or pepsin. When this is done two different domains are formed those being the constant region and variable region, see figure 1.9. The variable region contains the amino terminus of the antibody where the antigen binds. The structure of each different type of antibody varies only slightly from this model but the structure of the IgM antibody is worth commenting upon. IgM is a pentamer of five subunits (each of these being similar to a single IgG

molecule) connected by disulfide bonds. The half life of the IgM antibody is anywhere from four to six days whereas that of IgG is around three weeks.

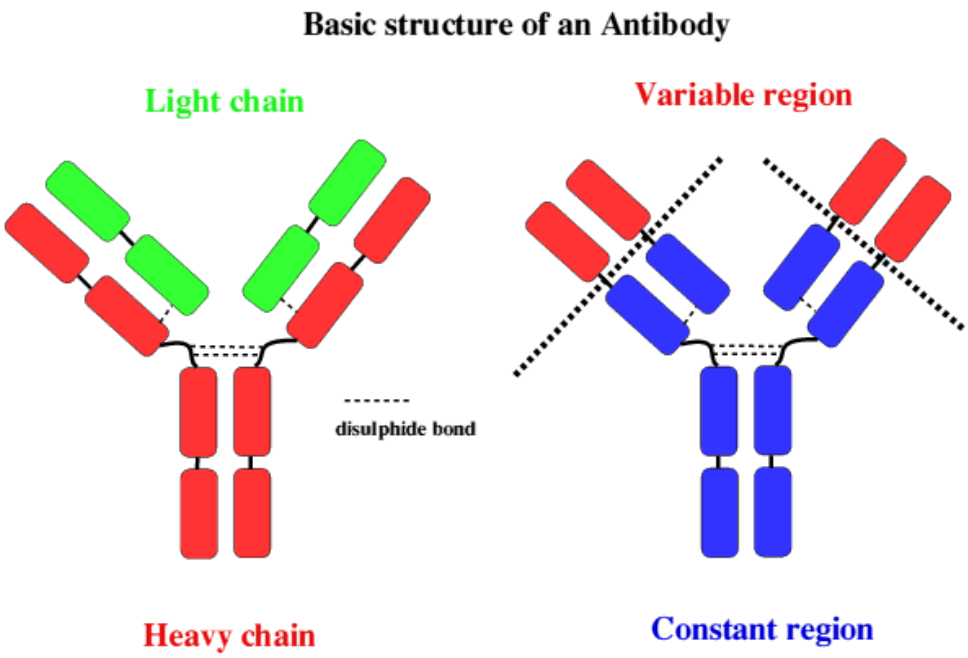


Figure 1.9 showing the basic structure of a typical antibody with the heavy and light chains illustrated on the left, and the variable and constant regions illustrated on the right. The variable region of the antibody is the site which recognises and binds to the antigen. (adapted from www.immuno.path.cam.ac.uk)

1.12.3.1 Polyclonal Antibodies.

This type of antibody is produced by different cells in the body and although they are basically the same in structure, slight differences in the variable portions of the heavy and light chains mean that they recognise different epitopes. Rabbit is the most common animal used for the production of this type of antibody followed by goat, pig, sheep, hoarse and guinea pig. The rabbit has become the most popular animal as it is both relatively easy to maintain and human antibodies to rabbit serum proteins are rare.

Antibodies of this type are manufactured by the process of generating an immune response from the animal, by injecting a sample of the antigen into the animal either intradermally or intraperitoneally. Once a response has happened blood is harvested from a major vein and the serum is isolated. The antibody can then be prepared, via various methods, into whole stabilised serum or fractions purified to certain degrees.

1.12.3.2 Monoclonal Antibodies.

These are produced by clones of plasma cells and are therefore identical and react with a specific epitope on the antigen surface. Mice are almost exclusively used for the production of this type of antibody. The antibodies are produced by stimulating the mouse spleen into producing B-cells specific to the antigen in question. These B-cells then produce a clone of antibodies against the antigen in question.

There is less batch to batch variation compared to polyclonal antibodies, they also have a lower probability of non-specific antibody binding. Each batch of monoclonal antibodies will only react to one specific epitope on the antigen, thus reducing any background signal. However, if this single target epitope does not survive the fixation process no staining will be apparent. With polyclonal antibodies having multiple target epitopes, the chance of this happening is much less.

1.12.3.3. Affinity.

In immunohistochemistry the term affinity of an antibody can be loosely defined by the time taken to reach equilibrium with the tissue antigen. The affinity of an antigen is related to its ability to form insoluble immune complexes. Polyclonal antisera contain a continuous spectrum of low to high affinity antibodies against several

epitopes on a particular antigen. This means that after incubation excessive washing is unlikely to result in loss of staining. Monoclonal antibodies, on the other hand, have a uniform affinity for a particular epitope, if this is low a loss of staining during the washing process is more likely.

1.12.3.4. Reaction Rates.

In ideal conditions antibodies and antigens react very rapidly. Unfortunately the conditions encountered in the immunohistochemical process are far from the ideal situation and the reaction rates can therefore take anything up to 48 hours. The reaction time can depend upon the type and method of tissue fixation, the concentration of the antibody and the ambient temperature. Shorter times occur when using high concentrations of high affinity antibodies (Boenisch et al., 1989a).

1.12.4 Fixation.

All histological techniques need to preserve tissues and cells in as reproducible and lifelike manner as possible. In order to achieve this specimens are usually fixed in a fluid. The aim of the fixative solution is to prevent autolysis, inhibit the growth of moulds and bacteria and to limit the damage to the structure of the sample during the subsequent staining process.

Unfortunately all these properties cannot be achieved without a cost and the dilemma in fixative techniques has always been that in order to preserve the tissues it is necessary to introduce some artefact. In performing their role the fixatives denature proteins thus some of the antigenic profile is lost. Under normal circumstances viable cells are normally encased in an impermeable membrane. The process of fixation breaks this down allowing relatively large molecules to enter and escape. Different

types of fixative result in different degrees of porosity with coagulant fixatives e.g. B5 resulting in larger pore sizes than non-coagulant fixatives e.g. formalin.

1.12.4.1. Formaldehyde based fixatives.

Almost all samples used for immunostaining are embedded in paraffin. Most of the popular fixatives contain formalin (40% w/v formaldehyde in water), a neutral salt to maintain tonicity and a buffering system to maintain pH. During the process of fixation or the subsequent paraffin embedding there may be shrinkage or distortion of the tissues.

Formalin fixes by the process of addition and not via coagulation. It reacts with basic amino acids to form cross linking methylene bridges. As a result of this there is relatively low permeability to macromolecules thus protecting the structures of the intracytoplasmic proteins. The process of protein cross linking leads to the masking of antigenic epitopes. This process is dependent upon factors such as the concentration of the fixative, the fixative contact time and the thickness of the tissues. If the tissue pieces to be fixed are small and fixed promptly in neutral buffered formalin for 6 – 24hrs there will be relatively little antigen masking. The thicker the tissue the longer the contact time is needed to fix the tissue at the centre of the specimen, and the more likely antigens are to be masked. This can be a problem with large tissue specimens obtained from resections from oral cancer, but should not cause too much difficulty with the relatively small biopsy specimens used in the thesis.

Careful consideration is needed when choosing a monoclonal antibody against an antigen when the specimen has been fixed in formalin. The formalin itself may

destroy the epitope in question but leave the rest of the antigen intact thus giving a false negative result. Formalin may also react with adjacent amino acids causing conformational changes in the antigen and thus inhibiting the binding of the antibody. This type of problem can be overcome by using proteolytic digestion. The very process of embedding the tissue in paraffin may also destroy the epitope as the tissue is heated to the melting point of wax (60°C) which can destroy the epitope on its own. Thus overheating of the tissue during embedding or during the drying process can adversely affect the staining process.

1.12.4.2 Other fixatives.

There are a number of other fixatives on the market, each with their own advantages and disadvantages. Mercuric-chloride based fixatives were designed with the aim in mind to better preserve the cytological appearance and better prevent tissue distortion than the formaldehyde based preparations. Whilst achieving this unfortunately they are very poor at penetrating tissues and as in the case of all coagulative fixants they cause considerable hardening of the tissues but are better at demonstrating intracytoplasmic antigens. Examples of this type include B5 and Zenker's solution.

Acetic acid-zinc chloride has become more popular with time as it is good at preserving membrane proteins. Periodate-lysine-paraformaldehyde is quite useful as it stabilises both proteins and carbohydrates. More recently potassium dichromate was added to preserve the lipid component of the tissue. Ethanol is not widely used as a fixative as it has very poor penetration into tissues and can cause conformational changes. However it fixes small pieces of tissue rapidly with good cytological

preservation. Acetone can also be used but again has very poor penetration into tissues (Boenisch et al., 1989b).

1.12.5 Antigen retrieval

Many methods of antigen retrieval exist including heating, enzyme digestion, use of ethylenediaminetetraacetic acid (EDTA), treatment with detergents and ultrasound. By far the most commonly used method is that of heating. This can be done using various pieces of equipment such as microwave ovens, pressure cookers, hot plates and water baths. Each method has its own advantages and limitations, but all are likely to be equally effective if used under optimal conditions.

There are a number of theories on the mechanism of how epitopes are unmasked. Some believe that the protein crosslinks formed during the formaldehyde treatment are broken down and others that the methods cause denaturing of the proteins to reveal previously masked epitopes. Antigen retrieval methods may work by either restoring the original protein structure previously altered during fixation or by breaking down the protein crosslinks formed thus unmasking the epitope.

1.12.5.1 Heating.

Most, if not all, crosslinks formed during formaldehyde fixation should be destroyed upon heating even at 37°C for 48 hours or for shorter times at higher temperatures e.g. a few minutes at 80 - 100°C. In theory any method of heating should unmask epitopes, but some of them are destroyed with heating and some still remain undetectable. Factors affecting the successful retrieval include the actual temperature used, the duration of heating and any variation in temperature during the process.

As previously mentioned many methods of heating exist but the three main ones employ either a microwave or a pressure cooker. Though they all have different advantages and disadvantages they obtain similar degrees of antigen retrieval. The choice of method depends upon the individual study and the equipment available.

One of the speediest methods involves the use of a standard, readily available microwave oven (900 watts 2450MHz) set on full power for two separate five minute cycles. The slides are placed in a non metallic rack in the buffer solution for the procedure. Care must be taken that they do not dry out as the buffer can boil over and be lost. While this method is quick, it is difficult to standardise. The second method involves heating in a microwave for a total of 20 minutes. Extending the time can improve the staining of many antigens but there is more chance of the fluid boiling over and of hot and cold spots developing.

The third method employs a pressure cooker as the source of heat. Microwaves do not raise the temperature of the buffer above 100°C, but in a pressure cooker temperatures of 115°C and higher can be achieved. The advantages of a pressure cooker include better reproducibility with large batches and relatively low cost of the equipment. Some comparative studies have indicated that pressure cooking or pressure cooking in combination with microwave heating produces more uniform, efficient, consistent and rapid immunostaining (Hayat, 2002a).

1.12.5.2 Ethylenediaminetetraacetic Acid (EDTA).

Exposing sections to high temperatures in combination with a calcium chelating or precipitating agent such as EDTA can increase the efficiency of antigen retrieval. The

combined treatment leads to the release of calcium ions previously tightly complexed to formalin fixed tissue sections. The use of EDTA both augments the intensity and number of cells stained. Variations in the pH of the EDTA has an influence on the intensity of staining with strong staining at pH 3 and neutral to high pHs but relatively weak staining at pH 4 and pH 5. Care must be taken when using EDTA as it is a strong oxidising agent and can thus alter the cell morphology.

1.12.5.3 Ultrasound.

There is only limited data on the mechanism by which ultrasound exerts a direct or indirect effect on the process of antigen retrieval. Treating the tissues with ultrasound causes enormous molecular agitation which both initiates and accelerates biochemical and physical reactions. This agitation also generates a considerable amount of heat which is rapidly lost from the tissues. The combination of the heat and the agitation is thought to release calcium ions thus enhancing the effect of the antigen retrieval solution. Ultrasound is also thought to break down the formalin induced cross links and expose the epitopes. This method is however difficult to reproduce as the exact parameters regarding the intensity at the acoustic frequency are difficult to set precisely (Hayat, 2002a).

1.12.6 Staining.

There are many staining methods available to localise antigens in immunohistochemistry. The choice depends on the type of specimen, the degree of sensitivity required, the processing time and the cost of the procedure. There are four main methods commonly described today the direct method, the indirect method, the soluble enzyme immune complex and the avidin-biotin method. Due to its increased

sensitivity of antigen detection the avidin-biotin, as detailed in section 1.12.6.4, method was utilised in this thesis

1.12.6.1 The Direct Method.

This is a relatively old technique which is rarely used today but on which many of the further techniques are based. It utilises a primary antibody which has been labelled with enzyme. This labelled antibody then reacts with the antigen in the tissue enabling it to be visualised. As this method only uses one antibody it is quick but because of this little signal amplification is achieved.

1.12.6.2 The Indirect Method.

In this method an unconjugated primary antibody is first attached to the antigen in question. Following this a second antibody (which has been labelled with an enzyme) against the primary antibody is added. The primary antibody is from a different species to the tissue that is being stained and antibodies raised in rabbits and mice are commonly used. This method is more sensitive than the direct method as a number of secondary antibodies can bind to various different epitopes on the primary antibody, as illustrated in figure 1.10. The staining intensity can be increased by modifying this method slightly into a three stage procedure. In this method a third labelled antibody against the second antibody is used therefore placing additional enzyme at the site of the tissue antigen and increasing the intensity of the stain.

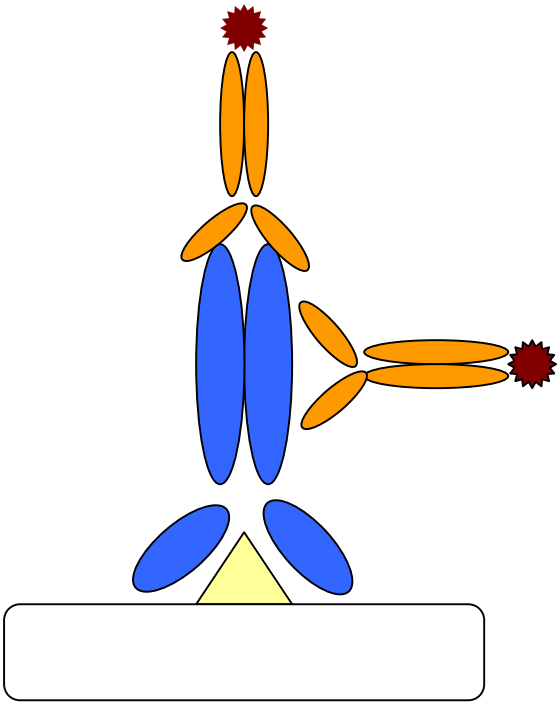


Figure 1.10 illustrating the indirect staining method. Primary antibody is represented in blue, secondary antibody represented in orange. The epitope is represented by the yellow triangle and the marker bound to the secondary antiboy is represented by the brown star. As illustrated, multiple secondary antibodies are able to bind to the primary antibody thus increasing the staining and hence the detection.

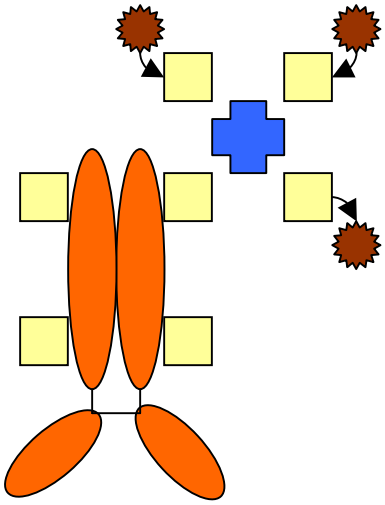
1.12.6.3 Soluble Enzyme Immune Complex Method.

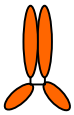
This is also referred to as the unlabelled antibody method and uses a preformed soluble enzyme-antienzyme immune complex where the enzyme is the antigen. To make this complex soluble the enzyme is added in excess and the precipitate removed. The sequence in this staining process begins with the addition of the primary unconjugated antibody, the secondary antibody is the added followed by the soluble immune complex and finally the substrate solution. The secondary antibody must be added in excess so that one of its antibody binding sites binds to the primary antibody

leaving the other free to bind the immune complex. This method is amongst the most sensitive immunochemical technique as many enzyme molecules are localised per antigen site.

1.12.6.4 Avidin-Biotin Method.

This method has a higher sensitivity of antigen detection. Avidin has four binding sites for biotin, and a high binding affinity. This method requires a so called “link” antibody which has been previously biotinylated, i.e. molecules of biotin have previously been covalently attached. One of the open sites on the avidin molecule then binds to the biotin on the link antibody. This leaves three binding sites available on the avidin molecule to further biotin binding. Once the avidin has bound, a further solution containing biotin with horseradish peroxidase is added. This biotin then binds to the three remaining “open” sites on the avidin molecule. Thus every biotin molecule attached to the secondary antibody has the potential to be amplified three-fold, as illustrated in figure 1.11. This method is said to be more sensitive than either the direct or indirect method. A solution of DAB is then added which reacts with the horseradish hydrogen peroxide, oxidises and turns brown enabling the detection of the epitope with a high degree of amplification.





secondary antibody

horseradish peroxidase

biotin

avidin

Figure 1.11 Illustrating the avidin biotin method of staining. As can be seen the secondary antibody has a number of molecules of biotin attached, avidin can then bind to this biotin molecule aswell as binding a further three biotin molecules with horseradish peroxidase attached. Thus the staining for each single epitope is greatly amplified.

1.13 Study Aims

Head and neck cancer comprises a large and diverse group of tumours. Different subsites are affected by different aetiological agents and respond differently to various curative therapies. This thesis aims to investigate the mechanism of radioresistance with regards to a single subset of head and neck cancer, namely the oral cavity. Thus leading to more accurate data not clouded by information relevant to other head and neck cancer subsites. Cell culture techniques along with a fractionated course of radiotherapy will be used with the aim of creating a novel radioresistant oral SCC cell

line. This will enable microarray analysis to be preformed on both the parent and daughter cell line to investigate radioresistant features of oral cancer. This will hopefully enable novel markers of radioresistance to be identified thus aiding in the decision making process for those tumours with adverse histological findings which may benefit from post operative radiotherapy. In the future microarray technology should enable the development of a genetic “fingerprint” by which the response radiotherapy can be predicted and, if necessary, identify appropriate genes which can be blocked sensitising the tumour to radiotherapy. The work done here on oral cancer should also help in identifying targets in other HNSCC subsites.

Chapter 2: Materials and Methods.

2.1 Cell Culture.

2.1.1 Cell lines.

Two commercially purchased cells lines were used in this experiment, both from the oral cavity and both squamous cell carcinomas. They were named “PE-CAPJ41” and “PE-CAPJ49” respectively and were obtained from the European Collection of Cell cultures, Salisbury, U.K. All cell culture reagents were from Invitrogen UK unless otherwise stated and all the plasticware used was from Sarstedt unless otherwise stated. The culture medium used was Dulbecco’s Modified Eagle’s Medium (DMEM) with the addition of 10% v/v foetal calf serum, 1% v/v penicillin and 1% v/v glutamine.

At all times during the handling of cells strict sterility was maintained and all surfaces and containers were wiped or sprayed with 70% v/v alcohol before processing took place. All work which involved manipulating the cells was undertaken in a class 2 microbiological safety cabinet equipped with high efficiency particulate air (HEPA) filters and UV sterilising lamp (Walker Safety Cabinets Ltd).

2.1.2 Thawing cells.

Before cells were thawed, the cell culture medium was warmed to 37˚C in a water bath for 30 minutes. The cells were then removed from the liquid nitrogen store, where they had been kept at -135ºC in sealed cryovials, and placed in a water bath at 37°C for ten minutes to ensure the medium was in liquid form before continuing. The vial was then sprayed with 70% v/v alcohol and transferred to the class 2 microbiological safety cabinet.

The cell suspension was removed and placed in a sterile 30ml polypropylene container. Cell culture medium was then added to make the suspension up to 10ml. This was centrifuged at 2500xg for three minutes. The supernatant was discarded and the remaining cell pellet was re-suspended in 12ml of cell culture medium. This was then split equally between three 75cm^2 flasks and placed in a humidified incubator kept at a constant temperature of 37°C with an atmosphere of 5% CO_2.

2.1.3 Culturing Cells.

Cells were grown in 75cm^2 tissue culture flasks incubated at a constant temperature of 37°C in an atmosphere of 5% CO_2. Four to five millilitres of DMEM was kept in the flask and changed as needed. The cells were grown in a monolayer, as shown is figure 2.1, and checked under a light microscope on a regular basis. When a confluence level of above 70% was reached the cells were split into three further flasks.

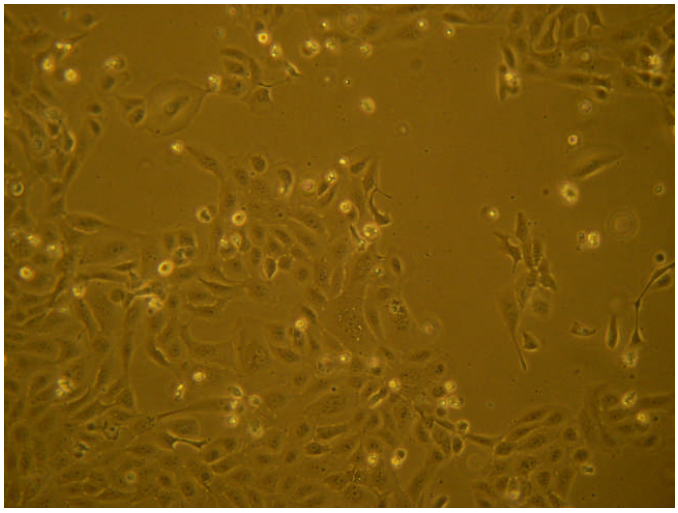


Figure 2.1 PE-CAPJ41 cells growing in a monolayer on the base of a tissue culture flask.

2.1.4 Passaging cells.

Once a 70% confluence level was reached the cells were split to facilitate further growth. The flask was removed from the incubator and transferred to the safety cabinet. The culture media was aspirated and discarded into a container of Virkon disinfectant. Four millilitres of trypsin (0.25% w/v) was added to the flask which was

then returned to the incubator. This was left for five minutes, removed and checked under the light microscope. Gentle tapping of the base of the flask was performed at this stage, and the flask was once again returned to the incubator. This procedure was repeated at five minute intervals until the majority of the cells had become detached from the base of the flask. The cells appeared more spherical in appearance under the light microscope after they had detached from the base of the flask, as illustrated in figure 2.2, and could be seen floating in the media. In total this process took between 15 to 20 minutes. Using trypsin alone to detach the cells avoids mechanical damage to cell structure from other methods such as the use of cell scrapers.

