E1B attenuated adenoviral therapy for recurrent squamous cell cancer of the head and neck.

Ian Ganly

This thesis was submitted to the University of Glasgow for the degree of Doctor of Medicine.

CRC Department of Medical Oncology and Dept of Plastic and Reconstructive surgery, Canniesburn hospital, Glasgow.
January 2001.

Contents

CHAPTER 3 : RESULTS

ABBREVIATIONS

A	adenine
bp	base pairs
BSA	bovine serum albumin
cdk	cyclin dependent kinase
cDNA	complementary DNA
C	cytosine
^{o}C	degrees centigrade
DEPC	diethylpyrocarbonate
DMEM	Dulbeccos modified Eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic acid triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GTP	guanosine 5-triphosphate
G	guanine
Gy	Gray
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
HNSCC	Head and neck squamous cell carcinoma.
HRP	horseradish peroxidase

IgG	immunoglobulin G
kb	kilo base pairs
kD	kilodaltons
KBM	keratinocyte basal medium
Lac Z	Beta galactosidase
mRNA	messenger RNA
ml	millilitre
mM	millimolar
mm	millimetres
mA	milliamps
NaCl	sodium chloride
NaOH	sodium hydroxide
PBS	phosphate buffered saline
PBF	phosphate buffered formalin
PCR	polymerase chain reaction
RNA	ribonucleic acid
Rnase	ribonuclease
SDS	sodium dodecyl sulphate
SLM	special liquid medium
SSC	sodium chloride/sodium citrate(buffer)
TAE	Tris/acetate buffer
Taq	Thermus aquaticus DNA polymerase
TBE	Tris/borate buffer
TBS	Tris buffered saline

TE	Tris/EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
T	thymine
UV	ultraviolet
uM	micromolar
ug	microgram
w/v	weight/volume
W	watts

TABLES AND FIGURES.

FIGURES.

TABLES.

ACKNOWLEDGMENTS

Over the past 3 years I have learned a considerable amount about cancer biology and medical oncology thanks to Bob Brown, Allan Balmain but especially Prof Stan Kaye. I would like to thank Prof Kaye for the trust and responsibility he gave me when handing this project to me. I would also like to thank Mr David Soutar, my surgical supervisor, who is an impressive figure to follow in the world of surgery. I would like to thank everyone at the CRC Beatson Institute and Department of Medical Oncology but especially groups O4 (previously R7) and O1 for teaching me all the laboratory techniques but special thanks are reserved for Spiros Linardopoulos, Sheelagh Frame, Debra Stuart and Francis Fee. All of the in vitro work utilised head and neck cell lines which had been established by Dr Ken Parkinson and I would like to thank him for allowing me to use these cell lines for these studies. Thanks are due to all the consultants in the West of Scotland who referred patients to the clinical trials, particularly Dr Robertson , Dr Yosef, Dr Junor. I also thank Irene Grant and Mary Anne Macham for helping with the ethical approval forms and data management, to Oirigh Park for her superb nursing care for all the patients and of course for her good "patter".....I will certainly miss working with her! The clinical trials would not have been possible if it were not for the patients and I am extremely grateful to them for participating and their relatives for their support. I also thank Onyx pharmaceuticals for financing the research,with particular thanks going to David Kirn, Chris Maack and Carla Heise at Onyx.

ABSTRACT

Recurrent head and neck cancer is a disease which causes significant morbidity and mortality. Despite advances in surgical reconstruction and in chemotherapy and radiotherapy, current treatments for this disease give poor responses which are of short duration. Because of this, there is a need for new biological therapies to be developed. An example of this is the E1B deleted adenovirus, Onyx-015, which has been shown to selectively replicate in and lyse cells with non-functional p53 (Bischoff et al,1996). This virus may be therapeutically useful in the treatment of a wide range of tumours since p53 abnormalities are very common in human cancer. The aims of this thesis were to determine if this virus would selectively replicate in and lyse head and neck squamous cell carcinoma cell lines with non-functional p53, to determine if the virus could cause tumour regression of subcutaneous tumours derived from human head and neck tumour cell lines in a nude mouse xenograft model, and lastly to determine if this virus could be given safely by intratumoural injection to patients with recurrent head and neck cancer and whether it could produce an anti-tumour response.

Using a panel of human head and neck squamous cell carcinoma cell lines of known p53 sequence and function, replication of the E1B attenuated adenovirus, Onyx-015, was tested by cytopathic effect assays, hexon protein staining and quantified by flow cytometry. These assays showed that replication was more efficient in cell lines with non-functional p53 confirming the previously published work in other cell types by Bischoff et al,1996. The cell line BICR16, derived from a patient with recurrent head and neck cancer, was used for in-vivo testing by intratumoural injection of

subcutaneous xenografts in nude mice. We showed tumour regression in all virus injected tumours. In contrast, diluent injected tumours continued to grow exponentially.

To determine the incidence of p53 inactivation in recurrent head and neck cancer, the p53 status of 22 recurrent head and neck tumours was evaluated by gene sequencing, mdm2 expression and HPV expression. Overall we have shown that there is a greater incidence of p53 mutations in recurrent disease compared to primary disease. However the incidence of HPV infection and mdm2 overexpression was similar to reported studies in primary disease. Nevertheless, we show that the overall incidence of p53 alterations is very high at 95%. This may be one factor which accounts for the poor response of this disease to radiotherapy and chemotherapy and implies new therapies which either restore p53 function or which act in a p53 independent manner may prove to be beneficial in this disease. Thus recurrent head and neck cancer was considered to be a suitable tumour type in which to carry out a phase I study using Onyx-015.

Two phase I trials were carried out - a single intratumoural injection protocol and a multiple (5 daily injections) intratumoural injection protocol. The phase I trial by single intratumoural injection was carried out in 22 patients with recurrent head and neck cancer. Six cohorts were investigated, with dose escalation from 10^7 -10^{11} pfu. 20 patients had received previous radiotherapy and 8 had received prior chemotherapy. Patients were eligible for retreatment four weekly. Treatment was well tolerated with the main toxicity being mild Grade I/II flu-like symptoms and occasional discomfort at the injection site. Dose limiting toxicity was not reached at the highest

dose of 10^{11} pfu. However, further dose escalation was not carried out because the maximum virus manufacturing capacity had been reached. In-situ hybridisation showed viral replication in 4 of 22 post-treatment biopsies all of which had mutant p53. However there was no statistically significant correlation between viral replication and p53 status. MRI scans were suggestive of central necrosis of injected tumours in 5 cases, 3 of whom were measured using non-conventional criteria as partial, and 2 as minor responses. A further 8 patients had stable disease in the injected tumours. The response duration for the 3 partial responses were 4, 8 and 12 weeks. Of the 5 patients showing a tumour response, 4 had mutant p53 on gene sequencing suggesting a selective response in p53 mutant tumours. However this was not statistically significant. Despite evidence of local response, in most cases progression of disease at uninjected sites eventually occurred. A multiple injection study was then carried out in 10 patients by 5 daily intratumoural injections. Toxicity was more frequent with 30% of patients reporting flu-like symptoms compared to 10% in the single injection protocol, but toxicity grade was not increased remaining at Grade I or II. There was improved viral distribution with 5 out of 7 tumours being positive for virus (3 patients did not have post-treatment biopsies done). However, only 1 of the 10 patients treated had a partial response though this was of longer duration than the single injection responses. To further evaluate the efficacy of a multiple injection protocol we are currently proceeding with a phase II study by multiple intratumoural injection in the same group of patients. In addition, recent work has shown additive cytotoxicity with virus combined with chemotherapy and radiotherapy (Heise et al,1997; Ganly et

al, unpublished results) and therefore a combination study with intratumoural virus and intravenous cisplatin/5FU is underway (Nemunaitis et al,1998).

PUBLICATIONS FROM THESIS.

The following papers have been accepted or submitted for publication from the research described in this thesis:

1. Ganly I, Kaye SB. Adenoviral therapy for tumours with non-functional p53. Surgery; Recent Advances Jan,ii-iii,1998.

2. Ganly I, Soutar D, Brown R, Kaye SB. p53 alterations in recurrent squamous cell carcinoma of the head and neck cancer refractory to radiotherapy. British Journal of Cancer (2000),82:392-398.

3. Ganly I, Kaye SB. Recurrent head and neck cancer- current therapy and future prospects. Annals of Oncology (2000),11:1-6.

4. Ganly I, Soutar DS, Kaye SB. Current role of gene therapy in head and neck cancer. European J of Surgical Oncology (2000),26:338-343.

5. Ganly I, Kirn D, Soutar D, Eckhardt G, Otto R, Robertson A G, Park O, Hiese C, Von Hoff D D, Kaye SB. A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumourally in patients with recurrent tumours of the head and neck. Clinical Cancer Research (2000), 6:798-806.

6. Khuri F, Nemunaitis J, Ganly I, Arseneau J, Tannock I, Romel L, Gore M, Ironside J, Heise C, Randlev B, Gillenwater A, Bruso P, Kaye SB, Hong WK, Kirn D.A controlled trial of intratumoural ONYX-015, a selectively replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. Nature Medicine (2000) ,6(8): 879-885.

Chapter 1
Introduction

1.1. GENERAL INTRODUCTION

Human adenoviruses have great potential as delivery vehicles for genes to treat many human diseases, including cancer (Ginsberg,1996; Ko et al,1996; Esandi et al,1997; Quillien et al,1997; Wilson ,1996). An alternative approach to cancer therapy involves the use of replication competent adenoviruses which can selectively replicate in and cause lysis of cells which lack wild type p53 function (Bischoff et al, 1996). The E1B attenuated adenovirus, Onyx-015, has an 800bp deletion in the E1B region which codes for the 55kDa protein of E1B. The normal function of this protein is to bind to and inactivate the p53 protein in infected cells. Since Onyx-015 lacks this protein it is unable to replicate in cells with functional p53 since it cannot inactivate p53. However, it will replicate effectively in cells with non-functional p53 causing cytolysis (**Figure 1**). This approach has potential advantages over the traditional therapies of chemotherapy and radiotherapy because of the possibility of targeting tumours at a molecular level leaving normal tissue unaffected.

p53 is the most important tumour suppressor gene and plays an important role in the negative regulation of the cell cycle (Lane et al,1992). Its normal function is to recognise and respond to DNA damage induced by radiation and other cytotoxic agents, causing either a G1 arrest or apoptosis (Levine,1997 for review). p53 is the most commonly mutated gene in human cancer and is inactivated in over 50% of human tumours (Hollstein et al, 1991). The incidence of p53 mutation in primary head and neck squamous cell cancer varies from 38% -53% (Boyle et al,1993; Brachmann et al,1992; Koh et al,1998; Mao et al,1996; Olshan et al,1997). In recurrent HNSCC the

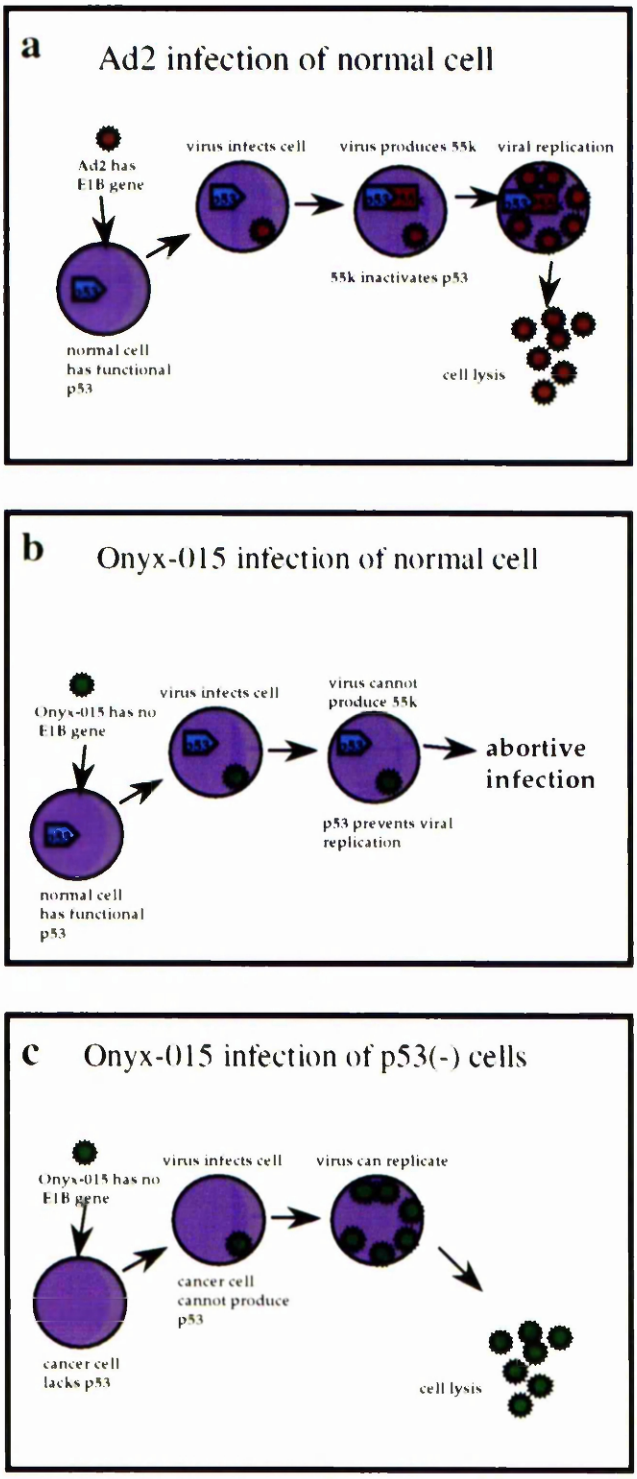


Figure 1. Schematic diagram showing the mechanism of action of the E1B55kDa deleted adenovirus,Onyx-015.

incidence may be even higher. The prognosis for patients with recurrent head and neck cancer is also very poor with average survival of between 3-6 months and current treatment options including brachytherapy (Nickers et al,1997) and chemotherapy (Clavel et al,1994) yield only short lasting responses with no significant survival benefit (Forastiere et al,1994). There is therefore a great need for new forms of therapy for this disease. Therefore a therapy such as Onyx-015, which selectively destroys tumours with mutant p53, may be particularly useful in the treatment of recurrent head and neck cancer. The objectives of this thesis were therefore to determine by in-vitro and in-vivo studies whether squamous cell carcinoma of the head and neck would be a suitable target tissue for Onyx viral therapy and to carry out a phase I dose escalation trial using Onyx-015 in patients with recurrent p53(-) squamous cell cancer of the head and neck.

1.2. SQUAMOUS CELL CANCER OF THE HEAD AND NECK .

1.2.1. Epidemiology.

Squamous cell carcinoma of the head and neck includes cancer of the oral cavity, pharynx, larynx and paranasal sinuses. It is a cancer which has a high mortality and which causes significant morbidity with problems with speech, swallowing and pain control. It is the 6th most common cancer world-wide (Parkin et al, 1984). In the USA , 45,000 new cases of head and neck cancer are diagnosed each year, and 14,000 people die from the disease (Boring et al, 1993). In white men, the incidence is decreasing but in black men it is increasing (Miller et al, 1992).

Epidemiological studies have shown a strong association with alcohol and tobacco abuse. Tobacco usage is the single most important aetiological factor and increases the risk of HNSCC by a factor of x5-25 (Rothman et al,1980). Recently it has been shown that tobacco carcinogens can induce specific mutations in the p53 tumour suppressor gene with G to A transitions being the most frequent mutation (Brennan et al, 1995). In patients who continue to smoke, there is a high incidence of second primary tumours (10-30% of patients) with a 2-3% risk each year (Silverman et al, 1983; Wynder et al , 1969). Smokeless tobacco (snuff) is also associated with the development of HNSCC due to the presence of carcinogens such as N-nitrosoamines, polonium-210 and benzopyrene (Hoffman et al, 1986). Alcohol increases the risk of HNSCC and there is a synergistic interaction with tobacco (Saracci et al, 1987). Dietary factors are also important and epidemiological and laboratory studies have shown that carotenoids (Vitamin A and synthetic retinoids) inhibit epithelial carcinogenesis. Studies have shown an inverse relationship with HNSCC formation and Vitamin A consumption (Mackerras et al,1988; Marshall et al, 1982). Chemoprevention trials with the synthetic retinoid 13-cis-retinoic acid have shown the effective inhibition of the development of second primary tumours (Hong et al, 1990). Poor oral hygiene increases the risk of oral cancer by a factor of x3 (Graham et al, 1977). Occupational factors are small but important aetiological factors. Wood dust, exposure to organic compounds and coal products are important factors (Maier et al, 1991). Nickel and asbestos exposure are also related mainly in laryngeal cancer (Burch et al, 1981). There is also a small familial risk with an increased risk of HNSCC in first

degree relatives (Cloos et al, 1996; Foulkes et al, 1995; Foulkes et al, 1996). Heritable factors which increase genetic susceptibility include DNA repair capability (Hsu et al, 1987), ability to metabolise carcinogens (Henderson et al,1998; Matthias et al,1998), altered oncogene and tumour suppressor gene function. DNA repair capability can be measured by a mutagen sensitivity test using bleomycin (Spitz et al, 1989; Schantz et al, 1990).

1.2.2. Classification and Staging of HNSCC.

Accurate staging in head and neck cancer is critical as it determines treatment modalities, survival and locoregional recurrence rate. The classical method of staging is the TNM classification system (Beahrs et al, 1988; Robbins,1991). This classification system is determined by direct visualisation of the tumour clinically or by endoscopy and palpation of the neck under a general anaesthetic, radiological visualisation by MRI, CT or U/S , and histological confirmation of cancer. T indicates the size, extent of invasion and site of invasion of the primary tumour. N describes the extent of cervical lymph node metastases and is determined by the location and size of the nodes. M indicates the presence or absence of distant metastases. For example, for oral cancer the TNM classification is as below:

<u>TNM classification of oral cavity cancer:</u>

T T1= <2cm.

T2= >2cm <4cm.

T3= >4cm.

T4= Extension to bone/muscle.

N N1= Ipsilateral single lymph node < 3cm

N2= Ipsilateral single lymph node > 3cm but < 6cm or

Ipsilateral multiple lymph nodes < 6cm or

Bilateral,contralateral lymph nodes < 6cm

N3= Lymph nodes > 6cm

M Distant metastases.

Metastatic spread to cervical lymph nodes is of poor prognosis since there is a 50% decrease in survival, increase in metastatic spread, and increase in locoregional recurrence (Snow et al, 1982). The presence of positive lymph nodes increases the chance of distant metastases. Leemans et al, 1993 showed that if extranodal spread is present the incidence is 19.1% and if 3 or more nodes are positive the incidence of distant metastases increases to 46.8%. Therefore in patients with 3 or more positive lymph nodes and/or extranodal spread there is an argument for adjuvent systemic therapy. Since the presence of positive nodes in the neck significantly affects the prognosis, the identification of clinically occult metastases is critical for staging. Unfortunately, light microscopy frequently misses micrometastases since the detection rate is only 1 cancer cell in 20 normal cells. Recent advances in molecular biology have lead to the development of PCR methods which detect 1 cancer cell in 10,000 normal cells. Brennan et al, 1995 used a molecular probe against mutant p53 to detect cancer cells in cervical lymph nodes. 21% of patients with negative nodes on light microscopy were positive by molecular probing resulting in a more advanced staging group in these patients. These molecular techniques give a

better prediction of survival, prognosis and a better determination of treatment modalities (Brennan et al, 1996).

1.2.3. Treatment of primary HNSCC.

Treatment of HNSCC depends on the stage of disease. Early stage disease is curative and consists of either surgical excision and reconstruction followed by adjuvant radiotherapy or radiotherapy alone. Late stage disease is considered noncurative and any treatment is for palliation. This may consist of external beam radiotherapy, local radiotherapy using radioactive implanted wires (Brachytherapy), systemic chemotherapy, surgery or a combination of these therapies.

The two most common and important causes of treatment failure are local recurrence and the development of second primaries. Only 10-15% of patients die of distant metastatic disease (Whitaker DC, 1987).

1.2.4. Recurrent disease.

a) Aetiology

There are many factors which predispose to the development of locoregional recurrence. The most important factor is incomplete surgical resection margins (Snow et al, 1989; Jones et al, 1992). This is determined not only by the experience of the surgeon but also by the site of the primary tumour which may limit the extent of surgical excision. For example, in larger tumours of the oropharynx and oral cavity it may be difficult to obtain adequate surgical resection margins and still preserve organ function. Recurrence in the neck is more likely if there are multiple nodes present and

if there is extranodal spread of disease. As well as site and extent of surgical resection, recurrence is also determined by the dose and timing of adjuvent radiotherapy. Delays in radiotherapy treatment beyond 6 weeks post surgery and radiation doses less than 54 Gy have been shown to result in poorer survival and disease free intervals (Peters et al,1993). Other factors which predispose to recurrence include poor differentiation of the primary tumour and evidence of neurogenic and vessel invasion. Woolley et al,1992 also showed an increase risk of recurrence if blood transfusion was carried out at the time of primary surgery. Molecular markers are also important. Amplification of the oncogene Cyclin D1 has been associated with recurrence and shortened survival (Michalides et al,1995). Brachman et al,1992 has shown that patients with mutations in the tumour suppressor gene p53 have a shorter time to recurrence. Overexpression of p53 has been shown to correlate with an increased incidence of recurrence and the development of second primaries and hence reduced survival (Shin et al,1996). Antibodies to p53 have also been shown to be of prognostic significance (Werner et al,1997). In a prospective study of primary head and neck squamous cell cancer (HNSCC), patients with positive serum antibodies to p53 had an increased incidence of tumour recurrence , second primaries and tumour related deaths (44.7% in antibody positive group compared to 21.1% in antibody negative group).

The single most important factor which predisposes to recurrence is incomplete surgical margins (Snow et al, 1989; Jones et al, 1992). The best treatment for incomplete surgical margins is reoperation (Zieske et al, 1986) or radiotherapy if the morbidity from further surgery is too great. The

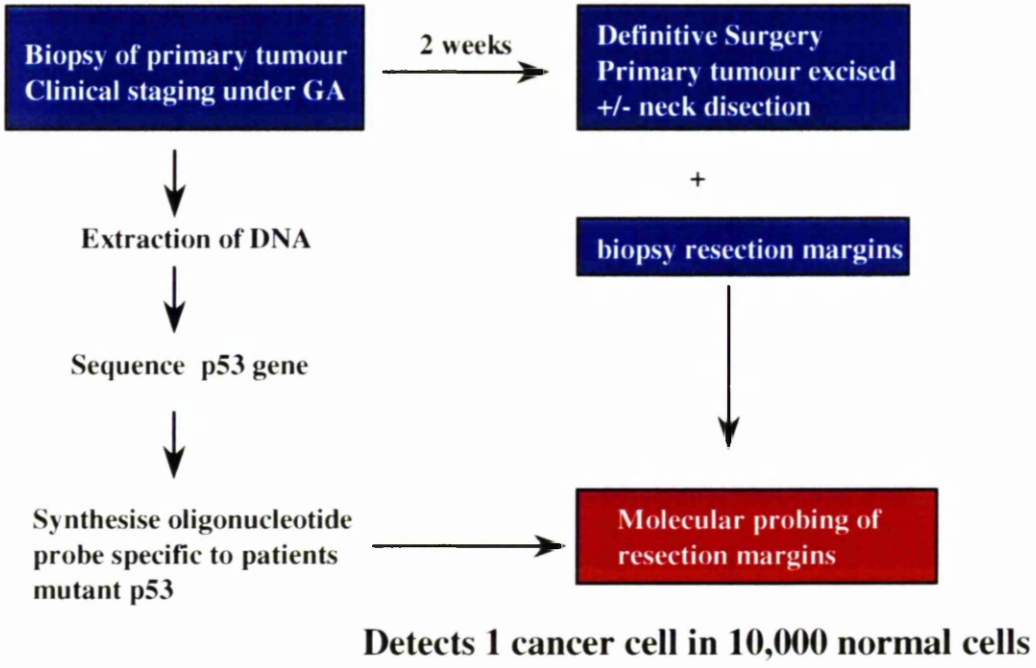


Figure 2.
Molecular analysis of surgical resection margins using a molecular probe to the mutant p53.

detection of tumour cells at resection margins is by light microscopy. However, light microscopy only detects 1 cancer cell in 20 normal cells (5%) yet <1% of tumour cells in the total cell population is associated with recurrence. More sensitive methods are therefore required to detect tumour cells at resection margins. Recently Brennan et al, 1995 used a molecular probe against the patients mutant p53 to detect tumour cells at resection margins. A schematic diagram of this method is shown in **Figure 2.** Using this technique, 25 patients with negative margins on light microscopy were evaluated. 13 were positive by molecular probing and 12 were negative. All those that were negative had no recurrence at follow up. Of the 13 positive, 5 developed recurrence and 8 did not. Moreover, the site of recurrence was at the site of positive tumour margin. As a consequence of this study a large multicenter trial has commenced examining the feasibility of this approach in the detection of incomplete resection margins. This is clearly important since these techniques would help to determine treatment. Patients with positive margins can be treated more aggressively by reoperation and adjuvant radiotherapy and closer follow up. Those with negative margins by molecular probing may not need adjuvant therapy thus reducing morbidity. At present these techniques are limited by cost and time but with advancing technology these will be overcome.

b) Management

Improvements in surgical reconstruction due to the development of microvascular free tissue transfer, better radiotherapy protocols such as hyperfractionation and chemoradiation (Zidan et al,1997; Adelstein et al,1997; Koness et al,1997; Lavertu et al,1999) have led to improvements in

locoregional control of head and neck cancer. As a result, more patients present with distant metastatic disease than before and therefore purely recurrent disease is less frequent. As a consequence, many clinical trials describing new treatments for recurrent head and neck cancer often include patients not only with locoregional recurrence but also with distant metastatic disease and locally advanced primary disease. Therefore when we discuss the management of recurrent disease it should be noted that many clinical trials include this mixed group of patients and this should be considered when interpreting any conclusions drawn from these clinical trials.

i) Surgery

Surgery plays a role in the treatment of both local and regional recurrence but only where curative surgery is possible or surgery results in significant palliation of symptoms. Recurrence in the neck can be treated surgically if the neck has not been previously treated either with surgery or radiotherapy. If a selective neck dissection has been carried out and recurrence has occurred outside the neck dissection field a complete radical neck dissection can be done in this case. However, usually the neck has already been treated with radiotherapy. In this case it is technically demanding to resect the tumour due to fixation of the tumour to local structures such as the carotid artery or vertebrae and it is also likely to be unsuccessful because of extranodal spread of disease. Surgical treatment of local recurrence is dependent on the site and previous treatment of the primary tumour. T1 and T2 tumours of the glottic larynx are treated with

radiotherapy and recurrence in this situation can be treated with curative intent with either a partial laryngectomy (Kooper et al,1995; Nibu et al,1997) or total laryngectomy. Surgical salvage can also be carried out for supraglottic tumours though the chance for cure is less than for glottic tumours (Tu et al,1997). Recurrence in the oral cavity (oral vestibule, buccal mucosa, gums, floor of mouth, oral tongue, hard palate, retromolar trigone) of small T1 and T2 tumours is difficult to detect early if the patient has had radiation treatment. However with close follow up these patients can be successfully salvaged surgically (Meyza et al,1991). With larger T3 and T4 oral cavity tumours, primary surgery and radiotherapy often fail and further surgery is rarely possible nor curative. In the oropharynx (base of tongue, faucial arch, tonsillar pillars and fossa and posterior pharyngeal wall) recurrence can be treated surgically if it involves the tonsillar pillars or fossa. However recurrence of the base of tongue is rarely curative since these tumours are hard to detect and therefore present late. Radical surgery in the form of a total glossectomy and laryngectomy is often required but even with such surgery further recurrence often occurs within 1 year and long term survival is very poor.

ii) Reirradiation.

Surgery and adjuvant radiotherapy or radiotherapy alone are the main therapies for primary head and neck cancer. For recurrent disease, the main concern with reirradiation is damage to previously irradiated tissues such as the spinal cord, skin and salivary gland. External beam radiotherapy can be carried out but is best when the time interval from primary radiotherapy is greater then 12 months to reduce any potential toxicites. Local control of

50% can be achieved if the radiation dose is greater than 60 Gy. Alternatively, lower doses of radiation can be given either with brachytherapy (Nickers et al, 1997; Harrison et al,1997) or by combining external beam radiotherapy with chemotherapy (chemoradiation).

Low dose radiotherapy in the form of brachytherapy has been shown to be effective in the treatment of primary head and neck cancer, especially in base of tongue tumours. However it also has a role in recurrent disease. Because the dose of radiation is lower (20-40Gy) and the depth of radiation penetration small, toxicity in previously irradiated tissue is limited. Nag et al,1996 have reported local control of 17% in patients using I^{192} implanted wires. The dose of external beam radiotherapy can also be reduced by combining with systemic chemotherapy (chemoradiation). Haraf et al, 1996 reported a 5 year survival of 22% and progression free survival of 26% using a combination of cisplatin/5FU/Hydroxyurea with radiotherapy (median dose 50Gy). Interstitial radiotherapy can also be combined with local tumour interstitial hyperthermia since hyperthermia kills hypoxic and S phase cells, neither of which are killed by radiation. Petrovic et al, 1989 reported a complete response of 68% and partial response rate of 32% in sites treated by this technique. Recently it was reported that gemcitabine (an analogue of deoxycytidine) was a potent radiosensitizer (Shewach et al,1996). Using a low dose of gemcitabine 2 hours pre-radiotherapy, a phase I trial by Eisbruch et al,1997 in 8 patients gave a complete response in 7 patients. However there was excessive mucosal and pharyngeal toxicity at the dose used and therefore studies using lower doses are planned.

iii) Chemotherapy

Chemotherapy may be given by systemic, intratumoural or intraarterial administration. Systemic chemotherapy may be given either as a single agent e.g. methotrexate or in combination e.g. Cisplatin/5FU. Treatment with combination chemotherapy increases overall response rates to 30-40% (Vokes,1996) compared with single agents where the response rate is only 10-20% (Jacobs et al,1992), but there is no improvement in survival (median survival is 6 months in both cases) (Forastiere et al, 1994). In a randomised trial carried out by the EORTC (European Organisation for Research and Treatment of Cancer), 382 chemotherapy naive patients with recurrent or metastatic head and neck cancer were randomly assigned to either a combination of CABO (cisplatin, methotrexate, bleomycin, and vincristine), cisplatin and infusional 5 flurouracil , or cisplatin alone (Clavel et al, 1994). Major responses were similar for CABO (34%) and cisplatin / 5FU (31%) but better than cisplatin alone (15%). However, there was no difference in progression free survival and overall survival between the 3 arms.

Intratumoural chemotherapy has been reported using a cisplatin/epinephrine gel suspension. In a study by Burris et al,1996, 45 patients had weekly intratumoural injection for 4 weeks. The response rate of treated tumours was good with 50% of tumours responding to therapy of which 40% were complete responses. However 50% of patients developed local toxicity with pain on injection, swelling, ulceration and reports of carotid artery blowouts and this has limited its use. However intratumoural electrochemotherapy (ECT) may provide improved responses with less local

tissue toxicity. Electrochemotherapy involves the intratumoural injection of low dose cytotoxic (cisplatin, bleomycin) and then the application of an electric current via electrodes which penetrate the tumour (electroporation). Electroporation causes a transient increase in cell membrane permeability and allows larger molecules to penetrate the cell. In vivo studies have been encouraging for a wide range of tumour types including head and neck (Hasegawa et al,1998; Nanda et al,1998). Studies have also been carried out in head and neck cancer patients. Panje et al,1998 reported 5 complete responses and 3 partial responses in 10 patients treated by electrochemotherapy using a low dose of bleomycin.

Intraarterial chemotherapy has also been reported. In a phase I trial carried out by Robbins et al, 1994, major responses were 86% (41% CR) in 22 untreated advanced stage patients and 62% (25% CR) in 16 recurrent disease patients.

Chemotherapy combined with biological agents such as interferon has been reported but with no beneficial effects (Benasso et al, 1993; Cascinu et al, 1994; Huber et al,1994). Benasso et al, 1993 treated 14 patients with cisplatin/5FU and recombinant interferon alpha-2b with a major response rate of 54% (31%CR). However, Cascinu et al, 1994 treated 34 recurrent or metastatic disease patients with a similar protocol with a major response rate of only 23%.

New chemotherapeutic agents are currently being evaluated. Docetaxel (a taxane) produced a response rate of 32% of median duration of 6.5 months when given to 37 patients with recurrent or metastatic disease (Catimel et al, 1994). Paclitaxel has produced an overall response rate of

40% in a phase II trial reported by Forastiere et al,1998. Interestingly response rates were increased to 67-77% when paclitaxel was combined with cisplatin (Schilling et al,1997; Hitt et al,1997). However when paclitaxel was combined with carboplatin (Fountzilas et al,1997) a response rate of only 23% was observed. These differences are presumably due to the differences in patients studied in respect to site of tumour and previous treatment. However, it is clear that paclitaxel has biological activity in head and neck cancer and has a role in the treatment of recurrent disease either as a single agent or in combination (Shin et al,1999).

Other new chemotherapeutic agents include the antimetabolite gemcitabine, an analogue of deoxycytidine. This has shown only 7 partial responses (13% response rate) in 54 patients with recurrent or metastatic disease (Catimel et al, 1994). However, when combined with Paclitaxel a response rate of 41% (11% CR and 30% PR) has recently been reported. Although this response rate is greater than combination therapy with cisplatin/5FU, the median time to progression of 4 months and median survival of 9 months are not significantly better than cisplatin/5FU therapy.

Other new agents include the antimetabolite thymetaq (Belani et al,1997), the Topoisomerase I inhibitor topotecan (Robert et al,1997) and Topoisomerase II inhibitor amonafide (Leaf et al,1997). Response rates from these agents have been poor at 14%, 14% and 3% respectively and it is unlikely that they will have any role in the treatment of recurrent disease.

Therefore, overall it is generally agreed that results of chemotherapy show only moderate response rates and these are of short duration producing little or no increase in survival. New chemotherapeutic agents have failed to

show any significant improvement in response with the possible exception of the taxanes. In addition, although different delivery systems in the form of intraarterial or intratumoural injection of chemotherapeutics have shown better responses, they are limited by their local tissue toxicity and also by the technical difficulty particularly in intraarterial administration.

iv) Biological therapies.

Biological therapies including immunotherapy and gene therapy have recently caused much interest. Such treatment has the potential to selectively target the tumour while leaving normal tissue relatively unaffected. These therapies are discussed in detail later (see 1.5. Gene therapy in head and neck cancer page 45).

1.2.5. Development of second primaries.

The other main cause of treatment failure is the development of second primaries in the aerodigestive tract including the oral cavity, lung and oesophagus. These are classified into synchronous (6 months from time of first primary) or metachronous (greater than 6 months). There are two current theories to the increased incidence of second malignancies. The first is the "field cancerisation theory" which is based on the hypothesis that the whole of the aerodigestive tract has been exposed to the same carcinogen (Slaughter et al, 1953). The second is the "common clonal origin theory" (Norwell,1976) in which all second and subsequent malignancies are believed to have developed from the same clone of malignant cells and these have proliferated to replace normal epithelium. There is evidence to support

both of these theories (Bedi et al,1996; Califano et al, 1996; Scholes et al, 1998). The overall incidence of second primaries is 10-20% (Schwartz et al, 1994; McGarry et al, 1992). However, the incidence varies with the site of the primary cancer. The 5 year second cancer incidence based on site of the primary cancer is 46% for base of tongue, 34% for pyriform sinus, 23% for larynx, 18% for oral cavity, 15% for tonsils and 10% for mobile tongue. The survival rate after the second cancer is also influenced by the site of the second cancer- the 5 year survival rate is 20% for second HNSCC, 3% for oesophageal cancer, 2% for lung cancer. Therefore the development of a second primary is almost always fatal. There is therefore a need for screening programs and chemoprevention trials in all HNSCC patients. Screening can be done by regular follow-up panendoscopy including laryngoscopy, bronchoscopy and oesophagoscopy. Chemoprevention of second cancers may be possible with retinoids (natural or synthetic derivatives of Vitamin A) since it has been reported that the synthetic retinoid 13-cis-retinoic acid (13cRA) can reverse oral premalignancy by inducing tumour cell differentiation and inhibiting proliferation (Braakhuis et al,1997). A clinical study by Benner SE et al, 1994 has also shown a reduced incidence of second primaries of 4% in those treated with 13cRA compared to 24% in untreated patients.

1.3. MOLECULAR GENETICS OF HEAD AND NECK CANCER

Head and neck carcinogenesis is a multistep process in which cells accumulate many genetic alterations followed by clonal expansion. These alterations include the activation of oncogenes and the inactivation of

tumour suppressor genes. It is estimated that between 6 to 10 independent genetic events are required for cancer formation (Renan et al,1994). A model for head and neck carcinogenesis has recently been proposed by Califano and Sidransky,1996. This is shown in **Figure 3**.

1.3.1.Cytogenetics

Progression in head and neck carcinogenesis is associated with increasing aneuploidy with complex chromosome abnormalities and rearrangements. Chromosomal losses occur on chromosomes 3p, 5q, 8p, 9p, 18q, 21q (Jin et al,1993; VanDyke,1994). Loss of 18q is associated with poor prognosis (VanDyke,1994) and loss of 3p21, 8p11, 14q are associated with reduced radiosensitivity (Cowan et al,1993). Areas of chromosomal gain or amplification occur at 3q, 5p, 7p, 8q and 11q13.

1.3.2.Tumour suppressor genes and allelic losses.

Generally, point mutation of one suppressor allele is followed by deletion of the 2nd allele and thus total inactivation of tumour suppressor function. Thus, chromosomal deletions (allelic losses) are markers for tumour suppressor gene loci. The advent of microsatellite markers, which are small DNA repeat units highly conserved throughout the human genome, has allowed the mapping of areas of chromosomal deletions and the location of tumour suppressor gene loci. This is because these microsatellites are able to distinguish the maternal and paternal alleles in normal DNA. This pattern can then be compared with tumour DNA in which deletion is represented by a loss of either the maternal or paternal allele. Using

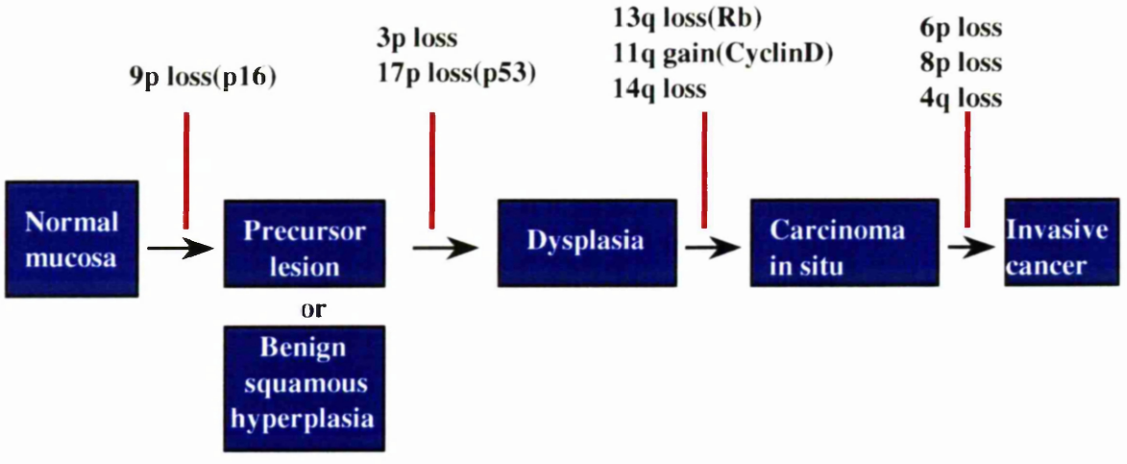


Figure 3.
Genetic progression model for squamous cell carcinoma of the head and neck.

microsatellite markers, allelotypes of head and neck cancer have now been reported (Nawroz et al, 1994; Ahsee et al,1994).

The most commonly deleted region (~66%) is on chromosome 9p21-22 which has been shown to harbour the tumour suppressor gene p16 (Serrano,1993). Allelic loss here occurs early and is present in early lesions such as dysplasias and carcinoma-in-situ (van der Riet,1994). Thus p16 inactivation is the first step in head and neck carcinogenesis. The mechanism of inactivation of p16 is only rarely by point mutation (Cairns et al,1994) and more commonly by homozygous deletion or methylation of the 5' CpG rich region of p16 (Merlo et al,1995; Herman et al,1995).

A 2nd commonly deleted locus is at chromosome 3p and again occurs early in head and neck carcinogenesis. Recently a new candidate tumour suppressor gene *FHIT* (fragile histidine triad) has been identified at 3p14.2 (Mao et al,1996). Over 80% of HNSCC cell lines have abnormalities in at least 1 allele of the *FHIT* gene suggesting it has a role in HNSCC carcinogenesis (Virgilio et al, 1996).

High loss also occurs on chromosome 17p at the location of the p53 tumour suppressor gene.This occurs after loss of 9p and 3p. Mutation of the p53 gene occurs in head and neck cancer in 38% -53% of patients (Boyle et al,1993; Brachmann et al,1992; Koh et al,1998; Mao et al,1996; Olshan et al,1997). Mutations are also found in premalignant lesions with a frequency of 33-35% (el-Nagger et al,1995; Mao et al,1996) indicating that p53 mutation occurs early in head and neck carcinogenesis and increases with tumour progression. Mutations are often G:T transversions located in exons 7 and 8. Other sites include codons 149, 274, 288, 296, 298. The prevalence

of mutations is also higher in patients who smoke tobacco or use alcohol chronically. The types of mutation in smokers is also different to non-smokers/non-drinkers (Brennan et al, 1995).

Loss of 13q occurs in 60% of HNSCC patients. The retinoblastoma gene is located at this site but analysis by immunohistochemical staining of HNSCC tumours shows that inactivation of Rb occurs in only a small percentage of tumours (Yoo et al, 1994). This suggests that there is another tumour suppressor gene at 13q.

Losses also occur at 18q and this is associated with a reduced survival (Rowley et al,1995). The highest incidence of LOH is at 18q21.1-21.3 using the microsatellite marker D18S35. 2 known tumour suppressor genes are located here - DCC and DPC4. However, these have not been shown to be implicated in HNSCC carcinogenesis (Kim et al, 1996). Other genes in this area are being investigated.

1.3.3. Oncogenes.

Amplification of chromosome 11q13 occurs in 30% of HNSCC patients. This is the location of the protooncogene Cyclin D1 (Somers et al, 1990). This amplification correlated with increased expression of the cyclin D1 gene (Jares et al, 1994). Moreover, DNA amplification of Cyclin D1 correlates with progression in head and neck cancer (Jares et al, 1994; Callender et al, 1994). Overexpression is associated with early recurrence and shortened survival (Michalides et al, 1995).

Mutations in the Ras oncogene occur rarely in HNSCC and thus Ras is not thought to be of importance in HNSCC carcinogenesis. The epidermal

growth factor receptor is a receptor for extracellular growth factors TGFα and EGF. Binding of EGF to the receptor stimulates the growth of epithelial tissues. In HNSCC, amplification of EGFR gene has been shown in cell culture and fresh tissues (Yamamoto et al,1986; Eisbruch et al,1987) and is higher in poorly differentiated compared to well differentiated tissues (Scambia et al, 1991). The c-erb-B2 oncoprotein is a 185kDa transmembrane protein homologous to the EGF receptor. The gene is located on chromosome 17q21. In breast cancer it is amplified in 25-30% of cases and this is associated with early relapse and reduced survival. In HNSCC , erb-B2 expression is common but is not of prognostic significance (Craven et al, 1992; Field et al,1992). The myc gene family c-myc, l-myc and n-myc are nuclear proteins important in cell proliferation and differentiation. Overexpression of c-myc prevents cell differentiation and increases cell proliferation. Overexpression of c-myc is present in 48% of HNSCC and is correlated with reduced survival (Field et al,1989).

1.3.4. Telomerase

The telomerase enzyme is associated with telomere elongation and cell immortalisation (Rhyu et al,1995). It is activated in many human tumours and is undetectable in normal tissues (Shay and Wright,1996 for review). In HNSCC, a study by Mao et al, 1996 showed telomerase expression in 90% of primary HNSCC and 100% of premalignant lesions such as dysplasias and hyperplasias. Normal epithelium showed no expression. Mutirangura et al, 1996 showed similar results.This suggested

that telomerase activation occurs early in HNSCC and may play a role in early carcinogenesis.

1.3.5. HPV

Human papilloma virus (HPV) infection is important in the aetiology of HNSCC. The prevalence of HPV16 infection is 20-31% (Chiba et al, 1996). It has been shown that the E6 gene product of HPV16 inactivates the tumour suppressor gene p53 and promotes its degradation (Scheffner et al,1990). In addition, the E7 gene product inactivates another tumour suppressor, the retinoblastoma gene (Dyson et al,1989). Both of these events lead to increased cell proliferation and cellular transformation accounting for the role of HPV infection in head and neck carcinogenesis (Shindoh et al, 1995; Haraf et al,1996).

1.4. p53 AND HUMAN CANCER.

1.4.1.Molecular Structure of p53.

a) Domains of p53.

The p53 gene is located on chromosome 17p.21 and codes for a 393 amino acid nuclear phosphoprotein. p53 was originally discovered as a protein complexed to SV40 T antigen in SV40 transformed cells (Lane and Crawford, 1979). Its structure can be divided into 3 domains - the transcriptional activation domain at the N terminus, the sequence specific DNA binding domain in the centre, and the oligomerisation domain at the C terminus. There are 5 conserved regions (conserved through evolution) , 4 of which are located in the DNA binding domain **(Figure 4).**

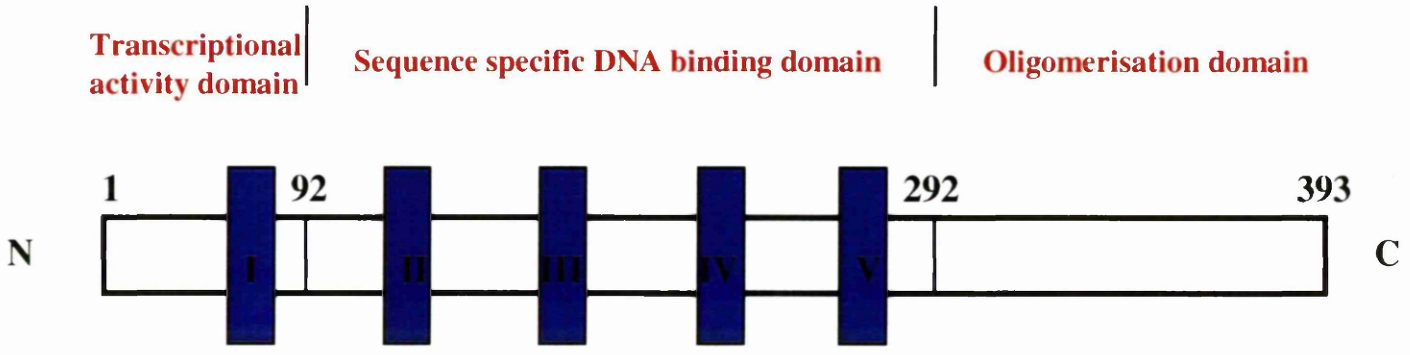


Figure 4.
Functional domains of the tumour suppressor p53. The 5 conserved domains are indicated in blue(I-V). 4 of the conserved regions are located at the DNA binding domain. p53 mutations occur mostly in these conserved domains

p53 is present predominantly as tetramers through the oligomerisation domain (Cho et al,1994; Jeffrey et al,1995). Dimers and monomers account for less than 5% of the total p53 molecules in a cell. The sequence specific DNA binding domain (Pavletich et al, 1993; Wang et al,1993) is very important as p53 regulates transcription through this region. p53 binds specifically to a p53 responsive element that contains repeats of the consensus binding sequence Pu-Pu-Pu-C-A/T-T/A-G-Py-Py-Py where Pu and Py are purine and pyrimidine nucleotides respectively (El-Deiry et al,1992).The N terminus of p53 is the transcriptional activation domain. This region can transcriptionally activate or repress other genes. p53 acts as a transcriptional activator of *mdm2, bax, IGF-BP3, p21,GADD45, Cyclin G, Thrombospondin* (Dameron et al, 1994) and acts as a transcriptional repressor for the genes *PCNA* (Proliferating Cell Nuclear Antigen), *c-fos ,c-jun, IL-6 and bcl2* (Horikoshi et al, 1995).

b) Phosphorylation of p53.

p53 protein is extensively post-translationally modified, mostly by phosphorylation and this is important in regulation of p53 function (Meek,1994). p53 is phosphorylated at sites within its N terminal and C terminal regions by several protein kinases. One such kinase is the double stranded DNA dependent protein kinase, DNA-PK (Lees-Miller et al, 1990). It is a nuclear serine/threonine protein kinase. Activation of DNA-PK requires DNA double strand breaks or other discontinuities in DNA (Gottlieo et al,1993). DNA-PK phosphorylates human p53 at serines 15 and 37 (Lees-Miller,1992). The DNA-PK phosphorylation sites are localised

within the transcriptional activation domain of p53. These sites also interact with transcription factors such as the TAFs and cellular coactivators p300/CBP (Lill et al,1997). These phosphorylation sites are also located next to the region where p53 binds to mdm2 (Kussie et al,1996). Recently it was shown that DNA damage induces phosphorylation of p53 at serine 15 via DNA-PK and that this results in the disruption of the p53/mdm2 interaction (Shieh et al,1997) thus alleviating mdm2 inhibition of p53. Phosphorylation of serine 15 of p53 in response to DNA damage from ionising radiation has also recently been shown to occur by the ATM (Ataxia telangectasia) protein (Banin et al,1998; Canman et al,1998).

Relatively little is known about the enzymes which catalyse the dephosphorylation of p53 *in vivo*. However protein phosphatase 2A has been shown to dephosphorylate several phosphorylated sites on p53 *in vitro* (Scheidmann, et al 1991).

1.4.2. Activation of p53.

p53 is activated by DNA damaging agents such as ionising radiation and cytotoxics, hypoxia and UV light (Kastan et al,1991). DNA damage from ionising radiation causes p53 protein levels to rise in cells with an increase in transcriptional activity (Kastan et al,1992; Kuerbitz et al, 1992). Ionising radiation causes DNA damage by causing strand breaks in the DNA and these strand breaks signal p53 activation (Hartwell et al, 1989). It has been suggested that as little as one double strand break per cell can initiate the p53 pathway (Huang et al 1996). It has recently been shown that activation of p53 in response to DNA damage involves phosphorylation of

p53 at serine 15 causing a conformational change in p53. This phosphorylation involves kinases including DNAPK (Shieh et al,1997) and ATM (Banin et al,1998; Canman et al,1998) as described above.

Exposure of cells to hypoxic conditions results in increased p53 protein levels and can lead to p53 dependent apoptosis (Graeber et al, 1996). It is widely believed that this induction of p53 occurs in the absence of DNA strand breaks. It has been shown that depletion of ribonucleotide triphosphates (rNTP) using inhibitors of purine and pyrimidine biosynthesis, is sufficient to induce p53 and p53 dependent G1 arrest (Linke, et al 1996). It may be the case that an inadequate supply of rNTPs results in changes in the RNA macromolecule population, analogous to the way that dNTP inhibitors lead to DNA strand beaks.

1.4.3. Normal function of p53.

p53 is the most important tumour suppressor gene and plays a central role in the negative regulation of the cell cycle (Lane,1992; Levine,1997 for review). In response to DNA damage or foreign DNA synthesis, wild type p53 is stimulated causing either a G1 arrest or apoptosis of the cells. **Figure 5** shows a schematic diagram of p53 activation and p53 dependent G1 arrest and apoptosis.

a) G1 arrest.

Ionising radiation induced DNA damage can result in a G1 arrest which is mediated mainly by the p21 protein (El-Diery et al, 1993; Harper el al 1993). p21 mRNA levels, as well as protein levels, increase following exposure of cells to ionising radiation in a p53 dependent manner (Xiong et

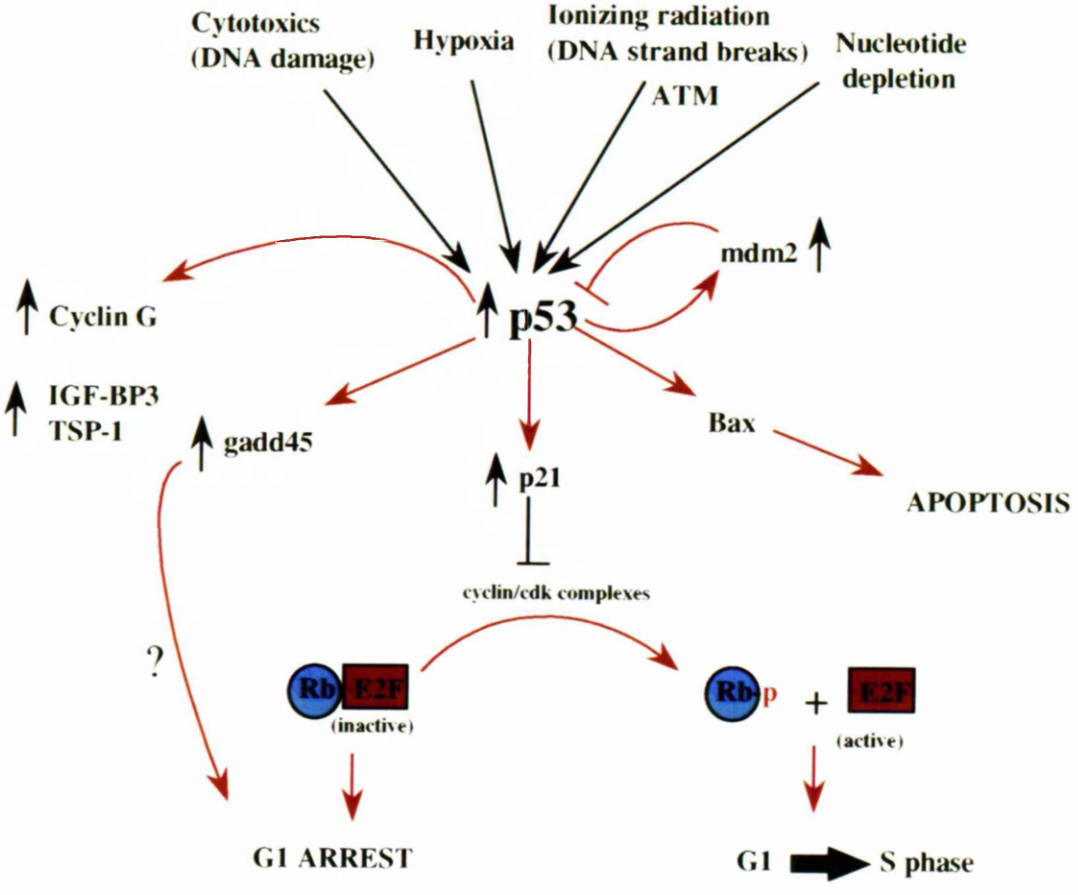


Figure 5.
Schematic diagram of p53 activation and p53 dependent G1 arrest and apoptosis.

al, 1993). p21 causes a G1 arrest by acting as a cyclin dependent kinase inhibitor preventing the phosphorylation of the Retinoblastoma gene product (pRb). In addition, p21 causes G1 arrest by inhibiting PCNA (proliferating cell nuclear antigen), a 36kDa protein capable of interacting with DNA polymerase α and which normally stimulates DNA replication and repair (Luo et al, 1995). The binding of p21 to PCNA does not affect the DNA repair activity of PCNA but is thought to alter the availability of PCNA for DNA replication. p21 is the most important mediator of p53 dependent cell cycle arrest. However other genes transcriptionally activated by p53 may play a role in DNA damage arrest. These include Cyclin G (Okamoto et al, 1996) and GADD45 (Kastan et al, 1992). Like p21, GADD45 also contains a p53 binding site and has been shown to be induced by p53 (Lu et al, 1993).

b)Apoptosis.

Apoptosis, or programmed cell death, is the process by which a cell actively commits suicide under a tightly controlled cascade of events. Apoptosis is recognisable microscopically by characteristic condensed basophilic cytoplasm, darkly staining nuclei, and the presence of apoptotic cell bodies. DNA from cells undergoing apoptosis is cleaved into fragments of roughly 200 base pairs. The signals that trigger apoptosis include the presence of DNA damage, growth factor or nutrient withdrawal, disruption of cell matrix interactions, altered expression of potent cellular oncogenes such as *myc*, and viral infection.

Apoptosis is controlled by the Bcl2 group of proteins which include the apoptotic factors bax (Oltavi et al 1993), bak and $bclx_s$ (Boise et al,

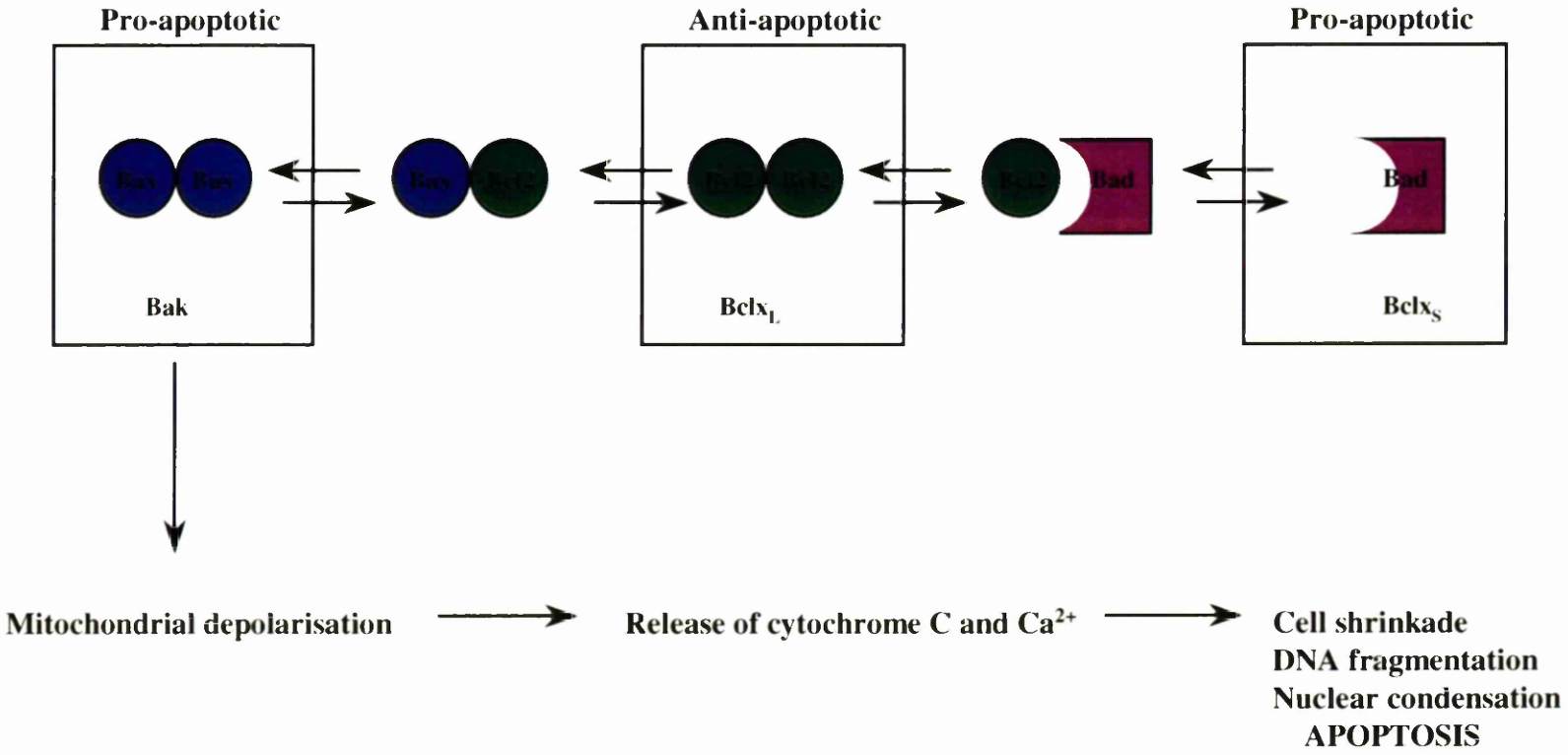


Figure 6. Control of apoptosis by the Bcl2 group of proteins.

1993) and the antiapoptotic (survival) factors Bcl2, bad and $bclx_L$. Bcl2 protein is an intracellular membrane protein that resides in the outer mitochondrial membrane, nuclear envelope and parts of the endoplasmic reticulum. The protein has been shown to block cell death induced by numerous stimuli including growth factor deprivation, Ca^{2+} ionophores, reactive oxygen species, some viruses, heat shock, and irradiation (Reviewed in White 1997).

p53 can transactivate *bax* (Selvakumaran et al, 1994) which contrasts with its ability to repress transcription of *Bcl2* (Miyashita et al, 1994). Thus in response to DNA damage, *p53* is activated causing activation of *bax* and repression of *Bcl2*. It has been proposed that when Bcl2 is in excess, Bcl2 homodimers predominate and cells are protected and when bax is in excess, bax homodimers predominate and cells are susceptible to apoptosis (Oltavi et al, 1993) **(Figure 6)**

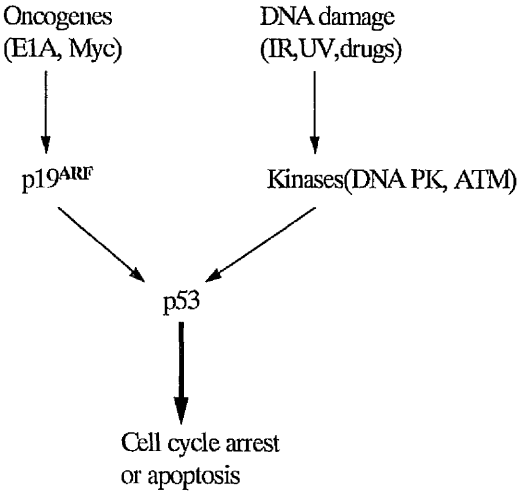
Although bax is the main mediator of apoptosis, the insulin-like growth factor binding protein 3 gene (*IGF-BP3*) may also be important in apoptosis (Buckbinder et al, 1995). IGF-BP3 protein has the ability to inhibit the signalling of insulin- like growth factor receptors and so plays an anti-mitogenic role in the cell. It can cause both G1 arrest and also apoptosis.

1.4.4. Regulation and stabilisation of p53.

p53 levels are controlled by the protein mdm2 (Barak et al, 1993). This protein has the ability to complex with p53 and inhibit its transcriptional activity. *Mdm2* is transcriptionally activated by wild type p53 in response to DNA damage. Thus an auto-regulatory feedback loop exists between mdm2 and p53. Overexpression of mdm2 inhibits p53 dependent G1 arrest in response to irradiation (Barak et al, 1993; Perry et al, 1993). It was been shown that mdm2 also promotes the degradation of p53. This is thought to be an important mechanism in ensuring that the p53 signal is quickly terminated (Haupt, et al 1997). It has been suggested that mutant p53 proteins have a longer half-life than wild type because they are not degraded by mdm2 (Midgley et al,1997). This is because mutant p53 is non-functional and is therefore not capable of activating mdm2 mediated degradation.

In response to DNA damage, p53 levels increase leading to increased transcription of mdm2. The auto-regulatory feedback loop between mdm2 and p53 would then lead to the early degradation of p53 and loss of p53 transcriptional activity. This is undesirable in a situation of DNA damage where prolongation of p53 transcriptional activity is required. Shieh et al,1997 recently showed that after DNA damage, N terminal phosphorylation of p53 occurs by DNA-PK at serine 15 and this disrupts the p53/mdm2 interaction. Thus p53 is stabilised and transcriptional activity is prolonged.

Another factor, $p19^{ARF}$-INK4a, has been reported to promote mdm2 degradation and therefore stabilise p53 (Zhang et al,1998; Pomerantz et al,1998). This mechanism of regulating p53 activity is distinct from that involving DNA damage since no phosphorylation of p53 occurs. It has been shown that oncogenes such as the adenoviral *E1A* and *Myc* regulate p53 dependent apoptosis by the induction of $p19^{ARF}$ (Zindy et al,1998; de Stanchina et al,1998). A schematic diagram showing the regulation of p53 is shown below.



1.4.5. Mutation of p53.

The most common somatic mutations in *p53* are single base substitutions (point mutations) in the conserved regions of the gene i.e. exons 5-8 (Hollstein et al, 1991) **(Figure 4)**. Most point mutations result in missense mutations which cause a change in a single amino acid. However a few result in frameshifts and premature termination of translation (Stop codons). These mutations are termed nonsense mutations. As well as single base substitutions, complete or partial deletions can also occur but these are not common. As a consequence of mutation, mutated p53 cannot bind or

regulate transcription through the specific DNA binding sequence. Therefore cells which sustain DNA damage are unable to respond by p53 mediated G1 arrest or apoptosis. Damaged DNA is retained leading to cellular transformation, genomic instability (Smith et al,1995) and the malignant phenotype. Germline mutations in p53 can also occur. Li-Fraumeni syndrome (LFS) is a rare autosomal dominant condition (Malkin et al,1990) in which patients are born with 1 mutated p53 allele and 1 wild type p53 allele. Mutation of the normal allele by somatic mutation results in p53 inactivation.These patients develop sarcomas in childhood (bone, soft tissues) and more rarely brain, lung, adrenocortical and bone marrow tumours.

1.4.6. Inactivation of p53.

As well as mutation and deletions, p53 may be inactivated by binding to cellular or viral proteins. The cellular factor mdm2 inhibits the transcriptional activity of p53 as previously described. Amplification or overexpression of mdm2 can therefore cause p53 inactivation mimicking p53 mutation. The mdm2 gene is frequently amplified in sarcomas (Oliner et al, 1992). Viral oncoproteins can also inactivate p53. Adenoviral E1B (55kDa) (Kao et al, 1990; Debbas et al, 1993; Sarnow et al, 1982) , HPV E6 (Lechner et al, 1992; Scheffner et al, 1990), and SV40 large T antigen (Gannon et al, 1987) all bind to p53 causing inactivation of the p53 protein. The same viruses also inactivate the other tumour suppressor gene Rb (retinoblastoma) by other proteins adenoviral E1A (Levine et al,1990), HPV E7 (Dyson et al,1989) and SV40 large T (Levine et al,1990).

1.4.7. p53 abnormalities in HNSCC.

a) p53 overexpression

p53 overexpression occurs in 60-68% of primary HNSCC (Watling et al,1992; Field et al,1991). Field et al, 1993 showed that overexpression correlated with smoking. p53 overexpression also correlates with an increased incidence of recurrence and the development of second primaries and hence reduced survival (Shin et al, 1996). Sauter et al, 1994 showed that over 50% of preinvasive lesions overexpress p53 suggesting that p53 overexpression is an early event and a marker for preinvasive lesions.

b) p53 inactivation

The p53 gene is frequently inactivated in HNSCC either by mutation, deletion or inactivation (by cellular factors mdm2 or viral factors HPVE6,E1B and SV40 proteins). The frequency of p53 mutations in primary HNSCC is 38% -53% (Boyle et al,1993; Brachmann et al,1992; Koh et al,1998; Mao et al,1996; Olshan et al,1997) and increases with progression. Tobacco carcinogens induce p53 gene mutations (Brennan et al, 1995; Lazarus et al,1996) and protein overexpression. Overexpression and mutation probably act at an early stage of squamous cell carcinogenesis (Shin et al, 1994; Sauter et al, 1995).