The cell suspension was removed from the flask and placed in a 30ml polypropylene container. Cell culture media was added to make the suspension up to 10ml in total, and the resultant mixture was centrifuged at 2500xg for three minutes. Once completed, this was returned to the safety cabinet, the supernatant removed and the remaining cell pellet re-suspended in 12ml of cell culture media. This was divided up into 4ml portions and each placed in a fresh 75cm^2 tissue culture flask and returned to the incubator.

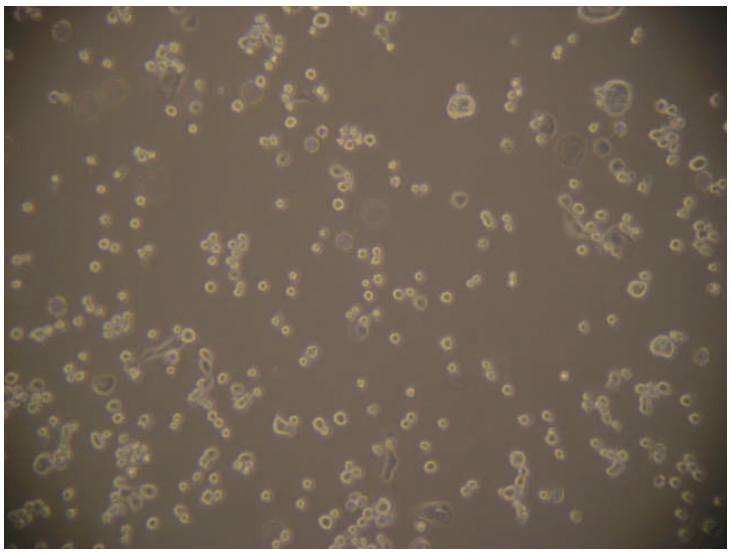


Figure 2.2. Microscopic view of trypsinised cells, as compared to fig 2.1 these cells are more circular in shape as they have lost their attachment to the base of the tissue culture flask.

2.1.5 Freezing cells.

Cells were removed from the flask in the same way as for passaging and the suspension was again spun in the centrifuge as described in section 2.1.4. Once the supernatant was removed however, the remaining cell pellet was suspended in 3ml of a freezing solution. This freezing solution was taken from a 50 ml stock made up of DMEM (46% v/v), Foetal calf serum (40% v/v), Dimethylsulfoxide (DMSO 10% v/v), penicillin (2% v/v) and glutamine (2% v/v). This was then divided up into three cryovials each containing one millilitre of solution. The vials were then placed upright into a freezer at -80°C overnight after which they were transferred into liquid nitrogen at -135°C for long term storage.

2.1.6 Counting Cells.

A cell pellet was prepared, as described in section 2.1.2, and re-suspended in cell culture media. Once done, 0.25µl was pippetted out into a sterile 1.5ml microcentrifuge tube and mixed with 0.25µl of trypan blue (0.4% v/v). As trypan blue has the ability to enter the cytoplasm of dead cells it was used to determine which of the cells were alive. After mixing, a small amount of the solution was placed under a glass cover slip on a haemocytometer and the number of cells in the grids labelled 1 – 5 in figure 2.3 were counted. Any cells which stained with the trypan blue were not deemed to be alive and so were not entered into the calculation. The average number of cells in each grid was calculated and multiplied by two. This figure was then multiplied by 10^4 to give the number of cells in 1ml of the solution.

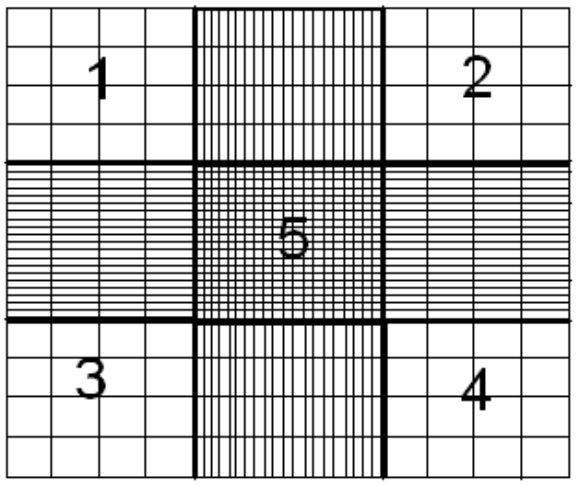


Fig 2.3. Grid on haemocytometer for counting cells.

2.1.7 Dose response curve.

The cells were counted using the method described in section 2.1.6 and $1x10^6$ cells were placed in 7ml polypropylene containers. A total of 6 containers were used and each container was filled with 5ml of the cell suspension. The containers were then labelled with the dose of radiation each was going to receive i.e. 0Gy, 2Gy, 4Gy, 6Gy, 8Gy and 10Gy. The samples were then irradiated using ionising radiation from a 6Mv radiotherapy machine (200cGy/min) at the Radiotherapy Department in Princess Royal Hospital, Hull.

A proportion of the suspension from each container, which corresponded to approximately 10 000 cells, was then removed and placed into six well tissue culture plates. This was done in triplicate. The plates were incubated at 37°C for 14 days, ensuring that the cell culture media did not dry out during this time. After 14 days the media was removed and the cells were fixed using a solution of methanol and glacial acetic acid (ratio 3:1 v/v). They were then air dried overnight before staining with eosin. This was achieved by covering the fixed layer of cells in each well with a

shallow layer of eosin. The cells were then left for five minutes before each well was rinsed with tap water.

In order to calculate the number of surviving cells after each dose of radiotherapy the stained colonies were scanned into a computer using a Biorad GS-800 calibrated densitometer. Colonies of cells of greater than 50 in number were deemed to represent surviving cells from the original cell line. The plates were examined under a light microscope and a colony of 50 cells was identified. This was then correlated with the image on the computer and a measurement taken. Any group of cells of this size or greater was then counted on the computer, and a graph of number of colonies against dose of radiotherapy was generated.

2.1.8 Incremental irradiation dose.

The parent cell line in this experiment refers to the cell line which had received no doses of radiotherapy from which a radioresistant cell line was hoped to be created. A sample of one million cells from the parent cell line was placed into a 7ml polypropylene container and made up to a volume of 5ml with culture medium. This was then taken to the Radiotherapy Department and given a dose of 4Gy. The cells were then split equally between two 75cm^2 tissue culture flasks, further media was added to a total of 5ml per flask, and returned to the incubator. The cells were checked under a light microscope, and when greater than 70% confluence was reached the cells were counted and a further one million cells were taken for another dose of 4Gy of radiation. This process was repeated until a final dose of 28Gy was reached for PE-CAPJ41 and 24Gy for PE-CAPJ49.

2.2. Expression microarray analysis

As mentioned in section 1.11.3 the basic methodology for all expression microarrays consists of generating cDNA from a sample of RNA, coupling this to a fluorescent dye and then hybridising this to a DNA probe. There are a number of methods in use today but in this instance we used the FairPlay® II Microarray Labelling Kit (Stratagene USA). One of the main problems when performing a microarray experiment is the interpretation of the results with regards to the intensity of the fluorescent dyes. An element which can be avoided with the use of this kit is the need to "dye swap" to allow for the uneven incorporation of labelled nucleotides during the reverse transcription reaction to produce labelled cDNA. This kit overcomes this by converting the labelling process into two parts. Firstly the amino allyl groups on the cDNA are modified and then the dye is chemically coupled to the cDNA. This removes the need for bulky CY-labelled dUTP to be incorporated during the reverse transcriptase reaction (Stratagene, 2004).

2.2.1. Extraction of RNA.

The Qiagen RNeasy® Mini animal cell RNA extraction kit (Qiagen UK) was used to provide all the necessary RNA extraction tubes and reagents except for the ß-mercaptoethanol (ß-ME) which was supplied by Sigma-Aldrich (UK). A stock solution of "Buffer RLT" was prepared with 990µl RLT and 10µl of ß-ME.

As described in section 2.1.4 the cells were pelleted and then re-suspended in 1.5ml PBS. They were transferred to 2ml centrifuge tubes and centrifuged at 3000g for 2 minutes. The resulting supernatant was removed and the pellet vortexed on high speed until the pellet returned to liquid form, thus allowing for adequate further mixing to

take place. The pellet was re-suspended in 660µl of the "RLT buffer" solution and gently mixed by pipetting. "RLT buffer" solution is used in order to disrupt the cell walls and plasma membranes of the cells, allowing the release of all the RNA contained in the sample. This mixture was then transferred to a QiaShredder® column (Qiagen) and centrifuged at 13000g for 2 minutes. The QiaShredder® column is used to homogenise the cells, this process shears the high molecular weight DNA and cellular components to create a homogenous lysate. Incomplete homogenisation leads to poor binding of the RNA to the RNeasy® column, used in later steps, and decreases the yield of RNA from the sample. To precipitate the RNA, the QiaShredder® was discarded and 600µl of RNase-free 70% ethanol was added to the flow through. The solution was once again mixed by pipetting and 700µl of the solution was added to an RNeasy® column. The 70% ethanol was added to create conditions appropriate for the binding of RNA to the column. This was then centrifuged at 8000g for 15 seconds and the flow-through discarded. We were then left with RNA and contaminants from the cell bound to the membrane in the column. Next we needed to remove all the contaminants from the column by applying a series of washes. Firstly, 700µl of "Buffer RW1" was added to the column and this was then centrifuged at 800g for 15 seconds. The column was then removed from the 2ml collection tube and transferred to a fresh identical container. To this column 500µl of "Buffer RTE" was added and the tube was then centrifuged at 8000g for 15 seconds. The flow through was discarded 500µl of "Buffer RPE" added and the column once again centrifuged at 8000g for 2 minutes. Once washing was completed, the column was placed into a fresh RNase-free 1.5ml microcentrifuge tube and 50µl of RNase-free water added. This was then incubated at room temperature for ten minutes and then centrifuged at 8000g for 1 minute to collect the RNA eludate. In order to increase

the concentration of the RNA the eluate was passed back through the column and centrifuged at 8000g for 1 minute for a final time. An overview of this process is represented in figure 2.4.

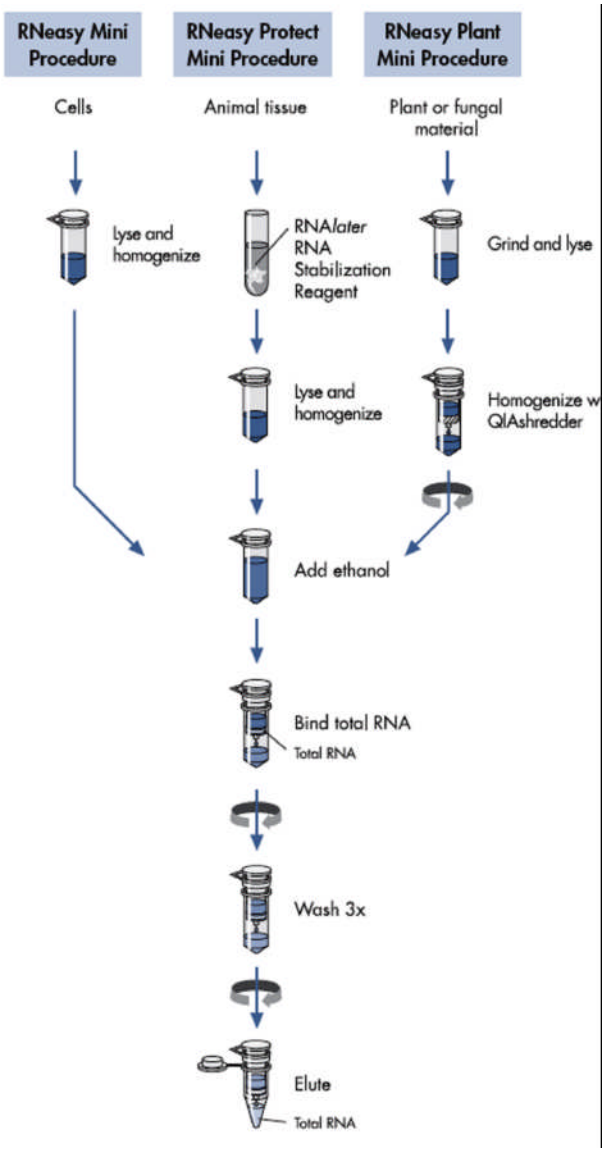


Figure 2.4. Representing an overview of the steps involved in isolating RNA from three different tissue types. Cells are initially lysed and homogenised, then RNA is bound to a membrane, the RNA is then washed three times to remove impurities then eluted in RNase free water. Reproduced from Qiagen RNeasy mini kit handbook. (Qiagen, 2006)

2.2.2. RNA Quantification and Purity assessment.

The quantification of RNA is more accurate when the sample to be measured is suspended in Tris rather than water. Water is not buffered and its' pH can therefore vary. This variation in pH affects the absorbance of the solution and thus alters the calculation of both purity and quantity of RNA from day to day. A solution of Tris buffer solution, with the addition of EDTA to remove any RNase activity, was prepared and RNA from the sample of RNA prepared in section 2.2.1 was added. This was achieved by adding 2µl of the RNA solution to 98µl of the Tris EDTA giving a concentration of 1 part in 50. The resulting 100µl was then added to a micro cuvette and a Biotech photometer® used to measure the absorbance at 260nm. This value was then used to calculate the concentration of RNA in one ml of solution using the equation shown below:

$$\text{Concentration} = \left(\frac{A260}{0.25x1.0} \right) xD$$

A260= absorption at 260nm
0.25= extinction coefficient of RNA
1.0= distance light travels in cm
D= dilution factor

The purity of the RNA to be used in the microarray experiment also needed to be checked. Again this was done using the Biotech photometer® with the same solution as before. The absorbance was tested at both 260nm and 280nm and the ratio of the two values indicated the purity of the sample of RNA. A value as close to 2 as possible was desirable as this indicates pure RNA sample.

2.2.3. Generation of amino allyl labelled cDNA.

All products used in the generation of amino allyl labelled cDNA were supplied by Stratagene unless otherwise stated. The first step in this process was to anneal the total RNA to an oligonucleotide d(T) primer. All RNA strands in the body have a poly-A tail at the 3' end of the molecule, the oligo d(T) binds to the poly-A tail allowing the reverse transcriptase to bind. The method described below was performed separately for both the parent and radioresistant cell line.

Firstly 10µg of the total RNA was suspended in 12µl of DEPC water. DEPC water was used to ensure no RNase is present and prevents RNA degradation during processing. Then 1µl of 500ng/µl oligonucleotide d(T) was added and the resulting solution incubated at 70ºC for 10 minutes in a heatblock. The tube was cooled on ice and a solution consisting of 2µl 10x StrataScipt™ reaction buffer, 1µl 20x dNTP mix, 1.5µl 0.1 M DTT and 0.5µl RNase block was prepared in a fresh sterile RNase/DNase free microcentrifuge tube. The RNA sample which was annealed to the oligo d(T) primer was added to this mixture. Finally, 3µl of StrataScript™ HC reverse transcriptase was added and the solution placed in an incubator at 42ºC for 60 minutes to allow reverse transcription to take place and generate the cDNA. The resultant mixture containing cDNA and RNA strands was then treated with 10µl of 1M NaOH at 70ºC for 10 minutes to hydrolyse any remaining RNA strands. The solution was cooled at room temperature and centrifuged briefly to collect the contents. Then 10µl HCl was added to neutralise the solution.

2.2.4. Purification of cDNA.

The solution from 2.2.3 contained not only cDNA but unincorporated excess nucleotides, buffer components and hydrolysed RNA which needed to be removed before the coupling reaction took place. The purification was achieved by first adding 4µl of 3M sodium acetate (pH 4.5), 1µl of 20mg/ml glycogen and 100µl of ice cold 95% ethanol and incubating this at -20ºC for 35 minutes to precipitate cDNA out of solution. The mixture was then centrifuged at 13,000g for 15 minutes at 4ºC to pellet the cDNA, and the resultant supernatant carefully removed. Then 0.5ml of ice cold 70% ethanol was added and the mixture centrifuged at 13,000xg for 15 minutes at 4ºC to wash the pellet. The resulting cDNA pellet was then carefully air dried at 37ºC to make sure all of the ethanol was removed before proceeding to the dye coupling reaction.

2.2.5. NHS-Ester containing dye coupling reaction.

The cDNA pellet was then re-suspended in 5µl of the 2x "Coupling Buffer", supplied with Fairplay® II microarray labelling kit, and placed in a heat block at 37ºC for 15 minutes to aid the process. Then 5µl of the dye (CyDye™ 3 or CyDye™ 5, Amersham Bucks UK) was added to the cDNA solution. The mixture was then gently mixed and the solution incubated at room temperature for 30 minutes in the dark, since fluorescent dyes can degrade when exposed to daylight.

2.2.6. Purification of the dye coupled cDNA.

At this stage the resultant mixture now contained uncoupled fluorescent dye and buffer salts which affect the hybridisation process if not removed at this stage. This was achieved by first adding 90µl of TE buffer (10mM Tris 1mM EDTA) to the dye

labelled cDNA from 2.2.5. A mixture of 100µl of DNA Binding Solution, supplied with Fairplay® II microarray labelling kit, and 100µl of 70%(v/v) ethanol was then prepared, added to the labelled cDNA and mixed by gentle vortexing. The mixture was then transferred to a Microspin Cup, supplied with Fairplay® II microarray labelling kit, seated in a 2ml receptacle tube and centrifuged in a microcentrifuge at 13000g for 30 seconds. The Microspin Cup is made up of a silica-based fibre matrix which, in the presence of the DNA-binding solution, binds to the cDNA, thus removing it from the solution. After centrifuging, the Microspin Cup was removed and retained and the contents of the tube were discarded. This process was then repeated. Once completed a solution of 750µl of 75% ethanol was added to the Microspin Cup and centrifuged in a microcentrifuge at 13000g for a further 30 seconds. The flow through was then discarded and the washing step repeated. The Microspin Cup was centrifuged once more at 13000g for 30 seconds, without the addition of further solutions, to remove any remaining wash buffer and then transferred to a fresh 1.5ml microcentrifuge tube. Next 50µl of 10mM Tris (pH 8.5) was placed on top of the fibre matrix and this was left to incubate at room temperature for five minutes. The addition of Tris to the matrix of the Microspin Cup creates conditions in which the cDNA is liberated from the matrix and becomes suspended in the Tris solution. The tube containing the Microspin Cup was then centrifuged at 13000g for 30 seconds in the microcentrifuge. The resulting fluid was recovered, reapplied to the fibre matrix and respun in the microcentrifuge for 30 seconds as before. This was done to maximise recovery of the labelled cDNA from the fibre matrix. The flow through now contained the purified labelled cDNA.

2.2.7. Microarray slide preparation.

Before the slides could be used for hybridisation they needed to be made as particle free as possible and any unbound target molecules or buffer substances needed to be removed to avoid any interference with the later hybridisation process. This was achieved by placing the microarray slide firstly into a 50ml centrifuge tube containing 40ml of 0.1% Triton X-100 and rinsing for five minutes. The slide was then removed from its container and placed in a fresh 50ml centrifuge tube containing 40ml of 1M HCl and rinsed for a total of two minutes. This rinse was repeated in a fresh tube before another was filled with 40ml of 100mM KCl solution, the slide placed inside and rinsed for ten minutes. The final rinse was performed using 40ml of distilled water for a duration of one minute. Once washed it was necessary to "block" the slides. This process ensures any reactive groups on the slide which may bind non-specifically to the added cDNA are unable to do so. The blocking was achieved by immersing the washed slides in Nexterion® Blocking Solution (Schott Germany) for 15 minutes at 50ºC. Once completed, they were rinsed in distilled water at room temperature for one minute. Excess solution was removed from the slides by centrifuging at 200g for a total of 5 minutes thus avoiding water stains. Once dried, a Hybriwell™ (Grace Bio-Labs USA) adhesive coverslip was applied over the printed area of the slide.

2.2.8. Hybridisation.

The purified labelled cDNA from section 2.2.6 was then re-suspended in Nexterion® Hybridisation solution (Schott Germany). This was done by first adding 50µl of the each cDNA in Tris buffer along with 400µl of Tris to a MicroCon spin cap. This was then spun in a centrifuge at 1000g for 15 minutes. The flow through was then

discarded and the cDNA remained in the MicroCon. The MicroCon was then inverted and 20µl of Nexterion® Hybridisation solution added. This was again centrifuged at 1000g for 2 minutes. The flow through now contained the cDNA in the correct hybridisation solution. To this 2µl of 1µg/µl human Cot-1 DNA (10ng/µl final concentration; Invitrogen, UK), 2µl of 1µg/µl Oligo d(A) (10ng/µl final concentration; Invitrogen, UK), 5µl of 7.5% w/v BSA (bovine serum albumin 0.2% final concentration; Invitrogen, UK) and 150µl of Nexterion™ Hybridisation buffer were added to minimise the cross-hybridisation between labelled targets and arrayed elements that share small regions of homology. This was heated at 95ºC for 3 minutes on a heat block to denature the target. At the same time a Hybriwell™ was placed over each slide to be used, as shown in fig 2.5, and once the solution had been denatured, 200µl was gently pippeted under the well, taking care to avoid any air bubbles, and the holes at each end sealed. The slide was then placed in an incubator for 16 hours at 65ºC to allow hybridisation to take place.

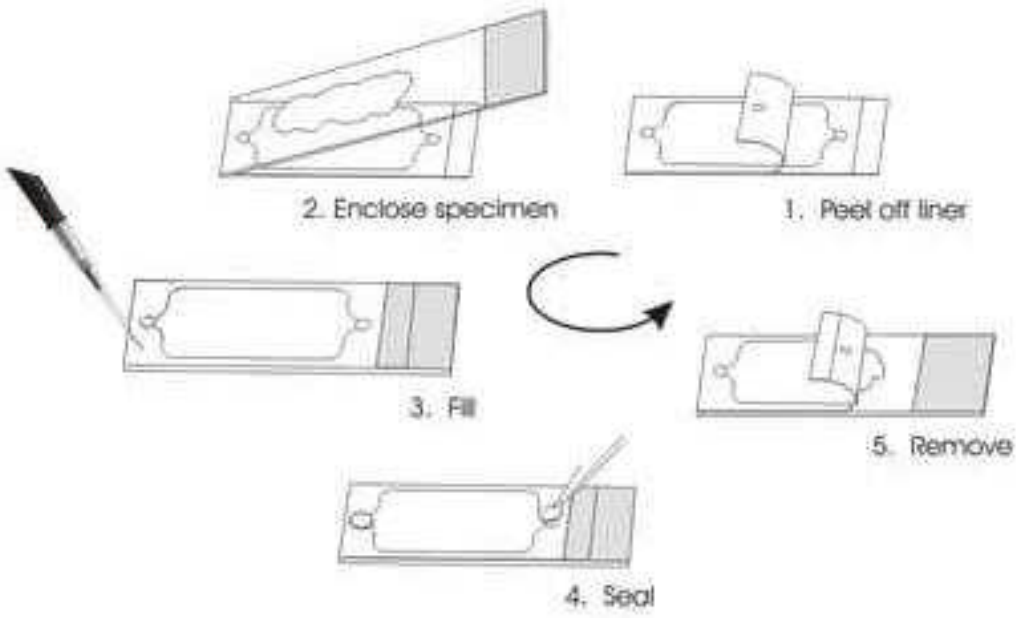


Fig 2.5. Showing method for application of and specimen placement under the Hybriwell™. Adapted from www.gracebio.com

2.2.9. Post hybridisation washing.

Once the labelled cDNA had been hybridised to the microarray all the excess buffer solution and unbound labelled cDNA needed to be removed so an accurate analysis of the staining could take place. This was done using differing concentrations of a solution containing SDS (Sodium Dodecyl Sulphate) and SSC (Sodium Salt Citrate). The slide was suspended in a conical flask which contained 2x SSC and 0.1% SDS. The flask was then placed in an incubator at a temperature of 42ºC whilst being gently stirred for five minutes. Next the slide was suspended in a fresh conical flask containing 2x SSC and 0.1%SDS and again stirred for five minutes at room temperature. The slide was then placed in another conical flask containing 0.2x SSC and stirred for one minute before a final wash in a flask containing 0.1xSSC again stirred for one minute. The slide was then spun in a centrifuge at 200g for five minutes to remove any excess solution. The slides were then ready for analysis.