Brachman et al,1992 showed those patients with p53 mutations have shorter time to recurrence. In tumours with wild type p53, the p53 protein may be inactivated by HPVE6 and targeted for degradation by the ubiquitin degradation pathway. Brachman et al,1992 showed a 10% incidence of HPV

in primary HNSCC as determined by PCR and all were in tumours with wild type p53.

In recurrent disease the incidence of mutation is predicted to be higher. This is one reason why treatment with a selectively replicating E1B adenovirus which targets mutant p53 tumour cells may be worthwhile in recurrent disease.

c) p53 serum antibodies.

Antibodies to p53 have been shown to be of prognostic significance (Werner et al,1997). In a prospective study of primary HNSCC, patients with positive serum antibodies to p53 had an increased incidence of tumour recurrence, second primaries and tumour related deaths (44.7% in antibody positive group compared to 21.1% in antibody negative group).

1.4.8. Clinical importance of p53 inactivation.

p53 dependent apoptosis is one of the main mechanisms of cell death by many cytotoxic agents. Thus loss of p53 function results in reduced sensitivity to chemotherapy (Lowe et al,1993; Lowe et al,1994; Eliopoulos et al,1995) and radiotherapy (Lowe and Schmitt,1993). p53 inactivation is also correlated with poor prognosis and disease progression (Lowe et al,1994). Therefore p53 inactivation is very important clinically. Because of this, new agents which either restore p53 function or which selectively target cells which lack p53 are being developed. Such genetic based therapy represents an area which is rapidly expanding in cancer therapy.

1.5. GENE THERAPY IN HEAD AND NECK CANCER.

Treatment failure in head and neck cancer is predominantly from local or distant relapse following primary treatment or from the development of second primaries. In stage III or IV disease, which represents two thirds of patients presenting with head and neck cancer, locoregional recurrence occurs in 50-60% of cases and distant metastatic disease occurs in 25% of cases (Stupp et al,1994). Distant metastases is most frequent in the lung followed by bone, liver, skin and brain (Merino et al,1977). Locoregional recurrence is usually treated with reirradiation, brachytherapy or chemotherapy whereas distant metastatic disease is treated with chemotherapy. In both cases only 35% of patients show any response to therapy and this is usually of short duration lasting 6-9 months (Forastiere et al,1994). Because of this poor response to current therapy, there has been much interest in the development of new therapies such as gene therapy (Breau et al,1996) since these therapies have the potential to specifically target tumour tissue leaving normal tissue relatively unaffected thus limiting local and systemic toxicity. As well as treating recurrent disease, these forms of therapy may also be useful in the treatment of minimal residual disease, either by local injection of surgical resection margins and the surgical bed to kill local residual disease, or by systemic therapy to treat microscopic deposits of tumour at distant sites.

1.5.1. Principles of gene therapy.

Gene therapy involves the introduction of foreign DNA into somatic cells to produce a therapeutic effect (Fujiwara and Roth,1994 for review). The therapeutic gene is transferred into the tumour cells using a vector.

Transfer may either be *in-vivo* in which the DNA and vector are directly introduced into the body, or *ex-vivo* in which cells are removed from the body, transfected with DNA and then reintroduced into the patient.

1.5.2. Gene delivery systems.

The mode of gene transfer can be classified into chemical, physical and viral. In chemical transfection DNA is introduced into cells using calcium phosphate, liposomes or DNA/protein complexes. Although useful for laboratory transfections, these are inefficient in vivo due to their poor efficiency of transfection. In addition, liposomes and protein DNA complexes are rapidly cleared from the body via the immune system. Physical methods of DNA transfection are by electroporation, microinjection and the use of ballistic particles. Electroporation guns for the introduction of chemotherapeutics as well as genes are currently being tested in various cancers including head and neck. In addition guns which fire small gold particles coated with DNA have recently been developed and are being tested. However, the most important method of transfer of DNA currently being used is viral vectors. Viruses used include retroviruses, adenoviruses, adeno-associated viruses and herpes viruses. Retroviruses contain RNA genomes that are reverse transcribed after introduction to produce a double stranded cDNA. This integrates stably and heritably into random sites of the host genome. These viruses give a high efficiency of transfer. The host range of cells which they infect includes fibroblasts, epithelial cells and smooth muscle cells. Cells of lymphoid origin are more resistant. They give permanent gene expression which is advantageous when

we require long term expression of the transgene such as in cystic fibrosis. They are currently limited due to the low production titre that can be produced (~10^{7-8} pfu) which reduces transduction efficiency into solid tumours. Other disadvantages are that they only infect dividing cells, and that the stable random integration into the host genome may be dangerous if germ cell transfection occurs. They are also limited in the size of transgene which can be inserted (~7-8kb). Therefore, the use of retroviruses has recently been superseded by the use of adenoviral vectors (Wilson et al,1996; Ginsberg,1996).

Adenoviruses are double stranded DNA viruses. They can be made replication defective with deletion of the E1 and E3 regions of their genome. The transgene can then be introduced into the vacated site. Adenoviruses currently used can carry approximately 6-8kb of DNA. However because they have a larger genome than retroviruses they can potentially carry larger transgenes up to 36 kb DNA in size. They have a tropism for a broader range of cell types and because they can be produced at high titres, ~10^{12}pfu , they have a higher transduction efficiency. Another advantage is that they can infect both nondividing as well as dividing cells. There is no integration into the host cell genome and therefore only transient gene expression occurs. This is adequate for cancer therapy but would not be desired in situations where long term gene expression is required such as cystic fibrosis. Gene expression using adenoviruses usually lasts for 7-42 days. Other disadvantages are that they continue to express other viral gene products which are recognised by the immune system leading to an inflammatory response and short term gene expression, they may become

replication competent by genetic recombination, and because 90% of humans have antibodies to human adenoviruses pre-existing immunity may reduce the transduction efficiency to very low levels particularly on second exposure to the adenoviral vector.

1.5.3. Classification of cancer gene therapy.

There are four main types of genes which can be introduced into tumour cells:

1. genes which suppress the expression of an oncogene i.e. anti-oncogenes.
2. genes which restore a defective tumour suppressor gene(replacement gene therapy).
3. genes which enhance immune surveillance(immunotherapy)
4. genes which activate prodrugs into active chemotherapeutic agents(gene directed enzyme prodrug therapy i.e. GDEPT).

In head and neck cancer these therapies may be potentially useful in three clinical situations. Firstly, they may be useful for the treatment of locoregional recurrence by direct tumour injection or the treatment of distant disease by systemic therapy. Secondly, they may be used for the treatment of minimal residual disease by direct injection of the surgical resection bed and resection margins in order to prevent locoregional recurrence. Thirdly, they may be useful for the treatment of distant minimal residual disease by systemic therapy to target radiologically undetectable metastatic disease.

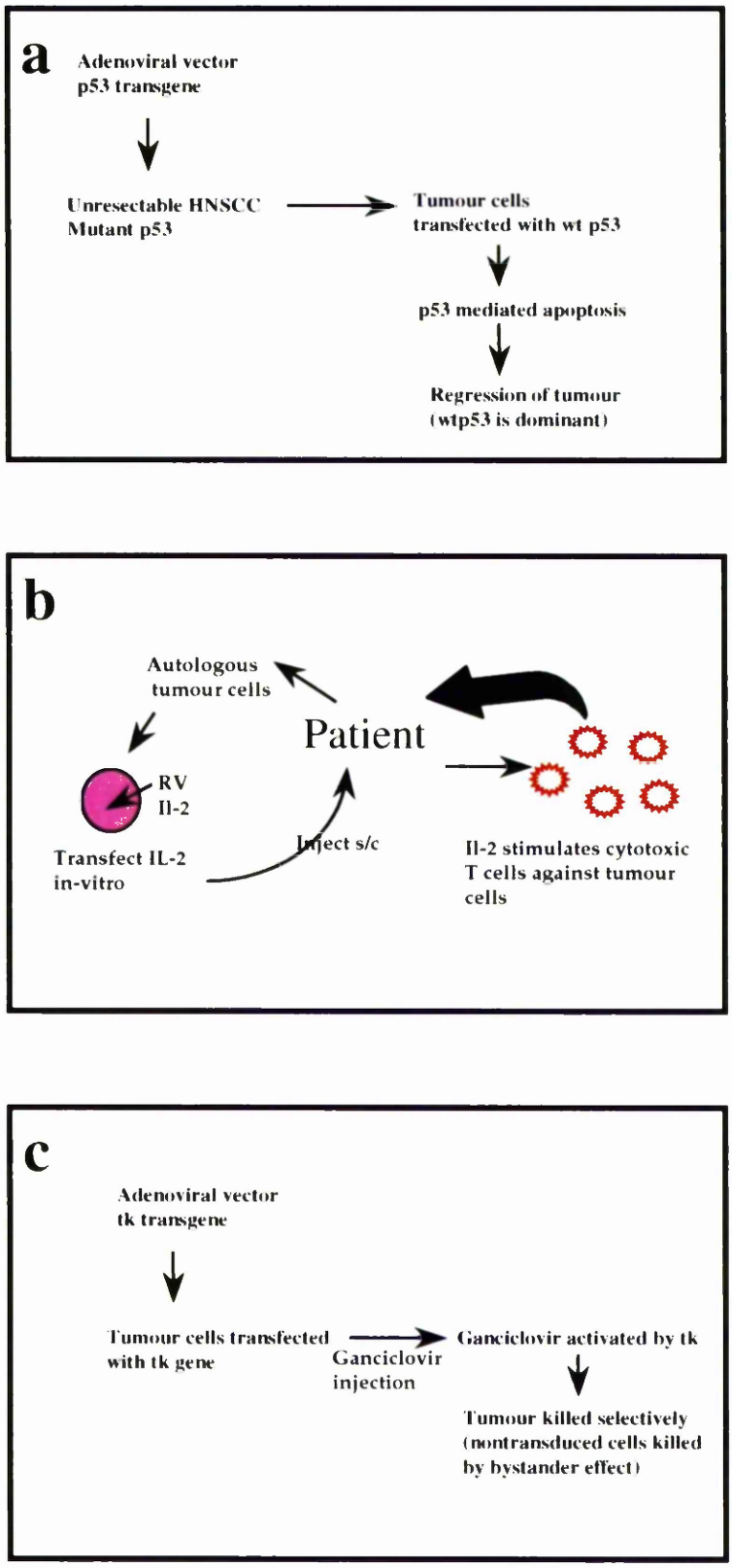


Figure 7. Examples of gene replacement therapy (7a), immunotherapy (7b) and GDEPT (7c) in HNSCC.

1.5.4. Current tumour suppressor gene replacement therapies in HNSCC.

There is currently one approved trial for squamous cell cancer of the head and neck. This utilises a replication deficient adenovirus for the transmission and overexpression of a wild type p53 gene (Clayman et al,1998). The hypothesis behind this is that the wild type p53 gene would be dominant over its mutant gene and select against proliferation and induce a p53 mediated apoptosis. In-vitro and in-vivo studies using such a technique have demonstrated growth suppression (Liu et al ,1994, Liu et al ,1995; Clayman et al, 1995). Phase I/II trials are underway at the MD Anderson Cancer Center, Texas, USA in patients with advanced local or regional cancer that is unresectable. Treatment is by direct intratumoural injection and is illustrated in **Figure 7a**. Results from the phase I trial showed no toxicity and evidence of tumour regression was found in some patients (Clayman et al, 1998). Analysis of tumour biopsies showed expression of the p53 transgene and evidence of apoptosis was also detectable. Another tumour suppressor gene that could be replaced is p16 since in HNSCC there is a very high frequency of p16 inactivation either by mutation, deletion or methylation (Reed et al,1996). It is believed to be one of the first steps in head and neck carcinogenesis and therefore would be a ideal target for gene replacement therapy. In-vitro and in-vivo studies using an adenoviral vector with a p16 transgene are encouraging (Rocco et al,1998) and a phase I trial using this system is due to commence in patients with recurrent HNSCC at the Johns Hopkins Hospital, USA.

1.5.5. Immunotherapy in HNSCC.

In immunotherapy, the aim is to either increase the immunogenicity of the tumour (Townsend et al, 1993) or increase the effectiveness of tumour infiltrating lymphocytes (Rosenberg et al, 1991). Non-specific immunostimulants (such as BCG, levamisole (an antihelminthic imidazole) and OK-432 (an inactivated low virulence strain of Streptococcus pyogenes), can be used to stimulate cytotoxic T lymphocytes. Both BCG (Taylor et al,1979) and levamisole (Olivari et al,1979) have failed to show any tumour response. However OK-432 has been reported to cause tumour reduction in some cases (Kitahara et al,1996). The immunogenicity of the tumour can be increased by transfecting into the tumour cells MHC class I and II genes to increase antigen presentation (Plautz et al,1993). Nabel et al,1993 have reported reduction of tumour size in a melanoma patient after gene transfer of MHC class I gene. The presence of antigen and MHC alone are insufficient for immune induction, and a class of molecules called costimulatory molecules are also required. These are expressed on antigen presenting cells and the best studied are called B7.1 and B7.2. Transfection of B7 into tumour cells should provide the necessary costimulation required for immune stimulation in immunogenic tumours and Chen et al,1994 have shown that B7 transduction into immunogenic lymphoma and melanoma cell lines using a retroviral vector decreased their tumourgenicity and induced protective immunity. Lang et al,1999 have shown that there is impairment of T cell activation in head and neck cancer patients and this is partly due to lack of B7.1 expression. They also showed that transduction of

the B7.1 gene into head and neck cell lines resulted in T cell activation. Thus gene therapy using the B7.1 gene as the transgene may be a potentially useful method for immunogenic therapy in head and neck cancer patients. Gleich et al,1998 recently reported a phase I study using Allovectin-7 (i.e. B7 gene complexed to a cationic lipid) by direct intratumoural injection in patients with advanced head and neck cancer who did not express HLA-B7. This study showed no toxicity and a partial response to therapy in 4 out of 9 patients treated. HLA-B7 expression was detected in 2 of the 9 patients and there was extensive apoptosis in both these patients.

The effectiveness of tumour infiltrating lymphocytes can be improved by cytokines interleukins 2 and 4 (Lin et al,1993), tumour necrosis factor, interferon alpha and gamma, and granulocyte-macrophage colony stimulating factor. These cytokines can stimulate the production of both tumour infiltrating lymphocytes and lymphokine activated killer cells in-vitro and these expanded cells are then used in adoptive immunotherapy (Whiteside et al,1996; Boscia et al,1988; Sacchi et al,1990) (**Figure 7b**). Alternatively interleukin 2 can be injected directly into the patient either peritumourally (Whiteside et al,1993) or perilymphatically (Cortesina et al,1988). However, although immune activation is seen, responses have been very poor.

1.5.6. GDEPT in HNSCC.

GDEPT involves the introduction of a gene coding for an enzyme capable of activating an inactive prodrug into an active cytotoxic drug. The most common system used is the herpes simplex virus thymidine

kinase/ganciclovir system. Herpes simplex virus thymidine kinase renders cells sensitive to the nucleoside analogue ganciclovir by converting ganciclovir into an active phosphorylated compound that terminates DNA synthesis. Since cancer cells divide more rapidly than normal cells, this treatment selectively kills tumour cells transduced with the gene rather than normal cells. The antitumour effect is dependent upon a biochemical bystander effect which is mediated by gap junctions between cells (Elshami et al,1996). The toxic metabolite is able to pass from a transduced cell into a nontransduced cell via gap junctions. Therefore only 10-20% of tumour cell transduction is required for efficacy. Selectivity can be achieved if the tk gene is linked to a tissue or tumour specific promoter. Adenovirus mediated gene transfer of the herpes simplex virus thymidine kinase gene has shown a reduced growth of squamous cell cancer in a nude mouse model (O'Malley et al, 1995; O'Malley et al, 1993) and a phase I clinical trial is currently underway at the Johns Hopkins hospital, USA (**Figure 7c**).

1.6. REPLICATING VIRUSES AS ANTICANCER AGENTS.

Despite bystander effects, the main disadvantage of current gene therapy vectors is the poor tumour transduction. One method to overcome this is to use a vector which is a replication competent virus which selectively replicates in tumour but not normal tissues. The tumour specific replication would increase the number of cells infected with virus and therefore deliver more therapeutic gene to the tumour. In addition, a replicating virus itself would cause lysis of any tumour cells infected. Recently, replication competent parvoviruses (Berns,1990; Rommelaere et

al, 1991) and herpes simplex (Yazaki et al, 1995; Mineta et al, 1995) viruses have been used to achieve viral oncolysis. Parvovirus has the advantage that it infects a wide range of tissue types but are non-pathogenic in humans. Thus viral toxicity is limited. Attenuated herpes simplex viruses have been used for the treatment of brain neoplasms in in-vivo mouse models (Yazaki et al, 1995; Mineta et al, 1995) and human trials are underway (Rampling et al,1998). These replicate only in dividing tumour cells leaving normal brain tissue unaffected. No studies have been reported in head and neck cancer to date.

The use of adenoviruses in the treatment of cancer was described by Smith et al,1956 where tumour necrosis was shown by direct intratumoural injection in patients with cervical cancer. Recently there has been much research in developing adenoviruses which selectively replicate in and destroy tumours. Two main strategies have been developed. In the first strategy the adenoviral E1A gene promotor is replaced by a tumour specific promotor. Rodriguez et al,1997 developed an adenovirus with the promotor for prostate specific antigen (PSA) to selectively replicate in prostate cancers, whereas Hallenbeck et al,1999 has developed a similar virus with the alpha feto protein (AFP) promotor to replicate in hepatocellular cancers. The second strategy is to remove the adenoviral genes whose normal function is to inactivate genes regulating the cell cycle i.e p53 and Rb. For example, deletion of the E1A gene results in an adenovirus which replicates selectively in cells with no functional retinoblastoma (Rb) gene. Such a virus has shown efficacy in vivo in a glioma model (Fueyo et al,2000). Alternatively, deletion of the E1B 55kDa adenoviral gene may result in a

virus which selectively replicates in cells with non functional p53. Indeed, the E1B 55kDa deleted adenovirus, Onyx-015, has recently been shown to selectively replicate in and lyse tumour cells with non functional p53 both in-vitro and in-vivo (Bischoff et al,1996; Heise et al, 1997). The E1B 55kDa protein binds directly to the N terminus of the p53 tumour suppressor gene and causes transcriptional inactivation of p53 (Dobner and Shenk et al,1996; Teodoro et al,1997). In addition, E1B 55kDa also mediates the degradation of p53. This degradation of p53 is believed to be mediated by complex formation between p53, E1B and E4ORF6 (Dobner and Shenk ,1996; Steeganga et al,1997) which targets p53 for degradation via the ubiquitin degradation pathway. Since Onyx-015 lacks this protein it is unable to replicate in cells with functional p53 since it cannot inactivate p53. However, it will replicate effectively in cells with non-functional p53 causing cytolysis (**Figure 1**). In squamous cell cancer of the head and neck there is a high incidence of p53 abnormalities and therefore an E1B deleted adenovirus, such as Onyx-015, may be potentially useful to treat this disease.

1.7. AIMS OF RESEARCH STUDY.

The aims of this research study were :

1. To determine whether Onyx-015 would selectively replicate in and lyse head and neck cell lines with non-functional p53.

2. To determine if Onyx-015 had efficacy in-vivo in human head and neck tumour xenografts in nude mice.

3. To determine the incidence of p53 mutations and p53 inactivation in recurrent head and neck tumours.

4. To carry out a phase I dose escalation trial of an intratumoural injection of Onyx-015 in patients with recurrent p53(-) tumours of the head and neck.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals

All chemicals were of AnalaR grade and were obtained from BDH Chemicals Ltd, Poole, Dorset or Sigma Chemicals. Ltd, Poole, Dorset except those obtained from the suppliers listed below.

Advanced Protein Products

Foetal calf serum

Beatson Institute Central Services

sterile PBS (Phosphate buffered saline)

PE (PBS and EDTA)

Biogenex

Universal blocking solution

J.Burrough(FAD) Ltd, Witham, Essex

ethanol

ENZO Diagnostics Inc. Farmingdale. NY

Biotinylated adenovirus DNA probe

FMC, Rockland, ME

Nuseive agarose

Gateway plc, Glasgow

Marvel dried non-fat milk powder

Gibco Europe, Life Technologies Ltd, Paisley

10 x DMEM

10 x RPMI

200mM glutamine

2.5% trypsin

penicillin

streptomycin

RNA ladder

DNA ladders

Promega, Madison,WI

Taq DNA polymerase

dNTPs

Rathburn Chemicals Ltd, Walkerburn, Peebleshire

phenol (water saturated)

Sigma Chemical Co.Ltd, Poole, Dorset

agarose

Tween 20

ethidium bromide

β- mercaptoethanol

orange G

bromophenol blue

xylene cyanol

proteinase K

Rnase

Pepsin

Propidium iodide

Vector Laboratories Inc, Burlington,USA

Vectashield antifade mounting medium

BCIP/NBT substate

DAB substrate

Nuclear fast red

ABC Mouse peroxidase kit

ABC Rabbit peroxidase kit

2.1.2. Equipment and plasticware

Applied Biosystems Ltd

PCR reaction tubes (thin walled)

Becton Dickinson Labware, Plymouth, Devon

tissue culture dishes (90mm,60mm)

Eastman Kodak Co, Rochester, New York

Ektachrome colour slide film

Fuji Photo Co.Ltd, Japan

x-ray film

Gibco Europe, Life Technologies Ltd, Paisley

Nunc 1ml cryotubes

Nunc 8-well chamber slides (permanox)

Nunc $25cm^2$, $75cm^2$, $175cm^2$ tissue culture flasks

Griener Labortechnik Ltd, Dursley

eppendorf tubes

Hybaid Ltd, Teddington, Middlesex

Omnislide in situ system

Labsystems, Basingstoke

pipette tips

Molecular Bioproducts, San Diego, CA

aerosol- resistant tips

Pharmacia Ltd, Milton Keynes, Buckinghamshire

Spin columns

Whatman International Ltd, Maidstone

3MM paper

2.1.3. Antibodies

Oncogene Science

p53 DO-1(mouse monoclonal)

Sigma Chemical Co.Ltd, Poole,Dorset

Fluorescein (FITC) conjugated AffinitiPure Goat Anti-Mouse IgG

DAKO

mdm2 (mouse monoclonal) Clone SMP14.

Antibody diluent solution.

Chemicon

Adenovirus anti-hexon protein (mouse monoclonal #MB805)

2.2. PRECLINICAL IN VITRO STUDIES

2.2.1. Cell cultures.

All the neoplastic keratinocytes (kindly donated by Dr K Parkinson) were cultured using lethally irradiated Swiss 3T3 fibroblast feeder layers (Rheinweld et al,1975) in DMEM (Dulbecci's modified Eagles Medium) containing 10% FCS , 10% L-Glutamine and 0.4μg/ml hydrocortisone. The p53 sequence and protein expression of all the neoplastic cell lines have been previously published (Burns et al,1993). The ovarian adenocarcinoma

cell lines A2780 and its cisplatin resistant derivative A2780Cp70 were grown in RPMI medium containing 10% FCS and 10% L-Glutamine. A2780 has wild type p53 sequence and function and A2780Cp70 has wild type p53 sequence which is non-functional (Behrens et al, 1987).

2.2.2. Viruses

Onyx-015 is a chimeric human group C adenovirus (Ad2 and Ad5) which has a deletion between nucleotides 2496 and 3323 in the E1B region encoding the 55kDa protein. In addition, there is a C to T transition at position 2022 in E1B which generates a stop codon at the third codon position of the protein. These alterations eliminate the expression of the 55kDa protein in Onyx-015 infected cells (Barker et al, 1987).

All adenoviruses were grown on the human embryonic kidney cell line HEK293 which expresses the E1 region of Ad2 (Graham et al,1977) and band purified on a CsCl gradient. Stocks were stored at -70^{0}C after addition of glycerol to a concentration of 50%vol/vol.

2.2.3. Plaque assays

Plaque assays were performed as described by Graham and Prevec,1991 to determine the quantity of infectious viral particles in viral stocks and burst assays. Briefly, plates were scraped into 0.5ml of media and frozen. Lysates were prepared by 3 cycles of freezing and thawing. Serial dilutions on the lysates were titred on HEK293 cells.

2.2.4. Replication assay

Cell monolayers were grown to 50% confluence on 90mm plates. One plate was infected with Ad2 at 10pfu/cell, one plate with Onyx-015 at 10pfu/cell and one plate left uninfected as a control. Nonabsorbed virus was removed after 90 minutes and then medium with 2% FCS added. After 48 hours incubation, the cells were harvested and collected into a cell pellet by centrifugation at 2,000revs/min.The cells were fixed in ice cold 70% ethanol for at least 1 hour, washed in PBS and then resuspended in 200 μl of anti-hexon protein antibody (Chemicon #MB805) at1/1000 dilution in PBS for 1 hour at 37^0C. The cells were washed in PBS and then resuspended in 100μl of FITC labelled anti-mouse IgG (Sigma) at 1/40 dilution at room temperature for 30 minutes. Cells were washed in PBS and then resuspended in propidium iodide solution (10 μg/ml) for 30 minutes at room temperature. The cells were then analysed by flow cytometry. The percentage of FITC staining cells was determined for Ad2 and Onyx-015 infected cells using the control cells as a negative control for FITC. Each infection was determined 3 times and the mean percentage calculated.

2.2.5. Cytopathic effect(CPE) assay

Briefly, cells were grown to 70-90% confluence and then infected with either Onyx-015 or Ad2 for 90 minutes at MOI of 1 and 10pfu/cell. Plates were monitored for CPE and the assay terminated when total cytolysis was observed at MOI of 1 pfu/cell with wild type adenovirus.

2.2.6. Immunofluorescence for adenovirus hexon protein

Cells were grown to 70-90% confluence and then infected with either Onyx-015 or Ad2 for 90 minutes at MOI of 10pfu/cell. After 72 hours, the cell sheet was scraped into the medium and pelleted by centrifugation at 2000revs/min for 10 minutes. The cell pellet was washed in PBS and then resuspended in a small volume of PBS. 25 μl aliquots were pippetted onto 6mm wells on teflon coated microscope slides (DAKO. Product code No S6114), air dried and fixed in acetone at room temperature for 10 minutes. Cells were then stained with 25μl of IMAGEN^{TM} Adenovirus reagent containing an FITC labelled mouse monoclonal antibody to adenovirus hexon protein for 15 minutes at 37^0C in a moist chamber, washed with PBS, air dried, then one drop of IMAGEN mounting fluid added and then a cover slip added. The 6mm well was then scanned using a confocal fluorescent microscope. Positive hexon staining was indicated by bright green fluorescence in the cytoplasm and/or nucleus of the cells.

2.3. PRECLINICAL IN VIVO STUDIES

2.3.1. Direct intratumoural injection efficacy studies.

Tumour cells (10^7 cells in 200μl of PBS) were injected into the flanks of 6 week old female athymic nude mice and allowed to grow into palpable tumours (5-8mm maximal diameter). Ad2 treated tumours were injected with 10^8 pfu Ad2 suspended in 100μl of PBS daily for 5 days. Control tumours were injected with vehicle (PBS) in an identical fashion. Onyx-015 treated tumours were injected with 10^8 pfu Onyx-015 in the same

way. Tumour measurements were taken twice weekly. The animals were humanely killed once tumours were greater than 15mm maximal diameter.

2.3.2. Detection of virus in tumours by in-situ hybridisation.

In-situ hybridisation was performed on formalin fixed paraffin embedded tumours cut into 5μm sections. Slides were deparaffinised in xylene, hydrated through ethanols 100%, 90%, 70% and then H_20. The tissue was digested with proteinase K (Sigma) and post fixed in 4% paraformaldehyde. Hybridisation was carried out overnight at 37^0C with 0.5μg/ml biotinylated adenovirus DNA probe (Enzo Diagnostics, Inc. Farmingdale. NY). After 3 successive washes in 1x SSC at 55^0C, an alkaline phosphatase conjugated antibiotin antibody (Vector Laboratories) was applied. NBT/BCIP was used as the chromagen and slides were counterstained with nuclear fast red (Vector).

2.3.3. Animals and animal care.

Female athymic nu/nu mice were obtained from Harlan Orlac at 4-6 weeks of age. Mice were housed 4 per cage in cages fitted with sterile filter tops and fed with sterile food and sterile water. Guidelines for animal care were strictly followed.

2.3.4. Analytical and statistical methods.

Mean tumour volumes in treated animals versus controls were compared at a given time point using the unpaired two-tailed t-test. Survival of animals in each group was analysed using the method of Kaplan and

Meier, and Kaplan-Meier plots for treated and control groups were compared for statistical significance using the Log rank test.

2.4. PRECLINICAL p53 STUDIES

2.4.1. Tumour collection.

Patients with recurrent squamous cell cancer of the head and neck gave consent for biopsies to be taken from the recurrent tumours. Core biopsies were taken using a 14G tru-cut needle under local anaesthetic. One sample was snap frozen in liquid Nitrogen for DNA extraction. One sample was fixed in phosphate buffered formalin , embedded in paraffin from which 5μm sections were cut for immunohistochemical analysis.

2.4.2. Immunohistochemistry.

a) Immunohistochemistry for p53.

Immunohistochemistry was performed on formalin fixed paraffin embedded tumours cut into 5μm sections. Slides were deparaffinised in xylene, hydrated through ethanols 100%, 90%, 70% and then H_20, then washed in PBS (Phosphate buffered saline). Antigen retrieval was carried out by microwaving in citrate buffer pH6.0 at 500W for 25 minutes and then allowed to cool over 20 minutes. The slides were washed in PBS for 5 minutes and then endogenous peroxide activity blocked with 3% v/v hydrogen peroxide in methanol for 10 minutes. After washing in PBS for 5 minutes, the slides were blocked with Universal blocking solution

(Biogenex) for 10 minutes, and then primary antibody (DO-1 Oncogene Science) at a dilution of 1/1000 in DAKO antibody diluent solution, added for 1 hour at room temperature. Antigen detection was done using a biotinylated secondary antibody followed by streptavidin. The chromogen used for detection was diaminobenzidine (Vector) for 3-10 minutes. The sections were counterstained with haematoxylin , dehydrated in graded alcohols followed by xylene, and then mounted in DPX mounting medium (BDH Chemicals). The immunohistochemistry pattern was scored using a histoscore based on intensity of staining and percentage of positive cells staining and given a score out of a maximum of 6.

Intensity of staining	*Score*	*%cells staining positive*	*Score*
None	0	0	0
Mild	1	5-20%	1
Moderate	2	20-80%	2
Severe	3	>80%	3

b) Immunohistochemistry for mdm2

The protocol used was as described for p53 immunohistochemistry. The primary antibody used was a mouse monoclonal antibody clone SMP14 (DAKO) at a dilution of 1/50 in DAKO antibody diluent solution for 1 hour at room temperature. Mdm2 staining was then scored by the percentage of cells staining positive and by the intensity of staining.

2.4.3. DNA extraction.

Genomic DNA was isolated directly from frozen tumours by lysis buffer containing 10mM Tris-HCl, 10mM EDTA, 10mM NaCl, 4% N-lauryl sarcosine and 2.75mg/ml proteinase K, followed by overnight digestion at 55^0C and EtOH precipitation. The resulting pellet was washed with 70% EtOH , dried, and resuspended in 100µl TE and stored at 4^0C.

2.4.4. p53 sequencing.

Exons 4 to 9 were sequenced using DNA extracted from tumour biopsies. This was done by Oncormed Corporation,Gaithersburg,Maryland.

2.4.5. HPV analysis.

The presence of HPV in tumour DNA was assessed by PCR. The primers for the amplification of HPV-16 and HPV-18 have been described previously (Yeudall et al,1991) and amplify fragments of the E6/E7 (HPV-16) or E6 regions (HPV-18) which are 165 and 99bp respectively. HPV 33 primers were those as described by Haraf et al,1996.

The HPV16 primers were

5' TTAATTAGGTGTATTAACTG3'

5' TGCATGATTACAGCRGGGTT3'

The HPV18 primers were

5'ATCTGTGTGCACGGAACTAAC3'

5'AATGCAAATTCAAATACCTC3'

The HPV33 primers were

5'GTGCCAAGCATTGGAGACAA3'

5'GATAAGAACCGCAAACACAG3'

SiHa DNA (single copy HPV16) and HeLa (HPV18) were used as positive controls. HPV amplification was carried out at 95^0C for 3 minutes, then 30 cycles of 95^0C for 30s, 55^0C for 30s, 72^0C for 30s, followed by a final extension time of 10 minutes at 72^0C. An aliquot was run on a 1% agarose gel (Nu-Sieve) containing 0.5μg/ml ethidium bromide in TBE buffer and then photographed under UV light to check for correct amplification.

2.5. PHASE I CLINICAL TRIAL - Single injection protocol.

2.5.1. Enrollment criteria.

A total of 22 patients with recurrent head and neck cancer were entered into the trial from 2 centres – 11 from Glasgow, Scotland and 11 from San Antonio, USA. Eligibility requirements included histologically confirmed squamous cell carcinoma of the head and neck which was recurrent and refractory to radiotherapy and/or chemotherapy. The tumour had to be amenable to direct injection and measurement either clinically or radiographically. The tumours had abnormal p53 by immunohistochemistry. All patients had a Karnofsky Performance Status of greater than or equal to 60%, life expectancy of greater than 3 months and were over 18 years of age. All patients had adequate haematological, renal and hepatic function. The maximum allowed creatinine was 1.5mg/dl, maximum allowed Aspartate transaminase (AST) and Alanine transaminase (ALT) was 2.5 fold the upper limit of normal, minimum allowed haemoglobin 9gm/dl,

minimum allowed white cell count of 3,000/μl (neutrophils 1,500/μl) and minimum platelet count of 100,000/μl. Patients had not received any chemotherapy or radiotherapy within 4 weeks of study entry. All patients gave written informed consent. The protocol was approved by the US Food and Drug Administration (FDA), the UK Gene Therapy Advisory Committee (GTAC), and the local institutional review board ethics committees.

2.5.2. Onyx-015.

Onyx-015 is a chimeric human group C adenovirus (Ad2 and Ad5) which has a deletion between nucleotides 2496 and 3323 in the E1B region encoding the 55kDa protein. In addition, there is a C to T transition at position 2022 in E1B which generates a stop codon at the third codon position of the protein. These alterations eliminate the expression of the 55kDa protein in Onyx-015 infected cells (16). Sterile purified lots of virus were produced for human clinical use by MAGENTA Corporation (Rockville,MD) and tested for titre, sterility and general safety by Microbiological Associates using FDA approved test methods.

2.5.3. Treatment Protocol.

Pre-treatment evaluation included complete blood cell count with differential, coagulation screen, routine biochemistry profile, urinalysis, CXR and ECG. All patients had a core biopsy of the recurrent tumour for p53 evaluation by immunohistochemistry and gene sequencing. CD4 lymphocyte counts were carried out to determine the immune status of the

patients. The size of the recurrent tumours was measured clinically and also by ultrasound, CT scan or MRI scan.

The volume of the injected tumour was determined either by clinical measurement or radiological measurement, depending on the site of the tumour. Vials of Onyx-015 were then thawed and diluted with diluent (electrolyte 48 solution) to a volume equivalent to 30% of this estimated tumour volume.The tumour to be injected was mapped into $1cm^2$ areas and then equal volumes of solution were injected into each area. Subcutaneous injection of local anaesthetic was used in some patients to prevent pain on injection. Patients then had vital signs recorded every 20 minutes for 2 hours, 2 hourly for the next 22 hours , then 6 hourly for the next 24 hours. Patients were then discharged home if vital signs were normal.

Follow up was twice weekly. Blood counts and biochemistry were carried out weekly. Blood samples were taken weekly for PCR for adenoviral DNA and to determine neutralising antibody titers to adenovirus. Swabs of the injection site and oropharynx taken pre-treatment and 8 days post-treatment were assessed for adenovirus by a direct immunofluorescent assay against adenoviral hexon protein. Tumour core biopsies were taken at days 8 and 22 and examined for adenoviral replication by *in-situ* hybridisation and for evidence of necrosis. Tumour measurement was carried out clinically and radiographically at 4 weeks. Patients were eligible for re-treatment with virus injections at 4 weeks (up to a maximum of 5 cycles) if measurements indicated response or stable disease in the injected tumour (see below), there was no dose-limiting toxicity (see below) and there was no evidence of disease progression at other sites. Ethical approval

was given for an additional injection of diluent alone into separate lesions using the same injection technique as for Onyx-015. The aim was to assess the volume effect of control intratumoural injection and this was carried out in 3 patients.

2.5.4. Evaluation of toxicity and response.

Toxicity was assessed using NCIC Toxicity Criteria. The Maximum Tolerated Dose (MTD) was defined as the dose at which 2 patients experienced a Dose Limiting Toxicity (DLT) after the first treatment with Onyx-015. The Dose Limiting Toxicity was defined as either a Grade 4 toxicity for flu-like symptoms due to Onyx-015, a Grade 4 toxicity for local reaction at the Onyx-015 injection site or any other toxicity of grade 3 severity due to Onyx-015. A minimum of 3 patients were treated at each dose level. If one of the 3 patients had a DLT, a total of 6 patients would be treated at that cohort. An escalation scheme was devised to permit rapid but safe increase in dose with a maximum of 10^{11} pfu set. The 10^{11} pfu limit was based on manufacturing limits. No intra-patient dose escalation was permitted.

Response to therapy was assessed after each cycle by clinical and radiological tumour measurement. All radiological measurements were made by the same radiologist. Since Onyx-015 caused substantial tumour necrosis centrally rather than uniform tumour shrinkage peripherally, the non-necrotic tumour area was determined and used to assess injected tumour response. These injected tumour responses were then classified as partial response (PR) if there was a greater than 50% reduction in the treated

tumour, minor response (MR) if there was a greater than 25% but less than 50% reduction in the treated tumour, stable disease (SD) if there was less than a 25% increase or decrease in the treated tumour and progressive disease if there was a greater than 25% increase in the injected tumour or the appearance of new lesions. Detailed responses are reported on a case-by-case basis because of the use of this non-conventional measurement methodology. To control for the effect of diluent, 3 patients who had more than 1 tumour had 1 tumour injected with Onyx-015 and another injected with diluent only.

2.5.5. Evaluation of p53 status.

1.Immunohistochemistry.

As described previously.

2.Gene sequencing.

Exons 4 to 9 were sequenced on pre-treatment tumour biopsies by Oncormed Corporation, Gaithersburg, Maryland.

2.5.6. In-situ hybridisation.

In-situ hybridisation was performed on formalin-fixed paraffin embedded tumours cut into 5μm sections. Slides were deparaffinised in xylene, hydrated through ethanols 100%, 90%, 70% and then H_20. The tissue was digested with proteinase K and post-fixed in 4% paraformaldehyde. Hybridisation was carried out overnight at 37^0C with 0.5μg/ml biotinylated adenovirus DNA probe (Enzo Diagnostics, Inc. Farmingdale, NY). After 3 successive washes in 1x SSC at 55^0C, an alkaline

phosphatase conjugated antibiotin antibody (Vector Laboratories) was applied. NBT/BCIP was used as the chromagen and slides were counterstained with nuclear fast red (Vector).

2.5.7. Adenoviral PCR.

The presence of adenovirus in plasma samples from patients was determined by PCR using primers to the E1A region of the adenoviral genome. This was carried out by Onyx pharmaceuticals.

2.5.8. Direct immunofluorescent assay for hexon protein.

Swabs from the injection site and oropharynx were placed in viral culture medium. Human embryonic kidney cells (HEK293) were grown to 70-90% confluence and then inoculated with this medium. After 72 hours, the cell sheet was scraped into the medium and pelleted by centrifugation at 2000revs/min for 10 minutes. The cell pellet was washed in PBS and then resuspended in a small volume of PBS. 25 μl aliquots were pippeted onto 6mm wells on teflon coated microscope slides (DAKO. Product code No S6114), air dried and fixed in acetone at room temperature for 10 minutes. Cells were then stained with 25 μl of IMAGEN^{TM} Adenovirus reagent containing an FITC labelled mouse monoclonal antibody to adenovirus hexon protein for 15 minutes at 37^{0}C in a moist chamber, washed with PBS, air dried, then one drop of IMAGEN mounting fluid added and then a cover slip added. The 6mm well was then scanned using a fluorescent microscope. Positive hexon staining was indicated by bright green fluorescence in the cytoplasm and/or nucleus of the cells.

2.5.9. Determination of neutralising antibody titres

Patient and control samples were incubated at 55^0C for 30 minutes to inactivate complement. Clinical plasma samples previously determined to produce high, midrange and negative titres were designated as plasma controls. Each dilution was mixed with adenovirus stock at a titre pre-qualified to produce 15-20 plaques per well of a 12 well dish in DMEM growth medium. The patient's samples and controls were inoculated for 1 hour at room temperature, and applied to 70-80% confluent JH393 cells in 12 well dishes. After 2 hours of incubation at 37^0C, 5% CO_2 plasma-virus mix was removed and 2ml of 1.5% Agarose in DMEM was added to each well. Plates were read on day7 post-inoculation by counting the number of plaque forming units (pfu) per well. The titre of neutralising antibody for each sample was reported as the dilution of plasma that reduced the number of plaques to 60% of the number of plaques in the virus control without antibody.

2.6. PHASE I TRIAL - multiple injection protocol.

A total of 10 patients with recurrent head and neck cancer were recruited. Eligibility criteria were identical to the single injection protocol. The injection procedure was identical except that patients received 5 daily injections at the same viral dose per injection. 4 patients were treated at 5 x 10^9 pfu and 6 patients at 5 x 10^{10} pfu. The total dose of virus administered in each of these cohorts was less than the maximum dose given in the single injection protocol (10^{11}pfu) and therefore we did not expect to see any

increase in toxicity. Evaluation of toxicity, tumour response, p53 status and viral replication assays were as described for the single injection protocol.

Chapter 3

Results

3.1. PRECLINICAL IN VITRO STUDIES.

3.1.1. Onyx-015 causes lysis of p53 deficient head and neck squamous cell cancer cell lines.

A panel of head and neck squamous cell cancer cell lines were tested for sensitivity to cytopathic effects induced by Onyx-015 and wild type adenovirus. The p53 sequences and protein expression for all cell lines has previously been carried out and are shown in **Table 1** (Burns et al,1993). All HNSCC cell lines tested have mutant p53 except cell line BICR19 which has a wild type p53 allele and a mutant p53 allele. The ovarian adenocarcinoma cell line A2780, which has wild type p53 sequence and function as determined by radiation induced G1 arrest, was used as a negative control for CPE. The isogenic cisplatin resistant ovarian adenocarcinoma cell line A2780Cp70, which has wild type p53 sequence but is non-functional, was used as a positive control for CPE. We have previously shown that both cytolysis and viral replication is x 5-10 greater in A2780Cp70 compared to A2780 (Kim Tae Young,unpublished results). **Table 2** shows the cell lines tested and results of CPE assays. **Figure 8** shows examples of CPE in different cell lines tested 5 days following virus infection. From **Table 2**, all HNSCC cell lines with mutant p53 showed cytolysis with Onyx-015. The cell line A2780 showed no CPE with Onyx-015 but was positive with wild type virus. Interestingly, the cell line BICR19 , which has 1 allele with wild type p53 showed no cytolysis with Onyx-015 suggesting that the wild type allele is still functional and is able to prevent Onyx-015 replication and cytolysis.

Cell line	Site	Stage	p53 sequence	amino acid change	p53 protein expression
SCC4	Tongue	Unknown	Codon 151 CCC>TCC	Pro>Ser	+
SCC9	Tongue	Unknown	Codon 274-285 32bp del^{n}	285+ out of frame	-
SCC15	Tongue	Unknown	Codon 336 frameshift mut^{n}	Stop	-
BICR16	Tongue(R)	$T_2N_0M_0$	Codon 146 TGG>TGA	Trp>Stop	-
BICR19	Epidermis	Not applicable	Exon 10 del^{n} 107bp del^{n}	332+out of frame	-
BICR78	Alveolus	$T_4N_1M_0$	Codon 176 Cys>phe	Cyst>Phe	+
A2780	Ovary	Not applicable	wild type	none	-
A2780Cp70	Ovary	Not applicable	wild type	none	+

Table 1.

p53 sequences and protein expression of head and neck squamous cell cancer lines and ovarian adenocarcinoma cell lines used for cytopathic effect assays and hexon protein staining.

Cell line	p53	CPE	CPE
		Onyx-015	Ad2
A2780	wt	-	+
A2780Cp70	wt	+	+
SCC4	mut	+	+
SCC9	mut	+	+
SCC15	mut	+	+
BICR16	mut	+	+
BICR19	wt/mut	-	+
BICR78	mut	+	+

Table 2

Cytopathic effect (CPE) of wild type adenovirus (Ad2) and Onyx-015 infection on HNSCC cell lines. The CPE studies were carried out using multiplicities of infection (MOI) of 1 and 10 pfu/cell to infect cell monolayers. Infections with Onyx-015 were scored for CPE on the day in which wild type adenovirus had induced CPE in all cells at an MOI of 1 pfu/cell. The "+" and "-" refer to the presence or absence of CPE.

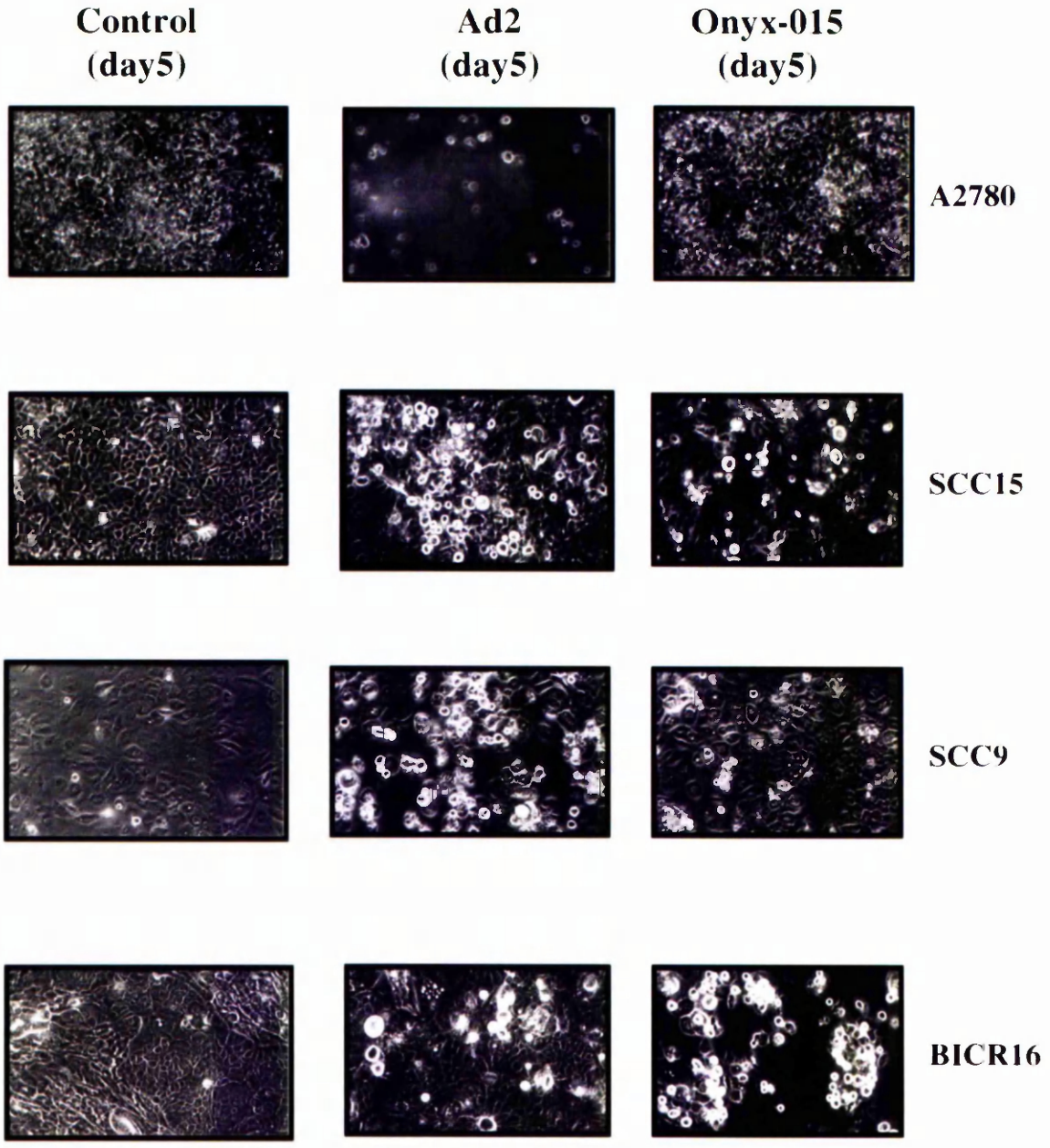


Figure 8. Cytopathic effect(CPE) of wild type adenovirus (Ad2) and Onyx-015 infection on HNSCC cell lines at day 5 post virus infection. Positive CPE is indicated by the typical "bunches of grapes" appearance of adenovirus infected cells. Cell line A2780 showed cytolysis with Ad2 but not Onyx-015. The cell lines SCC9, SCC15 and BICR16 show cytolysis with both Ad2 and Onyx-015.

3.1.2. Mutant p53 HNSCC cell lines infected with Onyx-015 allow viral replication.

To determine if cytolysis induced by Onyx-015 was due to replication of virus, cells were harvested 48 hours post infection and stained with an anti-hexon protein antibody. Hexon protein is a capsid protein which is synthesised in the late phase of viral DNA replication and is therefore a marker for viral replication. **Table 3** shows the results of hexon protein staining assays. **Figure 9** shows confocal microscopy pictures of hexon staining in the cell lines tested. All cell lines which were positive for CPE were also positive for hexon protein indicating that cytolysis and viral replication selectively occurred in the cell lines with mutant p53.

3.1.3. Quantification of viral replication of Onyx-015 in HNSCC cell lines by FACS analysis.

To quantify the amount of viral replication occurring in the cell lines, a FACS analysis assay was developed by measuring the number of hexon protein positive cells using an FITC labelled anti-hexon protein mouse monoclonal antibody. The percentage of positive cells with Onyx-015 was determined and expressed as a ratio in relation to the percentage positivity with wild type adenovirus. Each infection was carried out 3 times for each cell line and the mean percentage hexon positivity determined. **Table 4** shows the results of the cell lines tested and **Figure 10** shows typical FACS analysis plots for cell lines A2780 and A2780cp70. From **Table 4**, we can conclude that all mutant p53 cell lines show a x2-6 fold increased replication relative to the control cell line A2780. The cell line BICR19,

Cell line	p53	Hexon staining	
		Onyx-015	Ad2
A2780	wt	-	+
A2780Cp70	wt	+	+
SCC4	mut	+	+
SCC9	mut	+	+
SCC15	mut	+	+
BICR16	mut	+	+
BICR19	wt/mut	-	+
BICR78	mut	+	+

Table 3

Hexon protein staining of wild type adenovirus (Ad2) and Onyx-015 infected HNSCC cell lines. Cell monolayers were infected at a MOI of 10 pfu/cell and cells harvested 48 hours postinfection. Cells were stained for hexon protein expression using an FITC labelled anti-hexon protein mouse monoclonal antibody and then visualised using a fluorescent confocal microscope. Cells positive for hexon protein stained green and negative cells stained red with Evans blue counterstain.

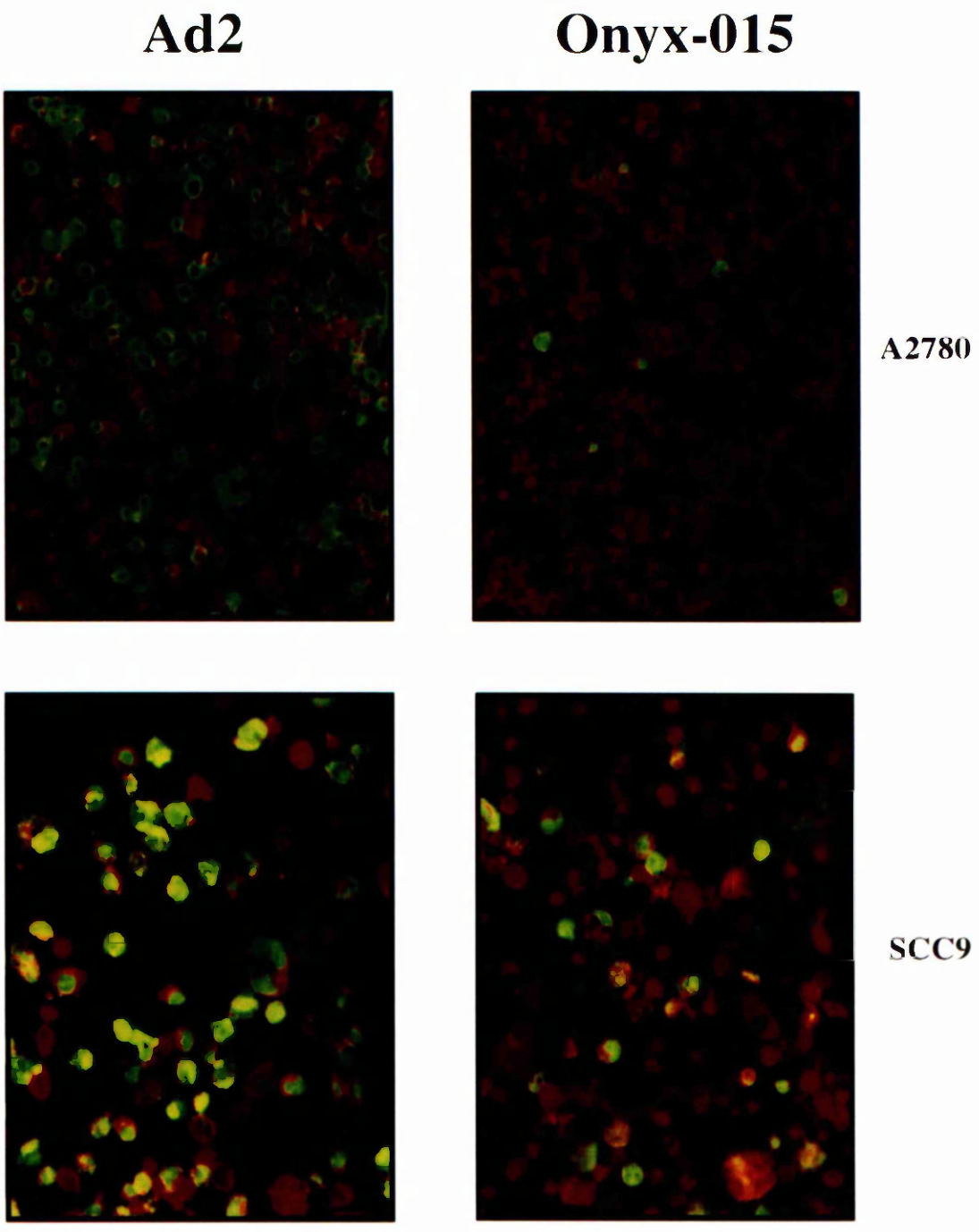


Figure 9.

Hexon protein staining of wild type Ad2 and Onyx-015 infected cells. Cells are stained with an FITC labelled anti-hexon protein mouse monoclonal antibody. Green staining cells represent hexon positive cells. Noninfected cells are stained red with the counterstain Evans blue.

Cell line	p53	Ad2	s.e.	Onyx-015	s.e.	Ratio Onyx/Ad2
						%
A2780	wt-funct[1]	65.7	15	8.7	4.7	13.2
A2780Cp70	wt-nonfunct[1]	84	5.1	54	2.7	64.3
SCC4	mutant	56	12.8	15.3	1.9	27.3
SCC9	mutant	78.2	8.2	30.8	7.6	39.4
SCC15	mutant	81.1	8.8	33.1	2.8	40.8
BICR16	mutant	52.1	3.9	25.1	4.3	48.2
BICR19	mutant	94.1	0.9	2.3	0.01	2.4
BICR78	mutant	48.8	15.4	40.3	21.8	82.6

Table 4

Percentage hexon positive cells quantified by FACS at a MOI of 10pfu/cell for 48 hours. Cell monolayers were infected at MOI of 10 pfu/cell and the cells then harvested at 48 hours post infection. Cells were stained with anti-hexon protein antibody linked to FITC . Cells were counterstained with propidium iodide. Mock infected cells were used for each cell line to determine the baseline FITC signal. The percentage of cells staining positive in infected cells was determined relative to the mock infected FITC signal. Each infection was carried out 3 times per cell line and the mean percentage hexon positivity determined.

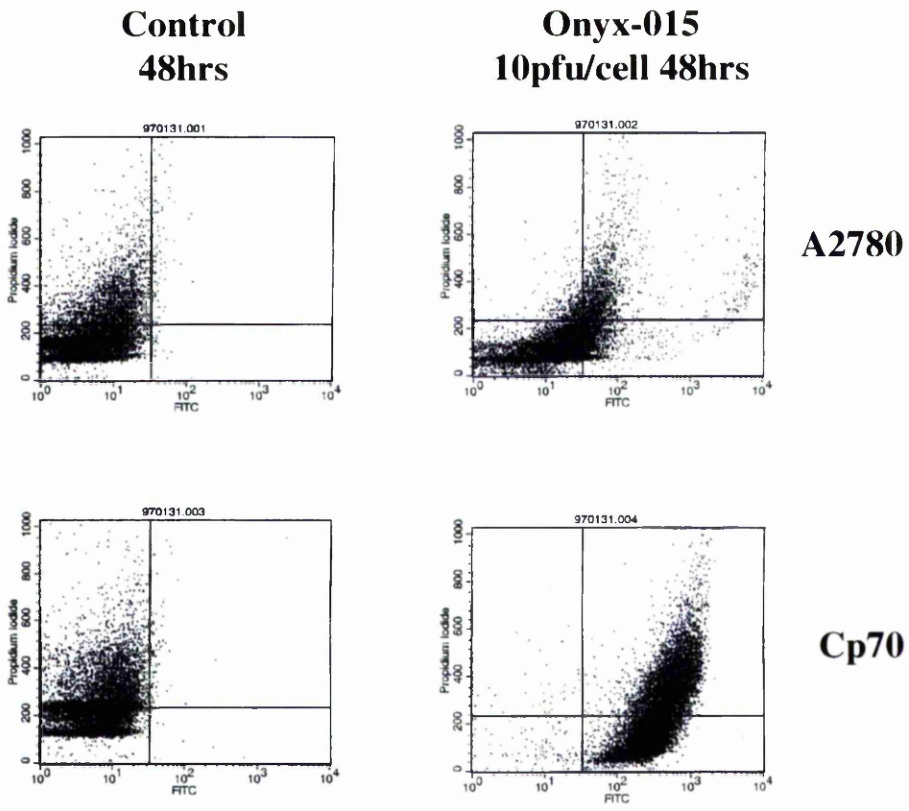


Figure10. FACS analysis of viral replication of Onyx-015 in A2780 and Cp70 by FITC labelled hexon protein quantification.

Cell monolayers were infected with Onyx-015 at a MOI of 10pfu/cell for 48hours and then cells were harvested and stained with an anti-hexon protein antibody linked to FITC. The cells were then counterstained with propidium iodide and then analysed by flow cytometry. The percentage of cells positive for hexon protein is indicated by the right upper and right lower quadrants. Nonspecific staining is indicated by cells to the left of the vertical line- the left upper and left lower quadrants. For A2780(functional p53), the hexon positivity is 13% and for Cp70 (nonfunctional p53) the hexon positivity is 82%. This indicates the selective replication for Onyx-015 for cells with nonfunctional p53.

which has a functional p53 allele, showed very high replication with Ad2 infection but very low replication with Onyx-015. The replication ratio was much lower than the control cell line A2780. This again indicated that only one functional p53 allele is necessary to prevent Onyx-015 replication.

In conclusion, these in-vitro studies showed that the E1B attenuated adenovirus, Onyx-015, replicated more efficiently in head and neck tumour cell lines with non-functional p53. This supported the previous published work in other tumour types (Bischoff et al,1996).

3.2. PRECLINICAL IN VIVO STUDIES.

3.2.1. Anti-tumour activity of Onyx-015 in BICR16 subcutaneous xenografts.

The mutant p53 HNSCC cell line BICR16 was selected for in-vivo testing. This is a cell line derived from a patient with recurrent head and neck cancer and was therefore representative of tumours in the phase I trial. Subcutaneous xenografts were formed in nude mice and once tumours were 5-10mm in maximum diameter, the tumours were injected at 10^8pfu with either wild type adenovirus or Onyx-015 daily for 5 days. Control tumours were injected with diluent of PBS. Tumour diameters were then measured twice weekly and growth curves plotted. **Figure 11a** shows the growth curve for Ad2 and **Figure 11b** shows the growth curve for Onyx-015 (also called dl1520). Control tumours continued to grow exponentially whereas Ad2 and Onyx-015 injected tumours underwent tumour regression. Both curves were statistically analysed using unpaired two-tailed t-test and found to be statistically significant. The p-value for Ad2 versus Control was 0.002

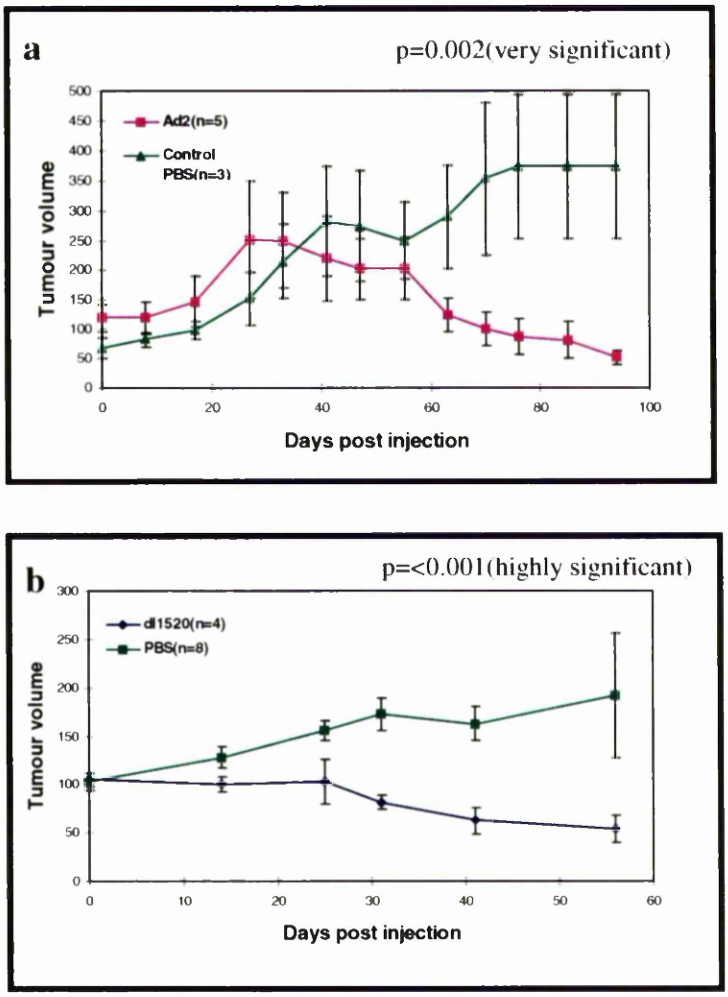


Figure 11.

Direct intratumoural efficacy studies in BICR16 nude mouse xenografts. Subcutaneous xenografts were formed in the flanks of 6 week old female nude mice. Once tumours were 5-10mm max. diameter, they were injected with either wild type adenovirus (Fig 11a) or Onyx-015 (Fig 11b) daily for 5 days. Control tumours were injected with PBS. Tumour size was measured twice weekly . p-values were calculated using unpaired two-tailed t-test.

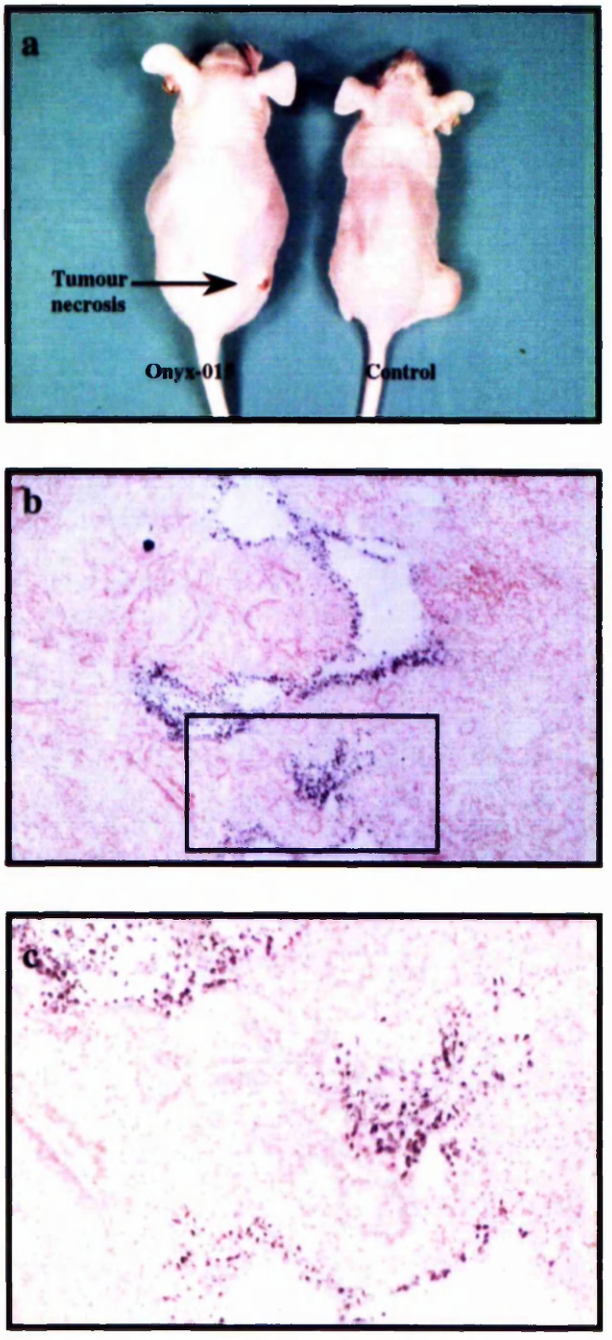


Figure 12.

Tumour regression and viral replication in BICR16 nude mouse xenografts. Figure 12a shows macroscopic tumour necrosis in virus injected tumours compared to controls. Control mice all showed evidence of cachexia. Viral replication was determined by insitu hybridisation. Figures 12b and 12c show low power and high power photographs of typical staining found by in situ hybridisation. Cells expressing viral DNA are stained dark blue and negative staining cells are stained red with the counterstain nuclear fast red. Virally infected cells were always located at the interface between necrosis and viable tumour.

(very significant) and the p-value for Onyx-015 versus Control was <0.001 (highly significant). **Figure 12a** shows control and virus injected mice. Control mice showed evidence of weight loss as tumours continued to grow exponentially. In contrast, the virus injected tumours underwent necrosis often with some ulceration of the overlying skin. In addition, there was no evidence of cachexia in the virus injected mice.

3.2.2. Anti-tumour activity of Onyx-015 is due to viral replication.

To determine whether or not tumour regression was due to viral replication, it was necessary to show the presence of viral replication in the injected tumours. Tumours were stained by in-situ hybridisation using a probe against adenoviral DNA. **Figures 12b and 12c** show examples of typical staining seen. The cells staining positive were always located at the border between necrosis and viable tumour. All viral injected tumours stained positive by in-situ hybridisation. These results suggested that the tumour regression observed in the mouse model was due to viral replication.