2.3 Immunohistochemistry.

2.3.1 Case selection

Following ethical approval, archival tissue blocks were identified retrospectively from the Histopathology and Head and Neck departments at Hull Royal Infirmary. Cases were identified from 1995 to 2005 and a database of the findings was constructed. All cases with squamous cell carcinoma of the lateral border of tongue, floor of mouth, mandibular alveolus, buccal mucosa and retromolar trigone were included in the study. Details including patient age, gender, smoking and alcohol history, stage and site of tumour, date of diagnosis, dates of radiotherapy, dose of radiotherapy, date of recurrence, site of recurrence and survival were recorded.

2.3.2 Definition of radioresistant cases.

Cases had to satisfy a set of criteria before they were considered to be radioresistant. Only cases that had a recurrence within 12 months of completing radiotherapy, which was at the original anatomical site, were deemed to be radioresistant. Thus any recurrence that consisted of neck disease was excluded as this may have represented original tumour that had not been included in the radiotherapy fields. Any "recurrence" at a site other than the original was also not included to remove the possibility of this simply being a second primary tumour.

2.3.3 Slide Preparation.

Paraffin embedded archival tissue blocks were retrieved from the Histopathology department at Hull Royal Infirmary. A total of 12 sections were prepared from each block using a Reichert-Jung microtome. Sections of 4 microns thickness were floated onto a water bath at 40ºC and placed onto Superfrost^{R} Plus glass microscope slides (Merck UK). The slides had been pre-labelled in pencil with the corresponding histology number and the antibody to be used. Once prepared in this way the slides were placed in an incubator at 37ºC overnight to dry.

The wax was removed, after the slides had been placed into a metal slide rack, by treating in a solution of Histo-clear™ (National Diagnostics, UK) warmed to a temperature of 37ºC. They were left in this solution for ten minutes then transferred to two further solutions of cold Histo-clear™ for ten seconds in each case.

The slides were then re-hydrated in three solutions of 100% ethanol, being left for ten seconds in each and then rinsed for one minute under running tap water. This was

done to return the cells on the slide to a state as near as possible to their in vivo condition. The endogenous peroxidase of red blood cells was blocked by placing the slides in 400ml of methanol to which 8ml of 30% hydrogen peroxide had been added, and leaving the slides for 20 minutes in the solution. Once removed, the slides were rinsed in running tap water.

2.3.4 Antigen retrieval.

A solution consisting of 1500 ml of distilled water with 15ml of Antigen Unmasking Solution (Vector UK) was heated to boiling point in a pressure cooker. When boiling vigorously, the slides were added and the solution brought to pressure of 103KPa. After three minutes the pressure cooker was cooled and the slides quickly transferred to Tris-Buffered Saline (TBS - 0.05 mol/l Tris HCL, 0.15mol/l NaCl, pH 7.6) to prevent any drying.

2.3.5 Blocking of endogenous avidin and biotin.

The cooled slides were assembled with coverplates and TBS solution and placed in a Sequenza slide holder ensuring no air bubbles were formed. Each reservoir was then filled with TBS solution and left for five minutes to prevent dehydration. A solution containing 1ml of casein and 9ml of TBS was prepared and 100µl of this was added to the middle of each slide reservoir to block any non-specific protein reactions. The slides were rinsed in TBS for five minutes to remove any excess casein.

Three drops of 0.1% avidin (Vector) was then added to each slide to block any endogenous biotin. After fifteen minutes the slides were rinsed with TBS for a further

five minutes. This process was then repeated using three drops of 0.01% biotin (Vector) to block any endogenous avidin.

2.3.6 Antibody Binding and Detection.

The primary antibodies, listed in section 6.2.2, were diluted with 0.2x casein in TBS and added to each slide. The slides were then covered and left for a total of two hours at room temperature. The ABC method of staining was used in this instance and the reagents were taken from the Duet kit (Dako UK). A solution consisting of 1 in 100 biotinylated goat antibody was prepared using TBS, and 100µl of this was added to each slide. The slides were then covered and left for half an hour. A preformed solution of avidin and biotinylated peroxidase was diluted to a concentration of 1 in 100 with TBS. To each slide 100µl of this was added, and the slides were then left covered at room temperature for half an hour.

2.3.7 Chromogen reaction.

The slides were removed from the sequenza and placed into a pot of fresh TBS solution. A solution of 400ml of TBS, 3ml DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma) and 15 drops of hydrogen peroxide (30% v/v) was then made up and the slides immersed in the solution until they stained brown for a maximum of 30 minutes. The slides were then rinsed in running tap water to terminate the reaction. To enhance the staining the slides were then immersed in a solution of 0.5% w/v copper sulphate solution for a total of five minutes and then rinsed in running tap water.

2.3.8 Counterstaining nucleus.

The cell nuclei were counterstained blue with Harris Haematoxylin (BDH Laboratory Supplies UK). After the slides had been rinsed in section 2.3.7, they were immediately immersed in a solution of Harris Haematoxylin for a total of 20 seconds and then washed in running tap water for a total of 30 seconds. The haematoxylin was then differentiated by dipping the slides in acid alcohol ten times and rinsing again in running tap water. The slides were then immersed in Scotts tap water substitute for one minute and finally rinsed in running tap water.

2.3.9 Mounting Slides.

The tissue sections were dehydrated by immersing them in three different containers of 100% ethanol for ten seconds in each. The slides were then dipped in three changes of Histoclear II (National Diagnostics) solution and then mounted by applying a cover slip with Histomount (National Diagnostics). The slides were then left overnight to dry at room temperature.

Chapter 3: Results – Development of Novel Radioresistant Oral Squamous Cell Carcinoma Cell Lines.

Aims:

- To establish the response of oral squamous cell carcinoma cell lines PE-CAPJ41 and PE-CAPJ49 to radiotherapy.
- To establish a novel sub-colony of radioresistant oral cancer cells using a course of fractionated radiotherapy.
- To confirm and quantify this radioresistance by comparing dose response curves.
- To enable further analysis to take place on the radioresistant cells lines in order to identify novel markers of radioresistance.

3.1 Introduction.

In order to obtain information about potential predictive markers of radioresistance it is necessary to study radioresistant tumours. As the inherent response to radiotherapy is only known after a tumour has already undergone a course of radiotherapy, samples which are then collected are often from the tumour which is now radioresistant, thus it is difficult to link any predictive value to markers elucidated from them. Pre-treatment tumour samples of this nature are difficult to come by and even if a pre-treatment biopsy specimen is found it is impossible to know whether this sample came from part of the tumour which was inherently radioresistant or not. Processing, transport and storage of tumour tissue also leads to variations in the quality and usefulness of such samples.

There is no standard definition for a radioresistant tumour and quantification of just how sensitive a tumour is in real life is almost impossible. Other studies have already shown that it is possible to create a cell line which shows a greater resistance to drug therapy than its' parent cell line (Losada et al., 2004) and that certain head and neck cell lines are more radioresistant than others (Schwartz, 1994). There are also a number of experiments that have created radioresistant cell lines of cervical (Chung et al., 2005a), oesophageal (Fukuda et al., 2004), lung (Guo et al., 2005), breast (Li et al., 2001) and pancreatic cancer (Ogawa et al., 2006). In vivo it is very difficult to distinguish whether the radioresistance was inherent in the patient or the tissue itself or if it was acquired after the cancer therapy. In any given tumour there may be a certain degree of heterogeneity (Fukuda et al., 2004) with regards to radiotherapy response therefore it is impossible to tell whether any pre-treatment specimens are from a radioresistant subpopulation or not. Using cell lines which have previously

been proven to be radioresistant overcomes these problems and allows the investigator to be confident that conclusions drawn from further studies are applicable to radioresistance.

This experiment aims to create a cell line which is significantly more resistant to the effects of radiotherapy than its parent, thus enabling quantification of the radioresistance and direct comparison of the resistant cell line to the parent in order to gain valuable information on how the genetic expression of the cell line has changed to enable it to become more resistant.

3.2 Materials and methods.

3.2.1 Cell Lines

An aliquot of PE-CAPJ41 and PE-CAPJ49, human oral squamous carcinoma cell lines obtained from the European Collection of Cell Cultures, were defrosted and cultured as per section 2.1.2 and 2.1.3. These cell lines were chosen due to their ease of culture, ready availability and as they came from the most common site for oral cancer i.e. floor of mouth/ lateral border of tongue. Each cell line was split into a test and control sample and exposed to the same conditions throughout the experiment.

3.2.2 Determination of Inherent Response to Radiotherapy.

Before a radioresistant cell line could be established, the inherent sensitivity of each was determined. A clonogenic assay was performed and dose response curves for each cell line were then constructed. For each dose response curve the cells were harvested and counted as per section 2.1.6 and 100 000 cells were placed in 7ml polypropylene containers. Six separate containers were used for each dose response

curve. These were then transported to the Radiotherapy Department at Princess Royal Hospital and given a dose of 0Gy, 2Gy, 4Gy, 6Gy, 8Gy and 10Gy to the respective container. The cells were then returned to the lab and 10 000 cells were removed from each container and placed into six well tissue culture plates. They were cultured for 14 days and then stained with eosin to enable a survival assay to be carried out as per section 2.1.7. Each curve was repeated on three separate occasions for confirmatory purposes. A final curve was constructed using the mean number of surviving cells at each dose of radiotherapy across the three curves.

3.2.3 Development of radioresistant cell lines.

The cells were cultured and passaged as per sections 2.1.3 and 2.1.4, and a total of one million cells were taken to the Radiotherapy Department at Princess Royal Hospital for treatment with fractionated doses of radiotherapy. This was done at two weekly intervals as described in section 2.1.8. Initially the cell line was exposed to 2Gy of radiotherapy at each visit as from the initial dose response curves this dose seemed to strike a balance between cell death and giving the cells enough time to carry out repair processes. However, it soon became apparent that the surviving cells were outgrowing the tissue culture flasks sooner than the planned two weekly dose of radiotherapy, thus further passages would be needed leading to potential differences in the expression of genes which at the final analysis may be due to the different passage number not the effects of radiotherapy. An incremental dose of 4Gy was then chosen which enabled us to use a single passage to return to the radiotherapy department for a further dose of radiation. The novel cell line derived from PE-CAPJ41 was called PE-CAPJ41“**RR**” and that cell line derived from PE-CAPJ49 was called PE-CAPJ49“**RR**”.

3.2.4 Confirmation of radioresistance.

In order to determine whether the novel cell lines were more resistant to radiotherapy a dose response curve was constructed, as per section 2.1.7, and compared to the dose response curve for PE-CAPJ41 and PE-CAPJ49. The dose response curve for the novel cell lines, PE-CAPJ41**RR** and PE-CAPJ49**RR** were repeated for confirmatory purposes. To compare the response to radiotherapy between the parent and novel cell lines graphs were constructed showing the surviving fraction of cells in percentage terms for each cell line at each dose of radiotherapy. This was done by taking the number of cells surviving from the sample which had received 0Gy to represent a survival fraction of 100%. Each further number of colonies was then calculated as a fraction of this and a curve constructed.

3.2.5 Physiological parameters of cell lines.

The plating efficiencies for each cell line were calculated to determine what effect the transport, passage and subsequent culture had on the cells. The number of cells originally transported to Princess Royal Hospital was known and after the dose response curve was constructed the number of cells which had survived the process that had received 0Gy was also known. The plating efficiency was calculated by determining what fraction of the cell had survived the whole process. From the dose response curves it was also possible to determine what dose of radiation was needed to kill 50% of the cells and the fraction that survived after doses of 2Gy and 4Gy. These figures allowed a comparison to be made about the relative response to radiotherapy of each cell line.

3.3 Results.

3.3.1 Dose response curves of PE-CAPJ41.

As expected the greatest number of cells survived when exposed to no radiation, however, as we plated out 10 000 cells in each of the wells for the survival assay it was surprising to see only a fraction of these had survived after receiving no radiotherapy. Figure 3.1 shows the three dose response curves for parental PE-CAPJ41 cells the majority of which were killed with 2Gy with only a small fraction surviving 4Gy. Figure 3.2 shows the resulting curve constructed by plotting the mean number of surviving cells at each dose of radiotherapy.

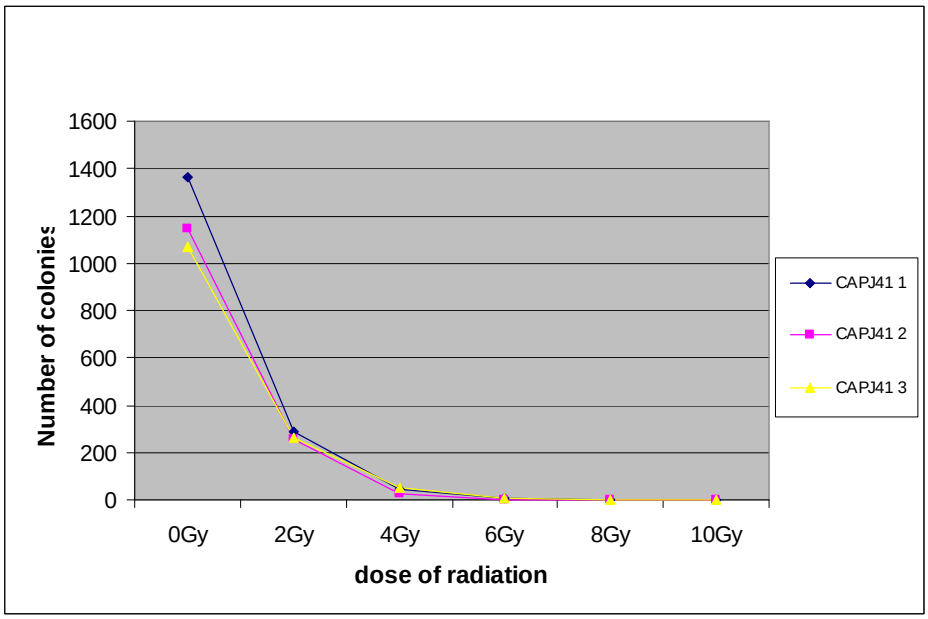


Fig 3.1 Initial dose response curves for parent PE-CAPJ41 cells performed on three separate occasions with the surviving number of cells plotted on the y-axis against the dose of radiotherapy given on the x-axis. Each line on the graph represents a separate experiment.

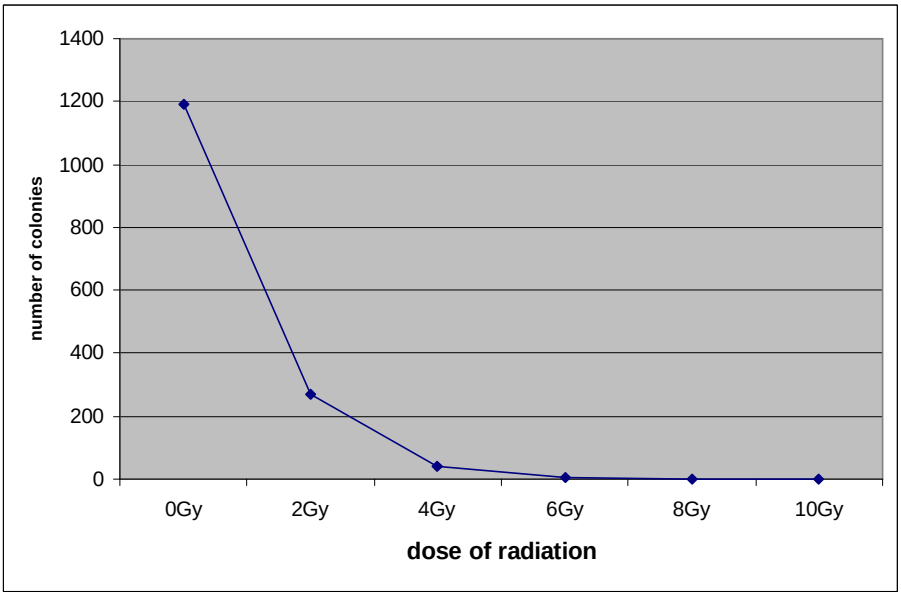


Fig 3.2 Final dose response curve of parent PE-CAPJ41 cells calculated by plotting the mean number of surviving cells at each dose of radiation on the y-axis against the dose of radiation on the x-axis.

3.3.2. Dose response curve of PE-CAPJ49.

Figure 3.3 shows the response curves generated for parental PE-CAPJ49 cells through the three separate experiments. As with PE-CAPJ41 the greatest number of cells survived when exposed to no radiation, and only a fraction of the cells that were exposed to 0Gy survived. When comparing figure 3.2 with 3.4 more cells of PE-CAPJ49 survived at 0Gy, 2Gy and 4Gy than those of PE-CAPJ41.

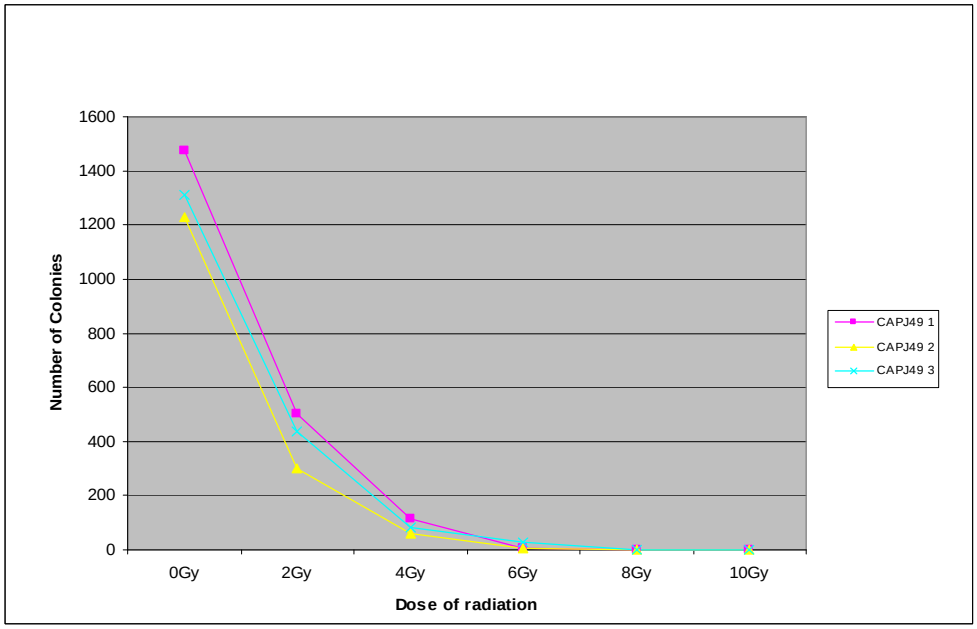


Fig.3.3 Initial dose response curves for parent PE-CAPJ49 cells performed on three separate occasions with the surviving number of cells plotted on the y-axis against the dose of radiotherapy given on the x-axis. Each line on the graph represents a separate experiment.

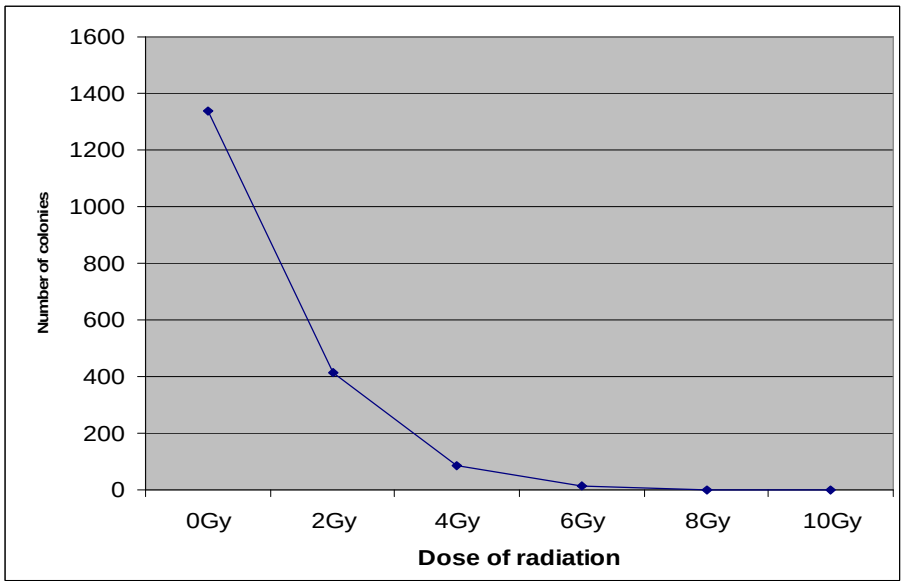


Fig. 3.4 Final dose response curve of parent PE-CAPJ49 cells calculated by plotting the mean number of surviving cells at each dose of radiation on the y-axis against the dose of radiation on the x-axis.

3.3.3 Incremental dose of radiotherapy.

Over a period of three months PE-CAPJ41 received a total dose of 28Gy radiation at incremental doses of 4Gy. The novel cell line was named PE-CAPJ41**RR**. During the initial doses of radiation CAPJ41**RR** proliferated at a slower rate than PE-CAPJ41. This continued until a dose of 24Gy was reached when the proliferation rate increased. This was taken to indicate a change in the inherent resistance to radiotherapy and the incremental dosage regimen was stopped one dose later. PE-CAPJ49 received a total of 24Gy over the same period and the new cell line was named CAPJ49**RR**. Again a change in the rate of proliferation was noted, as in PE-CAPJ41, but this time it was noticed at 20Gy. The irradiation was then stopped after one further dose and the cell line analysed.

3.3.4. Confirmation of radioresistance.

Figure 3.5 shows the resulting dose response curves for PE-CAPJ41**RR** as compared to PE-CAPJ41. As can be seen from the graph cells from PE-CAPJ41**RR** were not killed by radiotherapy until a dose of 6Gy was reached and some cells survived at a dose of 8Gy. This is in contrast to PE-CAPJ41 were no cells survived from a dose of 6Gy onwards. The graph therefore confirms the increased resistance to the effects of radiotherapy of PECAPJ41**RR**. Figure 3.6 shows the resulting dose response curves for PE-CAPJ49 as compared to PE-CAPJ49**RR** and once again a greater number of cells survived at all doses of radiotherapy other than 10Gy. Figures 3.7 and 3.8 show a direct comparison between each parent and each resistant cell lines in terms of surviving fraction of cells at each dose of radiotherapy.