3.3. PRECLINICAL p53 STUDIES

Currently available therapies for recurrent head and neck cancer including reirradiation (Nickers,1997) and chemotherapy (Clavel,1994) all have poor response rates which are short lasting (Forestiere,1994). The reason for this is unclear but is likely to be multifactorial in nature. One factor which may be important in resistance to therapy is loss of function of the tumour suppressor gene p53. It has been shown that inactivation of the p53 tumour suppressor gene is one of the major predictors of failure to

respond to radiotherapy and chemotherapy in many tumour types (McIlwrath et al,1994; Buttitta et al, 1997; Cutilli et al, 1998). This is because p53 plays a major role in the induction of apoptosis in response to genotoxic agents such as radiotherapy and chemotherapy (Huang et al,1996; Lowe et al,1993). Therefore one possible explanation for the poor response rates in recurrent HNSCC to radiotherapy and chemotherapy could be due to a high incidence of p53 inactivation in this disease. No previous studies have been reported on the incidence or mechanism of p53 inactivation in recurrent head and neck cancer. We therefore wanted to determine the incidence of p53 mutations in this disease and also gain insight into other mechanisms of p53 inactivation such as binding by the cellular protein mdm2 (Haupt et al,1997; Oliner et al,1992) and viral protein HPV E6 (Lechner et al, 1992; Scheffner et al, 1990). To determine the incidence of p53 inactivation in recurrent HNSCC, 22 recurrent tumours from patients previously treated with radiotherapy +/- surgery were analysed. The p53 status of each tumour was analysed by sequencing and by immunohistochemistry. Tumours were further analysed for mdm2 overexpression by immunohistochemistry and for HPV infection by PCR of tumour DNA.

3.3.1. Primary tumour, site of recurrence and previous treatment.

The primary tumour, site of recurrence and previous therapy for each patient studied are shown in **Table 5**. The mean age was 64 and the male to female ratio was 14:8. All patients had received radiotherapy. In addition, 4 patients had received chemotherapy and 14 patients had had surgery. The site of

Pt no.	Sex	Age	Primary	Site of recurrence	Prior therapy		
					Chemotherapy	Radiotherapy	Surgery
1	M	61	Piriform fossa	Left cervical	Yes	Yes	No
2	M	74	Hypopharyx	Bilateral cervical	Yes	Yes	No
3	M	32	Tongue	Right submandibular	No	Yes	Yes
4	F	80	Palate	Right clavicular area	No	Yes	Yes
5	F	68	Floor of mouth	Right neck-post triangle	No	Yes	Yes
6	F	38	Left auditory canal	Left cervical	Yes	Yes	No
7	M	53	Left temple	Left preauricular area	No	Yes	Yes
8	F	72	Right retromolar trigone	Right cervical	No	Yes	No
9	M	56	Floor of mouth	Left submandibular	No	Yes	Yes
10	M	88	Left palatoglossal fold	Bilateral cervical	No	Yes	Yes
11	M	76	Supraglottic larynx	Left cervical	No	Yes	No
12	M	82	Retromolar trigone	Left cervical	No	Yes	No
13	M	62	Tongue	Left cervical	No	Yes	Yes
14	M	75	Supraglottic larynx	Left submandibular	No	Yes	Yes
15	M	60	Larynx	Right cervical	No	Yes	Yes
16	F	64	Piriform fossa	Right cervical	Yes	Yes	No
17	M	59	Oropharynx	Left clavicular	No	Yes	Yes
18	F	58	Supraglottic larynx	Left cervical	No	Yes	Yes
19	F	59	Oropharynx	Left cervical	No	Yes	Yes
20	M	51	Tongue	Right cervical	No	Yes	Yes
21	F	75	Post cricoid	Left cervical	No	Yes	No
22	M	65	Supraglottic larynx	Left supraclavicular	No	Yes	Yes

Table 5.

Primary tumour, site of recurrence and previous treatment.

tumour recurrence was most commonly regional in the site of draining lymph nodes.

3.3.2. Incidence of p53 mutation in recurrent HNSCC is high and type of mutation correlates with immunohistochemistry.

The p53 sequence and immunohistochemistry for each tumour is shown in **Tables 6 and 7**. Of the 22 tumours studied, 15 had mutations in the p53 gene of which 6 were missense mutations and 9 were nonsense mutations. Generally, the immunohistochemistry pattern correlated directly with the type of p53 mutation i.e. missense mutations gave high scores and nonsense mutations gave low scores. One patient (patient 6) had a nonsense mutation yet stained positively by immunohistochemistry. The site of the mutation in this patient's p53 gene was in codon 9 and this would account for its detection by immunohistochemistry using the DO-1 antibody. Of the 7 patients with wild type p53 sequence, 5 were negative on immunohistochemistry (tumours 10, 11, 14, 17, 19) and 2 were positive indicating overexpression of wild type p53 (tumours 9, 21). Examples of p53 immunohistochemistry with the relevant histoscores are shown in **Figure 13.**

3.3.3. Incidence of HPV infection and mdm2 overexpression.

These results are shown in **Table 7.** 8 tumours were positive for HPV DNA, all of which were serotype HPV 16. Of these, 5 were in tumours with mutant p53 and 3 were in tumours with wild type p53. A typical positive PCR reaction for HPV16 is shown in **Figure 14**.

Pt no.	Exon	Codon	Base-pair change	Amino acid change	p53 Gene sequencing
					(Exons 5-9)
1	5	376	Splice site mutation	Truncated protein	Mutant-nonsense
2	8	273	CGT to TGT	Arginine to Cysteine	Mutant-missense
3	8	307	Deletion G	Stop signal	Mutant-nonsense
4	6		51 splice site mutn AG to AA	Truncated protein	Mutant-nonsense
5	5	141	TGC to TAC	Cysteine to Tyrosine	Mutant-missense
6	9	336	Frameshift mutation	Stop signal	Mutant-nonsense
7	8	280	AGA to ATA	Arginine to Isoleucine	Mutant-missense
8	8	282	CGG to TGG	Arginine to Tryptophan	Mutant-missense
9	wt				Wild type
10	wt				Wild type
11	wt				Wild type
12	8	266	GGA to TGA	Glycine to Stop signal-Truncated protein	Mutant-nonsense
13	6	205	TAT to TGT	Tyrosine to Cysteine	Mutant-missense
14	wt				Wild type
15	7	233	11 bp insertion	Stop signal at codon 250	Mutant-nonsense
16	9	317	CAG to TAG	Glutamine to Stop signal-Truncated protein	Mutant-nonsense
17	wt				Wild type
18	6	222	Deletion G	Stop signal	Mutant-nonsense
19	wt				Wild type
20	5	163	TAC to TGC	Tyrosine to Cysteine	Mutant-missense
21	wt				Wild type
22	5	167	10 bp deletion	Stop signal	Mutant-nonsense

Table 6. p53 sequence of recurrent squamous cell cancers studied.

Of 22 tumours studied, 15 had mutant p53 and 7 had wild type p53. Of the mutant p53 sequences, 6 were missense mutations and 9 were nonsense mutations.

Patient no.	p53 gene seq.	p53 IHC	p53 histoscore	mdm2 IHC	mdm2 histoscore	HPV16	HPV18/33
1	Mutant-nonsense	-	0	+	2	-	-
2	Mutant-missense	+	4	-	0	-	-
3	Mutant-nonsense	-	0	-	0	+	-
4	Mutant-nonsense	-	0	+	2	-	-
5	Mutant-missense	+	6	-	0	-	-
6	Mutant-nonsense	+	6	+	4	-	-
7	Mutant-missense	+	6	-	0	-	-
8	Mutant-missense	+	5	+	2	-	-
9	Wild type	+	5	+	3	-	-
10	Wild type	-	0	-	0	+	-
11	Wild type	-	0	+	5	-	-
12	Mutant-nonsense	-	0	+	3	+	-
13	Mutant-missense	+	4	-	0	+	-
14	Wild type	+	2	-	0	-	-
15	Mutant-nonsense	-	0	-	0	+	-
16	Mutant-nonsense	-	0	-	0	+	-
17	Wild type	+	2	+	5	+	-
18	Mutant-nonsense	-	0	+	4	-	-
19	Wild type	-	0	-	0	+	-
20	Mutant-missense	+	4	-	0	-	-
21	Wild type	+	6	+	5	-	-
22	Mutant-nonsense	-	0	+	4	-	-

Table 7. p53 and mdm2 immunohistochemistry histoscores and HPV PCR analysis in squamous cell cancers studied.

p53 immunohistochemistry histoscore correlated with the type of gene sequence; nonsense mutations had low histoscores and missense mutations had high histoscores. Mdm2 protein expression was detected in 11 tumours and HPV 16 detected in 8 tumours. HPV 18 and 33 was not detected in any tumour.

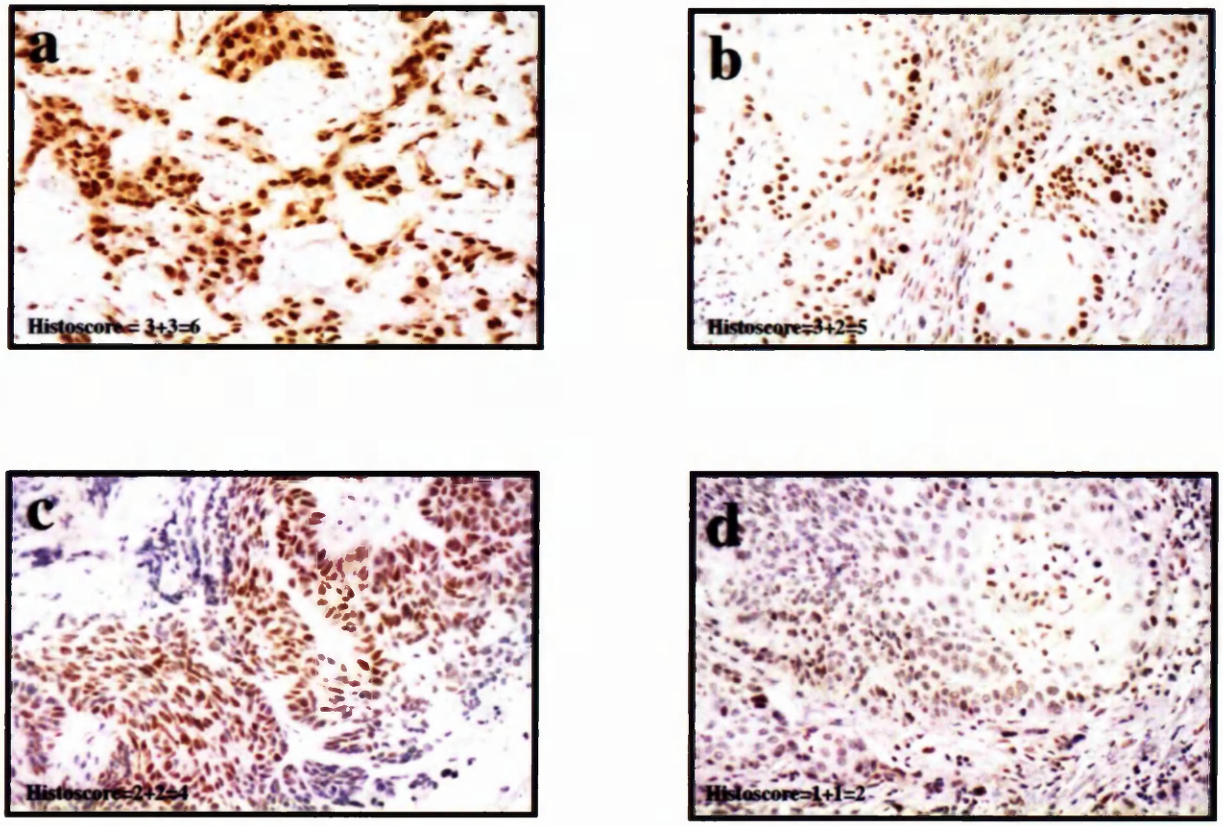


Figure 13.
Examples of p53 immunohistochemistry staining in HNSCC biopsies. Staining is graded using a histoscore grading system= intensity of staining + % of cells with positive staining.

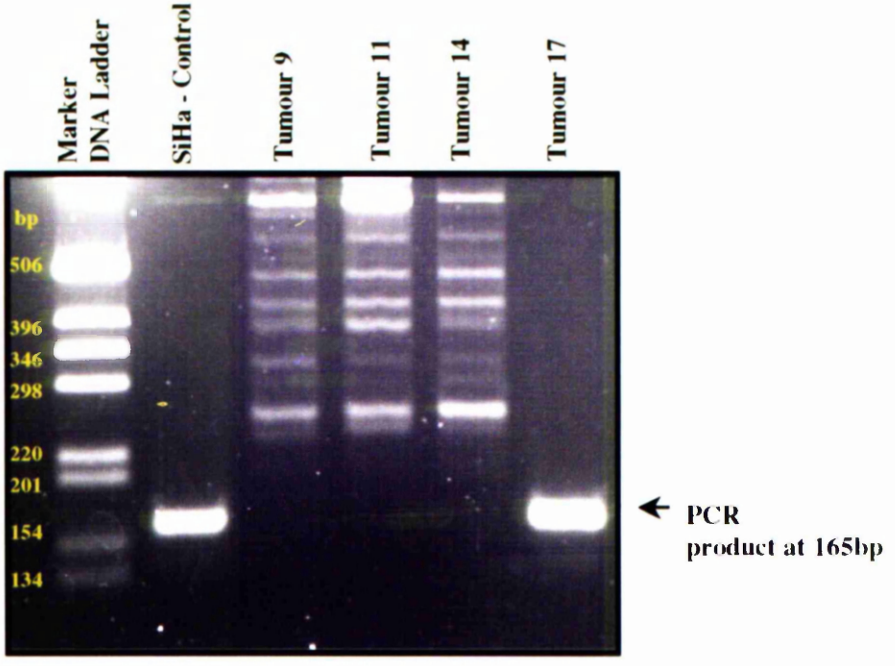


Figure 14. HPV 16 E6/7 analysis of tumour DNA by PCR.

DNA from SiHa cell line was used as a positive control for HPV16 E6/7. Tumour 17 is positive for HPV16 with the correct PCR product at 165 base pairs.

Mdm2 expression was detected in 11 of the 22 tumours (50%). Of these, 5 were weakly positive (histoscore=2, 3) and 6 were strongly positive (histoscore=4, 5, 6). Mdm2 expression was present in 7 tumours with mutant p53 and in 4 tumours with wild type p53.

Of the 5 tumours with wild type p53 sequence and negative or weakly positive p53 immunohistochemistry, 2 had HPV 16 expression (tumours 10, 19), 1 had high expression of mdm2 (tumour 11), 1 tumour had both (tumour 17), and 1 had neither. Of the 2 tumours with wild type p53 and strongly positive p53 immunohistochemistry (tumours 9, 21), both had high expression of mdm2 indicating inactivation by mdm2. Thus, 6 of the 7 tumours with wild type p53 had inactivation of p53 either by HPV 16 infection or by overexpression of mdm2. Examples of mdm2 immunohistochemistry in tumours 17 and 21 are shown in **Figure 15**.

Thus, overall we have shown that in recurrent HNSCC the incidence of p53 alterations is very high at 95%. We therefore considered recurrent head and neck cancer to be a suitable tumour type in which to carry out a phase I study using Onyx-015. Two phase I trials were carried out- a single injection protocol involving 22 patients and a multiple injection protocol involving 10 patients.

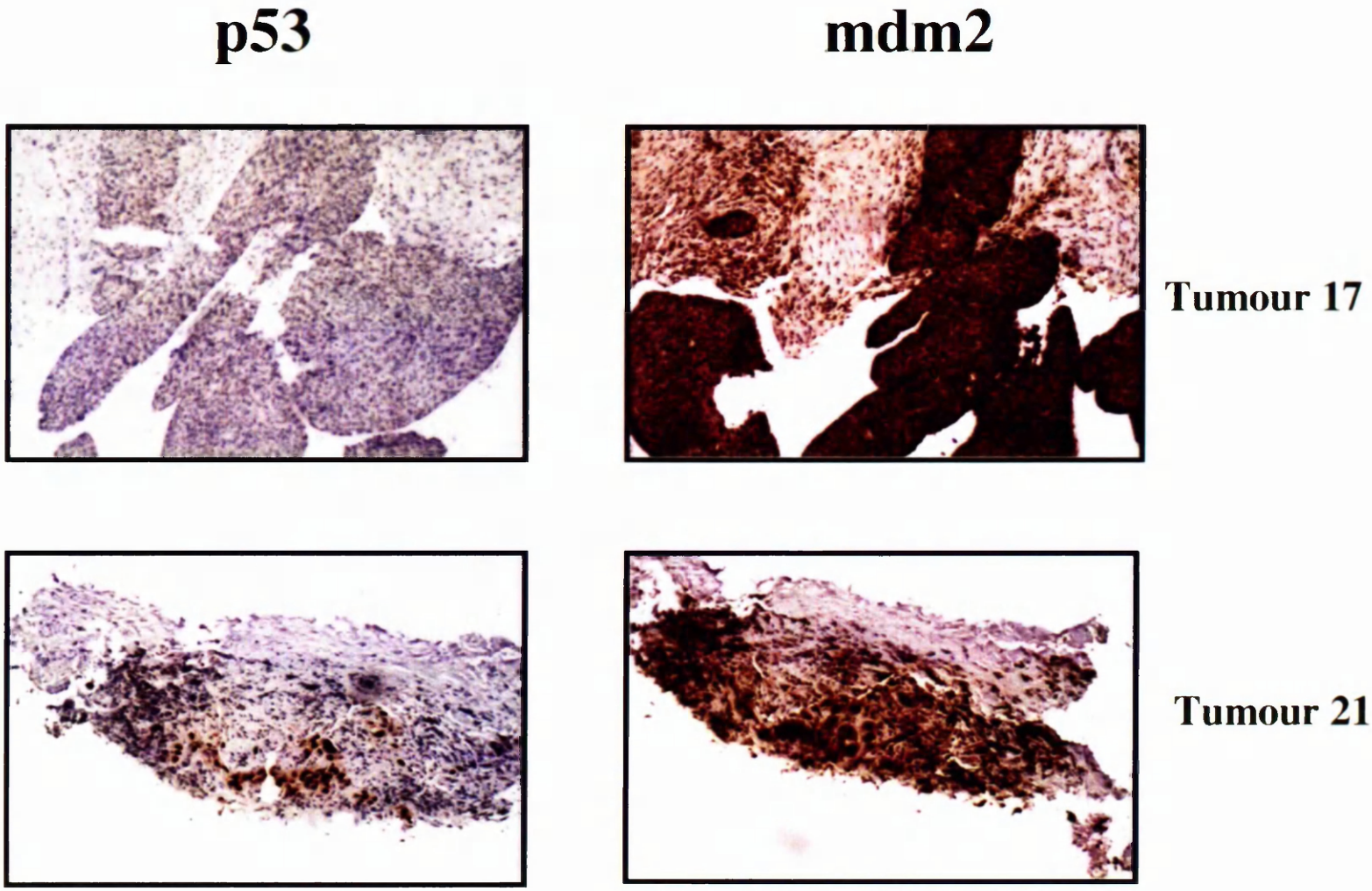


Figure 15. Examples of p53 and mdm2 immunohistochemistry in tumours with wild type p53 gene sequence.

3.4. CLINICAL PHASE I STUDY - single injection protocol.

3.4.1. Patient characteristics

The characteristics of the patients treated are shown in **Table 8.** All 22 patients treated were evaluable for toxicity and efficacy. 17 patients were male and 5 were female. The mean age was 63 and all patients had a performance score equal to or above 60% with a median of 80%. 21 patients had had prior therapy. 20 patients had received radiotherapy either alone or in combination with surgery and/or chemotherapy. 8 patients had received chemotherapy in combination with either radiotherapy or radiotherapy and surgery.

The most common site of recurrence was in the neck (regional recurrence) in the cervical area. Other sites included intraoral, preauricular, clavicular, facial and lip recurrences. The size of the recurrent tumours varied over a range from 2.2cm^2 to 20 cm^2 with the median being 11.8 cm^2. Examples of patients treated are shown in **Figure 16**.

A total of 22 tumours were positive for p53 by immunohistochemistry. Not all tumours had mutant p53 on gene sequencing however. 16 tumours had mutant p53 sequence, 5 had wild-type p53 sequence and 1 was non-interpretable. An example of typical positive p53 staining is shown in **Figure 17.** The pre-treatment immune status of each patient, as assessed by CD4 lymphocyte count, showed that most patients were immunosuppressed with 19 of the 22 patients having a CD4 count less than 500 and only 3 having a count greater than 500. 13 of the 22 patients had pre-existing neutralising antibodies to adenovirus.

Age(years)	Median	63
	Range	32-81
Sex	Male	17
	Female	5
Performance status	90%	3
	80%	11
	70%	5
	60%	3
Prior therapy	Surgery+XRT	10
	Chemo+XRT	4
	Surgery+XRT+Chemo	4
	Radiotherapy alone	2
	Surgery alone	1
	None	1
Location of recurrence	Cervical	10
	Supraclavicular	2
	Facial	2
	Tongue	2
	Oral	2
	Other	4
Tumour size-cm^2	Median	11.8
	Range	2.2-20
p53 gene sequence (exons 4-9)	Mutant	16
	Wild type	5
	Noninterpretable	1
Baseline neutralising antibody levels	Positive	13
	Negative	9
CD4 counts (range 152-1050)	<500	19
	>500	3

Table 8. Baseline patient characteristics.

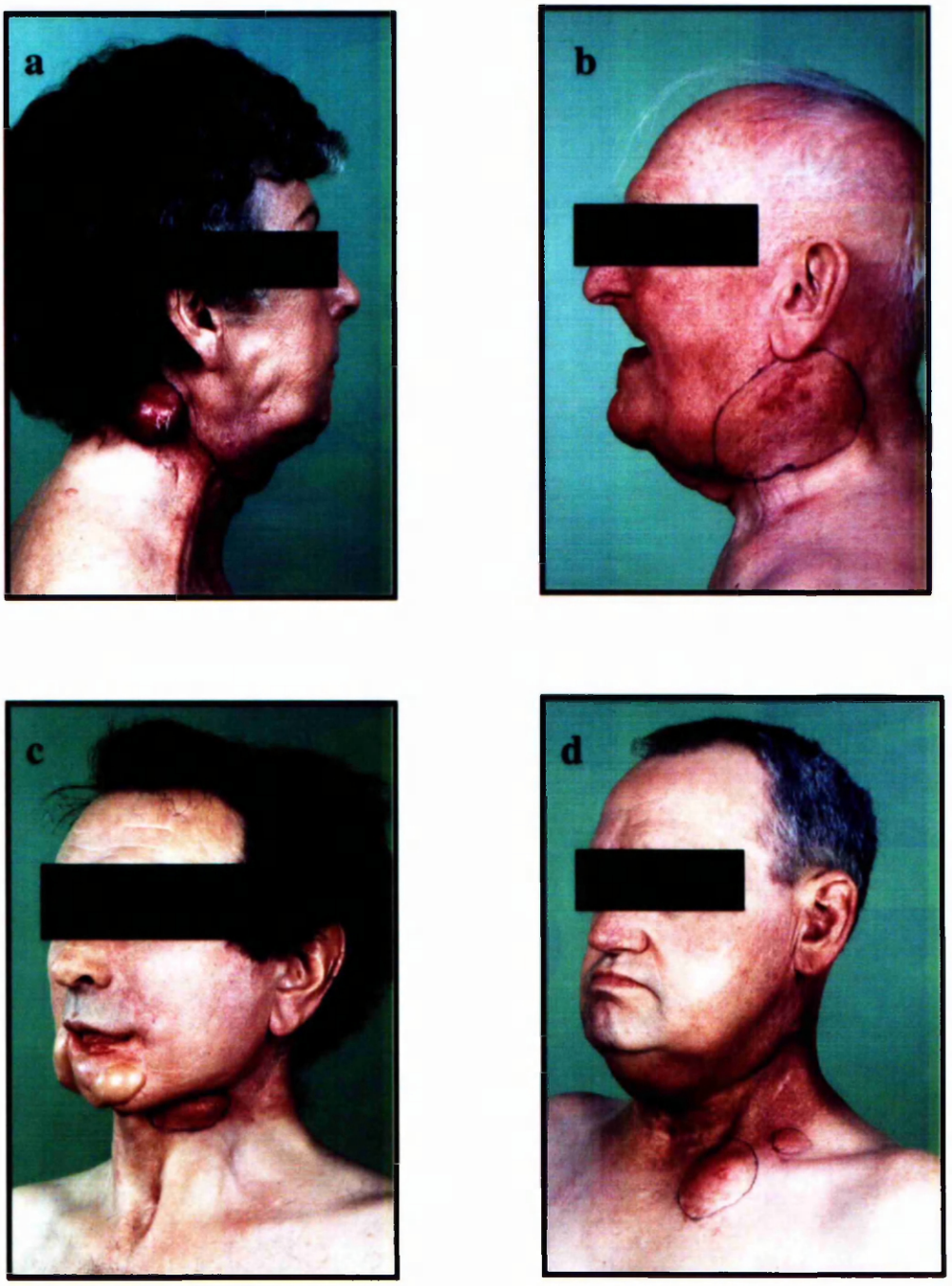


Figure 16.

Examples of recurrent head and neck cancers. Fig 16a shows a patient with a regional lymph node recurrence in the right neck from a floor of mouth primary tumour. Fig 16b shows a patient with local recurrence in the left neck from a left tonsillar primary tumour. Fig16c shows a patient with a left submandibular recurrence from an anterior floor of mouth primary tumour. Fig16d shows a patient with a left clavicular subcutaneous recurrence from a laryngeal primary tumour.

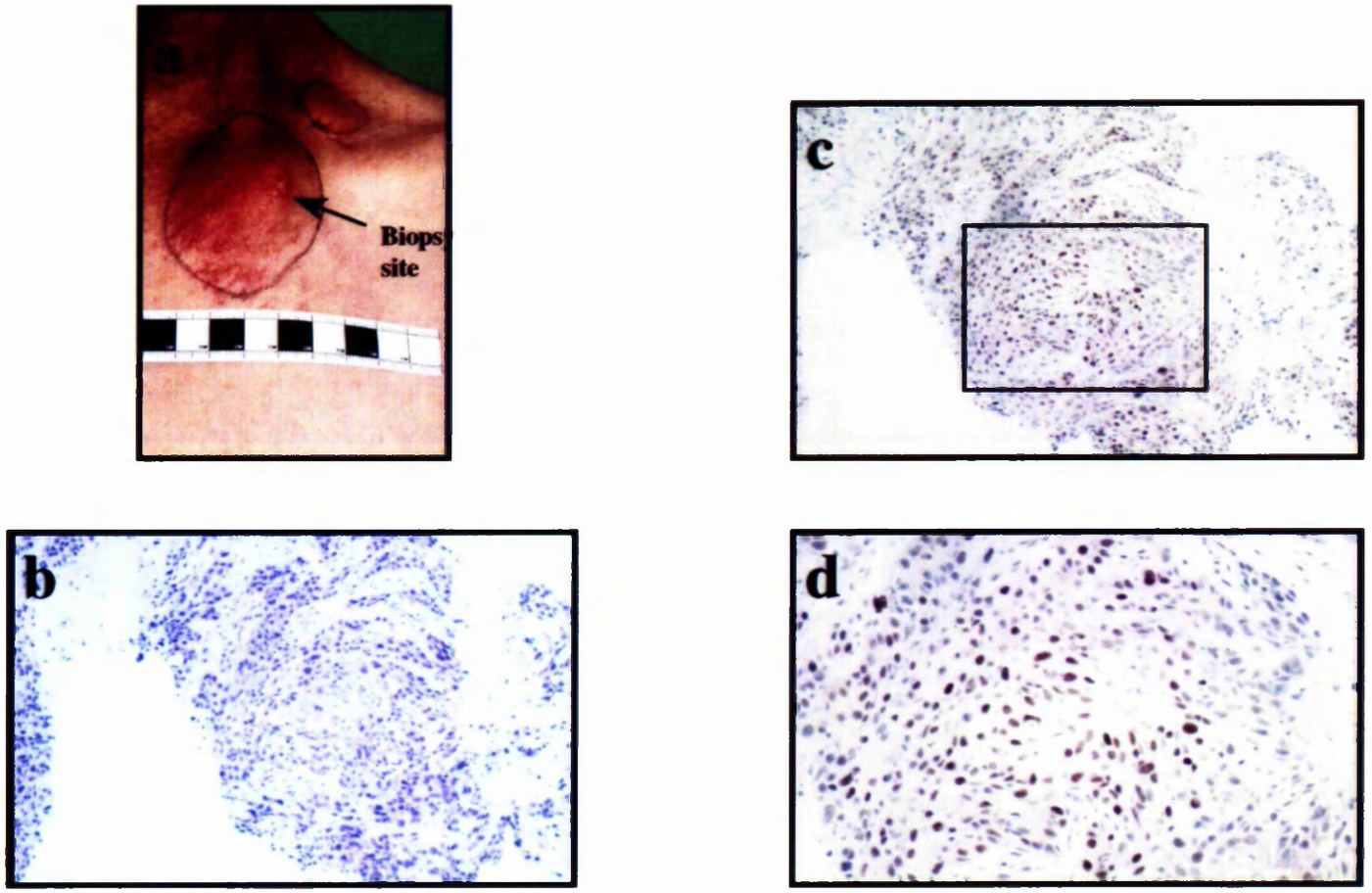


Figure 17.

Core biopsies were taken of the recurrent tumour (Figure 17a). Pathological diagnosis of squamous cell carcinoma was established on an haematoxylin and eosin stained section (Figure 17b). Immunohistochemistry for p53 was carried out as shown in Figure 17c/d. Dark brown nuclear staining indicates the cells which have mutant p53.

3.4.2. Toxicity and safety

Table 9 summarises the treatments given. Of the 22 patients, 14 received 1 cycle, 6 received 2 cycles, 1 received 3 cycles and 1 received 4 cycles. Therefore a total of 33 cycles were given. Intratumoural injection of Onyx-015 was well-tolerated with no dose-limiting toxicity being observed. Toxicities probably or possibly related to Onyx-015 are shown in **Table 10** and are all of grades I/II. The most frequent symptom was fever of Grade I/II. Two patients experienced discomfort during the injection which subsided within 1 hour in both patients. One patient had Grade II symptoms of tracheal obstruction which may have been related to Onyx-015. Serial blood counts showed no evidence of myelosuppression. Lymphocytopenia (Grade II - Grade IV) was seen in 5 patients, but this antedated virus injection and presumably related to pre-existing immunosuppression. There was no evidence of any other haematological or biochemical abnormality attributable to therapy. Patients who were retreated did not experience any further toxicity suggesting that neutralising antibody levels did not sensitise the patients to added toxicity.

All patients had blood plasma samples collected before and after virus injection (days 0, 3, 8, 15, 22 and 29). DNA was isolated from these plasma samples and PCR analysis was carried out using primers specific for adenovirus. All of these samples were negative suggesting Onyx-015 was not shed or did not persist in the circulation. All patients had swabs taken from the oropharynx and the injection site to detect virus shedding on days 0, 8, 15, 22 and 29. These swabs were analysed by a direct fluorescent hexon

Cohort	No.patients	No.cycles				Target tumour response			
		1	2	3	4	PR	MR	SD	Prog
10^7pfu	5	2	1	1	1	1	0	1	3
10^8pfu	4	4	0	0	0	0	0	2	2
10^9pfu	4	2	2	0	0	2	0	1	1
$3x10^9$pfu	3	2	1	0	0	0	1	1	1
10^{10}pfu	3	2	1	0	0	0	0	2	1
10^{11}pfu	3	2	1	0	0	0	1	1	1
Total	22	14	6	1	1	3	2	8	9

Table 9. Patients treated per cohort and target tumour response at >4 weeks using non-conventional measurements.

Toxicity profile - all Grade I/II		
Toxicity	**Cycle 1**	**All cycles, n=33**
Fever	7(32%)	7(21%)
Nausea	3(14%)	3(9%)
Chills	2(9%)	2(6%)
Flu syndrome	2(9%)	2(6%)
Diarrhoea	2(9%)	2(6%)
Pain(tumour)	2(9%)	2(6%)
Vomiting	1(4%)	1(3%)
Tracheal obstruction	1(4%)	1(3%)

Table 10. Toxicity profile for first cycle and all cycles.

protein assay and all were negative suggesting Onyx-015 was not readily shed from treated tumours even when the tumour was ulcerated.

3.4.3. Tumour response and correlation to p53 status.

All patients were evaluable for response and the data are summarised in **Table 9.** Ultimately, all patients in the study progressed due to the development of other neck lesions or due to distant metastases in the lung, liver or bone. However, if we consider only the tumours which were injected, then there was evidence of antitumour activity. A common finding following injection was that the injected tumour became soft and fluctuant, usually within 8 days following injection. Often the overlying skin became erythematous. MRI scans of injected tumours often showed a change in the signal from the tumour centre, in keeping with liquefaction of solid tumour. i.e. necrosis. Using non-conventional criteria for measuring the degree of tumour shrinkage (i.e. subtracting out central necrosis), 3 patients showed a partial response in the treated lesion and 2 showed a minor response. A further 8 patients had stable disease. There was no correlation between viral dose injected and tumour response.

Details of the 3 cases showing a substantial tumour shrinkage in the treated lesion are as follows:

1. Patient 1003: had a primary tongue tumour treated with surgery, radiotherapy and chemotherapy. He developed a recurrence in the right submandibular area with severe trismus and pain. This area was injected with 10^7pfu of virus. A 50% reduction in the size of the tumour occurred with 3 cycles of treatment given over 12 weeks. The MRI scans pre and

post-treatment showing changes suggestive of necrosis are illustrated in **Figure 18.** Symptomatically he improved with increased jaw mobility and reduced pain. This response was of 12 weeks duration before the patient was removed from study due to the development of lung metastases.

2. Patient 1007: had a left temple tumour treated initially with surgery and radiotherapy. He developed a recurrence in the left preauricular area extending deep into the pterygoid fossa. This was injected at 10^9pfu and a 75% reduction in size of the tumour, with radiological changes in keeping with necrosis, occurred after 2 cycles of treatment. This response was of 8 weeks duration before the patient was removed from study due to the development of orbital disease requiring radiotherapy.

3. Patient 2006: had a primary tongue tumour treated with radiotherapy. He developed a tongue recurrence and the right third of the tongue was injected at 10^9pfu. A large portion of tumour, measured as 80% of the original injected tumour, sloughed off on day 8. This response was of 4 weeks duration before the patient died from bacterial pneumonia (believed to be unrelated to virus injections).

Of the 2 patients with a minor response to treatment, 1 patient was removed from study at 4 weeks due to the development of a second locoregional recurrence and 1 patient was removed at 4 weeks for radiotherapy to the target treated tumour (This patient had not received prior radiotherapy and this had been planned prior to virus injection). Of the 8 patients with stable disease, 3 were removed from study due to the development of a second locoregional recurrence - 1 patient at 8 weeks and 2 patients at 4 weeks. The other 5 patients were removed from study due to

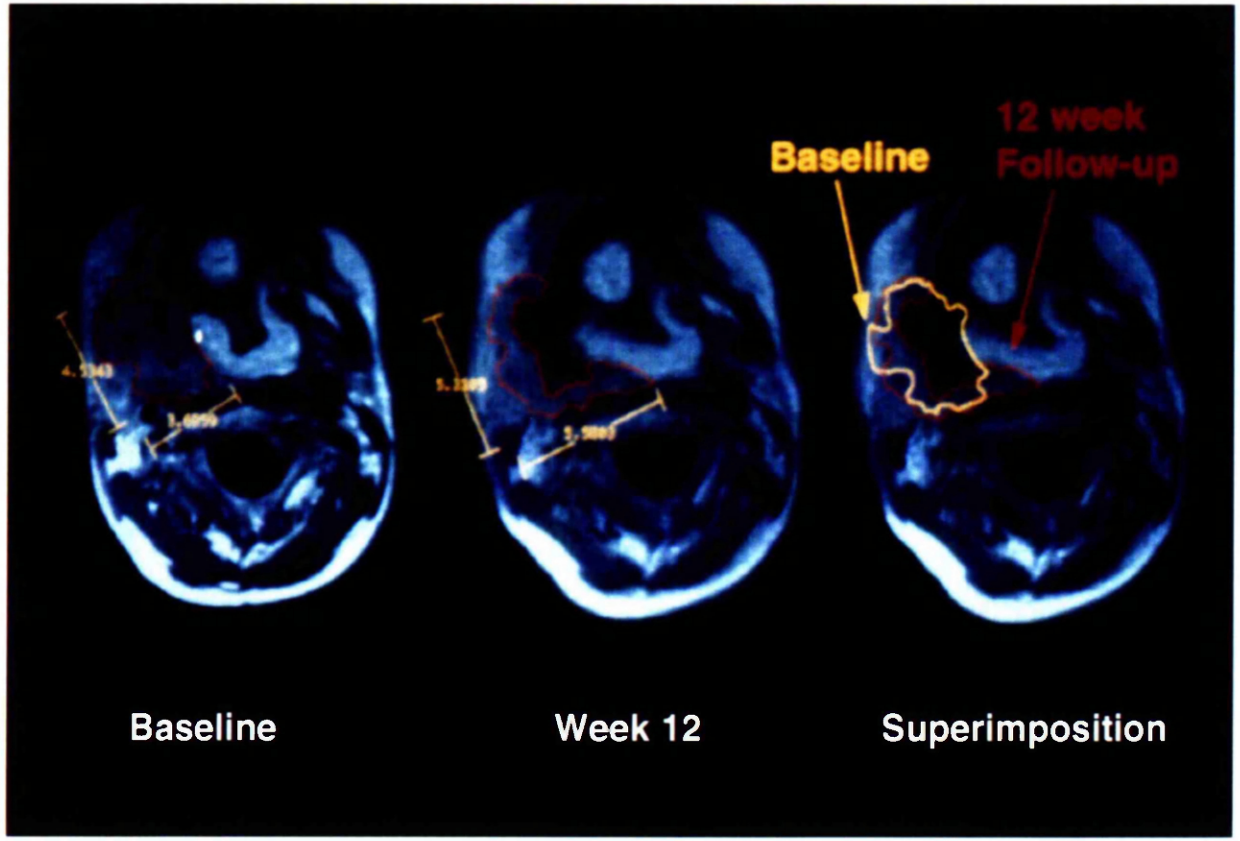


Figure 18. Patient 1003 had a primary tongue tumour and developed local recurrence in the right submandibular region. The depth of the tumour is shown in the baseline MRI scan outlined in red. 50% necrosis of the tumour occurred after 3 cycles of treatment. The area of necrosis and is shown on the week 12 MRI scan.

progression at the injected tumour at 4-6 weeks post virus injection. All other patients showed evidence of tumour progression locally and at other sites.

To control for the effect of diluent, 3 patients had satellite lesions injected with diluent alone. All of these tumours progressed with no clinical or radiological signs suggestive of necrosis.

The p53 status of tumours versus the tumour response is shown in **Table 11.** Of the 5 tumours which showed evidence of response, 4 had mutant p53 and 1 had wild type p53 suggesting that Onyx-015 may selectively replicate in mutant p53 tumours. However, if we compare the p53 status of tumours showing evidence of response (MR,PR) to those which did not (SD,Prog), statistical analysis using Fisher's exact test showed no significant correlation (p= 0.53).

3.4.4. Detection of Onyx-015 in tumour and correlation to p53 status.

Adenoviral DNA was detected in tumour biopsies by *in-situ* hybridisation. A typical example of staining is shown in **Figure 19.** 4 of the 22 patients showed positive evidence of viral replication on the biopsies obtained. All 4 of these patients had mutant p53 on gene sequencing. No patients with wild type p53 gene sequence showed virus replication. In addition, adenoviral DNA was only detected in tumour cells and normal skin and mesenchymal tissue within the biopsies were negative. This suggested that viral replication was selective for tumours with mutant p53. However, statistical analysis using Fisher's exact test **(Table 12)** showed no statistical significance (p= 0.53). Unfortunately due to the limited amount of clinical

		No.patients	Target tumour response				p-value
			PR	MR	SD	Prog	
Baseline neutralising	Positive	13	1	2	3	7	
antibody levels	Negative	9	2	0	5	2	0.96
p53 gene sequence	Mutant	16	2	2	4	8	
	Wild type	5	1	0	3	1	0.53
	Noninter.	1	0	0	1	0	

Table 11. Response to Onyx-015 related to p53 gene sequence and baseline neutralising antibody level.

p value based on comparison of tumours showing a response (PR,MR) versus no response (SD,Prog). PR,partial response; MR,minor response; SD,stable disease; Prog, progressive disease.

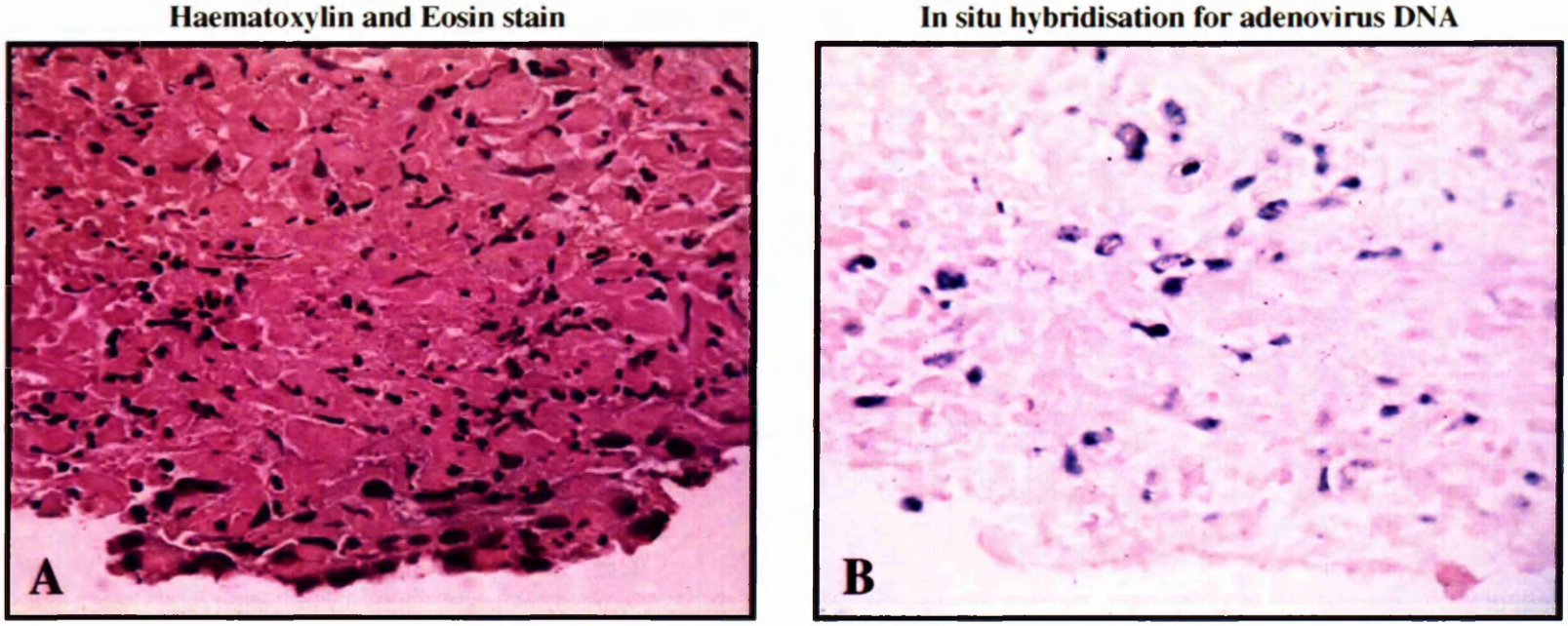


Figure 19. Detection of adenoviral DNA in tumour core biopsies by in situ hybridisation. Core biopsies were taken fromtumours at day 8 and day 22 post virus injection and stained for adenoviral DNA by in situ hybridisation. Figure 19a shows an H&E stained biopsy at high power. Figure 19b shows the same biopsy areas stained for adenoviral DNA. The dark blue staining cells represent cells expressing adenoviral DNA.

		No.patients	Viral replication by ISH		p-value
			Positive	Negative	
p53 gene sequence	Mutant	16	4	12	
	Wild type	5	0	5	0.53
	Noninter.	1	0	1	

Table 12. Viral replication related to p53 gene sequence.

p value based on comparison of tumours showing positive staining by in situ hybridisation versus no staining.

tissue available from the core biopsies, no attempt was made to culture virus from the biopsy specimens.

3.4.5. Humoral immune response and correlation to tumour response.

13 of the 22 patients treated (59%) had pre-existing neutralising antibodies to adenovirus and all but one patient developed increased antibody levels after treatment. If we compare baseline neutralising antibody levels of patients who had evidence of tumour response (MR,PR) to those who did not (SD,Prog) statistical analysis using Fisher's exact test (**Table 11**) showed no significant correlation (p=0.96). Therefore pre-existing neutralising antibody levels did not determine tumour response.

3.5. PHASE I TRIAL - multiple injection protocol.

3.5.1. Patient characteristics.

The characteristics of the patients treated and their prior therapy is shown in **Table 13**. All 10 patients treated were evaluable for toxicity and efficacy. 8 patients were male and 2 were female. The mean age was 66 and all patients had a performance score above 60% with a median of 80%. All patients had received radiotherapy either alone or in combination with surgery and/or chemotherapy. 5 patients had received chemotherapy in combination with radiotherapy and surgery. The most common site of recurrence was in the neck (regional recurrence) in the cervical area. The size of the recurrent tumours varied over a range from 4 cm^2 to 7.5 cm^2 with the mean being 6.2 cm^2.

Age(years)	Median	66
	Range	44-88
Sex	Male	8
	Female	2
Performance status	80%	5
	70%	3
	60%	2
Prior therapy	Surgery+XRT+Chemo	5
	Surgery+XRT	4
	Radiotherapy alone	1
Location of recurrence	Cervical	5
	Facial	2
	Oropharynx	3
Tumour size-cm^2	Median	6.2
	Range	4-7.5
p53 gene sequence (exons 4-9)	Mutant	5
	Wild type	5
Baseline neutralising antibody levels	Positive	7
	Negative	3
CD4 counts (range 152-1343)	<500	9
	>500	1

Table 13. Baseline patient characteristics in multiple injection protocol.

A total of 10 tumours were positive for mutant p53 by immunohistochemistry. Not all tumours were mutant p53 on gene sequencing however. 5 tumours had mutant p53 sequence and 5 had wild type p53 sequence. The pre-treatment immune status of each patient, as assessed by CD4 lymphocyte count, again showed that most patients were immunosuppressed. 9 of the 10 patients had a CD4 count less than 500 (range 152-1343).

3.5.2. Toxicity and safety

Table 14 summarises the treatments given. Of the 10 patients, 7 received 1 cycle, 2 received 2 cycles and 1 received 7 cycles. Therefore a total of 18 cycles were given. Intratumoural injection of Onyx-015 was again well tolerated with no dose limiting toxicity being observed. Toxicities probably or possibly related to Onyx-015 are shown in **Table 15** and are all of grades I/II. The most frequent symptom was flu syndrome (30%) of Grades I/II. Flu-syndrome was more frequent in the multiple injection study at 30% compared to approximately 10% in the single injection study. Nausea and diarrhoea were also more frequent with 30% of patients experiencing these symptoms compared to 10% in the single injection study. This may suggest there was more shedding of virus into the circulation in the multiple injection study. Serial blood tests showed no myelosuppression. Despite the increased number of injections, pain on injection was only reported in 1 patient. Safety studies were also encouraging since all blood samples to detect adenovirus by PCR were

Cohort	No.patients	No.cycles							Target tumour response			
		1	2	3	4	5	6	7	PR	MR	SD	Prog
$5x10^9$pfu	4	2	1	0	0	0	0	1	1	0	1	2
$5x10^{10}$pfu	6	5	1	0	0	0	0	0	0	0	4	2
Total	10	7	2	0	0	0	0	1	3	2	8	9

Table 14. Patients treated per cohort and target tumour response at >4 weeks using non-conventional measurements in multiple injection protocol.

Toxicity profile - all Grade I/II		
Toxicity	**Cycle 1**	**All cycles n=18**
Nausea	3(30%)	3(17%)
Flu syndrome	3(30%)	3(17%)
Chills	2(20%)	2(11%)
Diarrhoea	2(20%)	2(11%)
Lymphocytopenia	2(20%)	2(11%)
Fever	1(10%)	1(5%)
Vomiting	1(10%)	1(5%)
Pain(tumour)	1(10%)	1(5%)

Table 15. Toxicity profile for multiple injection protocol for first cycle and all cycles.

negative and all swabs of the oropharynx and injection sites were negative for adenovirus.

3.5.3. Tumour response and correlation to p53 status.

All patients were evaluable for tumour response and these are shown in **Table 14.** Using non-conventional criteria for measuring the degree of tumour shrinkage, 1 patient showed a partial response and 5 patients showed stable disease in the treated lesion. The patient with a partial response had a primary floor of mouth squamous cell cancer treated with surgery and radiotherapy. He developed a 4cm x 2cm recurrence in the left cheek and this was injected at 5×10^9 pfu. An 80% reduction in the tumour occurred over 7 cycles of treatment. This patient was removed from study at 28 weeks when the target tumour began to progress. Of the 5 patients with stable disease, 3 were removed from study at 4 weeks due to the development of a second locoregional recurrence. The other 2 patients were removed from study at 8 weeks again due to the development of a second locoregional recurrence. 4 patients progressed at the injected lesion at 4 weeks post virus injection.

The p53 status of tumours versus the tumour response is shown in **Table 16.** The tumour which showed a partial response had mutant p53 on gene sequencing. If we again compare the p53 status of tumours showing evidence of response (MR,PR) to those which did not (SD,Prog), statistical analysis using Fisher's exact test showed no significant correlation (p=1).

3.5.4. Detection of Onyx-015 in tumour and correlation to p53 status.

		No.patients	Target tumour response				p-value
			PR	MR	SD	Prog	
Baseline neutralising	Positive	7	1	0	5	1	
antibody levels	Negative	3	0	0	0	3	0.98
p53 gene sequence	Mutant	5	1	0	3	1	
	Wild type	5	0	0	2	3	1

Table 16. Response to Onyx-015 related to p53 gene sequence and baseline neutralising antibody level.

p value based on comparison of tumours showing a response (PR,MR) versus no response (SD,Prog). PR,partial response; MR,minor response; SD,stable disease; Prog, progressive disease.

		No.patients	Viral replication by ISH			p-value
			Positive	Negative	No biopsy	
p53 gene sequence	Mutant	5	2	1	2	
	Wild type	5	3	1	1	0.99

Table 17. Viral replication related to p53 gene sequence.

p value based on comparison of tumours showing positive staining by in situ hybridisation versus no staining.

Adenoviral DNA was detected in tumour biopsies by *in-situ* hybridisation. 5 of 7 tumours showed positive evidence of viral replication on the biopsies obtained. 3 patients did not have post-treatment biopsies carried out. This increased frequency suggested that multiple injections resulted in better viral distribution. In contrast to the single injection study, 2 of the 5 tumours with evidence of viral replication had wild type p53 gene sequence. Statistical analysis using Fisher's exact test (**Table 17**) again showed no significant correlation between replication and p53 gene sequence however. (p=0.99).

3.5.5. Humoral immune response and correlation to tumour response.

7 of the 10 patients treated (70%) had pre-existing neutralising antibodies to adenovirus and all developed increased antibody levels after treatment. If we compare baseline neutralising antibody levels of patients who had evidence of tumour response (MR,PR) to those who did not (SD,Prog) statistical analysis using Fisher's exact test (**Table 16**) showed no statistically significant correlation (p=0.98). Therefore pre-existing neutralising antibody levels did not determine tumour response.

Chapter 4

Discussion

4.1. RATIONALE

The E1B 55kDa gene deleted adenovirus,Onyx-015, has been shown to selectively replicate in cells with non-functional p53 (Bischoff et al, 1996). The objective of this thesis was to determine if this virus could be used as a new form of therapy for the treatment of recurrent head and neck cancer. Current therapies for this disease including reirradiation, chemotherapy, chemoradiation have poor response rates and short progression free time intervals. It is also a disease which produces considerable morbidity with difficulties in speech, swallowing and pain control. Therefore a locoregional therapy for this disease, such as intratumoural injection of Onyx-015, would be an attractive alternative therapy for this disease. Before we could carry out any clinical trials with this virus it was first necessary to determine if Onyx-015 could replicate in head and neck cancer cell lines both in vitro and in vivo and to determine if this was selective for mutant p53 cell lines. For this therapy to be useful in head and neck cancer it was also necessary that recurrent head and neck cancer had a high incidence of p53 inactivation. No studies have examined the p53 status of recurrent disease before and therefore we carried out a small study in 22 patients to evaluate the incidence of p53 inactivation by mutation, deletion and inactivation by other mechanisms.

4.2. PRECLINICAL HNSCC STUDIES.

4.2.1. In-vitro and in-vivo studies.

Previous in-vitro and in-vivo studies had shown that Onyx-015 would selectively replicate in and lyse tumours with mutant p53 (Bischoff et

al,1996). No viral studies have been carried out on squamous cell cancer cell lines either in-vitro or in-vivo. Using a panel of HNSCC cell lines of known p53 sequence we tested for viral induced cytolysis and viral replication using CPE assays, hexon protein immunofluorescence and FACS analysis to quantitate replication. In agreement with Bischoff et al,1996 we have shown all HNSCC cell lines with mutant p53 were susceptible to Onyx-015. The only HNSCC cell line tested with wild type p53 was BICR19. This cell line has one wild type allele and one mutant p53 allele. FACS analysis showed that little replication occurred in this cell line suggesting that only one functional allele of p53 may be required to confer resistance to Onyx-015. This data was encouraging since recent papers by Ridgeway et al,1997 ; Goodrum and Ornelles,1997 and by Hall et al,1998 had suggested that replication of E1B deleted adenoviruses showed no correlation to p53 status. These papers were also controversial since in all three papers the p53 function of the cell lines used was not fully determined. Goodrum et al,1997 suggested that replication was cell cycle dependent and was more productive if cells were in S phase of the cell cycle and that this was independent of p53 status. Hall suggested that viral induced cell death was more efficient in wild type p53 cell lines but failed to show the mechanism of cell death, i.e. apoptosis or viral replication. Therefore, despite these papers, the hypothesis that E1B attenuated viruses can selectively replicate in cells with non-functional p53 has not been disproved. Indeed we have recently shown using the paired ovarian adenocarcinoma cell lines A2780 (wild type p53 sequence and function) and A2780Cp70 (wild type p53 sequence but non-functional) that replication is six fold greater in the Cp70 cell line. However

we also showed that Onyx-015 could induce apoptosis in A2780 and that this was mediated by E1A induction of functional p53. This observation could explain some of the findings in the papers by Hall and Goodrum.

The cell line BICR16, derived from a patient with recurrent HNSCC, has a point mutation in the p53 gene at codon 146. This cell line was used as the in-vivo model for recurrent HNSCC by forming subcutaneous xenografts in nude mice. The tumours were injected with 5 daily injections of either Ad2 or Onyx-015 against a control solution of PBS. Tumour regression occurred for both viruses in all tumours. Statistical analysis showed a p value of 0.002 (very significant) for the Ad2 group and a p value of <0.001 (highly significant) for the Onyx-015 group. Furthermore, in-situ hybridisation for viral DNA in these treated tumours showed the presence of adenoviral DNA which suggested that regression was due to the replication of virus in the tumours. Thus both the in-vitro and in-vivo data were very encouraging and suggested that Onyx-015 may be efficacious in patients with recurrent HNSCC .

4.2.2. Preclinical p53 HNSCC studies.

Our next objective was to determine the incidence of p53 inactivation in recurrent HNSCC since for Onyx viral therapy to be successful it was necessary that tumours had non-functional p53. We studied 22 recurrent tumours and have shown that in recurrent HNSCC there is a high incidence of p53 mutation. 15 of the 22 tumours (68%) had either a p53 mutation or deletion. Of these, 6 were missense mutations whereas 9 had nonsense inactivation. The immunohistochemistry results correlated

very well with the mutations seen. Previous studies in primary head and neck cancer have shown an incidence of p53 mutation of 38% -53% (Boyle et al,1993; Brachmann et al,1992; Koh et al,1998; Mao et al,1996; Olshan et al,1997). It has been suggested that p53 mutation is an early event in head and neck carcinogenesis. Boyle et al,1993 showed an incidence of mutation of 19% in preinvasive lesions increasing to 43% in invasive lesions. However, Chung et al,1993 showed that the frequency of p53 mutations did not increase with stage of disease with a reported incidence of 47% in stage I/II disease and 37% in Stage III/IV disease. In our study, we have reported an incidence of p53 mutation in recurrent HNSCC of 68% which is higher than that reported for primary disease. We did not carry out microdissection of tumour cells from surrounding normal cells in the preparation of tumour DNA so it is possible that some tumours with wild type p53 gene sequence are in fact false negatives and therefore the incidence may be higher than 68%. Our figures (and those reported for primary disease by other studies) are also based upon sequencing exons 4-9 of the p53 gene. Although 95% of mutations occur in these exons, some mutations occur in intron regions and also other exons. Therefore the incidence of p53 mutations in our study may be higher. Similarly the incidence in previously published studies on primary head and neck cancer may also be slightly higher than 38-53%. It is also possible that previous studies have underestimated the incidence of p53 mutation due to the heterozygous nature of primary tumours resulting in false negative results on gene sequencing. In recurrent tumours, clonal expansion of tumour cells refractory to radiotherapy occurs and therefore the pickup rate for detecting p53 mutation may be more accurate. However,

another possibility may be the induction of p53 mutations by radiotherapy treatment itself since radiation is a potent DNA damaging agent and induces single and multiple base pair deletions in DNA. It is difficult to distinguish between these 2 possibilities but one method could be to prepare molecular probes to the p53 mutation in each recurrent tumour and then to use these probes to screen the primary tumours to look for the mutation.

p53 can be inactivated by human papilloma virus (HPV) due to the ability of HPV E6 protein to bind to and promote the degradation of the product of the p53 gene (Levine,1990; zur Hausen,1994). Indeed inactivation of p53 by HPV E6 is the major mechanism of p53 inactivation in cervical carcinogenesis (zur Hausen,1994) with approximately 80-90% of cervical cancers containing HPV DNA (Yoshikawa,1991). Previous studies have shown HPV DNA in tumours of the head and neck but its prevalence varies from 10-58% (Paz et al,1997; Haraf et al,1996; Miguel et al,1998; Koh et al,1998). A metanalysis by McKaig et al,1998 showed an overall incidence of 34.5% with the predominant type being HPV 16. The incidence also varies with tumour site with the highest prevalence being in tonsillar carcinoma (Paz et al,1997). In our study in recurrent tumours of the head and neck, we found an overall incidence of 36% (8/22), all of which were HPV 16. These results were very similar to the metanalysis figures by McKaig for primary tumours and this would suggest that HPV infection is not a major aetiological factor in recurrent disease. One of the limitations of our study, and of previously published studies, is that HPV detection is based upon PCR analysis of HPV DNA and not mRNA of HPV E6-E7 regions. It is possible that PCR analysis of HPV DNA can overestimate the

presence of HPV due to cross contamination resulting in false positives. Since RNA is readily degradable, cross contamination is less likely to result in false positives. Another advantage of using RNA is that it proves that any HPV present in tumour cells is actually expressed at the mRNA level. Some studies have reported HPV detection by detecting mRNA using in-situ hybridisation of paraffin embedded samples (Stoler et al,1992). A more accurate method however is to use RTPCR as reported by Czegledy et al,1995. In our study, DNA was used because the samples used were too small to allow an adequate amount of RNA to be prepared. Of the 8 tumours which were positive for HPV, 5 tumours were in tumours with mutant p53 and 3 were in tumours with wild type p53. These figures are similar to those in primary tumours as reported by Koh et al,1998 and suggests that p53 mutation and HPV positivity are not mutually exclusive mechanisms of p53 inactivation. This is in contrast to cervical carcinogenesis where p53 mutations are rare in cases with HPV but common in malignancies devoid of HPV infection (Crook et al,1991; Park et al,1994).

Another factor which can inactivate p53 is the cellular protein mdm2. Mdm2 inhibits the transcriptional activity of p53 by binding to the N terminus of p53 (Haupt et al,1997) which in turn leads to the degradation of p53 via the ubiquitin degradation pathway. Thus amplification or overexpression of mdm2 can cause p53 inactivation (Oliner et al,1992). In primary HNSCC, mdm2 overexpression is reported to be 40% (Matsumura et al,1996). Girod et al,1995 showed an increase in mdm2 expression from premalignant to malignant lesions indicating that this may be a mechanism for inactivation of p53 early in head and neck carcinogenesis. Further

evidence to suggest this was reported by Pruneri et al,1997 who showed that out of 17 laryngeal tumours overexpressing mdm2, 10 of these also overexpressed p53 and all ten had wild type p53 sequence. The importance of mdm2 overexpression in recurrent HNSCC is unknown. In the small group of tumours which we have studied, we have shown an incidence of 50% of which 5/11 were weakly positive and 6/11 were strongly positive for mdm2. Mdm2 overexpression was present in 7 tumours with mutant p53 and in 4 tumours with wild type p53 and this also suggests that p53 mutation and mdm2 overexpression are not mutually exclusive mechanisms of p53 inactivation. A particularly intriguing observation is that of the 7 tumours which had wild type p53 sequence, 6 were positive for either HPV infection, mdm2 overexpression or both. Thus in wild type p53 tumours, the p53 gene is inactivated by other mechanisms making the overall incidence of p53 inactivation in recurrent HNSCC in this study to be 95% (21 out of 22 tumours). It is unclear whether or not this overall incidence is greater than primary HNSCC since no study has ever examined p53 mutation, HPV infection and mdm2 overexpression in the same group of tumours. The observation that the incidence of HPV infection and mdm2 overexpression are similar to primary disease indicates that these factors have a limited role in the aetiology of recurrent HNSCC but may be more important in tumours in which the p53 gene is not inactivated by mutation.

The observation that recurrent disease has a very high incidence of p53 inactivation is clearly important as p53 is central to apoptosis induced by radiation and many chemotherapeutic agents. This may account for the poor response of these tumours to both reirradiation and chemotherapy.

Therefore, new therapies which can restore p53 functionality, such as adenoviral mediated transfer of wild type p53 (Roth et al,1996), may be beneficial in this disease. Indeed, in-vitro and in-vivo studies using such a technique have demonstrated growth suppression in HNSCC cell lines (Liu et al ,1994, Liu et al ,1995; Clayman et al, 1995). Results of a phase I study in patients with recurrent HNSCC were also recently reported (Clayman et al,1998) and this showed evidence of tumour necrosis in some patients. Other methods of restoring p53 function could be to use small molecules to inactivate the mdm2 protein (Bottger et al,1998), or drugs which interfere with HPVE6 binding to wild type p53 thus restoring function of p53. Other approaches to the treatment of this disease may be to use drugs which act in a p53 independent manner. For example, Taxanes (Taxol) act by inducing p53 independent apoptosis and have been successful in the treatment of refractory cisplatin resistant ovarian cancer, a disease where there is also a high incidence of p53 inactivation (Righetti et al,1996; Buttitta et al,1997). Catimel et al,1994 has shown this agent to be effective in recurrent HNSCC but there is no evidence yet that it has increased benefit over cisplatin / 5FU chemotherapy. However newer agents which act in a p53 independent manner may prove to be more beneficial. Our approach has been to use the selectively replicating adenovirus Onyx-015 which has been shown to selectively destroy tumours with non-functional p53. Since we have shown a high incidence of p53 inactivation in this disease, Onyx-015 should theoretically replicate in and destroy these tumours in patients. We therefore carried out a phase I trial using this agent in recurrent HNSCC.

4.3. CLINICAL PHASE I TRIAL - single injection protocol.

4.3.1. Lack of toxicity

The primary objective of the phase I study was to determine the safety of a single injection of Onyx-015 by intratumoural injection in patients with recurrent HNSCC. The results obtained showed that toxicity was very minor, with the main symptoms being mild flu-like symptoms. Two patients did experience mild pain on injection and this was related to the volume of injected solution. The pain resolved within 1 hour and rarely required any further analgesia. The maximum dose injected was 10^{11}pfu and this did not produce any serious adverse effects. The maximum tolerated dose was clearly not reached in this study, but dose escalation ceased because the limit of virus manufacturing capacity had been reached. This lack of toxicity is very encouraging and suggests that perhaps more potent replicating viruses can be explored in future trials. Safety data were also encouraging since no virus was detected in either blood samples or injection site and oropharyngeal swabs and this suggested that virus shedding was not a major issue. This was in agreement with a previous study which involved the intratumoural injection of a replication incompetent adenovirus Ad5p53 in patients with recurrent head and neck cancer (Clayman et al,1998). In this study adenovirus was detectable in blood samples by PCR at 30-90 minutes post virus injection but was undetectable by 48 hours. In our study the earliest sample was at 72 hours post injection and all were negative for adenovirus. Thus both studies show virus is undetectable after 48-72 hours post virus injection.

4.3.2. Evidence for antitumoural activity.

One of the secondary objectives of the study was to determine if there was evidence of an antitumour effect. This was assessed both clinically and radiographically by either CT or MRI scans. Ultimately, all patients in the study progressed due to the development of other neck lesions or due to distant metastases in the lung, liver or bone. However, if we consider only the tumours which were injected, then there was evidence of antitumour activity. Using non-conventional measurements, 5 (23%) patients showed either a partial or minor response, while a further 8 (36%) showed stable disease. Of the 5 patients who showed evidence of tumour response, 4 had mutant p53 suggesting a correlation between p53 status and tumour response. 1 patient had wild type p53 sequence, but it is possible that in this patient's tumour the p53 protein was inactivated by either HPV infection (Lechner,1992; Scheffner et al,1992), mdm2 overexpression (Barak et al,1993; Oliner et al,1992) or by some other factors. Alternatively, a mutation of the p53 gene may have been present outside exons 4-9 or in the intron regions. Despite this observation, statistical analysis of p53 status and tumour response showed no significant correlation however.

The cause of the clinical and radiological changes in keeping with necrosis in responding patients is most likely as a result of injection of Onyx-015 since diluent injected tumours showed no evidence of necrosis. These changes may be due to viral replication and spread. However, it may also be possible that some of the observed response is due to an immune response against virally infected tumour cells.