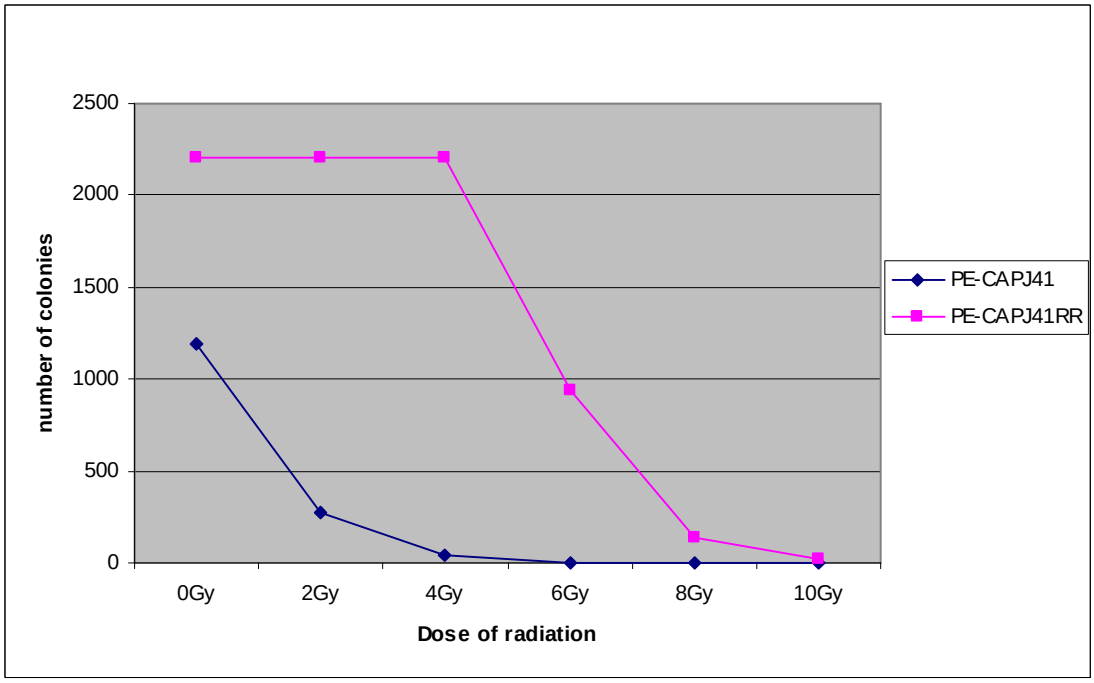


Fig. 3.5 Dose response curve showing PE-CAPJ41 plotted on the same axes as PE-CAPJ41**RR**. Number of surviving colonies is plotted on the y-axis against dose of radiation on the x-axis.

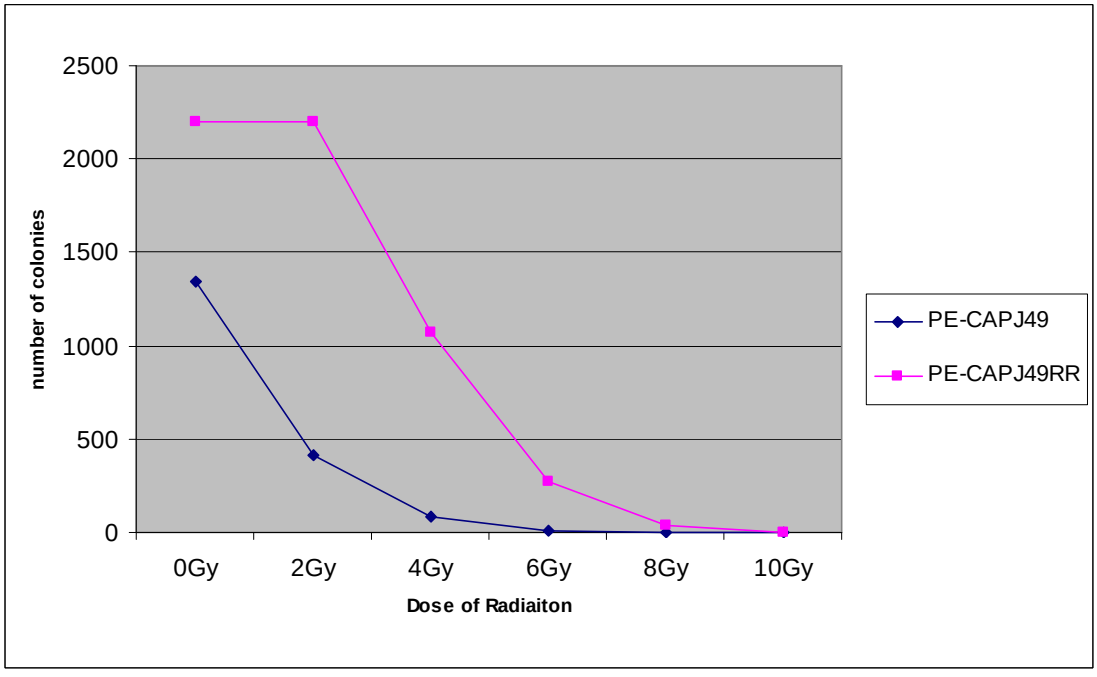


Fig. 3.6 Dose response curve showing PE-CAPJ49 plotted on the same axes as PE-CAPJ49**RR**. Number of surviving colonies is plotted on the y-axis against dose of radiation on the x-axis.

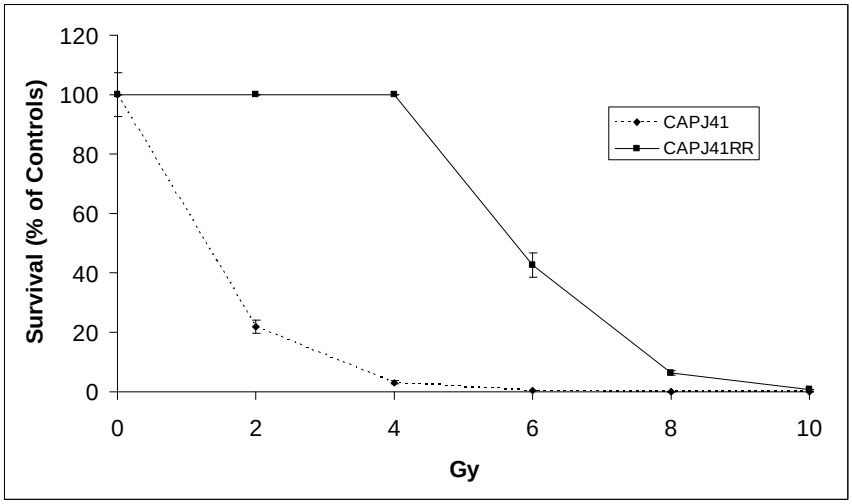


Fig 3.7 Survival curve comparing PE-CAPJ41 and PE-CAPJ41**RR**. Dose of radiotherapy is shown on the x-axis and % of surviving cells is shown on the y-axis.

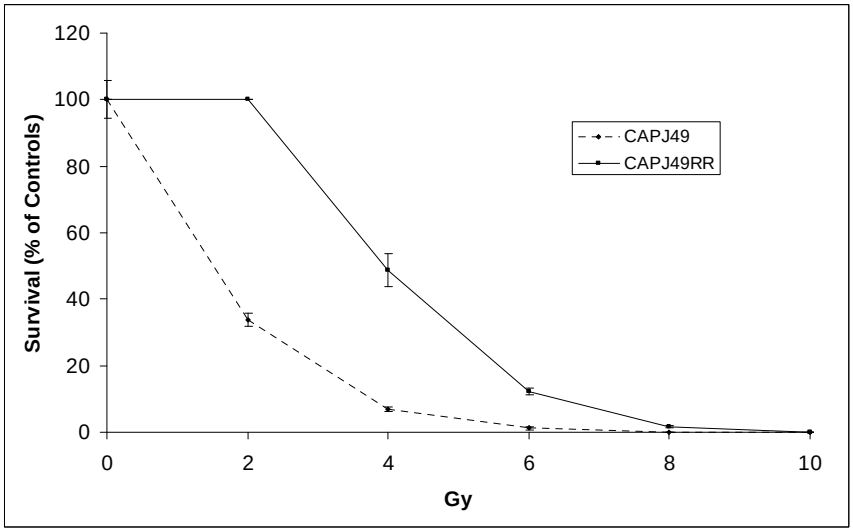


Fig 3.8 Survival curve comparing PE-CAPJ49 and PR-CAPJ49**RR.** Dose of radiotherapy is shown on the x-axis and % of surviving cells is shown on the y-axis.

3.3.5 Physiological parameters of cell lines.

Cell Line	Plating Efficiency (%)	LD_{50} (Gy)	SF2 (%)	SF4 (%)
CAPJ41	12.60±0.92	0.91±0.05	21.80±2.25	3.10±0.48
CAPJ49	13.93±0.77	1.26±0.05	33.78±1.93	7.01+0.70
CAPJ41**RR**	22.00±0.00	5.85±0.07	100.00±0.00	100.00±0.00
CAPJ49**RR**	22.00±0.00	3.96±0.12	100.00±0.00	48.74+4.96

Table 3.1 Physiological parameters of the cell lines. LD_{50} refers to the dose of radiotherapy needed to kill fifty percent of the cells. SF2 and SF4 refer to the Surviving Fraction of cells after a dose of 2Gy and 4Gy of radiotherapy.

3.4 Discussion.

Radiotherapy has been used for successfully treating oral squamous cell carcinoma for many years but with varying degrees of success with control rates varying from 18-76% (Carvalho et al., 2003). Radioresistance is therefore a major obstacle which affects the ability of the clinician to offer a potential cure. To enable the mechanism of radioresistance to be elucidated it is necessary to study radioresistant tumours. In these experiments we have created two radioresistant oral squamous cell carcinoma cell lines to allow further analysis to be performed on readily available physiologically proven radioresistant oral cancer cell lines.

PE-CAPJ41 and PE-CAPJ49 were chosen due to their availability and as they represented the most common site for oral cancer. Each cell line was irradiated with a dosing regimen of 4Gy increments. During the initial dosing increments it was noted that the irradiated cells for both PE-CAPJ41**RR** and PE-CAPJ49**RR** proliferated at a

slower rate than their parental counterparts. As they had been receiving doses of radiotherapy this was not unexpected as time would be needed to undergo DNA repair, cells would be paused in the cell cycle as this took place and a proportion of cells would die as a result of the radiotherapy. However, after PE-CAPJ41**RR** had received a dose of 24Gy the rate of proliferation was significantly increased as the tissue culture flask in which the cell line was being cultured became visibly more confluent after the two week incubation. A similar phenomenon was noticed with PE-CAPJ49**RR** but after a dose of 20Gy. This may have been noticed earlier than with PE-CAPJ41**RR** as PE-CAPJ49**RR** was irradiated on a schedule behind PE-CAPJ41**RR** and we were therefore more aware of potential changes in proliferation around this time.

Each cell line showed similar characteristics throughout the experiments and produced the corresponding radioresistant cell lines. PE-CAPJ41 and PE-CAPJ49 had an LD_{50} of 0.91Gy and 1.26Gy respectively so a similar dose of radiotherapy was needed to kill half of the surviving cells, whilst in contrast PE-CAPJ41**RR** and PE-CAPJ49**RR** had an LD_{50} of 5.85Gy and 3.96Gy respectively. PE-CAPJ41**RR** may have developed a higher LD_{50} as a result of the larger dose of fractionated radiotherapy it received (28Gy as opposed to 24Gy) which could have resulted in further changes in its' phenotype. We may have expected PE-CAPJ49**RR** to have shown a greater resistance to radiotherapy in the final analysis as PE-CAPJ49 was initially more resistant to radiotherapy. The fractionated dose of radiotherapy given during the experiment seems to have had more effect on the final outcome than the inherent radiosensitivity of the cell line. This could lead us to conclude that it is not

the inherent radiosensitivity of the tumour that is the greatest determinant of response but the actual fractionated regimen undertaken.

As can be seen from figures 3.5 (PE-CAPJ41 Vs PE-CAPJ41**RR**) and 3.6 (PE-CAPJ49 Vs PE-CAPJ49**RR**) there is an obvious difference in the response to radiation at all the tested doses from 0Gy to 10Gy. These figures not only show a difference in colony formation at 2Gy, 4Gy, 6Gy, 8Gy and 10Gy, but somewhat unexpectedly show a significant change in response at the 0Gy exposure which was acting as a control. This maybe due to the difference in proliferation rates between the parent and resistant cell lines with the resistant cell lines having developed a much faster rate of growth and proliferation. Genes which regulate proliferation may have been over expressed to give these cells a survival advantage. It is well known that the cell cycle has a role in the response to radiotherapy and if there are an increased number of cells in a tumour then it is more likely that there will be an increased number of cells in that part of the cell cycle that is relatively resistant to radiotherapy. This may well be one of the major ways in which a tumour avoids the effects of radiotherapy, or a reason why tumours which have failed radiotherapy have a worsened prognosis as those cells which are "left behind" are now proliferating at a much faster rate than previously, thus any delay in treatment leads to a much greater tumour bulk than would previously have been evident.

The two novel cell lines created in this experiment, PE-CAPJ41**RR** and PE-CAPJ49**RR**, need further analysis on a genetic level in an effort to identify those genes responsible for their increased radioresistant properties. In Chapter 4 and 5 we will aim to use cDNA microarray technology to compare the difference in expression

of genes between these novel cell lines and their parent counterparts. In the future it would be of interest to continue the dosing regimen we have employed here using an incremental dose of 6Gy or even 8Gy. The current dose of 4Gy may now not be enough to induce further changes in the novel cell lines as they have become relatively resistant to this degree of radiotherapy with 100% of PE-CAPJ41**RR** surviving and 50% of PE-CAPJ49**RR** at a dose of 4Gy.

Chapter 4 Results – Identification of novel molecular markers of radioresistance in oral squamous cell carcinoma cell line PE-CAPJ41 using microarray technology.

Aims:

- To extract RNA from the radioresistant oral cancer cell line PE-CAPJ41**RR**, established in chapter 3, and its parent counterpart PE-CAPJ41.
- To compare the gene expression of PE-CAPJ41**RR** and its' parental cell line PE-CAPJ41 using microarray technology.
- To identify altered gene products between the parent cell line and the radioresistant equivalent.
- To discuss the possible role these altered genes may have in radioresistance.

4.1 Introduction.

Microarray technology was first conceived in the late 1980s (Sasik et al., 2004) and since that time has grown in its use and popularity. The concurrent advances in bioinformatics together with the Human Genome Project have now enabled us to analyse the expression of thousands of genes simultaneously and also provide us with a method of identifying differentially expressed proteins in two distinct types of tissue (Gershon, 2002). The potential for microarray technology to identify new molecular markers is huge. Microarrays have already been used to identify clusters of genes which may help to predict the likely prognosis of HNSCC (Belbin et al., 2002), the risk of nodal metastasis (Warner et al., 2004) and the response to radiotherapy (Hanna et al., 2001). Belbin et al., (2002) used microarray technology to identify a cluster of 375 genes, such as CEA (carcinoembryonic antigen) and caspase-4, which they used to group head and neck cancer patients into on the basis of prognosis. Warner et al., (2004) identified CLDN1 as predicting for nodal metastasis in oral cancer again by using a cDNA microarray. Hanna et al., (2001) used biopsy samples from two radioresistant tumours and compared them with samples from clinically radiosensitive tumours to identify differentially expressed genes. They were able to use a group of 60 genes to predict the response to radiotherapy. However, the definition used for radioresistance in this paper was tumour regression of less than 40% after a six week course of radiotherapy thus later recurrences were excluded. Utilising biopsy samples from patients also has the inherent problem that there is no way of ensuring these are taken from a clone of cells resistant to radiotherapy within the tumour.

The study described here utilised cell lines in an effort to overcome these problems. As it can be very difficult to distinguish whether radioresistance was inherent in a

patient/tissue itself or if it was acquired after radiotherapy, using established cell lines has the advantage that the response to radiotherapy is known, and is quantifiable, before the microarray analysis starts, and as the whole clone of cells has been shown previously to be radioresistant no error in sampling can occur. However, as with any in-vitro model the environment in which the cells are kept will not be the same as those experienced in vivo, with the influence of such things as the immune system removed, which may lead to results which do not translate directly to real life.

Given the complexity of radiation-induced responses, microarray analysis offers new opportunities to identify a wider range of genes and signalling pathways involved in the response to radiation (Guo et al., 2005). The technique of using radioresistant cell lines and microarray technology has previously been used to predict novel markers of radioresistance in cervical (Chung et al., 2005a), oesophageal (Fukuda et al., 2004; Matsuyama et al., 2001), lung (Guo et al., 2005), breast (Li et al., 2001) and pancreatic cancer (Ogawa et al., 2006).

In this thesis we have attempted to identify differentially expressed genes between previously established radiosensitive and radioresistant oral cancer cell lines. The radioresistant cell lines used were established in chapter 3 and cDNA microarrays were then employed to investigate differential gene expression.

4.2 Materials and Methods.

The head and neck squamous cell carcinoma cell line PE-CAPJ41 was used in this experiment along with the radioresistant counterparts PE-CAPJ41**RR** which had been derived from the parent cell line using the method described in section 2.1.8. In order

to decrease the chances of altered gene expression between different flasks of cells, RNA was only extracted from flasks which had reached a confluence of 80% or more. Total RNA was extracted using the Qiagen RNeasy® Mini animal cell extraction kit, from both the radioresistant cell line and the parent cell line as described in section 2.2.1.

The Human V3 Cancer Subset (http://omad.operon.com/download/index.php), consisting of approximately 3000 genes, was purchased from Operon (Germany) and microarrays were printed on pre prepared glass slides in house. The FairPlay® II microarray labelling kit, purchased from Strategene®, was used to generate cDNA from total RNA extracted from the parent and radioresistant cell line, as described in sections 2.2.3 and 2.2.4. The parent cell line was then labelled using CyDye™ 5 and the radioresistant cell line was labelled with CyDye™ 3 as described in section 2.2.5 and 2.2.6. The labelled cDNA was then hybridised to the microarray slide for 18 hours then washed to remove any unbound labelled cDNA as described in section 2.2.8 and 2.2.9. Once this was completed it was scanned using an Axon GenePix™ 4100A scanner purchased from Axon instruments and the data analysed using the Acuity 4.0 software (Axon Molecular Devices USA). The full microarray experiment was performed on three separate occasions to validate the results.

Each microarray slide had a total of 2908 different genes represented on its surface and each oligonucleotide (feature) representing the gene was repeated three times on each slide. Thus there were almost 9000 features on each array to be analysed using the Acuity™ software. Each feature on each microarray was scanned as above with a resolution of 5µm/pixel. These images were then analysed by applying an array

specific feature (gene) location file (.gal file). This gal file was used to generate a results file (.gpr) which contained the feature name, individual dye intensities at each feature and the dye ratio at each feature. These results files were then imported into the Acuity™ v4.0 software. The collective data from each microarray was then normalised using the Lowess method thus allowing for any variation between the same features throughout the slide. From this a data set was created which only included those features with the parameter "flags ≥0" thus ensuring only those features found were included in the analysis. Once the data had been filtered it was analysed to identify those genes which had altered in a significant way across all features on all of the microarrays. Only those with a small intra- or inter-array variance were included. As three features were printed on each slide the combined intra- and inter-array variance was calculated based upon the mean of the three features per slide (technical replicate) across the three slides (biological replicate), so 9 features in total. Intra- and inter-array variance was analysed using the one sample t-test and only those features with $p \leq 0.03$ were considered for further analysis. Calculating the variance in this way meant that only those features which did not vary from day to day or from slide to slide were considered for further analysis. From these features, only those which had a mean expression change of at least 2-fold were selected. This was achieved by selecting the features whose log ratio value was ≥1.0 or ≤-1 as a log value of 1.0 corresponds to a real life fold change of 2.

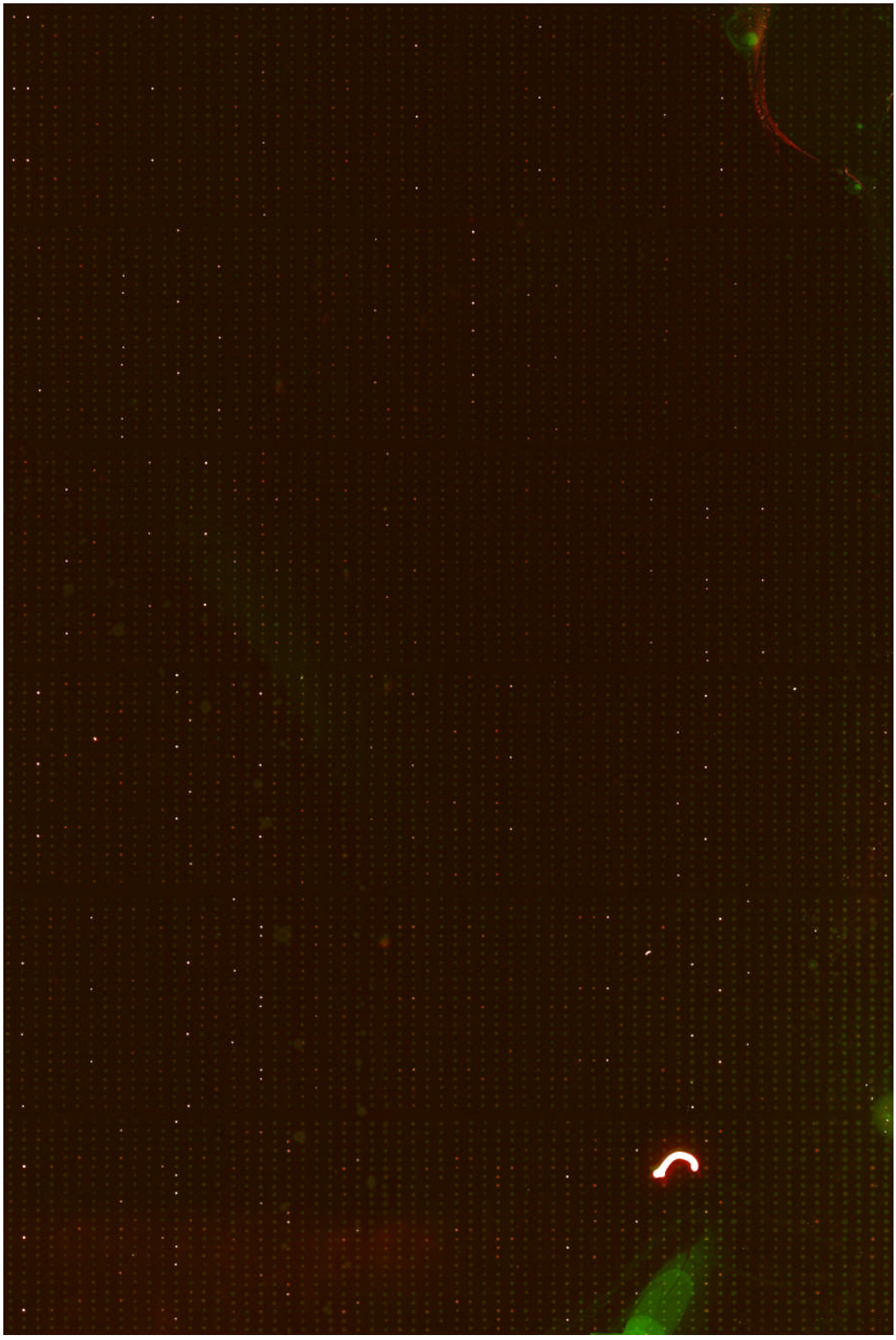


Fig 4.1 Microarray of PE-CAPJ41 parent cell line versus PE-CAPJ41**RR**. Green features represent genes over expressed in radioresistant cell line, red features represent genes over-expressed in radiosensitive cell line and white features represent those which have been saturated with markers.