4.3.3. Efficiency of viral replication

Viral replication was detected in patient biopsies by *in-situ* hybridisation techniques. Although the presence of viral DNA is not definitive evidence of viral replication it is suggestive that replication is occurring. Positive evidence of viral DNA was found in 4 patients, all of whom had mutant p53 tumours. No replication was detected in tumours with wild type p53 or in surrounding normal tissue. This supported the hypothesis that Onyx-015 selectively replicated in mutant p53 cells. However the correlation between p53 status and viral replication was not statistically significant. Interestingly other tumours with mutant p53 did not show evidence of virus by in-situ hybridisation. There are several possible explanations for this. It may be that Onyx-015 does not have the rigid selectivity for p53 mutant tumours as suggested by Bischoff et al,1996. Indeed recent studies (Goodrum et al,1997; Hall et al,1998) have shown no correlation between p53 status and viral replication in cell lines of known p53 status. Even these studies were controversial however since the p53 functional status of the cell lines used had not been fully determined and the mechanism of cell death (i.e. apoptosis versus viral replication) had not been fully established. Using the matched colon cancer cell lines RKO/RKOp53.13, Bischoff et al,1996 reported reduced replication of Onyx-015 virus in the p53 transfectant cell line using burst assays as the measure of replication. In contrast, Hall et al,1998 reported no difference in replication in the same cell lines, and a second matched pair of cell lines HT1080/HT1080-6TGc5, using cytopathic effect assays as their measure of replication. The authors however, did not determine the mechanism of cell

death i.e. viral induced cytolysis versus apoptosis and did not produce a quantitative measure of viral replication. These different methods of assaying viral induced cell death may account for the apparently conflicting results obtained in these papers. The clinical study presented in this thesis on head and neck cancer patients has not resolved this controversial issue. It is also possible that the low detection rate of viral replication in mutant p53 tumours may also have been due to the sensitivity and specificity of the technique used. For example, the tumour biopsies were very small compared to the size of the injected tumours (<1%) and therefore the area of tumour which was injected may not have been biopsied in the majority of cases. In animal experiments, replication was evident in nude mouse human tumour xenografts in the cells at the watershed between necrosis and viable tumour (Heise et al,1999). Since it is not possible to biopsy necrotic tissue effectively, it is not surprising that biopsies of viable tumour did not show replication. In addition, animal studies in nude mice have shown that distribution is better with a multi-injection protocol (Heise et al,1999). It is also possible that viral spread is limited by the fibrotic nature of these tumours since the majority of patients had previously been treated with external beam radiotherapy.

Lastly, it is possible that viral spread is affected by systemic or local immune effects. Adenovirus can produce two systemic immune effects - cell mediated and humoral immunity. Cell mediated immunity is mediated by cytotoxic T lymphocytes and is stimulated by adenovirus antigens produced in the host cell and presented along with MHC moities on the cell surface. This can cause early elimination of virus but may also be beneficial as it

causes the immune mediated killing of tumour cells. In this clinical study, all patients were immunosuppressed at baseline with low total lymphocyte counts and low CD4 counts. A total of 19 patients had a CD4 count less than 500 with a median range of 200-300. To determine whether or not a cellular immune response occurred, biopsy specimens were stained for helper T lymphocytes (CD4), cytotoxic T lymphocytes (CD8) and for macrophage infiltration (CD68). However, because the biopsies were so small it was not possible to make any conclusions as to the relevence of a cellular response to overall tumour response. We are currently planning to carry out a pre-operative study on patients with early stage HNSCC in which patients receive a single intratumoural injection of virus within the first 2 weeks prior to definitive surgery. It will then be possible to examine whole tumour sections once the tumour is excised to determine the extent of viral spread and immune effector cell response.

The neutralising antibody response (humoral response) to adenovirus occurs later. Theoretically, this should reduce the ability to reinfect host cells with adenovirus after first inoculation. In this study, all patients developed a rising neutralising antibody response despite being immunosuppressed. Statistical analysis showed no correlation between pretreatment antibody levels and tumour response. In addition, patients who were retreated continued to show a response despite rising antibody levels. This data would suggest that neutralising antibody to adenovirus may not be clinically relevant to intratumoural efficacy. There is also some evidence that suggests that antibody penetration into these tumours may be minimal (Jain,1994; Dvorak et al,1988), in which case a rising antibody would have

no effect in a repeat intratumoural administration procedure and would only be of importance in repeat systemic viral injection. If humoral immunity proved to be troublesome, it is possible to suppress this with γ-interferon and IL-12 (Yang et al,1995), by using viruses with non-crossreactive serotypes (Mastrangeli et al,1996), or by creating viruses which are encapsulated in poly(lactic-glycolic) acid copolymer (PLGA) to evade the immune system (Beer et al,1997).

Local immunity, as well as systemic immunity, is also a factor which may limit viral spread. Tumours secreting interferon may neutralise viral spread. It has recently been shown that adenovirus infection stimulates the activation of the transcription factor NFκB which results in a downstream inflammatory response (ICAM-I up-regulation) (Clesham,1998). It has also been shown that adenovirus infection causes stimulation of the Raf/MAPK pathway and IL-8 secretion (Bruder et al,1997). Experiments to investigate the role of the immune system are planned with a pre-operative study in patients with early stage HNSCC.

4.4. CLINICAL PHASE I TRIAL - multiple injection protocol.

Preclinical data suggested that multiple injections of virus may be more effective (Heise, unpublished results). A daily x 5 multiple intratumoural injection study was therefore carried out following the single injection study.

The primary objective of the multiple injection protocol was to determine the safety of Onyx-015 injection by intratumoural injection and to compare this to toxicity observed in the single injection protocol. The results showed that there was an increase in flu-like symptoms in the multiple injection study. 30% of patients reported these symptoms compared to 10% in the single injection study. However, although more patients reported these symptoms, the grade of toxicity remained at Grade I/II. The increase in flu-like symptoms suggested that this may be due to viral shedding into the circulation. However, PCR analysis of blood from all patients did not detect any evidence of adenovirus. These flu-like symptoms again only lasted for a period of 48 hours before they settled. Surprisingly, there was no increase in the reported incidence of pain on injection despite the fact that the patients received repeat injections into the same site over 5 successive days. Therefore, overall the multiple dose injection procedure was well tolerated and was as safe as the single injection procedure.

When we examined tumour biopsies for viral replication by in-situ hybridisation, virus was detected in 5 of 7 patients (3 patients did not have post-treatment biopsies). This was substantially greater than the single injection protocol where only 4 of 22 patients had positive biopsies. In addition, the percentage number of cells staining positively for virus was

greater and the distribution of virus more uniform compared to the single injection protocol. Therefore, overall the multiple injection protocol gave better viral distribution compared to the single injection protocol. Unlike the single injection study, 2 of the 5 patients with evidence of viral replication had wild type p53 on gene sequencing. This does not discount the selectivity for Onyx-015 to replicate only in mutant p53 tumours since it is possible that the 2 tumours with wild type p53 had mutations outside exons 4-9 or p53 may have been inactivated by other mechanisms such as mdm2 overexpression or HPV infection.

Although the multiple injection study produced better viral distribution, this did not result in an increase in efficacy in the limited number of patients studied. Of the 10 patients treated, only 1 patient had a partial response and this was at a dose of 5×10^9 pfu. This patient received 7 cycles of treatment resulting in an 80% reduction in tumour size but was eventually removed from study at 28 weeks when the target tumour began to progress. Because only a limited number of patients were studied with multiple injections, we cannot draw any conclusions regarding efficacy and therefore a larger phase II study is planned to further evaluate efficacy with the multiple injection protocol.

4.5. FURTHER DEVELOPMENTS.

In summary we have shown that intratumoural injection of the E1B attenuated replicating adenovirus, Onyx-015, is feasible and safe with very limited toxicity. Although no objective tumour responses were observed, evidence for biological activity was observed. However, response did not

correlate significantly with the p53 status of injected tumour. In addition, although viral replication was found only in tumours with mutant p53 and not in normal cells, the relationship between p53 status and viral replication was not statistically significant. This may have been due to limitations in the techniques used to assess replication but does not exclude the possibility that the selective replication of Onyx-015 for p53 mutant tumours is not as stringent as first thought. Further studies in patients are therefore required to resolve this issue. Nevertheless the observation of biological activity of this agent in recurrent head and neck cancer patients is encouraging. Current available therapies for recurrent head and neck cancer, such as tumour debulking surgery, further radiation and chemotherapy have all produced poor responses of limited duration. All these therapies also produce significant morbidity. An agent such as Onyx-015, which has very little toxicity and which can be given as an outpatient without the need for hospitalisation, may be an attractive alternative to these therapies but only if responses are comparable. Further studies are therefore warranted to further evaluate this form of therapy for this disease. Several potential clinical applications exist for this new form of therapy. Since p53 mutation is the commonest genetic alteration in human cancers, E1B 55kD gene- deleted adenoviruses may be of importance in the treatment of a wide range of human cancers. It may be of use in the local control of recurrent HNSCC, a disease for which currently available therapies are of little benefit. In addition, it may be useful for the treatment of minimal residual disease in head and neck cancer. The commonest cause of treatment failure in head and neck cancer is locoregional recurrence and the single most important factor

responsible for this is incomplete surgical margins (Snow,1989). It is now possible to detect 1 tumour cell out of 10,000 in resection margins using a molecular probe against the patient's mutant p53 (Brennan et al,1995). Therefore it may be possible that patients with positive margins using this molecular probe could be treated by direct injection of the surgical margins or bathing the surgical bed with Onyx-015 to prevent local tumour recurrence.

Malignant glioma and ovarian cancer are other tumours in which p53 mutations are frequently seen and which may be amenable to local or regional injections. Phase I studies in these cancers and others are currently underway. Recent work has also shown that combination therapy with Onyx-015 and chemotherapeutic agents such as cisplatin and 5-Fluorouracil (5FU) was superior to treatment with either agent alone when tested in nude mouse xenograft tumours (Heise et al,1997). A clinical trial of cisplatin/5FU combined with Onyx-015 has recently started in patients with recurrent head and neck cancer and early results are encouraging (Nemunaitis et al,1998).

Much research is also underway to try to improve the efficacy of these oncolytic adenoviruses in cancer therapy, particularly in the areas of delivery, intratumoural spread and modulating the immune system (as mentioned before). Although direct intratumoural injection of virus is useful for the treatment of local tumour disease, tumour targeting is required for the systemic treatment of distant metastatic disease. Some targeting occurs naturally because the capillaries present in tumours are more leaky than in normal tissue and this allows adenoviral particles to selectively concentrate

in the tumour. However, active tumour targeting can also be achieved by attaching ligands, such as epidermal growth factor or basic fibroblast growth factor, to the capsid of the adenovirus (Gu et al,1999; Curiel,1999). Alternatively, the adenovirus fibre protein can be modified to present a tumour specific ligand such as the RGD (Arg-Gly-Asp) motif (Dmitriev et al,1998). As well as delivery, much research is currently being carried out to improve intratumoural spread of adenovirus. This can be enhanced by increasing diffusion of the virus, increasing tropism or increasing the lytic effect of the virus. Diffusion can be enhanced by combining virus injection with hyaluronidase, heat, radiation or vasodilator drugs. Tropism can be enhanced by inserting peptides onto the adenoviral fibre, such as stretches of lysines to bind heparin sulphate receptors as well as RGD motifs to bind to αυ integrins (Dmitriev et al,1998; Staba et al,2000). However such viruses may also lose some of their selectivity causing unwanted toxicity. The lytic efficiency of the adenovirus can also be improved by deleting the anti-apoptotic E1B-19kDa adenoviral gene as well as the E1B 55kDa gene. Such a virus has been shown to induce more apoptosis, is released earlier, and spreads faster than wild type virus (Sauthoff et al,2000). However, again toxicity in normal cells may be increased using this approach. Therefore, rather than deleting viral inhibitors of cell death, one could try to enhance the lytic effect of adenovirus by increasing the expression of the adenoviral E3 11.6kDa death protein (Tollefson et al,1996).

In conclusion, the results presented in this thesis are encouraging for the future development of adenoviruses in cancer treatment. However much more research is required to improve viral replication, targeting,

intratumoural spread as well as modulation of the immune system before these therapies could be described as the new cancer magic bullet.

References

Adelstein DJ, Saxton JP, Lavertu P, Tuason L, Wood BG, Wanamaker JR, et al. A phase III randomized trial comparing concurrent chemotherapy and radiotherapy with radiotherapy alone in resectable stage III and IV squamous head and neck cancer: preliminary results. *Head Neck* 1997; **19**:567-575.

Ahomadegbe JC, Barrios M, Fogel S, Le Bihan ML, Douc-Rasy S, Duvillard P, et al. High incidence of p53 alterations (mutation, deletion, overexpression) in head and neck primary tumours and metastases; absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions. *Oncogene* 1995;**10:**1217-1227.

Ahsee KW, Cooke TG, Pickford IR, Soutar D, Balmain A. An allelotype of squamous cell carcinoma of the head and neck using microsatellite markers. *Cancer Res.* 1994;**54:**1617-1621.

Banin S, Moyal L, Shieh S-Y, Prives C, Reiss Y, Shiloh Y, et al. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* 1998;**281:**1674-1677.

Barak Y, Juven R, Haffner OM. mdm2 expression is induced by wild type p53 activity. *Embo J* 1993;**12:**461-468.

Barker DD, Berk AJ. Adenovirus proteins from both E1B reading frames are

required for transformation of rodent cells by viral infection and DNA transfection. *Virology* 1987;**156:**107-121.

Beahrs OH, Henson DE, Hutter RVP, Myers MH, eds. Manual for staging of cancer. Philadelphia: J.B.Lippincott, 1988.

Bedi GC, Westra WH, Gabrielson E, Koch W, Sidransky D. Multiple head and neck tumours: Evidence for a common clonal origin. *Cancer Res.* 1996;**56:**2484-2487.

Beer SJ, Matthews CB, Stein CS, Ross BD, Hilfinger JM, Davidson BL. Poly(lactic-glycolic) acid copolymer encapsulation of recombinant adenovirus reduces immunogenicity in vivo. *Gene Therapy* 1998;**5:**740-746.

Behrens BC, Hamilton TC, Masuda H, Grotzinger KR, Whang-Peng J, Louie KG, et al. Characterisation of a cis-diamminedichloroplatinum resistant human ovarian cell line and its use in evaluation of platinum analogues. *Cancer Res.* 1987;**47:**414-418.

Belani CP, Aqarwala S, Johnson J, Cohn A, Bernstein J, Langer C, et al. A phase II trial of Thymetaq in patients with squamous cell carcinoma of the hed and neck. *Proc Am Soc Clin Oncol* 1997;**16:**387a.

Benasso M, Merlano M, Blengio F, Cavallari M, Rosso R, Toma S. Concomitant alpha-interferon and chemotherapy in advanced squamous cell

carcinoma of the head and neck. *Am. J. Clin. Oncol.* 1993;**16:**465-468.

Benner SE, Lippman SM, Hong WK. Retinoid chemoprevention of second primary tumours. *Semin Hematol* 1994;**31**(4)**:**26-30.

Berns KI. Parvovirus replication. *Microbiol Rev* 1990;**54:**316-329.

Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, et al. A mutant adenovirus which selectively replicates in tumour cells with non-functional p53. *Science* 1996;**274:**373-376.

Boise LH, Gonzalez-Garcia MPCE, Ding L, Lindsten T, Turka LA, Mao X, et al. bcl-x, a bcl-2 related gene that functions as a dominant regulator of apoptotic cell death.*Cell* 1993;**74:**597-608.

Boring CC, Squires TS, Tong T. Cancer Statistics. *CA Cancer J. Clin.* 1993;**43:**7-26.

Boscia R, Chen K, Johnson JT, Whiteside TL. Evaluation of therapeutic potential of interleukin 2 expanded tumour-infiltrating lymphocytes in squamous cell carcinoma. *Ann Otol Rhinol Laryngol.* 1988;**97:**414-421.

Bottger A, Bottger V, Sparks A, Liu WL, Howard SF, Lane DP. Design of a synthetic mdm2-binding mini protein that activates the p53 response in vivo. *Current Biol.* 1997;**7:**860-869.

Boyle JO, Koch W, Hruban RH, van der Riet P, Sidransky D. The incidence of p53 mutations increases with progression of head and neck cancer. *Cancer Res.* 1993;**53:**4477-4480.

Braakhuis BJM, Klaassen I, Vanderleede BM, Cloos J, Brakenhoff RH, Copper MP. Retinoid metabolism and all-trans retinoic acid induced growth inhibition in head and neck squamous cell carcinoma cell lines. *Br J Cancer.* 1997;**76:**189-197.

Brachman DG, Graves D, Vokes E, Beckett M, Haraf D, Montag A, et al. Occurrence of p53 gene deletions and human papilloma virus infection in human head and neck cancer. *Cancer Res* 1992;**52**(17)**:**4832-4836.

Breau RL, Clayman GL. Gene therapy for head and neck cancer. *Curr. Opin. Oncol.* 1996;**8:**227-231.

Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, et al. Molecular assessment of histopathological staging in squamous cell carcinoma of the head and neck. *N. Engl. J. Med.* 1995;**332:**429-435.

Brennan JA, Boyle JO, Koch WM, Goodman SN, Hruban RH, Eby YJ, et al. Association between cigarette smoking and mutation of the p53 gene in head and neck squamous carcinoma. *N. Engl. J. Med.* 1995;**332:**712-717.

Brennan JA, Sidransky D. Molecular staging of head and neck squamous carcinoma. *Cancer Metastasis Rev. [0167-7659]* 1996;**15:**3-10.

Bruder JT, Kovesdi I. Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. *J Virol.* 1997;**71:**398-404.

Buckbinder L, Taalbott R, Velasco-Migeuel S, Takenaka I, Faha B, Seizinger BR, et al. Induction of the growth inhibitor IGF binding protein 3 by p53. *Nature* 1995;**377:**646-649.

Burch JD, Howe GR, Miller AB. Tobacco,alcohol,asbestos, and nickel in the aetiology of cancer of the larynx:A case-control study. *J. Natl. Cancer Inst.* 1981;**67:**1219-1224.

Burns JE, Baird MC, Clark LJ, Burns PA, Edington K, Chapman C, et al. Gene mutations and increased levels of p53 protein in human squamous cell carcinomas and their cell lines. *Br. J. Cancer.* 1993;**67**

Burris H, Vogel CL, Schwarz M, Castro D. Intradose(Cisplatin/Epinephrine) injectable gel for intratumoural therapy of accessible solid tumours including head and neck squamous cell carcinoma(abstract 593). *Ann Oncol.* 1996;**7(suppl 5):**123.

Buttitta F, Marchetti A, Gadducci A, Pellegrini S, Morganti M, Carnicelli V,

et al. p53 alterations are predictive of chemoresistance and aggressiveness in ovarian carcinomas:a molecular and immunohistochemical study. *Br J Cancer* 1997;**75**(2)**:**230-5.

Cairns P, Mao L, Merio A, Lee DJ, Schab D, Eby Y, et al. Low rate of p16(MST1) mutations in primary tumours with 9p loss. *Science* 1994;**265:**415-417.

Califano J, Riet P, Westra W, Nawroz H, Clayman G, Piantadosi S, et al. Genetic progression model for head and neck cancer: Implications for field cancerisation. *Cancer Res.* 1996;**56:**2488-2492.

Callender T, El-Nagger AK, Lee MS, Frankenthaler R, Luna MA, Batsakis JG. PRAD-1(CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. *Cancer* 1994;**74:**152-158.

Canman CE, Lim D-K, Kastan MB, Siliciano JD. Activation of the ATM Kinase by ionising radiation and phosphorylation of p53. *Science* 1998;**12:**1677-1679.

Cascinu S, Fedeli A, Fedeli SL, Catalano G. Cisplatin, 5-FU and interferon alpha-2b for recurrent or metastatic head and neck cancer. *Br. J. Cancer* 1994;**69:**392-393.

Catimel G, Vermorken JB, Clavel M, DeMulder P, Judson I, Sessa C, et al.

A phase II study of gemcitabine in patients with advanced squamous cell carcinoma of the head and neck. *Ann. Oncol.* 1994;**5:**543-547.

Catimel G, Verweij J, Mattijssen V, Hanauske A, Piccart M, Wanders J, et al. Docetaxel(taxotere) : an active drug for the treatment of patients with advanced squamous cell carcinoma of the head and neck. *Ann. Oncol.* 1994;**5:**533-537.

Chen L, McGowan P, Ashe S, Johnston J, Li Y, Hellstrom I, et al. Tumour immunogenicity determines the effect of B7 costimulation on T cell-mediated tumour immunity. *J. Exp. Med* 1994;**179**(2)**:**523-532.

Chiba I, Shindoh M, Yasuda M, Yamazaki Y, Amemiya A, Sato Y, et al. Mutations in the p53 gene and human papillomavirus infection as significant prognostic factors in squamous cell carcinomas of the oral cavity. *Oncogene* 1996;**12:**1663-1668.

Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumour suppresser-DNA complex: understanding tumourigenic mutations. *Science* 1994;**265:**346-355.

Chung KY, Mukhopadhyay T, Kim J, Casson A, Ro JY, Goepfert H, et al. Discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. *Cancer Res* 1993;**53:**1676-1683.

Clavel M, Vermorken JB, Cognetti F, Cappelaere P, de Mulder PHM, Schornagel JH, et al. Randomised comparison of cisplatin, methotrexate, bleomycin and vincristine (CABO) versus cisplatin and 5-flurouracil versus cisplatin in recurrent or metastatic squamous cell carcinoma of head and neck. *Ann. Oncol.* 1994;**5:**521-526.

Clayman GL, El-Naggar AK, Roth JA, Zhang WW, Goepfert H, Taylor DL, et al. In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. *Cancer Res.* 1995;**55:**1-6.

Clayman GL, El-Naggar AK, Merritt J, Bruso P, Roth JA, Lippman S, et al. Adenovirus mediated p53 gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. *J of Clinical Oncology* 1998;**16**(6)**:**2221-2232.

Clesham GJ et al. High adenoviral loads stimulate NFkB dependent gene expression in human vascular smooth muscle cells. *Gene Therapy* 1998;**5:**174-180.

Cloos J, Spitz MR, Schantz SP, Hsu TC, Zhange ZA, Tobi H, et al. Genetic susceptibility to head and neck squamous cell carcinoma. *J. Natl. Cancer Inst.* 1996;**88:**530-535.

Cortesina G, De Stefani A, Giovarelli M, Barioglio M, Cavallo GP, Jemma

C, et al. Treatment of recurrent squamous cell carcinoma of the head and neck with low doses of interleukin 2 injected perilymphatically. *Cancer.* 1988;**61:**2482-2485.

Cowan JM, Beckett MA, Weichselbaum RR. Chromosome changes characterising in vitro response to radiation in human squamous cell carcinoma lines. *Cancer Res.* 1993;**53:**5542-5547.

Craven JM, Pavelic Z, Stambrook PJ. Expression of c-erb-B2 gene in human head and neck carcinoma. *Anticancer Res.* 1992;**12:**2273-2276.

Crook T, Wrede D, Vousden KH. p53 point mutations in HPV negative human cervical carcinoma cell lines. *Oncogene* 1991;**6:**873-875.

Curiel DT. Strategies to adapt adenoviral vectors for targeted delivery. *Ann. NY Acad. Sci.*1999; **886:**158-171.

Cutilli T, Papola F, Di Emidio P, Leocata P, Corbacelli A. p53 mutations and chemoresistance in oromaxillofacial squamous cell carcinomas. The results of a molecular genetics study of p53 in metastatic oromaxillofacial tumours and an evaluation of the response to neoadjuvant chemotherapeutic treatment. *Minerva Stomatol.* 1998;**47**(1-2)**:**1-9.

Czegledy J, Iosif C, Hanson BG, Evander M, Gergely L, Wadell G. (1995). Can a test for E6/E7 transcripts of human papillomavirus type 16 serve as a

diagnostic tool for the detection of micrometastases in cervical cancer. *Int. J. Cancer.* (Pred Oncol): **64**,211-215.

Dameron KM, Volpert OV, Tainski MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994;**265:**1353-1584.

de Stanchina E, McCurrach ME, Zindy F, Shieh S, Prives C, Roussel MF, et al. E1A signaling to p53 involves the p19 tumour suppresser. *Genes & Dev* 1998;**12:**2434-2442.

Debbas M, White E. Wild type p53 mediates apoptosis by E1A which is inhinited by E1B. *Genes Dev.* 1993;**7:**546-554.

Dmitriev I, Krasnykh V, Miller CR, Wang M et al. An adenovirus vector with genetically modified fobers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol.* 1998;**72:**9706-9713.

Dobner, T., N. Horikoshi, et al. Blockage by adenovirus E4ORF6 of transcriptional activation by the p53 tumour suppressor. *Science* 1996;**272**: 1470-1473.

Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM. Identification and characterisation of the blood vessels of solid tumours that are leaky to

circulating macromolecules. *Am. J. Pathol.* 1988;**133:**95-109.

Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus 16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989;**243:**934-937.

Eisbruch A, Blick M, Lee JS. Analysis of the epidermal growth factor receptor gene in fresh human head and neck tumours. *Cancer Res.* 1987;**47:**3603-3605.

Eisbruch A, Shewach DS, Urba S, Wolf GT, Bradford CR, Marentette LJ, et al. A phase I trial of radiation concurrent with low-dose gemcitabine for head and neck cancer: high mucosal and pharyngeal toxicity(abstract 1377). *Proc Am Soc Clin Oncol.* 1997;**16:**386a.

El-Diery WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. *Nat. Genet.* 1992;**1:**45-49.

El-Diery WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, et al. WAF1: a potential mediator of p53 tumour suppression. *Cell* 1993;**75:**817-825.

el-Nagger AK, Lai S, Luna MA, Zhou XD, Weber RS, Goepfert H, et al. Sequential p53 mutation analysis of pre-invasive and invasive head and neck squamous carcinoma. *Int. J. Cancer* 1995;**64:**196-201.

Eliopoulos AG, Kerr DJ, Herod J, Hodgkins L, Krajewski S, Reed JC, et al. The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. *Oncogene* 1995;**11:**1217-1228.

Elshami AA, Saavedra A, Zhang H, Spray DC, Fishman GI, Kaiser LR, et al. Gap junctions play a role in the bystander effect of the herpes simplex virus thymidine kinase / gancyclovir system in vitro. *Gene Ther* 1996;**3**(1)**:**85-92.

Esandi MC, van Someren GD, Vincent AJ, van Bekkum DW, Valerio D, Bout A, et al. Gene therapy of experimental malignant mesothelioma using adenovirus vectors encoding the HSVtk gene. *Gene Ther* 1997;**4**(4)**:**280-287.

Field JK, Spandidos DA, Stell P. Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma. *Oncogene* 1989;**4:**1463-1468.

Field JK, Spandidos DA, Malliri A, Gosney JR, Yiagnisis M, Stell PM. Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinoma of the head and neck. *Br. J. Cancer* 1991;**64:**573-577.

Field JK, Spandidos DA, Yiagnisis M. C-erb-B2 expression in squamous

cell carcinoma of the head and neck. *Anticancer Res.* 1992;**12:**613-620.

Field JK, Pavelic ZP, Spandidos DA, Stambrook PJ, Jones AS, Gluckman JL. The role of the p53 tumour suppresser gene in squamous cell carcinoma of the head and neck. *Arch. Otolaryngol. Head Neck Surg.* 1993;**119:**1118-1122.

Forastiere AA. Overview of platinum chemotherapy in head and neck cancer. *Semin. Oncol.* 1994;**21:**20-27.

Forastiere AA, Shank D, Neuberg D et al. Final report of a phase II evaluation of paclitaxel in patients with advanced squamous cell carcinoma of the head and neck: An Eastern Cooperative Oncology Group Trial (PA 390). *Cancer* 1998;**82:**2270-2274.

Foulkes WD, Brunet JS, Kowalski LP, Narod SA, Franco EL. Family history of cancer is a risk factor for squamous cell carcinoma of the head and neck in Brazil: a case-control study. *Int. J. Cancer* 1995;**63:**769-773.

Foulkes WD, Brunet JS, Sieh W, Black MJ, Shenouda G, Narod SA. Familial risks of squamous cell carcinoma of the head and neck:retrospective case-control study. *BMJ* 1996;**313:**716-721.

Fountzilas G, Skarlos D, Athanassiades A, Samantas E, Nicolaou A, Dombros N, et al. Paclitaxel by three hour infusion and carboplatin in

advanced carcinoma of the nasopharynx and other sites of the head and neck. A phase II study conducted by the Hellenic Cooperative Oncology Group. *Ann Oncol* 1997;**8:**451-455.

Fueyo J, Gomez-Manzano C, Alemany R, Lee PS et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene.* 2000;**19:**2-12.

Fujiwara T, Grimm EA, Roth JA. Gene therapeutics and gene therapy. *Current Opinion in Oncology* 1994;**6:**96-105.

Ganly I, Kirn D, Rodriguez GI, Soutar D, Eckhardt G, Otto R, et al. Phase I trial of intratumoural injection with an E1B attenuated adenovirus,ONYX-015, in patients with recurrent p53(-) head and neck cancer. *J of Clinical Oncology* 1997;**16:**1362.

Gannon JV, Lane DP. p53 and DNA polymerase alpha compete for binding to SV40 T antigen. *Nature* 1987;**329:**456-458.

Ginsberg HS. The ups and downs of adenovirus vectors. *Bull N Y Acad Med* 1996;**73**(1)**:**53-58.

Girod SC, Cesarz D, Fischer U, Krueger GR. Detection of p53 and mdm2 protein expression in head and neck carcinogenesis. *Anticancer Res* 1995;**15**(4)**:**1453-1457.

Gleich LL, Gluckman JL, Armstrong S, Biddinger PW, Miller MA, Balakrishnan K, et al. Alloantigen gene therapy for squamous cell carcinoma of the head and neck:results of a phase I trial. *Arch Otolaryngol Head Nneck Surg* 1998;**124**(10):1097-1104.

Goodrum FD, Ornelles DA. The early region 1B 55-kilodalton oncoprotein of adenovirus relieves growth restrictions imposed on viral replication by the cell cycle. *J. Virol.* 1997;**71**(1):548-561.

Gottlieo TM, Jackson SP. The DNA dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell* 1993;**72:**131-142.

Graeber TG. Hypoxia mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996;**379:**88-91.

Graham FL. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 1977;**36:**59-72.

Graham S, Dayal H, Rohrer J. Dentition, diet, tobacco and alcohol in the epidemiology of oral cancer. *J. Natl. Cancer Inst.* 1977;**59:**1611-1618.

Graham FL, Prevec L. Manipulation of adenovirus vectors. *The humane press, Clifton,NJ* 1991;**7:**109-128.

Gu DL, Gonzalez AM, Printz MA, Doukas J, Ying W et al. Fibroblast growth factor 2 retargeted adenovirus has redirected cellular tropism: evidence for reduced toxicity and enhanced antitumour activity in mice. *Cancer Res.* 1999;**59:**2608-2614.

Hall AR, Dix BR, O'Carroll SJ, Braithwaite AW. p53 dependent cell death/apoptosis is required for a productive adenovirus infection. *Nature Med* 1998;**4**(9)**:**1068-1072.

Hallenbeck PL, Chang YN, Hay C, Golightly D, Stewart D, Lin J, Phipps S, Chiang YL. A novel tumour-specific replication restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Hum Gene Ther* 1999;**10:**1721-1733.

Haraf DJ, Nodzenski E, Brachman D, Mick R, Montag A, Graves D, et al. Human papilloma virus and p53 in head and neck cancer: clinical correlates and survival. *Clin Cancer Res* 1996;**2**(4)**:**755-762.

Haraf DJ, Weichselbaum RR, Vokes EE. Re-irradiation with conconotant chemotherapy of unresectable recurrent head and neck cancer: a potentially curable disease. *Ann. Oncol.* 1996;**7:**913-918.

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin dependant kinases. *Cell* 1993;**75:**805-816.

Harrison LB. Applications of brachytherapy in head and neck cancer. *Semin Surg Oncol* 1997;**13**(3)**:**177-184.

Hartwell LH, Weinert TA. Checkpoints : controls that ensure the order of cell cycle events. *Science* 1989;**246:**629-634.

Hasegawa H, Kano M, Hoshi N et al. An electrochemotherapy model for rat tongue carcinoma. *Journal of Oral Pathology & Medicine* 1998;**27:**249-254.

Haupt Y, Maya R, Kazaz A, Oren M. mdm2 promotes the rapid degradation of p53. *Nature* 1997;**387:**296-299.

Heise H, Sampson-Johannes A, Williams A, McCormack F, Von Hoff DD, Kirn DH. Onyx-015, an E1B gene-attenuated adenovirus, causes tumour specific cytolysis and antitumoural efficacy that can be augmented by standard chemotherapeutic agents. *Nat. Med.* 1997;**3**(number 6)**:**639-645.

Henderson CJ, Smith AG et al. Increased skin tumourogenesis in mice lacking pi class glutathione S-transferase. Proc Natl Acad Sci. USA.**95(9)**:5275-5280.

Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.*

1995;**55:**4525-4530.

Hitt R, Hornedo J, Colomer R, Hidalgo M, Brandanz A, Pena M, et al. Study of escalating doses of paclitaxel plus cisplatin in patients with inoperable head and neck cancer. *Semin Oncol* 1997;**24:**58-64.

Hoffman D, Harley NH, Fisenne I. Carcinogenic agents in snuff. *J. Natl. Cancer Inst.* 1986;**76:**435-437.

Hollstein M, Sidransky D, Vogelstein B, Harris C. p53 mutations in human cancer. *Science* 1991;**49:**49-53.

Hong WK, Lippman SM, Itri LM. Prevention of second primary tumours with isoretinoin in squamous cell carcinoma of the head and neck. *N. Engl. J. Med.* 1990;**323:**795-801.

Horikoshi N, Usheva J, Chen AJ, Levine AJ, Wienmann R, Shenk T. Two domains of p53 interact with the TATA binding protein, and the adenovirus E1A protein disrupts the association, relieving p53 mediated transcriptional repression. *Mol. Cell. Biol.* 1995;**15:**227-234.

Hsu TC. Genetic predisposition to cancer with special reference to mutagen sensitivity. *In Vitro Cell. Dev. Biol.* 1987;**23:**591-603.

Huang LC, Clarkin KC, Wahl GM. Sensitivity and selectivity of the DNA

damage sensor responsible for activating p53-dependent G1 arrest. *Proc. Natl. Acad. Sci; USA* 1996;**94:**4827-4832.

Huber MH, Shirinian M, Lippman SM, Dimery IW, Frankenthaler RA, Hong WK. PhaseI/II study of cisplatin, 5-flurouracil and alpha-interferon for recurrent carcinoma of the head and neck. *Invest. New Drugs* 1994;**12:**223-229.