4.3 Results.

All genes that were over expressed by the radioresistant cell line when scanned fluoresced in the green channel and all those that had been under expressed by the radioresistant cell line fluoresced in the red channel. Thus each feature on the microarray either appeared green, red or a combination of both colours on the final image. This can be seen in Figure 4.1 which illustrates the final scanned image of the microarray slide. Any of the features which appear white on the image are those which have been saturated with labelled cDNA.

Using the methods described above seven genes were identified which had changed significantly in all three repeats of features and on all the arrays that had been performed, and these are represented in table 4.1.

Gene	Expression change			Mean	Fold	t-test
	Array 1	Array 2	Array 3	change	change	
CD74	4.607	9.84	9.519	7.989	-7.989	0.015
CLCN6	3.147	2.005	3.679	2.943	-2.943	0.029
CLU	2.596	4.978	2.748	3.441	-3.441	0.029
HRMT1L2	2.998	3.871	2.674	3.181	-3.181	0.009
PIT1	3.23	4.655	2.754	3.546	-3.546	0.015
PTK7	5.615	3.391	2.802	3.936	-3.936	0.024
TEAD4	2.822	2.988	3.186	2.998	-2.998	0.001

Table 4.1 Showing the raw data from all three microarrays and calculated fold change for all of the genes identified by the microarray analysis as being associated with radioresistance. The expression change is expressed as the ratio of the medians value at each pixel of the feature. A negative fold change corresponds to decreased expression of the gene in PE-CAPJ41RR.

4.3.1 Markers identified in the PE-CAPJ41 Cell line.

4.3.1.1 HRMT1L2.

As can be seen from Figure 4.2 HRMT1L2 was under expressed by the radioresistant cell line PECAPJ41RR, as the feature fluoresces in red, by a factor of just over three. This correlated well across all of the arrays with the one sample t-test proving the correlation to be significant (p=0.009).

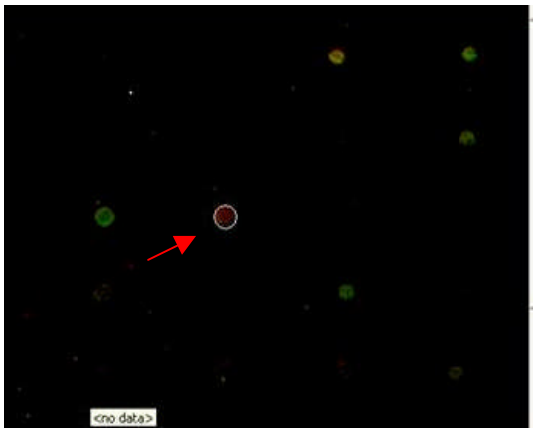


Fig 4.2 Magnified image of microarray slide showing the HRMT1L2 feature which was under expressed by the radioresistant cell line and the feature therefore is red. Feature marked by red arrow.

4.3.1.2. PITX1.

As can be seen in table 4.1, PITX1 was also under expressed by the radioresistant cell line by a factor of 3.5. This was found to be significant when compared across all three arrays (p=0.015). Figure 4.3 shows the scanned image of the feature.

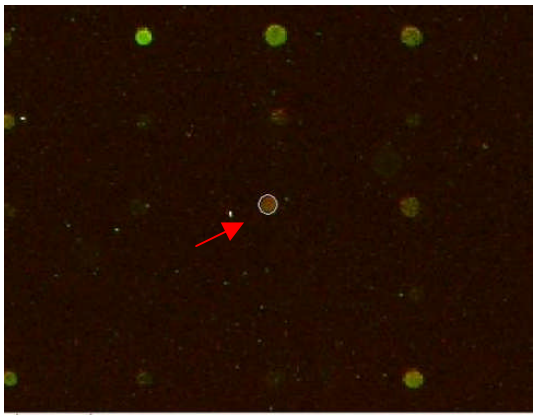


Fig 4.3 Magnified view of microarray slide highlighting the feature PITX1 as indicated by the arrow. As can be seen the feature is predominantly red, unlike those surrounding it, indicating its decreased expression.

4.3.1.3. PTK7

PTK7 was under-expressed by the radioresistant cell line by a factor of almost 4 across all three microarrays. This was found to be a significant change in expression when comparing the data across all three arrays using the one sample t-test (p=0.024).

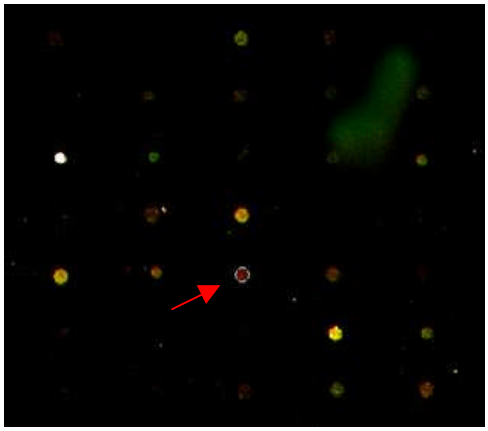


Fig 4.4 Magnified image of microarray showing PTK7 feature as illustrated by the arrow. Other features can be seen in the image, some in green showing they have been overexpressed by the radioresistant cell line.

4.3.1.4. TEAD4

TEAD4 was underexpressed by the radioresistant cell line by a factor of 2.9 across all three microarray experiments. This change was found to be significant when comparing data from all three arrays (p=0.001).

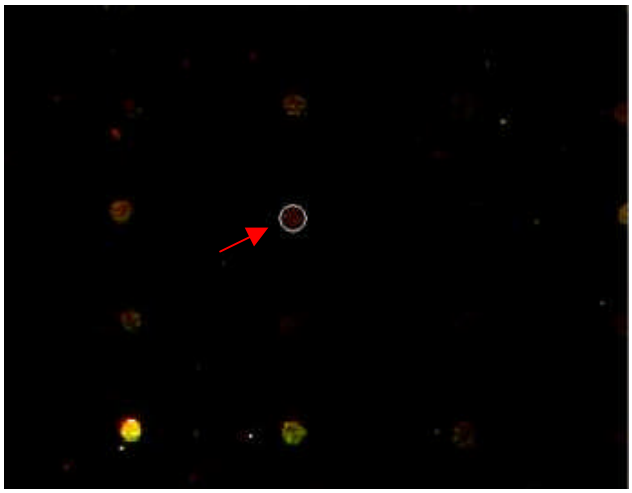


Fig 4.5 Magnified image of a section of microarray showing the feature TEAD4 in red as pointed out by the arrow.

4.3.1.5 CD74

CD74 was underexpressed by a factor of almost eight across all the repeats and the change was found to be significant as the p-value was 0.015 when comparing the data across all three arrays.

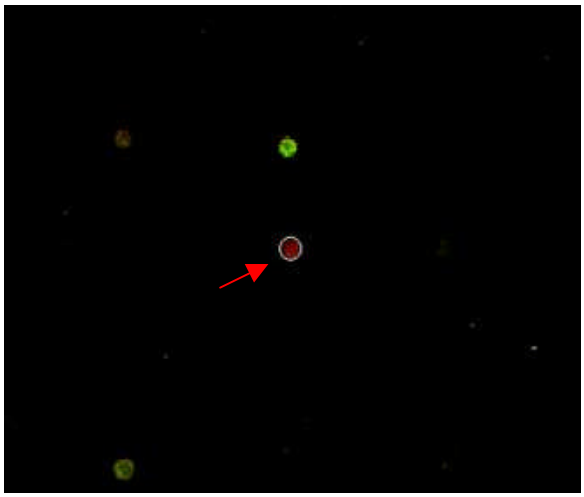


Fig 4.6 Magnified image of a section of microarray slide illustrating the feature corresponding to CD74.

4.3.1.6 CLCN6

CLCN6 was under-expressed by a factor of almost three across all three arrays with the change found to be statistically significant when comparing data across all three arrays (p=0.029).

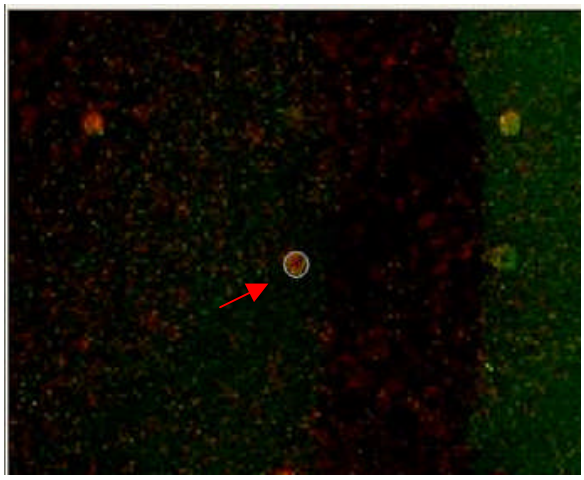


Fig 4.7 Magnified image of a section of microarray slide showing the feature which represents CLCN6.

4.3.1.7 CLU

CLU was under-expressed by a factor of nearly 3.5 across all three arrays. When comparing the data across all of the arrays this change was found to be statistically significant (p=0.029).

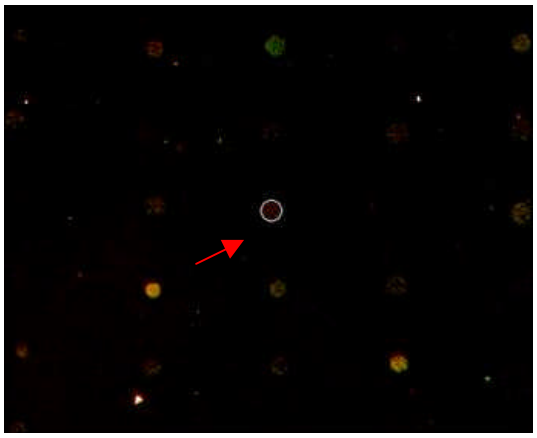


Fig 4.8 Magnified image of a section of a microarray illustrating the feature corresponding to CLU.

4.4. Discussion.

All of the genes identified in this experiment, illustrated in table 4.1, have been significantly under expressed by the radioresistant cell line. This could be explained by the fact that loss of the resultant proteins confers a survival advantage to these cells after a course of radiotherapy. However, it could be hypothesised that this group of genes are particularly more sensitive to the damaging effects of radiotherapy than the other genes represented on this microarray, and are thus consistently damaged by the effects of ionising radiation.

4.4.1 HRMT1L2

HRMT1L2, also known as heterogeneous nuclear ribonucleotein methyltransferase 1-like 2, protein arginine N-methlytransferase 1, PRMT1, and interferon receptor 1 bound protein 4, is a protein encoding gene for arginine methyl transferases located on the long arm of chromosome 19. Protein arginine methyltransferases (PRMTs) regulate mRNA processing and maturation by altering the activity of RNA binding proteins through methylation (Scorilas et al., 2000). There are three isoforms of HRMT1L2 in existence, resulting from alterative mRNA splicing, two of these have been shown to be down regulated in breast cancer (Scorilas et al., 2000). HRMT1L2 is also associated with the signal transducers and activators of transcription (STAT) family of proteins. HRMT1L2 methylates STAT 1 which is needed for any transcriptional activation. Inhibitors of HRMT1L2 are found in certain cancer cells thus STAT1 is not methylated and this in turn impairs the interferon mediated gene induction and antiproliferation (Mowen et al., 2001). STAT 1 has been implicated in both modulating pro and anti-apoptotic genes and there are studies that have shown that STAT1 deficient cells are more resistant to agents that induce apoptosis

(Stephanou and Latchman, 2003). As HRMT1L2 is under-expressed by the radioresistant cell line, the methylation of STAT1 will be impaired and its function reduced. The cell line will therefore be deficient in the active form of STAT1 and be more resistant to apoptosis induced by ionising radiation.

4.4.2 PITX1

PITX1 is also known as pituitary homeobox 1, paired-like homeodomain transcription factor 1, HGNC:9004, BFT, POTX and PTX1. It is a protein encoding gene found on the long arm of chromosome 5 and belongs to a family of transcription factors with the other members being PITX2 and PITX3 (Tremblay et al., 2000). PITX1 acts as a pan pituitary regulator of transcription and is associated with a large number of downstream regulators. The expression of PITX1 has been found to be decreased by more than 50% in pituitary adenomas (Skelly et al., 2000).

PITX1 has been found under expressed in prostate, bladder and colon cancer and a role has been identified in the regulation of the proto-oncogene RAS (Kolfschoten et al., 2005). The p21 RAS proto-oncogene is at the heart of a signalling network that mediates the control of cell growth, metabolism and differentiation. Transmembrane receptors initiate signals that are transduced via RAS and propagated by a phosphorylation cascade to the nucleus thereby altering the activation of specific transcription factors (Bradford et al., 1996a). In a recent study by Kolfsschoten et al., (2005) it was found that PITX1 down regulates the RAS pathway and hence suppresses tumorigenicity.

Studies have shown that cell lines in which Ras activity has been inhibited are more susceptible to the effects of radiotherapy than their wild type counterparts (Brunner et al., 2004a; Brunner et al., 2003b) although the exact mechanism by which this is achieved is as yet not fully understood. In our radioresistant cell line the expression of PITX1 was reduced thus enabling activity of the RAS pathway and conferring a relative resistance to the effects of ionising radiation.

4.4.3 PTK7

PTK7 is a protein encoding gene found on the short arm of chromosome 6 also known as protein tyrosine kinase 7 and colon carcinoma kinase 4 (CCK4). Receptor protein tyrosine kinases transduce extracellular signals across the cell membrane and some of the more well known members include the four EGFRs. They play a role in cell signalling and transduce signals regulating growth, differentiation, adhesion, migration and apoptosis (Muller-Tidow et al., 2004). EGFR has been shown to have a relationship with radiotherapy response (Huang et al., 1999), and there have already been a number of clinical trials showing that blocking EGFR improves outcome after radiotherapy (Harris, 2004).

PTK7 belongs to a subgroup of this family that has lost its catalytic tyrosine kinase activity whilst retaining its role in signal transduction (Mossie et al., 1995), they are known to associate with various transmembrane receptors mediating tyrosine phosphorylation events in response to various ligands (Easty et al., 1997). A number of PTKs, including PTK7, are oncogenes and have been implicated in human cancer (Wilks, 1993). PTK7 has been found to be frequently down regulated during malignant melanoma progression implying that loss of PTK7 correlates with

tumorigenecity (Easty et al., 1997). It has been found to be over expressed in acute myeloid leukaemia (Muller-Tidow et al., 2004) and used to aid in determining the prognosis of pulmonary adenocarcinoma (Endoh et al., 2004).

The exact role PTK7 plays interacting with EGFR is as yet unknown. This study has shown that under expression of PTK7 correlates with radioresistance, it may be that loss of PTK7 allows the antiapoptotic cascade to be triggered by EGFR.

4.4.4 TEAD4

TEAD4 is a protein encoding gene found on the long arm of chromosome 12 and is also known as transcriptional enhancer factor 3, RTEF1, TEF-3, EFTR-2, RTEF-1, TEFR-1, MGC9014, TCF13L1 and hRTEF-B. It belongs to a family of transcription factors which contains at least four distinct members, TEF-1, EFT, EFTR-1 and TEAD 4 itself (Yasunami et al., 1996). A transcription factor is a regulatory protein that binds to a specific region of DNA resulting in either an increased or decreased rate of gene transcription. Each particular gene has its own specific binding site which ensures that the gene is only transcribed in the proper cell type and at the correct time during development. These family of transcription factors have been associated with the S100B (Tsoporis et al., 2003) gene and with papillary renal cell carcinoma (Weterman et al., 1996).

TEAD4 activates the S100B promoter causing an increase in its activity. S100B has a wide variety of both intra and extracellular functions. These include regulation of the cells morphology and cell cycle progression. Increased levels of S100B also inhibit the calcium dependent phosphorylation of the p53 tumour suppressor mechanism which results in uncontrolled tumour growth (Harpio and Einarsson, 2004). In this

experiment TEAD4 was under-expressed by the radioresistant cell line, which seems to contradict what would be expected as this would lead to a more controlled tumour growth rate, however, rapidly dividing cells are more susceptible to the effects of radiotherapy than more quiescent cells, so a loss of this transcription factor may actually confer a survival advantage with regards to response to radiotherapy.

4.4.5 CD74

The CD74 gene is located on the long arm of chromosome 5 and is also known as DHLAG, HLADG, Ia-Gamma and protein 41. It encodes for a protein that is associated with the major histocompatabilty complex class II molecules (MHC-II) (Claesson et al., 1983). MHC-II molecules bind peptide antigens and internalise and process them ready for presentation to T cells. They are expressed on the cell surface as heterodimers composed of an α and β chain. For these chains to unfold, assemble and be expressed correctly on the cell surface they need to be associated with the invariant chain during their synthesis (Elliott et al., 1994). CD74 encodes for this "invariant chain". If this "invariant chain" is not present in the cell the MHC-II will not unfold correctly and will therefore be trapped inside the cytoplasm of the cell. As MHC-II molecules play a role in antigen presentation and identification of tissues it may be that the loss of this gene enables the radioresistant cell line to avoid detection by the host immune system, thus conferring a survival advantage. However, as these cells were cultured in vitro, no immune system was present and the resultant ability to avoid an immune response would not have enabled these cells to survive the effects of radiotherapy.

4.4.6 CLCN6

CLCN6 is located on the short arm of chromosome 1 and is also known as chloride channel 6, RP5-934G17.1, CLC-6 and KIAA0046 (Nomura et al., 1994). It is a member of a family of nine voltage dependent chloride channel genes which have diverse functional characteristics whilst sharing significant sequence similarity (Brandt and Jentsch, 1995). Relatively little evidence exists as to the exact role CLCN6 has in the pathogenesis of cancer but it was found to be under-expressed in more aggressively behaving breast cancer (Han et al., 2006). As it was also significantly under-expressed in the more rapidly proliferating and radioresistant cell line in this experiment it may be of future interest to further investigate the mechanism whereby this is achieved.

4.4.7 CLU

CLU is a protein encoding gene situated on the short arm of chromosome 8 (Purrello et al., 1991). It is also known as clusterin, AAG4, APOJ, CLI, KUB1, MGC24903, SGP-2, SGP2, SP-40, TRPM-2 and TRPM2. Clusterin is a glycoprotein that has been implicated in various cell functions involved in carcinogenesis and tumour progression (Shannan et al., 2006). It has been shown to be over-expressed in lung (Cao et al., 2005), bladder (Yamanaka et al., 2005), prostate (Zellweger et al., 2002) and squamous cell carcinomas (Zhang et al., 2005). It is usually induced during apoptosis and is considered to be a stress inducible pro-survival factor (Caccamo et al., 2006). Clusterin has been shown to inhibit apoptosis by interacting with the pro-apoptotic activities of BAX (Zhang et al., 2005). Clusterin can be found in two sub cellular locations either closely associated with the nucleus, nCLU, or in the cytoplasm, sCLU. This difference in sub cellular location seems to have a profound

effect on the role clusterin plays on the survival of the cell population. nCLU is pro-apoptotic whilst sCLU functions as a pro-survival protein. In the radioresistant cell line PE-CAPJ41**RR** clusterin was significantly under-expressed when compared with its parent cell line. This finding may be considered as somewhat unexpected as clusterin seems to be a protein more often associated with the increased survival of a cell population and an overall inhibition of apoptosis. However, one possible explanation for this under-expression may be understood by remembering the fact that it is usually induced during apoptosis (Caccamo et al., 2006). The radioresistant cell line after been exposed to a total of 28Gy is relatively resistant to the effects of radiotherapy and so apoptosis of the cells of this lineage will be a relatively rare event when compared with the parent cell line PE-CAPJ41.

This experiment is, as yet, a preliminary study and all the markers discussed above require validation to ensure they are under-expressed in reality and not just a result of artefacts in the microarray process or selected out by this particular method of statistical analysis. This could be achieved by utilising real time PCR on the cell lines to confirm their under-expression in PE-CAPJ41**RR**. However, there are some encouraging preliminary findings into the mechanism by which a squamous cell carcinoma evades the effects of radiotherapy.

Chapter 5 Results – Identification of Novel Molecular Markers of Radioresistance In The Oral Squamous Cell Carcinoma Cell Line PE-CAPJ49 Using Microarray Technology.

Aims:

- To extract RNA from the radioresistant oral cancer cell line, PE-CAPJ49**RR**, established in chapter 3, and its parent counterpart.
- To compare the gene expression PE-CAPJ49**RR** with the parental cell line PE-CAPJ49 using microarray technology.
- To identify altered gene products between the parent cell line and PE-CAPJ49**RR**.
- To discuss the possible role these altered genes may have in radioresistance.
- To compare differentially expressed genes identified in Chapter 4 from PE-CAPJ41**RR**.

5.1 Introduction.

Microarray technology has become a more widespread and accessible tool for examining the differential gene expression between two types of tissue over recent years (Tilstone and Aldhous, 2003). Each array has large numbers of genes represented on their matrix and hence analysis of these arrays produces large quantities of data. Powerful statistical analysis software is then needed before sense can be made of the raw data available (Sasik et al., 2004). Confirmatory processes are always advisable in any experiment and microarray technology is no different in this respect. This experiment utilizes a separate oral cancer cell line from chapter 4, which was exposed to the same conditions and analyzed in the same fashion, in an attempt to identify some markers of radioresistance which are common to radioresistant tumours.

5.2 Materials and Methods.

The head and neck squamous cell carcinoma cell line PE-CAPJ49 was used in this experiment along with the radioresistant counterparts PE-CAPJ49**RR** which had been derived from the parent cell line using the method described in section 2.1.8. In order to decrease the chances of altered gene expression between different flasks of cells, RNA was only extracted from flasks which had reached a confluence of 80% or more. Total RNA was extracted using the Qiagen RNeasy® Mini animal cell extraction kit, from both the radioresistant cell line and the parent cell line as described in section 2.2.1.

The human V3 cancer subset (http://omad.operon.com/download/index.php), consisting of approximately 3000 genes, was purchased from Operon and microarrays

were printed on pre prepared glass slides in house. The FairPlay® II microarray labelling kit, purchased from Strategene®, was used to generate cDNA from total RNA extracted from the parent and radioresistant cell line, as described in sections 2.2.3 and 2.2.4. The parent cell line was then labelled using CyDye™ 5 and the radioresistant cell line was labelled with CyDye™ 3 as described in section 2.2.5 and 2.2.6. The labelled cDNA was then hybridised to the microarray slide for 18 hours then washed to remove any unbound labelled cDNA as described in section 2.2.8 and 2.2.9. Once this was completed it was scanned using a GenePix™ 4100A scanner (Axon instruments) and the data analyzed using the Acuity 4.0 software. The full microarray experiment was performed on three separate occasions to validate the results.

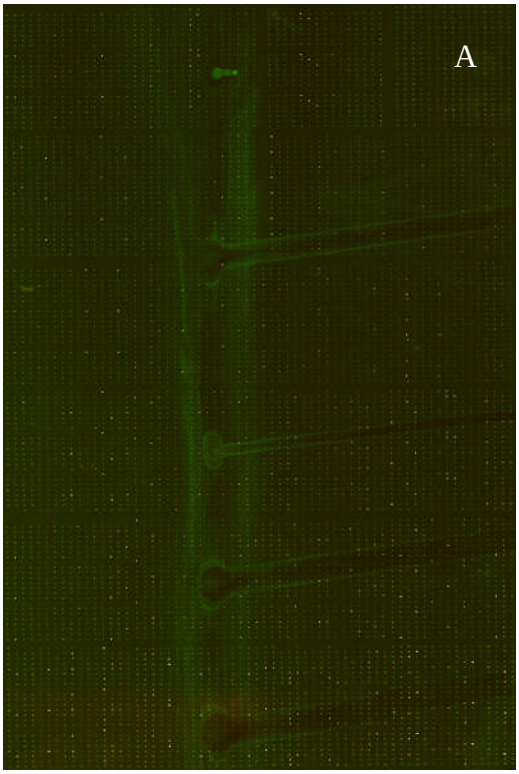
Each microarray slide had a total of 2908 different genes represented on its surface and each oligonucleotide (feature) representing the gene was repeated three times on each slide. Thus there were almost 9000 features on each array to be analyzed using the Acuity™ software (Molecular Devices Corporation U.S.A). Each feature on each microarray was scanned as above with a resolution of 5µm/pixel. These images were then analyzed by applying an array specific feature (gene) location file (.gal file). This gal file was used to generate a results file (.gpr) which contained the feature name, individual dye intensities at each feature and the dye ratio at each feature. These results files were then imported into the Acuity™ v4.0 software. The collective data from each microarray was then normalized using the Lowess method thus allowing for any variation between the same features throughout the slide. From this a data set was created which only included those features with the parameter "flags ≥0" thus ensuring only those features found were included in the analysis.

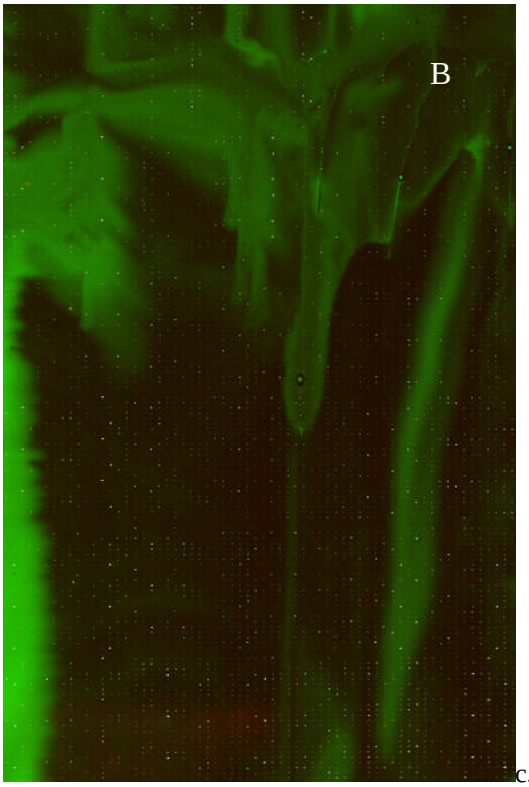
Once the data had been filtered it was analyzed to identify those genes which had altered in a significant way across all features on all of the microarrays. Only those with a small intra- or inter-array variance were included. As three features were printed on each slide the combined intra- and inter-array variance was calculated based upon the mean of the three features per slide (technical replicate) across the three slides (biological replicate), so 9 features in total. Intra- and inter-array variance was analyzed using the one sample t-test and only those features with $p \leq 0.03$ were considered for further analysis. Calculating the variance in this way meant that only those features which did not vary from day to day or from slide to slide were considered for further analysis. From these features, only those which had a mean expression change of at least 2-fold were selected. This was achieved by selecting the features whose log ratio value was ≥ 1.0 or ≤ -1 as a log value of 1.0 corresponds to a real life fold change of 2.

5.3. Results.

The final microarray scanned images comparing PE-CAPJ49 and PE-CAPJ49**RR** can be seen in Figure 5.1. All the features represented by green spots on the slides represent those genes which have been over-expressed by PE-CAPJ49**RR** and all the features represented by the red spots represent those genes that have been under-expressed by PE-CAPJ49**RR**.

From the statistical analysis described above 40 genes were identified which had been significantly over or under expressed by PE-CAPJ49**RR**. Of these 40 genes one had been under expressed and the rest over-expressed by PE-CAPJ49**RR**. These genes can be found in Table 5.1.





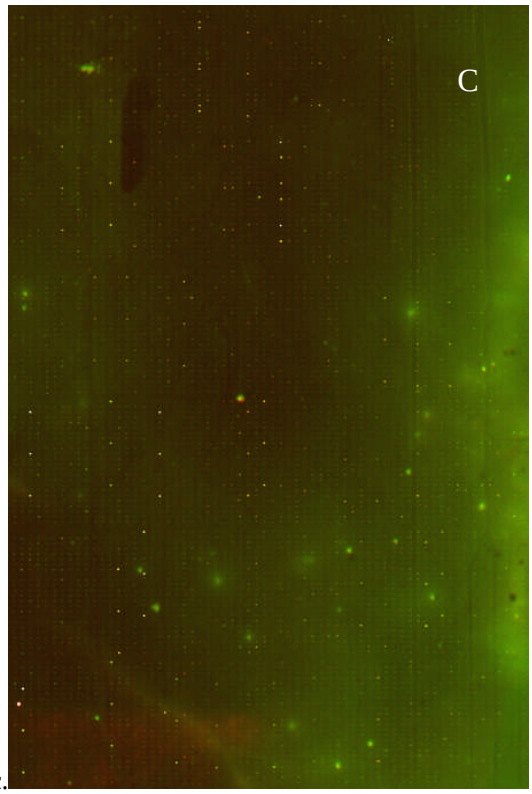


Fig 5.1. Showing scanned images of all three microarrays performed in this experiment. The variations in experimental conditions can be seen when comparing the three slides. Slides “a” and “b” show different patterns of streaking from the drying process, whilst slide “c” has a number of large bright green spots due to particulate contamination either during the hybridisation or drying process. As each feature is represented three times on each slide, enough good data exists to allow for such losses.

Gene name	Expression change			Mean change	Fold change	t-test
	Array 1	Array 2	Array 3			
EPHA4	0.104	0.089	0.06	0.084	11.86	0.004282
CD3E	0.069	0.163	0.069	0.100	9.967	0.014188
ABCC2	0.049	0.088	0.166	0.101	9.901	0.020594
BMP5	0.132	0.131	0.089	0.117	8.523	0.003583
CYP3A4	0.089	0.188	0.08	0.119	8.403	0.014705
MAD	0.193	0.134	0.159	0.162	6.173	0.003346
FCGR3B	0.135	0.138	0.221	0.165	6.073	0.007645
ZNF46	0.110	0.14	0.246	0.165	6.048	0.015935
NOL1	0.113	0.241	0.197	0.184	5.445	0.016504
CCL11	0.116	0.232	0.203	0.184	5.445	0.014809
GMEB1	0.141	0.225	0.207	0.191	5.236	0.007199
RBM6	0.099	0.27	0.207	0.192	5.208	0.02865
CASP8	0.176	0.255	0.146	0.192	5.199	0.009716
TGFB2	0.125	0.290	0.250	0.222	4.511	0.026495
H2AFG	0.148	0.276	0.244	0.223	4.491	0.015081
KLK8	0.212	0.203	0.263	0.226	4.425	0.00287
MAP3K12	0.170	0.218	0.334	0.241	4.155	0.017462
RREB1	0.183	0.338	0.257	0.259	3.856	0.016295
RUN1	0.293	0.283	0.284	0.287	3.488	0.000068
PMS2L5	0.262	0.422	0.227	0.304	3.293	0.022575
PAWR	0.283	0.347	0.287	0.306	3.272	0.003168
OT2	0.328	0.391	0.219	0.313	3.198	0.019979
VEGFC	0.279	0.372	0.319	0.323	3.093	0.005291
KRT9	0.215	0.426	0.336	0.326	3.071	0.028797
NFIC	0.327	0.251	0.409	0.329	3.040	0.015292
TGFA	0.391	0.279	0.318	0.329	3.036	0.007635
MAPKAPK3	0.226	0.456	0.307	0.330	3.033	0.029928
PPAT	0.379	0.257	0.362	0.333	3.006	0.011917
CCNE1	0.258	0.394	0.386	0.346	2.890	0.0161
TRIP11	0.302	0.489	0.283	0.358	2.793	0.025717
SART3	0.300	0.289	0.491	0.360	2.778	0.025349
DD3	0.265	0.403	0.423	0.364	2.750	0.020177
↓EGR1	↓2.841	↓2.782	↓2.505	↓2.709	↓2.709	0.001528
PSMD9	0.389	0.401	0.354	0.381	2.622	0.00151
BECN1	0.301	0.423	0.466	0.397	2.521	0.019195
SAS	0.488	0.361	0.418	0.422	2.368	0.009956
ERBB2	0.460	0.464	0.348	0.424	2.358	0.011608
IGHMBP2	0.374	0.479	0.425	0.426	2.347	0.00703
ICAM3	0.371	0.436	0.489	0.432	2.315	0.008829
LRRFIP1	0.424	0.404	0.496	0.441	2.266	0.005654

Table 5.1 Showing the raw data from all three microarrays, with the expression change shown as the ratio of the medians, and calculated fold change for all of the genes identified by the microarray analysis as being associated with radioresistance along with the p-value as calculated by the one sample t-test. The "↓" symbol represents a gene that has been under-expressed by PE-CAPJ49**RR**.

5.4 Discussion.

A relatively large number of genes, with significant expression change in PE-CAPJ49**RR** when compared to parental cells, have been identified using the methods described. They have a wide range of functions throughout the cell being involved in proliferation, cell cycle regulation, apoptosis and autophagy. PE-CAPJ49**RR** was exposed to 24Gy of ionising radiation in 4Gy increments to produce a radioresistant cell line. During this process DNA will have been damaged and cellular processes will have been initiated to repair this damaged DNA or initiate cell death should this repair not be possible. The genes identified above are a group of genes that are involved with this process. Some of these genes are capable of increasing the survival of the cells involved, thereby avoiding the effects of radiotherapy, whilst others are involved in initiating cell death due to damaged DNA.

As we have shown PE-CAPJ49**RR** is relatively more resistant to radiotherapy and the resulting cell death is much reduced compared to PE-CAPJ49. We would expect the over-expression of pro-survival genes to outnumber those genes over-expressed that have a role in cell death. From the genes identified, 16 are so called pro-survival genes (section 5.4.1) and nine are involved in cell death (section 5.4.2). The exact role of the remaining 15 genes (section 5.4.3) in the pathway conferring increased radioresistance has yet to be determined.

5.4.1 Pro-survival genes.

Gene Name	Function
RUN1	Transcription factor - promotes cell cycle progression - suppressive effect on apoptosis.
KLK8	Serine protease which binds epidermal growth factor
PAWR	Leads to an up regulation of Bcl-2 and decreased levels of the pro-apoptotic Bax
BMP5	Part of TGFβ superfamily responsible for accelerating cell cycle
EPHA4	Part of protein tyrosine kinase receptor family
VEGFC	Member of vascular endothelial growth factor family – stimulates cell growth
IGHMBP2	Involved in repair of DNA
GMEB1	Inhibits caspase mediated apoptosis
TGFA	Binds to EGFR initiating anti-apoptotic pathway
ICAM3	Increases activity of FAK which inhibits apoptosis
ERBB2/HER-2	Associated with radioresistance, inhibits apoptosis
RREB1	Transcription factor involved in activation of RAS
NOL1	Proliferation associated antigen – associated with increased proliferation
TGFB2	Accelerates cell cycle progression
RBM6	Regulates apoptosis ?through Bcl-2/Bax pathway
MAPKAPK3	Member of serine threonine protein kinase family

Table 5.2 showing the pro-survival genes over-expressed by PE-CAPJ49**RR** and a brief description of their function.

Of the pro-survival genes ERBB2, also known as HER-2 and c-erb B2, is the most well known. This gene is located on the long arm of chromosome 17 and encodes for a member of the epidermal growth factor family of receptor tyrosine kinases. It has been shown to be over-expressed in a wide variety of human tumours including HNSCC (Do et al., 2004), oral SCC (Khan et al., 2002), breast (Siziopikou and Khan,

2005), prostate (Fossa et al., 2002) and oesophageal cancer (Akamatsu et al., 2003). The role this receptor has on cell function is wide and varied and includes regulating apoptosis, cell proliferation and the response to a number of stresses (Yarden, 2001). More recently its role in response to radiotherapy has been extensively studied and it has been found to play a part in the effect radiotherapy has on HNSCC (Uno et al., 2001), breast (Juranic et al., 2004), oesophageal (Akamatsu et al., 2003) and prostate cancer (Fossa et al., 2002). In each case the over-expression of HER2 has been associated with an increased resistance to radiotherapy and in some cases monoclonal antibodies or small molecule tyrosine-kinase inhibitors have been used to block the effects of HER2 leading to a decrease in the relative radioresistance of the original tumour (Mundy et al., 2006; Azria et al., 2003). The exact pathway by which HER2 is able to overcome the effects of radiotherapy has not yet been fully discovered but a number of important genes or gene products have been found to play some part. Among them are VEGFC (Yang et al., 2002), which was found to be over-expressed in breast cancer and correlated with the over-expression of HER2, and the MAPK family of protein kinases which are up-regulated by the downstream effects of HER2 activation (Liang et al., 2003) and play a role in radioresistance.

PE-CAPJ49**RR** demonstrated over-expression of HER2 by a factor of over two in all three microarrays further strengthening the evidence to support the role this gene has on the effect of radiotherapy. Interestingly the analysis also revealed an over expression of VEGFC, by a factor of over three, and a number of members of the MAPK family, namely MAPKAPK3 and MAP3K12. From the studies already performed on breast cancer (Liang et al., 2003; Yang et al., 2002) it would appear that HER2 may work in a similar way in radioresistant oral cancer.

VEGFC is a protein encoding gene situated on the long arm of chromosome 4. The protein it encodes is a member of the vascular endothelial growth factor family. Its over-expression has been linked with an unfavourable prognosis in a wide variety of human tumours including tongue (Lopez de et al., 2004), oesophageal (Byeon et al., 2004), cervical (Gombos et al., 2005), gastric (Hachisuka et al., 2005), thyroid (Hung et al., 2003) and prostate cancer (Jennbacken et al., 2005). It is thought to have its effect by increasing the expression of Bcl-2 (Dias et al., 2002), thus decreasing the apoptotic effect of radiation and increasing the survival of the cell population. A number of other genes in our analysis, PAWR and RBM6, increase the survival of the cell population via interactions with the Bcl-2/Bax ratio to up or down regulate the respective gene to have an overall anti-apoptotic effect.

PAWR is a protein encoding gene found on the long arm of chromosome 12 (Johnstone et al., 1998). It is also known as apoptosis response protein and over-expression of PAWR has been found to lead to an increase in the anti-apoptotic protein Bcl-2 with a converse reduction in the pro-apoptotic protein Bax (Boehrer et al., 2004). RBM6 is a protein encoding gene situated on the short arm of chromosome 3 (Timmer et al., 1999) also known as RNA binding protein 6. It has been shown to have a role in regulating apoptosis through interaction with Bcl-2 (Sutherland et al., 2005).

ICAM3 was over-expressed by a factor of over 2 across all three microarrays. It is a protein encoding gene situated on the short arm of chromosome 19 (Bossy et al., 1994). Whilst it is named intracellular adhesion molecule 3 and has a role in cell to cell interaction and leukocyte migration it has also been shown to be a potent

signalling molecule relevant in cancer (Simmons, 1995). A recent study, utilising microarray technology, on radioresistant cervical cancer showed that ICAM3 was significantly over-expressed by radioresistant cervical cancer (Chung et al., 2005b) which would correlate well with our findings here. Further more this study also found that increased levels of ICAM3 lead to an increase in the phosphorylation of FAK which results in a decrease in apoptosis (Chung et al., 2005b).

Two of the members of the transforming growth factor β family were found to be over-expressed by PE-CAPJ49**RR**, TGFB2 and BMP5. BMP5, also known as bone morphogenic protein 5, is a protein encoding gene situated on the short arm of chromosome 12 (Hahn et al., 1992). TGFB2 is located on the long arm of chromosome 1 (Barton et al., 1988) and is one member of a family of proteins involved in various cell functions including cell differentiation and proliferation (Motegi et al., 2003). The TGFB family act by accelerating cell cycle progression from the G0/G1 phase to the S phase thus increasing the rate at which cells proliferate (Motegi et al., 2003). As PE-CAPJ49**RR** over-expressed two of this family of genes the subsequent increase in the cells proliferation rate would have helped to overcome the effects of ionising radiation and increase the survival of the cells.

Two other genes over-expressed by PE-CAPJ49**RR** also have a role in increasing the progress of a cell through the cell cycle namely NOL1 and RUN1. RUN1 is a gene situated on the long arm of chromosome 22 (Avramopoulos et al., 1992), it encodes a transcription factor which has been shown to promote progress through the cell cycle (Peterson et al., 2005) and induces S-phase entry via cyclin D3 (Bernardin-Fried et al., 2004). It has also been shown to be an effective suppressor of apoptosis (Abe et

al., 2005). Thus working in two ways to enhance the radioresistance of PE-CAPJ49**RR**. NOL1, also known as proliferation associated nucleolar protein, is a gene located on the short arm of chromosome 12 (Baens et al., 1994) it is expressed in the G1 phase of the cell cycle and peaks in early S-phase (Valdez et al., 1992) and is associated with an increase in cell proliferation (Fonagy et al., 1993).

As previously discussed a member of the EGFR family, HER-2, was significantly over-expressed by PE-CAPJ49**RR**. A number of other genes were also over-expressed by PE-CAPJ49**RR** and exert their effect via interactions with the EGFR family of receptor protein kinases namely KLK8 and TGFA. KLK8, also known as kallikrein 8, is a gene located on the long arm of chromosome 19 (Harvey et al., 2000) and encodes for a member of the kallikrein family which are a subgroup of the serine proteases. Its role in cancer is not yet fully understood and it has been found to be over-expressed in colon (Yousef et al., 2004a), cervical (Cane et al., 2004) and ovarian cancer (Yousef et al., 2003) whilst being under-expressed by breast cancer tissue (Yousef et al., 2004b). Whilst it seems to have a number of different functions the one which seems most pertinent to PE-CAPJ49**RR** is the ability it has to bind epidermal growth factor (EGF) (Mason et al., 1983) thus enabling it to be transported more efficiently and aid in its eventual binding to EGFR with the initiation of the subsequent pro-survival cascade. TGFA is a gene situated on the short arm of chromosome 2 (Tricoli et al., 1986) it encodes for the polypeptide transforming growth factor alpha. It has been shown to bind to and activate EGFR to promote cellular growth (Werneburg et al., 2003). This method of activation of EGFR has been shown to produce a mitogenic response via the ERK/MAPK pathway (Sawhney et al.,

2003). The interaction of these two proteins seems to be another part of the pathway by which PE-CAPJ49**RR** evaded the effects of radiotherapy.

A transcription factor is a protein that controls whether a particular gene is "switched" on or off, thus increasing or decreasing the gene product. IGHMBP2 and RREB1 are two such transcription factors that were over-expressed by PE-CAPJ49**RR**. IGHMBP2, also known as immunoglobulin Mu binding protein, is a gene located on the long arm of chromosome 11 (Fukita et al., 1993). It is a transcription factor (Sebastiani et al., 1995) that is involved in DNA repair and replication (Shen et al., 2006). RREB1, also known as Ras responsive element binding protein 1, is a gene located on the short arm of chromosome 6 (Thiagalingam et al., 1997). The RAS proto-oncogene is at the heart of a signalling network that mediates the control of cell growth, metabolism and differentiation. Transmembrane receptors initiate signals that are transduced via RAS and propagated by a phosphorylation cascade to the nucleus (Bradford et al., 1996b). Increase in the activity of Ras has been associated with radioresistance in a number of cell line studies (Brunner et al., 2004b; Brunner et al., 2003a). The binding of RREB1 to its target, Ras responsive element, results in an up-regulation of this protein (Thiagalingam et al., 1996) and may well be another way in which PE-CAPJ49**RR** resists the effect of ionising radiation.

GMEB1, also known as glucocorticoid modulatory element binding protein 1, is a protein encoding gene situated on the short arm of chromosome 1 (Theriault et al., 1999). It is a potent inhibitor of caspase mediated apoptosis having its effect by binding to pro-caspase 2 (Tsuruma et al., 2004) and the pro-caspases 8+9 thereby disrupting their activation and the subsequent apoptotic effect (Tsuruma et al., 2006).

This gene was over-expressed by a factor of over 5 across all three microarrays. Interestingly caspase 8 was also over expressed by PE-CAPJ49**RR** by a factor of just over 5. The induction of caspase 8 to initiate apoptosis in the cells damaged by the irradiation may have been antagonised by a corresponding increase in the levels of GMEB1, thus enabling the cells to survive.

5.4.2 Genes associated with cell death.

Gene name	Function
Caspase 8	Initiator caspase involved in the initiation of apoptosis
CCNE1	Induces caspases
LRRFIP1	Represses TNFα
DD3	Decreases cell proliferation and increases apoptosis
PMS2L5	DNA mismatch repair gene
MAPK3K12	Inhibits cell growth
MAD	Transcriptional repressor, limits proliferation
EGR1	Increases clusterin – antiapoptotic and pro-survival
BECN1	Initiator of autophagy

Table 5.3. Showing genes associated with cell death with a brief description of their function.