Jacobs C, Lyman G, Velez-Garcia E, Sridhar KS. A phase III randomized study comparing cisplatin and fluorouracil as single agents and in combination for advanced squamous cell carcinoma of the head and neck. *J Clin Oncol* 1992;**10:**257-263.

Jain RK. Barriers to drug delivery in solid tumours. *Sci. Am.* 1994;**271:**58-65.

Jares P, Fernandez PL, Campo E, Nadal A, Bosch F, Aiza G, et al. PRAD-1/Cyclin D1 gene amplification correlates with messenger RNA overexpression and tumour progression in human laryngeal carcinomas. *Cancer Res.* 1994;**54:**4813-4817.

Jeffrey PD, Gorina S, Pavletich NP. Crystal structure of the tetramerization domain of the p53 tumour suppresser at 1.7 angstoms. *Science* 1995;**267:**1498-1502.

Jin Y, Mertens F, Mandahl N, Heim S, Olegard C, Wennerberg J, et al. Chromosome abnormalities in eighty-three head and neck squamous cell carcinomas: influence of culture conditions on karyotypic pattern. *Cancer Res.* 1993;**53:**2140-2146.

Jones KR, Lodge-Rigal RD, Reddick RL, Tudor GE, Shockley WW. Prognostic factors in the recurrence of stage I and II squamous cell cancer of the oral cavity. *Arch. Otolaryngol. Head Neck Surg.* 1992;**118:**483-485.

Kao CC, Yew PR, Berk AJ. Domains required for in vitro association between the cellular p53 and the adenovirus 2 E1B 55K proteins. *Virology* 1990;**179:**806-814.

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 in the cellular response to DNA damage. *Cancer Res* 1991;**52:** 6304-6311.

Kastan MB, Walsh WV, Plunkett BS, Kuerbitz SJ. Wild type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA.* 1992;**89:**7491-7495.

Kastan MB. A mammalian cell cycle checkpoint pathway utilising p53 and GADD45 is defective in Ataxia Telangiectasia. *Cell* 1992;**71:**587-579.

Kim SK, Fan Y, Papadimitrkopoulou V, Clayman G, Hittelman WN, Hong

WK, et al. DPC4, a candidate tumour suppresser gene , is altered infrequently in head and neck squamous cell carcinoma. *Cancer Res.* 1996;**56:**2519-2521.

Kitahara S, Ikeda M, Inouye T, Matsunaga T, Yamaguchi K, Takayama E, et al. Inhibition of head and neck metastatic and /or recurrent cancer by local administration of multi-cytokine inducer OK-432. *J Laryngol Otol.* 1996;**110:**449-453.

Ko SC, Gotoh A, Thalmann GN, Zhau HE, Johnston DA, Zhang WW, et al. Molecular therapy with recombinant p53 adenovirus in an androgen independent metastatic human prostate cancer model. *Hum Gene Ther* 1996;**7**(14)**:**1683-1691.

Koh JY, Cho NP, Kong G, Lee JD, Yoon K. p53 mutations and human papilloma DNA in oral squamous cell cancer: correlation with apoptosis. *Br. J. of Cancer.* 1998;**78**(3)**:**354-359.

Koness RJ, Glicksman A, Liu L, Coachman N, Landman C, Slotman G, et al. Recurrence patterns with concurrent platinum based chemotherapy and accelerated hyperfractionated radiotherapy in stage III and IV head and neck cancer patients. *Am J Surg* 1997; **174**:532-535.

Kooper DP, van den Broek P, Manni JJ, Tiwara RM, Snow GB. Partial vertical laryngectomy for recurrent glottic carcinoma. *Clin Otolaryngol*

1995;**20**:167-170.

Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA* 1992;**89:** 7491-7495.

Kussie PH, Gorina S, Marechel V, Elenbaas B, Moreau J, Levine AJ, et al. Structure of the MDM2 oncoprotein bound to the p53 tumour suppressor transactivation domain. *Science* 1996;**274:**948-953.

Lane DP, Crawford LV. T antigen is bound to a host protein in SV40 transformed cells. *Nature* 1979;**278:**261-263.

Lane DP. p53, guardian of the genome.*Nature* 1992;**358:**15-16.

Lang S, Whiteside TL, Lebeau A, Zeidler R, Mack B, Wollenberg B. Impairment of T cell activation in head and neck cancer in situ and in vitro:strategies for an immune restoration. *Arch Otolaryngol Head Neck Surg* 1999;**125**(1)**:**82-88.

Lavertu P, Adeltein DJ, Saxton JP, Secic M, Eliachar I, Strome M, et al. Aggressive concurrent chemoradiotherapy for squamous cell head and neck cancer: an 8 year single institution experience. *Arch Otolaryngol Head Neck Surg* 1999; **125**:142-148.

Lazarus P, Stern J, Zwiebel N, Fair A, Ritchie JP, Schantz S. Relationship between p53 mutation incidence in oral cavity squamous cell carcinomas and patient tobacco use. *Carcinogenesis* 1996;**17:**733-739.

Leaf AN, Neuberg D, Schwartz EL, Wadler S, Ritch PS, Dutcher JP, et al. An ECOG phase II study of amonafide in unresectable or recurrent carcinoma of the head and neck, Eastern Cooperative Oncology Group. *Invest New Drugs.* 1997;**15:**165-172.

Lechner MS. Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. *Embo J.* 1992;**11:**3045-3052.

Leemans CR, Tiwari R, Nauta JJ, Van der Waal I, Snow GB. Regional lymph node involvment and its significance in the development of distant metastases in head and neck cancer. *Cancer* 1993;**71:**452-456.

Lees-Miller SP, Chen YR, Anderson CW. Human cells contain a DNA activated protein kinase that phosphorylates Simian virus 40 T antigen, mouse p53 and the human Ku antigen.*Mol. Cell Biol* 1990;**10:**6472-6483.

Lees-Miller SP, Sakaguchi K, Ullrich SJ, Anderson CW. Human DNA activated protein kinase phosphorylates serines 15 and 37 in the amino terminal transactivation domain of human p53. *Mol. Cell. Biol.* 1992;**12:**5041-5049.

Levine AJ. The p53 protein and its interactions with the oncogene products of the small DNA tumour viruses. *Virology* 1990;**177:**419-426.

Levine AJ. p53. The cellular gatekeeper for growth and division. *Cell* 1997;**88:**323-331.

Lill NL, Grossman SR, Ginsberg D, Livingston DM. Binding and modulation of p53 by p300/CBP coactivators. *Nature* 1997;**387:**823-827.

Lin WC, Yasumura S, Whiteside TL. Transfer of interleukin 2 receptor genes into squamous cell carcinoma: modification of tumour cell growth. *Arch Otolaryngol Head Neck Surg* 1993;**119:**1229-1235.

Linke SP, Clarkin KC, Leonardo DI, Tsou A, Wahl GM. A reversible, p53 dependent G0/G1 arrest induced by ribonucleotide depletion in the absence of detectable DNA damage.*Genes & Dev* 1996;**10:**934-947.

Liu TJ, Zhang WW, Taylor DL, Roth JA, Goepfert H, Clayman GL. Growth suppression of human head and neck cancer cells by the introduction of a wild type p53 gene via a recombinant adenovirus. *Cancer Res.* 1994;**54:**3662-3667.

Liu TU, El-Nagger AK, McDonnell TJ, Steck KD, Wang M, Taylor DL, et al. Apoptosis induction mediated by wild-type p53 adenoviral gene transfer

in squamous cell carcinoma of the head and neck. *Cancer Res.* 1995;**55:**117-122.

Lowe SW, Schmitt EM, Smith SW, Osborne BAJT. p53 is required for radiation induced apoptosis in mouse thymocytes. *Nature* 1993;**36:**847-849.

Lowe SW, Riley HE, Jack T, Honsman DE. p53 dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cancer Res* 1993;**54:**3500-3505.

Lowe SW. p53 status and the efficacy of cancer therapy in vivo. *Science* 1994;**266:**807-810.

Lu X, Lane DP. Differential induction of transcriptionally active p53 following UV or ionising radiation: defects in chromosome instability syndromes. *Cell* 1993;**75:**765-778.

Luo Y, Hurwitz J, Massague J. Cell-cycle inhibition by independent Cdk and PCNA binding domains in P21 waf1. *Nature* 1995;**375:**159-164.

Mackerras D, Buffler PA, Randall DE. Carotene intake and the risk of laryngeal cancer in coastal Texas. *Am. J. Epidemiol.* 1988;**128:**980-988.

Maier H, Dietz A, Gewelke V. Occupational exposure to hazardous

substances and risk of cancer in the area of the mouth cavity, oropharynx, hypopharynx and larynx. A case-control study. *Laryngol. Rhinol. Otol. (Stuttg.)* 1991;**70:**93-98.

Malkin D, Li FP, Strong LC. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* 1990;**250:**1233-1238.

Mao L, Fan Y, Lotan R, Hong WK. Frequent abnormalities of FHIT , a candidate tumour suppresser gene in head and neck cancer cell lines. *Cancer Res.* 1996;**56:**5128-5131.

Mao L, El-Nagger AK, Fan YH, Hong WK. Telomerase activity in head and neck squamous cell carcinoma and adjacent tissues. *Cancer Res.* 1996;**56:**5600-5604.

Mao EJ, Schwartz SM, Daling JR, Oda D, Tickman L, Beckmann AM. Human papilloma viruses and p53 mutations in normal pre-malignant and malignant oral epithelia. *Int. J. Cancer.* 1996;**69:**152-158.

Marshall J, Graham S, Mettlin C. Diet in the epidemiology of oral cancer. *Nutr. Cancer* 1982;**3:**145-149.

Mastrangeli A, et al. Sero-switch adenovirus mediated in vivo gene transfer: circumvention of anti-adenovirus humoral immune defenses against repeat

adenovirus vector administration by changing the adenovirus serotype. *Human gene therapy* 1996;**7:**79-87.

Matsumura T, Yoshihama Y, Kimura T, Shintani S, Alcalde RE. p53 and mdm2 expression in oral squamous cell carcinoma. *Oncology* 1996;**53**(4)**:**308-312.

Matthias C, Bockmuhl U, et al. The glutathione S-transferase GSTP1 polymorphism: effects on susceptibility to oral, pharyngeal and laryngeal carcinomas. *Pharmacogenetic;* **8(1)**:1-6.

McGarry GW, MacKenzie K, Periasamy P, McGurk F, Gatehouse S. Multiple primary malignant tumours in patients with head and neck cancer: the implications for follow-up. *Clin. Otolaryngol.* 1992;**17:**558-562.

McIlwrath AJ, Vasey PA, Ross GM, Brown R. Cell cycle arrests and radiosensitivity of human tumour cell lines:dependence on wild type p53 for radiosensitivity. *Cancer Res* 1994;**54**(14)**:**3718-3722.

McKaig RG, Baric RS, Olshan AF. Human papillomavirus and head and neck cancer: epidemiology and molecular biology. *Head Neck* 1998;**20**(3)**:**250-265.

Meek DW. Posttranslational modification of p53. *Semin. Cancer Biol.* 1994;**5:**203-210.

Merino OR, Lindberg RD, Fletcher GH. An analysis of distant metastases from squamous cell carcinoma of the upper respiratory and digestive tracts. *Cancer* 1977;**40:**145-151.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, et al. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppresser p16/CDKN2/MTS1 in human cancers. *Nat. Med.* 1995;**1:**686-692.

Meyza JW, Towpik E. Surgical and cryosurgical salvage for oral and oropharyngeal cancer recurring after radical radiotherapy. *Eur J Surg Oncol* 1991;**17:**567-570.

Michalides R, van Veelen N, Hart A, Loftus B, Wientjens E, Balm A. Overexpression of cyclin D1 correlates with recurrence in a group of 47 operable squamous cell carcinomas of the head and neck. *Cancer Res.* 1995;**55:**975-978.

Midgley CA, Lane DP. p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. *Oncogene* 1997;**15:**1179-1189.

Miguel RE, Villa LL, Cordeiro AC, Prado JC, Sobrinho JS, Kowalski LP. Low prevalence of human papillomavirus in a geographic region with a high

incidence of head and neck cancer. *Am J Surg* 1998;**176**(5):428-429.

Miller BA, Ries LAG, Hankey BF. Cancer statistics review: 1973-1989. *Natl. Cancer Inst. Monogr.* 1992:92-2789.

Mineta T et al. Attenuated multimutated herpes simplex virus 1 for the treatment of malignant gliomas. *Nat Med* 1995;**1**:938-943.

Miyashita T, Krajewski M, Krajewska HW, Wang HKLDA, Liebermann B, Hoffman BRJC. Tumour suppresser p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* 1994;**9**:1799-1805.

Mutirangura A, Supiyaphun P, Trirekapan S, Sriuranpong V, Sakuntabhai A, Yenrudi S, et al. Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma. *Cancer Res.* 1996;**56**:3530-3533.

Nabel GJ, Nabel EG, Yang ZY, Fox BA, Plautz GE, Gao X, et al. Direct gene transfer with DNA -liposome complexes in melanoma: expression, biological activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. USA.* 1993;**90**:11307-11311.

Nag S, Schuller D, Pak V, Young D, Grecula J, Bauer C, et al. Pilot study of intraoperative high dose rate brachytherapy for head and neck cancer. *Radiother Oncol* 1996;**41**(2):125-130.

Nanda GS, Sun FX, Hofmann GA, Dev SB. Electroporation therapy of human larynx tumours HEp-2 implanted in nude mice. *Anticancer Research* 1998;**18:**999-1004.

Nawroz H, van der Riet P, Hruban RH, Koch WM, Ruppert JM, Sidransky D. Allelotype of head and neck squamous cell carcinoma. *Cancer Res.* 1994;**54:**1152-1155.

Nemunaitis J, Khuri F, Ganly I, Tannock I, Kuhn J, McCartey T, et al. Phase II trials of intratumoural ONYX-015, an E1B 55-kDa gene-deleted adenovirus, alone and in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Cancer Gene Therapy* 1998;**5**(6)**:**Suppl abstract O-78.

Nibu K, Kamatu S, Kawabata K, Nakamizo M, Nigauri T, Hoki K, et al. Partial laryngectomy in the treatment of radiation-failure of early glottic cancer. *Head Neck* 1997;**19**:116-120.

Nickers P, Kunkler I, Scalliet P. Modern Brachytherapy: Current State and Future Prospects. *European Journal of Cancer* 1997;**33**(11)**:**1747-1751.

Norwell PC. The clonal expansion of tumour cell populations. *Science* 1976;**94:**23-28.

O'Malley BWJ, Ledley FD. Somatic gene therapy in otolaryngology-head

and neck surgery. *Arch. Otolaryngol. Head Neck Surg.* 1993;**119:**1191-1197.

O'Malley BWJ, Chen SH, Schwartz MR, Woo SLC. Adenovirus mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res.* 1995;**55:**1080-1085.

Okamoto K, Beach D. Cyclin G is a transcriptional target of the p53 tumour suppresser protein. *EMBO J* 1996;**13:**416-4822.

Oliner JD, Kinzler KW, Meltzer PS, George D, Vogelstein B. Amplification of a gene encoding a p53 associated protein in human sarcomas. *Nature* 1992;**358:**80-83.

Olivari AJ, Pradier R, Califano L, Guardo A, Glait HM. Levamisole in squamous cell carcinoma of the head and neck. *Cancer Treatment Rep* 1979;**63:**983-990.

Olshan AF, Weissler MC, Pei H, Conway K. p53 mutations in head and neck cancer: new data and evaluation of mutational spectra. *Cancer Epidemiol Biomarkers Prev* 1997;**6**(7)**:**499-504.

Oltavi ZN, Millman CLKSJ. BCL-2 heterodimerizes in vivo with a conserved homologue, BAX that accelerates programmed cell death.*Cell* 1993;**74:**609-619.

Panje WR, Hier MP, Garman GR et al. Electroporation therapy in head and neck cancer. *Annals of Otology, Rhinology & Laryngology* 1998;**107:**779-785.

Park T, Wilezynski SP, Paquette RL, Miller CW, Koeffler HP. p53 mutations in HPV negative cervical carcinoma. *Oncogene* 1994;**9:**205-210.

Parkin et al. Cancer epidemiology. *Bullitin of the World Health Organisation* 1984;**62:**163-182.

Pavletich NP, Chambers KA, Pabo CO. The DNA binding domain of p53 contains four conserved regions and the major mutation hot spots. *Genes and Dev* 1993;**7:**2556-2564.

Paz IB, Cook N, Odom-Maryon T, Xie Y, Wilczynski SP. Human papillomavirus(HPV) in head and neck cancer. An association of HPV16 with squamous cell carcinoma of Waldeyer's ring. *Cancer* 1997;**79**(3)**:**595-604.

Perry ME, Piette JA, Zawadzki D, Harvey D, Levine AJ. The mdm2 gene is induced in response to UV light in a p53 dependent manner. *Proc. Natl. Acad. Sci. USA* 1993;**90:**11623-11627.

Peters LJ, Goepfert H, Ang KK, Byers RM, Maor MH, Guillamondegui O,

et al. Evaluation of the dose for postoperative radiation therapy of head and neck cancer: first report of a prospective randomized trial. *Int J Radiat Oncol Biol Phys* 1993; **26**:3-11.

Petrovich Z, Lam K, Langholz B, Astrahan M, Luxton G, Rice D. Interstitial thermoradiotherapy for recurrent head and neck tumours. *Am. J. Otolaryngol.* 1989;**10:**257-260.

Plautz GE, Yang ZY, Wu BY, Gao X, Huang L, Nabel GJ. Immunotherapy of malignancy by in vivo gene transfer into tumours. *Proc. Natl. Acad. Sci. USA.* 1993;**90:**4645-4649.

Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, et al. The Ink4a tumour suppresser gene product,p19,interacts with mdm2 and neutralizes mdm2's inhibition of p53. *Cell* 1998;**92:**713-723.

Pruneri G, Pignataro L, Carboni N, Luminari S, Capaccio P, Neri A, et al. Mdm2 oncoprotein overexpression in laryngeal squamous cell carcinoma : association with wild-type p53 accumulation. *Mod Pathol* 1997;**10**(8)**:**785-792.

Quillien V, Heresbach Le Berre N, Dufour T, Denais A, Guegan Y, Ferry N, et al. Gene therapy of a model of glioblastoma in rats using adenovirus vector encoding the HSVtk gene. *Bull Cancer* 1997;**84**(11)**:**1047-1052.

Rampling R, Cruickshank G, MacLean A, Brown M. Therapeutic replication-competent herpes virus (letter; comment). *Nat Med* 1998 Feb 4:2 133.

Reed AL, Califano J, Cairns P, Westra WH, Koch W, Ahrendt S, et al. High frequency of p16 inactivation in head and neck squamous cell carcinoma. *Cancer Research* 1996;**56**(16)**:**3630-3633.

Renan MJ. How many mutations are required for tumourogenesis? Implications from human cancer data. *Mol. Carcinog.* 1994;**7:**139-146.

Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single clones. *Cell* 1975;**6:**331-343.

Rhyu MS. Telomeres, telomerase and immortality. *J. Nat. Cancer Inst.* 1995;**87:**884-894.

Ridgway PJ, Hall AR, Myers CJ, Braithwaite AW. p53/E1B58kDa complex regulates adenovirus replication. *Virology* 1997;**237:**404-413.

Righetti SC, Torre GD, Pilotti S, Menard S, Ottone F, Colnaghi MA et al. (1996). A comparitive study of p53 gene mutations, protein accumulation and response to cisplatin-based chemotherapy in advanced ovarian carcinoma. *Cancer Res.* **56**:689-693.

Robbins KT. Neck dissection classification and TNM staging of head and neck cancer. *Am Academy of Otolaryngology-Head and Neck Surgery Foundation* 1991:1-29.

Robbins KT, Storniolo AM, Kerber C, Vicario D, Seagren S, Shea M, et al. Phase I study of highly selective supradose cisplatin infusions for advanced head and neck cancer. *J. Clin. Oncol.* 1994;**12:**2113-2120.

Robert F, Soong SJ, Wheeler RH. A phase II study of topotecan in patients with recurrent head and neck cancer. *Am J Clin Oncol* 1997;**20:**298-302.

Rocco JW, Li D, Liggett WH, Duan L, Saunders JK, Sidransky D, et al. p16INK4A adenovirus mediated gene therapy for human head and neck squamous cell cancer. *Clin Cancer Res* 1998;4(7):1697-1704.

Rodriguez R, Schuur ER, Lim HY, Henderson GA et al. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* 1997;**57:**2559-2563.

Rommelaere J, Cornelis JJ. Antineoplastic activity of parvoviruses. *J. Virol Meth* 1991;**33:**233-251.

Rosenberg SA. Immunotherapy and gene therapy of cancer. *Cancer Res.*

1991;**51:**5074-5079.

Roth J et al. Retrovirus mediated wild type p53 gene transfer to tumours of patients with lung cancer. *Nature Med* 1996;**2**(9)**:**985-991.

Rothman KJ, Cann CI, Flanders D. Epidemiology of head and neck cancers. *Epidemiol Rev* 1980;**2:**195-209.

Rowley H, Jones AS, Field JK. Chromosome 18: a possible site for a tumour suppressor gene deletion in squamous cell carcinoma of the head and neck. *Clin. Otolaryngol.* 1995;**20:**266-271.

Sacchi M, Snyderman CH, Heo DS, Johnson JT, d'Amico F, Herberman RB, et al. Local adoptive immunotherapy of human head and neck cancer xenografts in nude mice with lymphokine-activated killer cells and interleukin 2. *Cancer Res.* 1990;**50:**3113-3118.

Saracci R. The interactions of tobacco smoking and othe agents in cancer aetiology. *Epidemiol. Rev.* 1987;**9:**175-193.

Sarnow P, Ho YS, Williams J, Levine AJ. Adenovirus E1B-58kD tumour antigen and SV40 large tumour antigen are physically associated with the same 54kd cellular protein in transformed cells. *Cell* 1982;**28:**387-394.

Sauter ER, Cleveland D, Trock B, Ridge JA, Klein-Szanto AJ. p53 is

overexpressed in fifty percent of pre-invasive lesions of head and neck epithelium. *Carcinogenesis* 1994;**15:**2269-2274.

Sauter ER, Ridge JA, Trock B, Cleveland D, Whitley KV, Mohr RM, et al. Overexpression of the p53 gene in primary and metastatic head and neck carcinomas. *Laryngoscope* 1995;**105:**653-656.

Sauthoff H, Heitner S, Rom WN, Hay JG. Deletion of the adenoviral E1b 19kDa gene enhances tumour cell killing of a replicating adenoviral vector. *Hum Gene Ther.* 2000; **11:**379-388.

Scambia G, Panici PB, Battaglia F. Receptors for epidermal growth factor and steroid hormones in primary laryngeal tumours. *Cancer* 1991;**67:**1347-1351.

Schantz SP, Spitz MR, Hsu TC. Mutagen sensitivity in patients with head and neck cancers: A biologic marker for risk of multiple primary malignancies. *J. Natl. Cancer Inst.* 1990;**82:**1773-1775.

Schartz LH, Ozsahis M, Zhang GN, Touboul E, De Vataire F, Andolenko P, et al. Synchronous and metachronous head and neck carcinoma. *Cancer* 1994;**74:**1933-1938.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes

the degradation of p53. *Cell* 1990;**63:**1129-1136.

Scheidmann KH, Mumby MC, Rundell K, Walter G. Dephosphorylation of simian virus 40 large T antigen and p53 protein by protein phosphatase 2A:inhibition by small t- antigen. *Mol. Cell Biol* 1991;**11:**1996-2003.

Schilling T, Heinrich B, Kau R, Herzog M, Quasthoff S, Diergarten K, et al. Paclitaxel administered over 3 hour followed by cisplatin in patients with advanced head and neck squamous cell carcinoma: a clinical phase I study. *Oncology* 1997;**54:**89-95.

Scholes AGM, Woolgar JA, Boyle MA, Brown JS, Vaughan ED, Hart CA, et al. Synchronous Oral Carcinomas: Independent or Common Clonal Origin. *Cancer Research* 1998;**58:**2003-2006.

Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, et al. Immediate early up regulation of bax expression but not TGF beta: a paradigm for distinct apoptotic pathways. *Oncogene* 1994;**9:**1791- 1798.

Serrano M, Hannon GJ. A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993;**366:**704-707.

Shay JW, Wright WE. Telomerase activity in human cancer. *Curr Opin Oncol* 1996;**8**(1)**:**66-71.

Shewach DS, Lawrence TS. Gemcitabine and radiosensitization in human tumour cells. *Invest New Drugs* 1996;**14:**257-263.

Shieh S, Ikeda M, Taya Y, Prives C. DNA damage induced phosphorylation of p53 alleviates inhibition by mdm2. *Cell* 1997;**91:**325-334.

Shin DM, Kim J, Ro JY, Hittelman J, Roth JA, Hong WK, et al. Activation of p53 expression in premalignant lesions during head and neck tumorigenesis. *Cancer Res.* 1994;**54:**321-326.

Shin DM, Lee JS, Lippman SM, Lee JJ, Tu ZN, Choi G, et al. p53 expression: predicting recurrence and second primary tumours in head and neck squamous cell carcinoma. *J. Natl. Cancer Inst.* 1996;**88:**519-529.

Shin DM, Lippman SM. Paclitaxel based chemotherapy for recurrent and/or metastatic head and neck squamous cell carcinoma: current and future directions. *Semin Oncol* 1999; **26:**100-105.

Shindoh M, Chibas I, Yasuda M, Saito T, Funaoka K, Kohgo T, et al. Detection of human papillomavirus DNA sequences in oral squamous cell carcinomas and their relation to p53 and proliferating cell nuclear antigen expression. *Cancer* 1995;**76:**1513-1521.

Silverman SJ, Gorsky M, Greenspan D. Tobacco usage in patients with head and neck carcinomas: A follow-up study on habit change and second

primary oral/oropharyngeal cancers. *J. Am. Dent. Assoc.* 1983;**106:**33-35.

Slaughter DP, Southwick HW, Smejkal W. "Field cancerisation" in oral stratified squamous epithelium. Clinical implications of multicentric origin. *Cancer* 1953;**6:**963-968.

Smith R et al. Studies on the use of viruses in the treatment of carcinoma of the cervix. *Cancer* 1956;**9:**1211-1218.

Smith ML, Fornace AJ. Genomic instability and the role of p53 mutations in cancer cells. *Curr. Opin. Oncol.* 1995;**7:**69-75.

Snow GB, Annyas AA, van Slooten EA, Bartelink H, Hart AA. Prognostic factors of neck node metastasis. *Clin. Otolaryngol.* 1982;**7:**185-192.

Snow GB. Evaluation and staging of the patient with head and neck cancer. *Churchill Livingstone, New York* 1989**:**17-38.

Somers KD, Cartwright SL, Schechter GL. Amplification of the int-2 gene in human head and neck squamous cell carcinomas. *Oncogene* 1990;**5:**915-920.

Spitz MR, Fueger JJ, Beddingfield NA. Chromosome sensitivity to bleomycin-induced mutagenesis: An independent risk factor for upper aerodigestive tract cancers. *Cancer Res.* 1989;**49:**4626-4628.

Staba MJ, Wickham TJ, Kovesdi I, Hallahan DE. Modifications of the fiber in adenovirus vectors increase tropism for malignant glioma models. *Cancer Gene Ther.* 2000; **7:**13-19.

Steegenga, W. T., N. Riteco, et al. The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. *Oncogene.* 1997;**16:**3:349-357

Stoler MM, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT, Broker TR. Human papillomavirus type 16 and 18 gene expression in cervical neoplasms. *Hum. Pathol.* 1992;**23**:117-128.

Stupp R, Weichselbaum RR, Vokes EE. Combined modality therapy of head and neck cancer. *Semin. Oncol.* 1994;**21:**349-358.

Taylor SG, Sisson GA, Bytell DE. Adjuvant chemoimmunotherapy of head and neck cancer. *Recent Results Cancer Res.* 1979;**68:**297-308.

Teodoro, J. G. and P. E. Branton. Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55- kilodalton E1B protein of human adenovirus type 5. *J. Virol* 1997; **71**: 3620-3627.

Tollefson AE et al. The adenovirus protein (E3-11.6k) is required at very

late stages of infectionfor efficient cell lysis and release of adenovirus from infected cells. *J. Virol.* 1996;**70:**2296-2306.

Townsend SE, Alison JP. Tumour rejection after direct costimulation of CD8+ Tcells by B7-transfected melanoma cells. *Science* 1993;**259:**368-370.

Tu GY, Tang PZ, Jia CY. Horizontovertical laryngectomy for supraglottic carcinoma. *Otolaryngol Head Neck Surg* 1997;**117**:280-286.

van der Riet P, Nawroz H, Hruban RH, Corio R, Tokino K, Koch WM, et al. Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. *Cancer Res.* 1994;**54:**1422-1424.

Van Dyke DL, Worsham MJ, Benninger MS, Krause CJ, Baker SR, Wolf GT, et al. Recurrent cytogenetic abnormalities in squamous cell carcinoma of the head and neck region. *Genes Chromosom. Cancer* 1994;**9:**192-206.

Virgilio L, Shuster M, Gollin SM, Veronese ML, Ohta M, Huebner K, et al. FHIT gene alterations in head and neck squamous cell carcinomas. *Proc. Natl. Acad. Sci. U. S. A.* 1996;**93:**9770-9775.

Vokes EE, Athanasiadis I. Chemotherapy for squamous cell carcinoma of head and neck. *Ann Oncol* 1996;**7:**15-29.

Wang Y, Reed M, Wrang P, Stenger JE, Mayr G, Anderson ME, et al. p53

domains: identification and characterisation of two autonomous DNA binding regions. *Genes and Dev* 1993;**7:**2575-2586.

Watling DL, Gown AM, Coltera MD. Overexpression of p53 in head and neck cancer. *Head Neck* 1992;**14:**437-444.

Werner JA, Gottschlich S, Folz BJ, Goeroegh T, Lippert BM, Maass JD, et al. p53 serum antibodies as prognostic indicator in head and neck cancer. *Cancer Immunol. Immunother.* 1997;**44:**112-116.

Whitaker DC. Clinical evaluation of tumours of the skin. *WB Saunders, Philadelphia* 1987**:**1158-1172.

White E. Life, death and the pursuit of apoptosis. *Genes Dev.* 1997;**10:**1-15.

Whiteside TL, Letessier E, Hirabayashi H, Vitolo D, Bryant J, Barnes L, et al. Evidence for local and systemic activation of immune cells by peritumoural injection of i interleukin 2 in patients with advanced squamous cell carcinoma of the head and neck. *Cancer Res.* 1993;**53:**5654-5662.

Whiteside TL, Chikamatsu K, Nagashima S, Okada K. Antitumour effects of cytolytic T lymphocytes (CTL) and natural killer (NK) cells in head and neck cancer. *Anticancer Res* 1996;**16**(4C)**:**2357-2364.

Wilson J, Engelhardt J, Grossman M, Simon R, Yang Y. Gene therapy of

cystic fibrosis lung disease using E1 deleted adenovirus: a phase I trial. *Human Gene Therapy* 1994;**5:**501-519.

Wilson JM. Adenoviruses as gene delivery vehicles. *New England J of Med* 1996;**334**(18)**:**1185-1187.

Woolley AL, Hogikyan ND, Gates GA, Haughey BH, Schechtman KB, Goldenberg JL. Effect of blood transfusion on recurrence of head and neck carcinoma. Retrospective review and meta-analysis. *Ann Otol Rhinol Laryngol* 1992; **101**:724-730.

Wynder EL, Dodo H, Bloch DA. Epidemiologic investigation of multiple primary cancer of the upper alimentary and respiratory tracts. A retrospective study. *Cancer* 1969;**24:**730-739.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993;**366:**701-704.

Yamamoto T, Kamata N, Kawano H. High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. *Cancer Res.* 1986;**46:**414-416.

Yang Y, Trinchieri G, Wilson J. Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung. *Nat Med* 1995;**1:**890-893.

Yazaki T. Treatment of human malignant meningiomas by G207, a replication competent multimutated herpes simplex virus 1. *Cancer Res* 1995;**55:**4752-4756.

Yeudall WA, Campo MS. Human papillomavirus DNA in biopsies of oral tissues. *J. Gen. Virol.* 1991;**72:**173-176.

Yoo GH, Xu HJ, Brennan JA, Westra W, Hruban RH, Koch WM, et al. Infrequent inactivation of the retinoblastoma gene despite frequent loss of chromosome 13q in head and neck squamous cell carcinoma. *Cancer Res.* 1994;**54:**4603-4606.

Yoshikawa H, Kawana T, Kitagawa K, Mizuno M, Yoshikura H, Iwamoto A. Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. *Jpn J Cancer Res* 1991;**82:**524-531.

Zhang Y, Xiong Y, Yarbrough WG. ARF promotes mdm2 degradation and stabilizes p53: ARF-INK4a deletion impairs both the Rb and p53 tumour suppression pathways. *Cell* 1998;**92:**725-734.

Zidan J, Kuten A, Rosenblatt E, Robinson E. Intensive chemotherapy using cisplatin and fluorouracil followed by radiotherapy in advanced head and neck cancer. *Oral Oncology* 1997; 33:129-135.

Zieske LA, Johnson JT, Myers EN, Thearle PB. Squamous cell carcinoma with positive margins: surgery and postoperative irradiation. *Arch. Otolaryngol. Head Neck Surg.* 1986;**112:**863-866.

Zindy F, Eischen CM, Kamijo T, Sherr CJ, Roussel MF. Myc signaling via the ARF tumour suppresser regulates p53 dependent apoptosis and immortalisation. *Genes & Dev* 1998;**12:**2424-2433.

zur Hausen H. Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. *Curr. Topics. Microbiol. Immunol.* 1994;**186:**131-156.