Caspases are a large family of evolutionary preserved proteases that are synthesised as inactive precursors. They are divided up into initiator and effector caspases with the initiator caspases activating the effectors in response to cell death signals which bring about apoptosis (Chang and Yang, 2000). Dysregulation of caspases has been found to be a prominent feature of many human diseases including cancer (Thompson, 1995). Caspase 8 has been shown to be one of the initiator caspases (Tsuruma et al.,

2006) and to be activated by the Fas death receptor (Muzio et al., 1996). The gene for caspase 8 is situated on the long arm of chromosome 2 (Grenet et al., 1999) and was over-expressed by PE-CAPJ49**RR** by a factor of just over 5 times which may be associated with increased apoptosis caused by the ionising radiation. However, its function may have been blocked by the concomitant over expression of GMEB1, again over expressed by PE-CAPJ49**RR** by a factor of just over 5, which inhibits caspase 8. Such natural inhibitors have been found to be effective in blocking this pathway to apoptosis (Chang and Yang, 2000). Genes associated with promoting apoptosis would not normally be linked with a radioresistant cell line, and the over-expression of caspase 8 may well be a false discovery.

The inhibition of caspase 8 has been shown to lead to a rise in the gene product of BECN1 (Yu et al., 2004a) which was also over expressed by PE-CAPJ49**RR** by a factor of just over 2. BECN1, also known as beclin 1, is a protein encoding gene situated on the long arm of chromosome 17 (Aita et al., 1999). Beclin 1 has been shown to promote autophagy and autophagic induced cell death. Autophagy is the process by which components of the cytoplasm are degraded during nutrient deprivation (Yu et al., 2004b). The increase in the levels of beclin 1 in PE-CAPJ49**RR** could be an alternative mechanism to apoptosis to initiate cell death in those cells whose DNA is badly damaged by ionising radiation. As the cell line was stable when the microarray was performed it would not be expected to find a gene promoting cell death to be over-expressed, thus BECN1 may, as with caspase 8, be false finding.

CCNE1, also known as cyclin E, was over expressed by PE-CAPJ49**RR** by a factor of almost three. It is a protein encoding gene situated on the long arm of chromosome 19 (Demetrick et al., 1995). It is required in the G1/S transition of the cell cycle (Brzezinski et al., 2005) and has been associated with a number of human cancers including pancreatic (Yue and Jiang, 2005), adrenocorticoid (Tissier et al., 2004) and ovarian (Tsuda et al., 2004). A previous study showed an up regulation of cyclin E after haematopoietic cells were exposed to radiotherapy and in a further study went on to show that this increase in cyclin E lead to enhanced caspase activation (Mazumder et al., 2004). The clone of PE-CAPJ49**RR** cells may have induced this gene in an effort to rid itself of the damaged DNA via activation of the caspase cascade.

EGR1, also known as early growth response 1, was the only gene that was under-expressed by PE-CAPJ49**RR**. It is a protein encoding gene situated on the long arm of chromosome 5 (Chavrier et al., 1989). Increased expression of EGR1 is triggered by cell cycle arrest and not by damage to DNA (Quinones et al., 2003) which may be one of the reasons why it was not up regulated in PE-CAPJ49**RR**, as these cells were proliferating at a much higher rate than their counterpart PE-CAPJ49 (see chapter 3). EGR1 expression is required for the expression of the pro-survival/antiapoptotic protein clusterin (found to be under expressed in PE-CAPJ41**RR** see section 4.4.7) via a cascade of events initiated by ionising radiation (Criswell et al., 2005). Therefore loss of expression of this gene would lead to a decrease in clusterin and the overall balance of the system to be tipped towards cell destruction.

A number of genes over expressed by PE-CAPJ49**RR** have been shown to lead to an overall decrease in the proliferation rate of tumour cells; these being MAD, DD3 and

MAP3K12. MAD was over expressed by a factor of just over six times across all three microarrays, and is a gene situated on the short arm of chromosome 2 belonging to a subfamily of MAX interacting proteins (Edelhoff et al., 1994). MAD antagonises the effect of another member of the family, MYC, to hold the cells in the G0/G1 phase of the cell cycle thus slowing down cell proliferation (Zhao and Xu, 1999). DD3 was over expressed by a factor of just under three by PE-CAPJ49**RR** and is a protein encoding gene situated on the short arm of the X chromosome (Park et al., 1998). It is a member of the DEAD box protein family and is involved in cellular growth and division (Chang et al., 2006). Chang et al., (2006) showed that increased expression of DD3 had the effect of holding the cells in the G0/G1 phase thus having an inhibitory effect on cell proliferation. MAP3K12 was over expressed by a factor of just over four times by PE-CAPJ49**RR** and is a protein encoding gene situated on the long arm of chromosome 12 (Reddy et al., 1995). Expression of this gene leads to a dramatic reduction in the proliferative capacity of cells grown in culture (Bergeron et al., 1997). As cells are relatively radioresistant in early G1 of the cell cycle (Iliakis, 1997), genes which have the ability to pause the cell cycle at the G0/G1 stage would confer a degree of radioresistance.

PMS2L5 was over expressed by a factor of over three by PE-CAPJ49**RR** and is a gene situated on the short arm of chromosome 7. Activation of p53 in response to cell damage leads to cell cycle arrest allowing the cell to repair DNA and recover or to initiate cell death if the damage is too great. PMS2L5 plays a pivotal role in this decision making process, stabilising p73 which is a member of the p53 family required for p53-dependent apoptosis (Chen and Sadowski, 2005).

LRRFIP1, also known as leucine rich repeat interacting protein 1, was up regulated by a factor of just over 2 by PE-CAPJ49**RR**. It is a protein encoding gene situated on the long arm of chromosome 2 (Johnson et al., 1992). It can be categorised as a transcription repressor and not only inhibits the growth related effects of TGFα (Suriano et al., 2005) but also represses transcription of EGFR and the subsequent downstream antiapoptotic effects (Reed et al., 1998).

5.4.3. Genes whose role in radioresistance has yet to be determined.

Gene name	Role established in cell function
ABCC2	Transports molecules across intra and extra-cellular membranes
FCGR3B	Fc gamma receptor
PSMD9	Multicatalytic proteinase complex – degrades intracellular target proteins
SAS	Involved in growth related cellular processes
PPAT	Catalyses first step of purine nucleotide biosynthesis
CD3E	T-cell surface glycoprotein
CYP3A4	Member of cytochrome p450 enzymes
CCL11	Cytokine associated with eosinophilic inflammatory disease
H2AFG	Histone family member
NFIC	Cellular transcription factor
ZNF46	?transcription factor
OT2	Transcription factor
SART3	RNA binding nuclear protein
TRIP11	Associated with golgi apparatus
KRT9	A type 1 keratin

Table 5.4 Showing the 15 genes whose exact role in radioresistance in unknown along with a brief description of their role.

As can be seen from Table 5.4 there are a number of genes over expressed by PE-CAPJ49**RR** whose precise role in the pathway of radioresistance could not be determined. Three of these genes have been identified as transcription factors, OT2 (Gherzi et al., 1997), NFIC (Qian et al., 1995) and ZNF46 (Chardin et al., 1991), but their exact function is not yet determined. ABCC2 and CYP3A4, when over expressed, have been shown to be associated with the metabolism of various chemotherapy agents. ABCC2 is a member of the ABC transporters which functions in the transport of various molecules across intra- and extra-cellular membranes, and has a particular role in drug export in the kidney (Evers et al., 1998). Evers et el., (1998) showed that the gene product of ABCC2 led to a relative drug resistance to the anti-cancer drug vinblastine. CYP3A4 is a member of the cytochrome p450 family of enzymes and plays a major role in the activation of pro-carcinogens and the metabolism of several drugs including tamoxifen (Kapucuoglu et al., 2003).

Two of the other genes identified, PSMD9 and SAS, are involved in cellular growth responses. PSMD9 is an ATP dependent protease for selectively degrading intra-cellular target proteins which belongs to a family of proteases which play an essential role in the proliferation of cells. This role, however, has not been identified with PSMD9 and its exact function in cell growth and proliferation is as yet unclear (Watanabe et al., 1998). SAS the protein encoded by this gene is a member of the transmembrane 4 superfamily and is involved in growth related cellular processes (Jankowski et al., 1994). It is thought that it plays some role in cell growth response to external stimuli (Jankowski et al., 1995). CCL11, also known as eotaxin, is a gene encoding for a cytokine that is most commonly associated with eosinophilic inflammatory disease and has a role in fine tuning cellular responses at sites of

allergic inflammation (Ogilvie et al., 2001). The treatment of cells with ionising radiation has been shown to up-regulate eotaxin and a role for it in patients with eosinophilia after exposure to radiotherapy has been suggested (Huber et al., 2000).

5.4.4. Comparison of markers identified in PE-CAPJ41RR and PE-CAPJ49RR.

None of the genes identified using the statistical method described in section 5.2 and 4.2 respectively were the same when comparing the two cell lines and some genes that we might have expected to be altered were also not identified. The criteria used to identify the genes involved with radioresistance were quite strict and may have led in part to this discrepancy, particularly regarding the intra- and inter-array variance. No gene was considered for further analysis if the feature was not identified in all three technical replicates on the slide and in all biological replicates across the three different microarrays.

Bcl-2 has been shown to be related to the response a tumour has to radiotherapy in a number of different papers (Hara et al., 2005; Rupnow and Knox, 1999; Chin et al., 2005; Nix et al., 2005) and would have been expected to be involved in our radioresistant cell lines response to radiotherapy, yet was not highlighted in our analysis. When the data from the microarray was looked at more closely for PE-CAPJ49**RR** it was found to be over expressed across all three microarrays performed by a factor of 2.35 as seen in Table 5.5. However the change in expression varied considerably between array 1 and the other two performed leading to a p-value which did not support a statistically significant change through all the arrays. With regards to PE-CAPJ41**RR** again Bcl-2 was over-expressed but this time with a fold change of 1.38. In array 1 the feature was unfortunately unavailable for analysis due to a

technical problem with that part of the microarray slide leading to the feature being spoiled which again meant that the change was not considered to be consistent across all three biological replicates.

Gene	Expression change			Mean change	Fold change	t-test
	Array 1	Array 2	Array 3			
Bcl-2	0.113	0.649	0.517	0.424	2.35	0.184
Bcl-2(41)	-	0.686	0.759	0.723	1.38	0.098
Bad	1.944	0.526	2.375	-	-	0.596
Bad(41)	5.301	3.963	1.086	3.450	-3.450	0.165
ERBB2/Her-2	0.460	0.464	0.348	0.424	2.36	0.012
ERBB2/Her-2(41)	1.611	2.276	0.355	-	-	0.129
Caspase 8	0.176	0.255	0.146	0.192	5.12	0.009
Caspase 8 (41)	3.037	1.288	0.871	-	-	0.383
GMEB1	0.141	0.225	0.207	0.191	5.23	0.007
GMEB1 (41)	1.774	1.495	0.577	-	-	0.724

Table 5.5. Showing a comparison between the genes expression of PE-CAPJ41**RR** and PE-CAPJ49**RR**, with the expression change shown as the ratio of the median change across the numerical value of each picsel analysed in each microarray. Thus all values of greater than one correspond to an under expression in the "**RR**" cell lines and all values less than 1 correspond to an over expression in the "**RR**" cell lines. The genes shown in blue represent the data from PE-CAPJ41**RR**.

Bad is one of the Bcl-2 family members which promotes cell death by its pro-apoptotic nature (Schoelch et al., 1999) and was represented on the gene set used in this microarray experiment. Due to its pro-apoptotic function we may have expected this gene to exhibit decreased expression by both PE-CAPJ41**RR** and PE-

CAPJ49**RR**. Overall this was the case across the microarrays with 5 out of the six arrays performed across the two cell lines showing a reduction in its expression, as illustrated in Table 5.5. It was not identified in our analysis as the change was not a consistent one through all of the repeats with a resultant p-value which was not statistically significant.

ERBB2, also known as Her-2, was significantly over-expressed by a factor of 2.36 by PE-CAPJ49**RR** but this did not correlate with PE-CAPJ41**RR**. Here ERBB2 showed decreased expression in the first two arrays and was over-expressed in the third. This variability in results across different arrays highlights the importance of performing a number of technical repeats when dealing with microarray technology.

Caspase 8 was significantly over-expressed by PE-CAPJ49**RR** with a corresponding increase in GMEB1. These two genes were not highlighted in the analysis of PE-CAPJ41**RR**. When we look more closely at the data, shown in Table 5.5, we can see that both caspase 8 and GMEB1 were under-expressed by PE-CAPJ41**RR** in the first two arrays and both over-expressed in the final one. It is interesting to note that their expression seems to always correlate well between the two genes, further strengthening the argument that their function is linked. It could by hypothesised that these genes were under-expressed by PE-CAPJ41**RR** as its DNA was less susceptible to damage caused by ionising radiation due to the increased dose of radiotherapy it had been exposed to.

5.5 Conclusion.

In conclusion, we have successfully analysed the tumour cell line PE-CAPJ49**RR**, and from this we have identified 40 genes which have shown a significant change in their levels of expression. All of these targets require further evaluation to confirm these results. Interestingly no gene was found to show a significant change in expression in PE-CAPJ41**RR** and PE-CAPJ49**RR**. As there are over 200 OSCC cell lines available worldwide (Lin et al., 2007) an alternative way of investigating radioresistance using cell lines would be to establish the inherent response to radiotherapy of a number of these cell lines, using the clonogenic assay techniques described in chapter 3. Once the different response to radiotherapy was known, microarray analysis could then be performed comparing the expressed genes of each cell line. Using this method would eliminate those genes which are simply damaged by the radiotherapy, as may have happened in our study. As the tumour has an intrinsic resistance to radiotherapy, as opposed to the model used in this thesis where the resistance is radio-induced, the results may be more applicable to predictive markers. However, as each cell line comes from a different tumour it would be impossible to tell if the targets identified are simply due to the differing genotype of each tumour or whether they truly are responsible for the different response to radiotherapy.

Chapter 6 – Results Molecular markers of radioresistance in oral squamous cell carcinoma as detected by Immunohistochemistry.

Aims:

- To enable the prediction of response to radiotherapy utilising pre-treatment biopsy specimens.
- To confirm markers of radioresistance previously described in oral squamous cell carcinoma.
- To investigate previously described markers of radioresistance from other head and neck sites.
- To identify novel molecular markers of radioresistance in oral squamous cell carcinoma.

6.1 Introduction.

Despite oral cancer being the twelfth most common cancer world-wide (Parkin et al., 2005), there has been little improvement in the five year survival over the last three decades (Bettendorf et al., 2004). This is in part due to the difficulty in choosing the treatment modality that will be the most suitable for an individual patient, with the knowledge that there are a large proportion of tumours which recur after radiotherapy treatment.

The family of epidermal growth factor receptors (EGFR) consists of four tyrosine kinase transmembrane receptors which lie at the heart of a multitude of cell processes including cell proliferation, angiogenesis, migration and apoptosis (Harari, 2004) as pictured in figure 6.1. So far ten ligands have been identified in humans which bind to the receptor each causing slightly differing intracellular responses (Yarden, 2001). Over expression of EGFR has been demonstrated in 80-100% of head and neck malignancies, using methods such as immunohistochemistry or fluorescent in situ hybridization (Harari, 2004), and has been associated with a poorer prognosis (Nicholson et al., 2001). Over expression of EGFR has also been associated with an overall decrease in the response a tumour has to the effects of radiotherapy, with an increase in functional EGFR demonstrated in epidermal keratinocytes and increased resistance to radiotherapy demonstrated in vivo and in vitro (Peter et al., 1993).

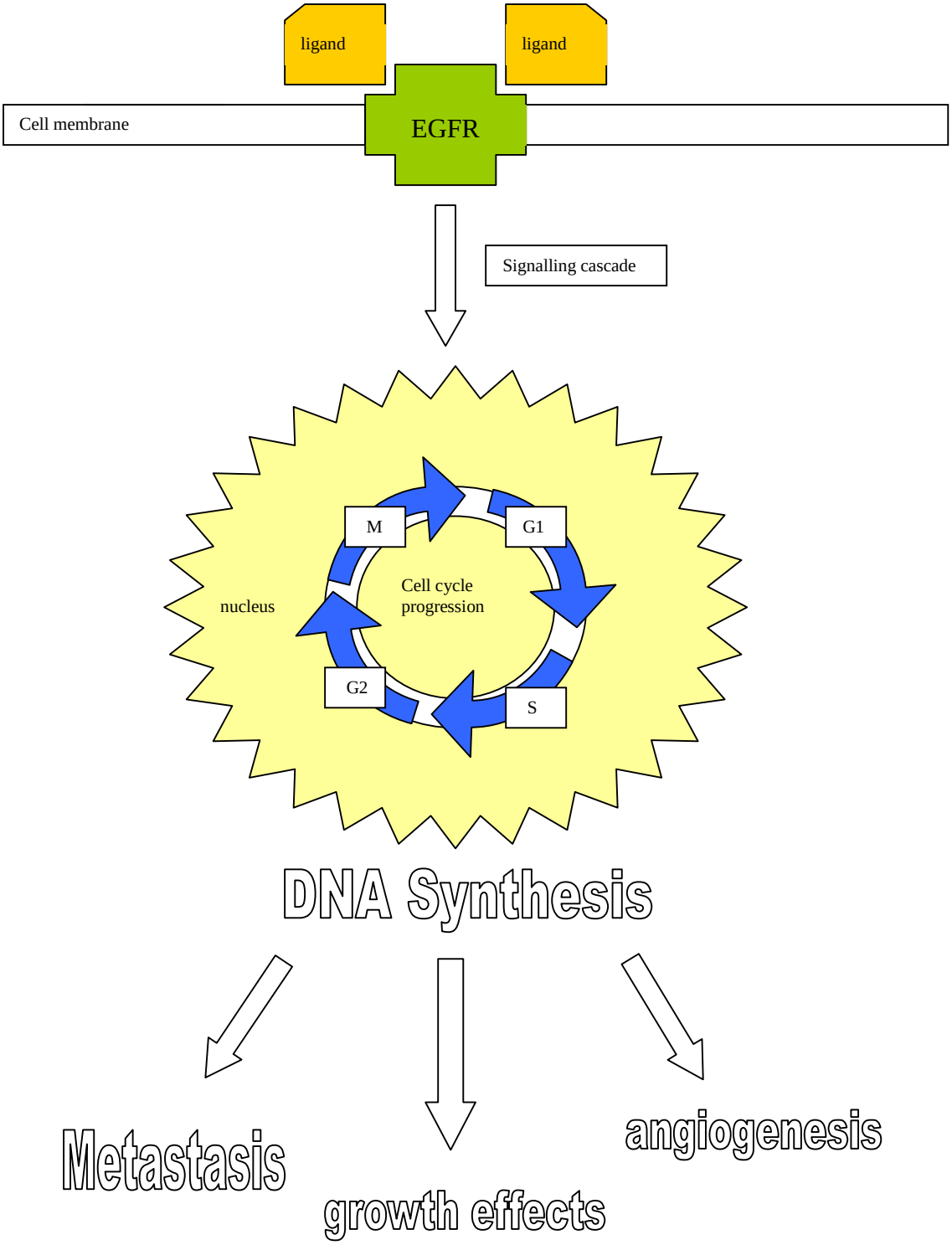


Figure 6.1 Effects of EGFR adapted from (Harari, 2004). After ligands bind to the receptor a number of effects can take place including angiogenesis, proliferation and metastasis.

The mechanism by which EGFR exerts its effect depends on which particular ligand binds and the subsequent cascade initiated. It has been found that activation of EGFR by binding of either epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) leads to an increase in COX-2 expression. Increased levels of EGFR have also been found together with over expression of COX-2 in breast cancer cells (Dannenberg et al., 2005). A mechanism where-by activation of EGFR leads to an increase in prostaglandins which then stimulate COX-2 expression has also been described in a number of papers (Lippman et al., 2005; Mohan and Epstein, 2003).

The cyclo-oxygenase enzyme catalyzes the conversion of arachidonic acid into various prostaglandins. These prostaglandins exert their effect by binding to G-protein coupled cell surface receptors leading to changes in the cellular level of cyclic adenosine monophosphate and calcium ions. Prostaglandins play a role in cell proliferation, angiogenesis, immune surveillance and apoptosis all of which are important in the pathogenesis of cancer (Lin et al., 2002). Two isoforms of the enzyme exist and are referred to as COX-1 and COX-2. Whilst COX-1 is present in normal tissue COX-2 is an inducible enzyme which is expressed in relation to inflammation and carcinogenesis (Lin et al., 2002). Over expression of COX-2 has been demonstrated by immunohistochemical analysis in many human malignancies including oral cancer (Lin et al., 2002; Mohan and Epstein, 2003; Altorki et al., 2004).

Inhibition of COX-2 with selective drugs such as celecoxib has been shown to be effective in reducing the number of adenomatous polyps in patients with FAP (familial adenomatous polyposis) (Brown and DuBois, 2005). Celecoxib was also

found to inhibit the growth of head and neck cancer cell lines and augment the efficacy of chemotherapy agents (Hashintani et al., 2003). Hashintani et al., (2003) showed that treatment of the head and neck squamous carcinoma cell line SCC25 with celecoxib enhanced the sensitivity of the cell line to vincristine, bleomicin and doxyrubicin. To date only one study exists looking at the relationship between radiotherapy and COX-2. In the study by Terakado et al (2004) 41 pre-treatment biopsy samples, taken from patients with oral cancer, all of whom had pre-operative radiotherapy, were analysed for expression of COX-2 utilising immunohistochemistry. A tumour was considered to be radioresistant by examining the resected tumour specimen for residual tumour. By comparing the response to radiotherapy (26 cases were deemed non-responders and 15 were deemed as responders) with the expression of COX-2 the conclusion was made that COX-2 predicts for radioresistance ($p=0.047$) (Terakado et al., 2004).

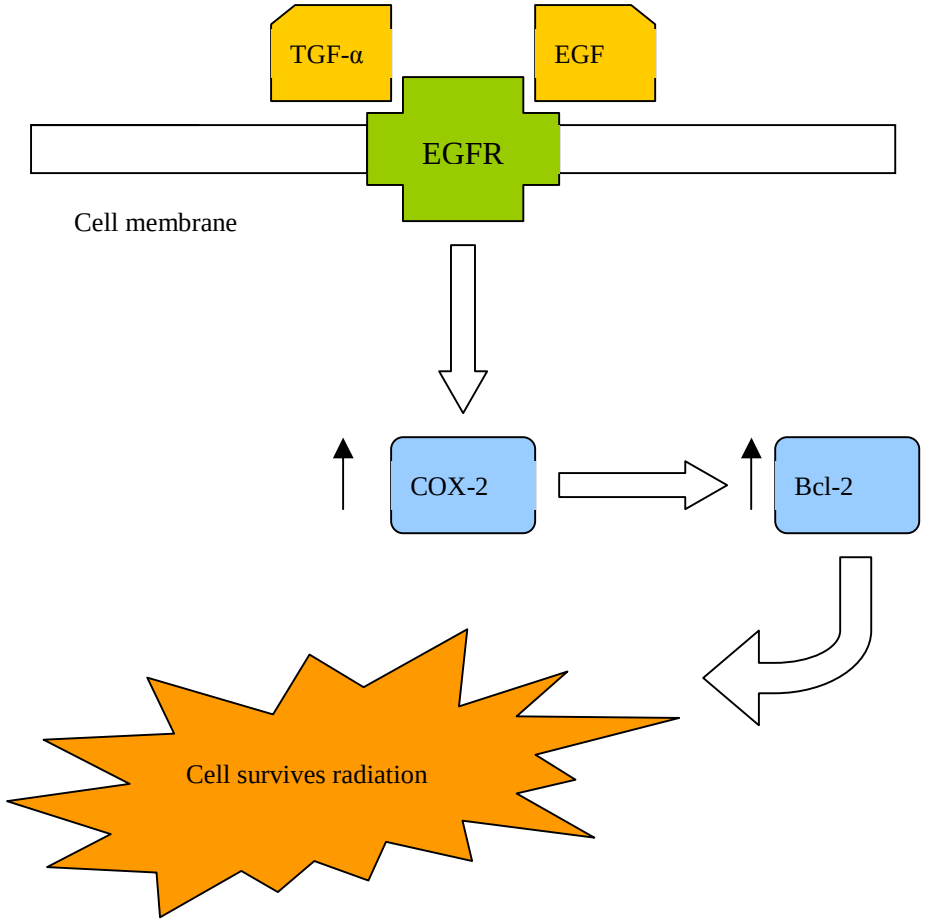


Figure 6.2 Interaction of COX-2, EGFR and Bcl-2. Binding of ligands to the receptor causes an increase in the expression of COX-2 leading to an up-regulation of Bcl-2 and suppression of apoptosis.

Increase in expression of COX-2 has also been shown to lead to an increase in intracellular levels of the antiapoptotic protein Bcl-2 (Brown and DuBois, 2005). It has been shown that COX-2 inhibits programmed cell death in human colon caner cells and produces an increase in expression of Bcl-2 (Sheng et al., 1998). A schematic diagram of the interaction of EGFR, COX-2 and Bcl-2 can be seen in Figure 6.2.

One of the major ways in which radiotherapy exerts its effects is through the induction of apoptosis in tumour cells (Rupnow and Knox, 1999). Thus if a tumour gains the ability to evade the effects of apoptosis the success of radiotherapy may be limited and the tumours' ability to survive will be enhanced. The Bcl-2 family of proteins have a major role in the apoptosis cascade and consist of both pro-apoptotic

members such as Bax and anti-apoptotic members such as Bcl-2 (Haupt et al., 2003). Bcl-2 was first recognised as a gene that was deregulated by the t(14;18) chromosomal translocation associated with B cell lymphoma in man (Bakhshi et al., 1985). Its anti-apoptotic role was soon recognised and is now well established (Vaux et al., 1988). It initiates apoptosis by stabilising the mitochondrial membrane, preventing cytochrome c release and its subsequent binding to apoptosis activating factor-1 (Thomadaki and Scorilas, 2006). In response to stress Bax forms a homodimmer causing release of cytochrome c from the mitochondria. This results in caspase 9 activation which eventually leads to apoptosis (Adams and Cory, 1998). Overall the ratio of pro-apoptotic (e.g. Bax) to pro-survival (e.g.Bcl-2) proteins seems to determine the cells sensitivity or resistance to apoptotic stimuli such as radiotherapy (Thomadaki and Scorilas, 2006).

The reported prevalence of bcl-2 in oral cancer varies quite widely from study to study with recent studies finding bcl-2 positive oral cancers in 55%, 26%, 17% and 7% of cases respectively on immunohistochemical analysis (Lo Muzio et al., 2005; Lo Muzio et al., 2003; Schildt et al., 2003; Vora et al., 2003). Bax has not been as extensively studied in the literature as its' counterpart Bcl-2 but it has been reported to be over-expressed in 46% of oral cancer specimens (Teni et al., 2002).

A review by Smith and Hafty, (1999) looked at molecular markers of radioresistance, including EGFR, Bcl-2, p53 and VEGF, in head and neck cancer and concluded that there where no markers at present which reliably predicted the response a tumour has to radiotherapy. This review looked at head and neck cancer as a whole, grouping all the individual subsites together (Smith and Haffty, 1999). As head and neck cancer

consists of a diverse group of tumours, from the tongue to the hypopharynx, it would seem more sensible to study each subsite separately. This approach may yield more rewarding results and is the approach adopted in this chapter. As EGFR is over expressed in 80-100% of head and neck cancer it would seem unlikely that this marker alone will aid in the prediction of a tumours response to radiotherapy. This thesis attempts to use the knowledge that EGFR is linked with the expression of COX-2 and Bcl-2 to see if these three markers can be used either alone or in conjunction to predict the response to radiotherapy of oral squamous cell carcinoma.

6.2 Materials and Methods.

6.2.1 Patient details.

The Local Research Ethics Committees' approval was obtained to allow the data and archival tissue blocks to be collected on patients who had been treated with radiotherapy for oral squamous cell carcinoma. Using the local head and neck cancer database in tandem with the local histopathology database, 254 cases of oral squamous cell carcinoma were identified in the time period 1996 to 2004. Of these, 185 case notes were retrieved and 88 of these had been treated with radiotherapy as the primary modality. Tumours were classed as radioresistant if biopsy proven squamous cell carcinoma recurred at the primary tumour site within twelve months of the initial treatment. In the present literature no universally accepted definition of radioresistance exists. A recurrence should have the same histology as the original tumour and occur at the original site within a specified time frame. These characteristics prevent second primaries or occult metastasis being classified as radio recurrence. The definition used in this thesis has been used in other head and neck case series (Nix et al., 2005).

Ten such cases were identified from the notes, but only nine tissue samples were available for retrieval. To reduce confounding factors the nine cases were matched with a control case that was deemed radiosensitive, as there had not been a recurrence of the original tumour within the first year of follow-up. They were also matched for age, gender, tumour subsite, T-stage and smoking and alcohol history. The dose of radiotherapy given ranged from 50Gy – 60Gy in a fractionated regimen. Patients had been followed up in the ENT head and neck clinic on a monthly basis for the first year, on a bimonthly basis for the second year, every three months for the third year, six monthly for the forth year and yearly thereafter. At each visit patients had undergone a clinical examination of their oral cavity for evidence of tumour recurrence.

6.2.2 Immunohistochemistry

The tissue sections were stained for EGFR, COX-2, Bcl-2 and Bax, as described in section 2.3, using 100µl of the primary antibodies anti EGFR (Ab-5 clone H11, Neomarkers USA), anti cox-2 (BD Transduction laboratories UK), anti Bcl-2 (Ab-3 clone Stratech Scientific Ltd K) and anti Bax (Ab-5 clone Stratech Scientific) respectively. This was used at a dilution of 1:50, (diluted in 0.2x casein in TBS, with the negative control being 100µl of 0.2x casein in TBS) for EGFR, COX-2 and Bcl-2 and 1:100 for Bax.

6.2.3 Interpretation and assessment of staining.

The slides were independently scored by two investigators who were blinded as to the outcome of radiotherapy. If a difference of opinion was encountered the slide in

question was discussed, and if no consensus could be reached a third scorer, also blinded to the outcome of radiotherapy, was asked to adjudicate. During the scoring process for all the markers assessed a consensus of opinion was reached in each case. As EGFR, COX-2 and Bcl-2 are not usually detected in normal oral mucosal (Hou et al., 1992), sections were regarded as positive if greater than 10% of the tumour cells stained positive for their presence. The pro-apoptotic marker was scored in the context of loss of expression, so if ≥50% of the cytoplasm stained for Bax then the section was deemed to be positive.

6.2.4 Statistical Analysis

Statistical analysis was performed using the statistics programme SPSS v11.5 for windows (SPSS inc. Illinois, USA). Bivariate Pearson correlation analysis was performed and p-values generated for correlations between the radioresistant group and other variables. A p-value of less then 0.05 was taken to indicate a significant relationship.

6.3. Results.

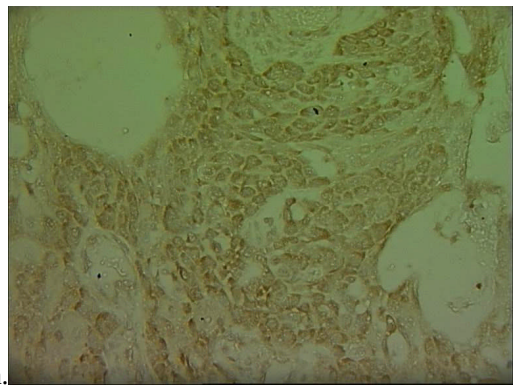
From our data, ten patients were identified who satisfied our criteria for a radioresistant tumour. Of these, nine tissue blocks were available for analysis. All had been treated with radiotherapy as the primary treatment modality. The characteristics of this patient group are shown in table 6.1 along with the characteristics of the controls. The age range of the radioresistant group was from 33 years to 79 years with a mean age of 53 years. The radioresistant samples were matched with seven controls for gender, age, smoking and alcohol history and tumour subsite. The controls were considered radiosensitive if there was no reported recurrence at the site of the first

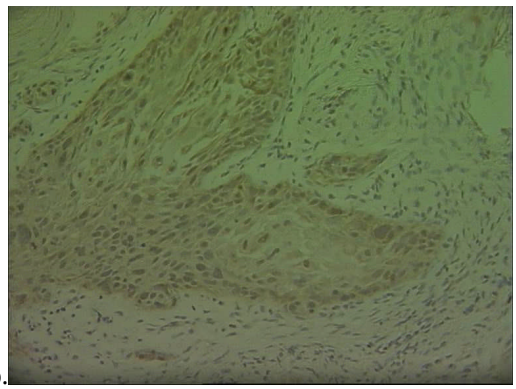
tumour after the first year of follow-up. Samples of the slides staining positive and negative are shown in figure 6.2 with summary of data shown in table 6.3. As can be seen in Table 6.1 a correlation was found between EGFR and response to radiotherapy, with positive staining correlating with a radioresistant tumour (p=0.0439), a significant correlation was also found between BAX and smoking history, with positive staining correlating with smokers (p=0.0004).

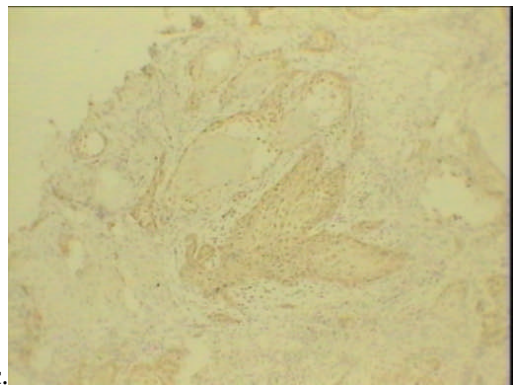
Sample	Age	Gender	Smoking	Alcohol	Subsite	Response
1	33	♀	Smoker	Nil	Tongue	Radioresistant
2	42	♀	Nil	Nil	Tongue	Radioresistant
3	44	♀	Nil	Drinker	Tongue	Radioresistant
4	47	♂	Nil	Drinker	Tongue	Radioresistant
5	50	♂	Smoker	Drinker	Oral cavity	Radioresistant
6	55	♂	Smoker	Drinker	Floor of mouth	Radioresistant
7	57	♂	Smoker	?	Tongue	Radioresistant
8	70	♂	Smoker	?	Oral cavity	Radioresistant
9	79	♀	Smoker	Nil	Floor of mouth	Radioresistant
10	37	♀	Nil	Drinker	Tongue	Radiosensitive
11	45	♂	Nil	Drinker	Tongue	Radiosensitive

12	46	♀	Smoker	Nil	Floor of mouth	Radiosensitive
13	55	♂	Smoker	Drinker	Floor of mouth	Radiosensitive
14	59	♂	Smoker	Nil	Tongue	Radiosensitive
15	73	♂	Smoker	Drinker	Tongue	Radiosensitive
16	78	♀	Nil	?	Tongue	Radiosensitive

Table 6.1. Showing the patient characteristics of the radioresistant and radiosensitive groups. Where "?" appears the information was not available from the notes. ♂ = male patient and ♀ = female patient. Oral cavity refers to a tumour which involves both the lateral border of tongue and the floor of mouth.

a. 

b. 

c. 

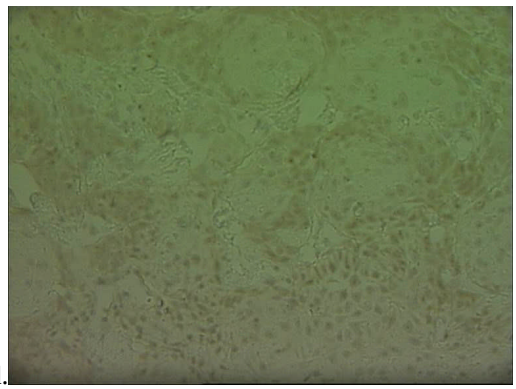
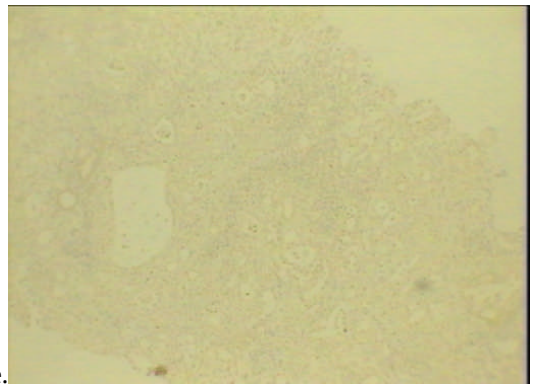
d. 

Figure 6.3. Showing positive staining results for (a) Bax, (b) EGFR, (c) Bcl-2 and (d) COX-2. Cells stained brown represent a positive result for the

e. 

	Positive staining	Negative Staining
EGFR	4 (25%)	12 (75%)
COX-2	6 (38%)	10 (62%)
Bcl-2	15 (94%)	1 (6%)
BAX	8 (50%)	8 (50%)

Table 6.2. Showing the number of samples staining positive for the markers investigated in this chapter with corresponding percentages in brackets.

	COX-2 +	EGFR +	Bcl-2 +	BAX+
Radioresistance	0.5473	0.0439	0.8609	0.6419
Age	0.5717	0.0716	0.4006	0.7389
Smoking history	0.4577	0.5816	0.2711	0.0004

Alcohol history	0.6059	0.5463	0.4533	0.4710
Gender	0.7192	0.7889	0.2070	0.1489

Table 6.3. Showing p-values for correlations between positive staining for marker, tumour and patient characteristics. P-values of less than 0.05 were considered significant. Values highlighted in blue are significant correlations.

6.4. Discussion.

All of the markers examined have previously been studied in relation to head and neck cancer, either on a specific subsite or taking head and neck cancer as a whole. EGFR has been shown to be over-expressed in 80 – 100% of head and neck cancer (Harari, 2004) and studies have also shown an over-expression in 100% or oral squamous cell carcinoma (Storkel et al., 1993). An over-expression of COX-2 has been reported in 100% of head and neck cancer (Chan et al., 1999) and in a study on oral cancer alone the over-expression of COX-2 was reported as 60% (Terakado et al., 2004). Bcl-2 and its counterpart Bax have not been as extensively studied with the reported over-expression varying from 7-55%, for Bcl-2, and 46%, for BAX, in cases of oral cancer (Lo Muzio et al., 2005; Lo Muzio et al., 2003; Schildt et al., 2003; Vora et al., 2003; Teni et al., 2002; Teni et al., 2002).

When comparing the current literature to our results there are some clear differences. In our study only 25% of the specimens stained positive for EGFR (table 6.2) as opposed to the near 100% of oral cancer specimens found in previous studies (Storkel et al., 1993). Positive staining for COX-2 was found in 38% of cases which compares with 60% of cases described in other reports (Terakado et al., 2004). Bcl-2 was expressed by 94% of our sample, a much larger fraction than previously reported (Lo Muzio et al., 2003; Schildt et al., 2003; Vora et al., 2003), and Bax stained positive in 50% of cases, a figure much more akin to the other study on the expression of Bax in oral cancer (Teni et al., 2002). These discrepancies highlight the problems associated with immunohistochemistry as there are many variables which can alter the potential staining of a tumour for a particular antigen including the fixative used and the fixation process (Farmilo et al., 2003). The different scoring systems used in the literature also have an effect on the results. Terakado et al., (2004) allowed for a positive result with as little as 5% of the tumour cells staining for COX-2 thus leading to a potential increase in the positive samples as compared to our study. The number of independent scorers along with their ability to interpret histological slides has a bearing on the outcome of a positive result with a consultant histopathologist being more skilled than some investigators at interpreting staining results.

Of the markers studied in this chapter only EGFR showed a correlation with a radioresistant tumour (Table 6.3) with positive staining predicting for a radioresistant tumour. This finding is supported by an in vitro study on head and neck cancer cell lines, in which a monoclonal antibody, C225 also known as cetuximab, was used to block the effects of EGFR. When this was combined with radiotherapy the effects

were greater than those of radiotherapy alone (Harari and Huang, 2001) suggesting a role for EGFR in radioresistance.

As previously stated the EGFR family is at the centre of a cascade of events and can activate a number of different responses depending on the ligand which binds to it including increased expression of Bcl-2 and COX-2. Whilst 25% of cases stained positive for EGFR in our study the percentage of cases staining positive for the other markers (COX-2, Bcl-2 and BAX) was always higher than this ranging from 38-94% (Table 6.2). These other markers may play a role in the overall genotype of oral cancer and not just in the response to radiotherapy. Other stimuli may lead to their over expression, such as smoking or alcohol intake, as well as EGFR status.

Positive staining for BAX was found to correlate with the smoking history in the cases selected in our study ($p=0.004$). Teni et al., (2002) found a similar correlation with Bax and chewing tobacco induced oral cancer again utilizing the method of immunohistochemistry to detect the expression of Bax. Thus suggesting that the carcinogen in the tobacco causes an up regulation of the protein Bax in an effort to induce apoptosis in the cells with tobacco induced DNA damage.

Whilst only EGFR status was found to predict for response to radiotherapy in this study other studies on COX-2 and Bcl-2 in head and neck cancer have found a positive correlation (Nix et al., 2005; Terakado et al., 2004). The relatively small sample size of oral SCC specific cases in this study means that any correlation has to be particularly strong for a statistically significant relationship to be found. So whilst there may indeed by a relation ship between COX-2, Bcl-2, Bax and response to

radiotherapy in oral SCC, it is not strong enough to overcome the small number in this study. It would be interesting to study a larger group of homogeneous oral SCC tumours to try and identify a link between Bcl-2, COX-2, Bax and radioresistance.

Chapter 7 – Concluding remarks.

Radiotherapy has been, and continues to be, a major treatment modality used in the treatment of oral squamous cell carcinoma. The prognosis of oral cancer has failed to show significant improvement over the past three decades. Resistance of oral cancer cells to the effects of ionising radiation remains an obstacle to improvement in the prognosis of this type of cancer. Radiotherapy in itself has a number of unpleasant side effects and when a tumour fails to respond to radiotherapy the prognosis is worsened further. The overall aim of this Thesis was to investigate the mechanism of radioresistance and attempt to identify markers that could predict the response to radiotherapy. To achieve this, the Thesis had a number of secondary aims:

- To establish a novel subclone of radioresistant oral cancer cells using a course of fractionated radiotherapy.

- To use expression microarrays to investigate the mechanism by which oral cancer avoids the effects of radiotherapy.
- To identify potential markers of radioresistance in oral cancer using microarrays.
- To investigate previously described markers of radioresistance in head and neck cancer with specific focus on oral cancer via immunohistochemistry.

Investigation of radioresistance in a tumour is hampered by a number of factors. The definition of radioresistance itself in a clinical setting varies widely from study to study affecting the final outcome of the study and any realistic comparison between studies. Sampling error can occur when a biopsy is taken from a tumour as this biopsy only represents a small fraction of the overall tumour population. This fraction may or may not have responded to radiotherapy in the patient and so the predictive value of studies utilizing such methods is called into question. This Thesis has overcome these problems by creating a novel, physiologically proven, radioresistant cell line from a previously established oral squamous carcinoma cell line. This enables the characteristics of a radioresistant population of cells to be studied and extraction of large quantities of pure nucleic acids for further genetic analysis. Thus far it is our understanding that no other proven radioresistant oral cancer cell line has been created for study. A whole host of techniques can now be used on this "stock" of radioresistant oral cancer cells to both investigate mechanisms of radioresistance and trial therapies for eventual clinical use.

There have been a number of research studies in the past which have looked at individual markers of radioresistance (Terakado et al., 2004) or markers in tandem

such as p53 and Ki67 (Couture et al., 2002) in an effort to predict response of oral cancer to radiotherapy. The literature on the whole can be quite contradictory and a reliable marker is yet to be fully evaluated. This Thesis aimed to look beyond a single indicator of radioresistance by utilizing the relatively new technology of cDNA microarrays.

The utilization of cDNA microarray technology has been used to study a wide variety of cancers and in many different aspects of cancer development. To date they have not been used to search for markers of radioresistance in oral cancer. This Thesis aimed to fill this gap by comparing our radioresistant cell line to its parent cell line, shown to be considerably less resistant to the effects of radiotherapy. We were able to identify a number of genes which when present confer a radioresistant phenotype to the tumour. The most promising results came from the PE-CAPJ49 cell lines with a total of 40 genes being identified as having a significantly altered level of expression. Of these, one of the genes identified had shown decreased expression the radioresistant cell line and the rest had been over-expressed. When the function of these genes was looked at more closely, it soon became evident that the genes fell into two broad groups, those whose function was cytotoxic to the cell, and thus contradictory to expected findings, and those which exhibited a cytoprotective effect. As would be expected, the majority of the genes whose function was well known had an overall cytoprotective effect on the cell. Interestingly a number of the genes identified actually counteracted each other with this being best illustrated by the interaction of caspase 8 and GMEB1.

From the analysis performed on the two cell lines PE-CAPJ41**RR** and PE-CAPJ49**RR** it is clear that no single gene holds the key to the cascade of events that leads to a colony of cells becoming radioresistant. Instead, a complex interaction of many genes is involved in regulating the response each cell has to the harmful effect of ionising radiation and overcoming the inevitable damage to the DNA. These findings should enable us to eventually determine a panel of genes which will identify how a tumour is likely to respond to a course of fractionated radiotherapy using a routine assay such as Immunohistochemistry.

Alternatively in the future it may be possible to use a sample of tumour DNA, extracted from a patient, and by using cDNA microarrays create a “fingerprint” of the cancer DNA which will enable us to confidently predict whether this tumour will respond to radiotherapy and if not, which gene/gene product needs inhibiting to allow radiotherapy to work. This thesis aims to be the start of this process but before this can happen, further studies such as quantitative PCR need performing to confirm that the genes identified in this thesis are actually implicated in radioresistance.

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