

**MORPHOLOGICAL ALTERATIONS AND THE
EFFECT ON TUMOUR SUPPRESSOR GENE
EXPRESSION INDUCED BY HIGH DOSE RATE
INTRALUMINAL BRACHYTHERAPY IN
OESOPHAGEAL CARCINOMA**

MONALISA SUR

M.B., B.S., D.C.Path (Clin Path), F.C.Path.S.A. (Anat Path)

A thesis submitted to the Faculty of Health Sciences, University of the
Witwatersrand, Johannesburg, in fulfillment of the requirements for the
degree of Master of Medicine (Anatomical Pathology)

Johannesburg, February, 1999

Contents

1.	Title	1.1
2.	Contents	2.1
3.	Declaration	3.1
4.	Acknowledgments	4.1
5.	Preface	5.1
6.	Abstract	6.1
7.	Introduction	7.1
	a. The problem.....	7.2
	b. Brachytherapy.....	7.4
	c. <i>MDR-1 gene</i> : p-glycoprotein.....	7.6
	d. p53 (Tumour suppresser gene).....	7.8
	e. bcl-2 oncogene.....	7.11
	f. Apoptosis.....	7.15
	g. Rationale for the studies.....	7.20
8.	Aims and Objectives	8.1
9.	Hypothesis	9.1
10.	Material and Methods	10.1
	a. Staging and selection criteria.....	10.2
	b. Brachytherapy.....	10.4
	c. Surgery.....	10.6
	d. Rationale for preoperative HDR ILBT in Oesophageal cancer.....	10.6
	e. Morphological changes in oesophagectomy specimens.....	10.7
	f. <i>MDR-1 gene</i> product : p-glycoprotein.....	10.7
	g. p53, bcl-2 and apoptosis.....	10.9

Contents

1.	Title	1.1
2.	Contents	2.1
3.	Declaration	3.1
4.	Acknowledgments	4.1
5.	Preface	5.1
6.	Abstract	6.1
7.	Introduction	7.1
	a. The problem.....	7.2
	b. Brachytherapy.....	7.4
	c. <i>MDR-1 gene</i> : p-glycoprotein.....	7.6
	d. p53 (Tumour suppresser gene).....	7.8
	e. bcl-2 oncogene.....	7.11
	f. Apoptosis.....	7.15
	g. Rationale for the studies.....	7.20
8.	Aims and Objectives	8.1
9.	Hypothesis	9.1
10.	Material and Methods	10.1
	a. Staging and selection criteria.....	10.2
	b. Brachytherapy.....	10.4
	c. Surgery.....	10.6
	d. Rationale for preoperative HDR ILBT in Oesophageal cancer.....	10.6
	e. Morphological changes in oesophagectomy specimens.....	10.7
	f. <i>MDR-1</i> gene product : p-glycoprotein.....	10.7
	g. p53, bcl-2 and apoptosis.....	10.9

Contents

1.	Title	1.1
2.	Contents	2.1
3.	Declaration	3.1
4.	Acknowledgments	4.1
5.	Preface	5.1
6.	Abstract	6.1
7.	Introduction	7.1
	a. The problem.....	7.2
	b. Brachytherapy.....	7.4
	c. <i>MDR-1 gene</i> : p-glycoprotein.....	7.6
	d. p53 (Tumour suppresser gene).....	7.8
	e. bcl-2 oncogene.....	7.11
	f. Apoptosis.....	7.15
	g. Rationale for the studies.....	7.20
8.	Aims and Objectives	8.1
9.	Hypothesis	9.1
10.	Material and Methods	10.1
	a. Staging and selection criteria.....	10.2
	b. Brachytherapy.....	10.4
	c. Surgery.....	10.6
	d. Rationale for preoperative HDR ILBT in Oesophageal cancer.....	10.6
	e. Morphological changes in oesophagectomy specimens.....	10.7
	f. <i>MDR-1 gene</i> product : p-glycoprotein.....	10.7
	g. p53, bcl-2 and apoptosis.....	10.9

11.	Review of Literature	11.1
I.	Morphological changes induced by Radiotherapy in oesophageal carcinoma.....	11.2
a.	In vivo studies.....	11.2
b.	In vitro/animal studies.....	11.7
II.	<i>MDR-1</i> gene product: p-glycoprotein.....	11.8
III.	p53, bcl-2 and apoptosis.....	11.15
a.	Effect of chemo/radiotherapy on the expression of p53 in oesophageal cancers..	11.15
b.	Effect of chemo/radiotherapy on the expression of bcl-2 in oesophageal cancers.	11.22
c.	Role of apoptosis in treated oesophageal cancer.....	11.26
12.	Results	12.1
a.	Morphological changes.....	12.2
b.	Effect on expression of <i>MDR-1</i> gene product : p-glycoprotein.....	12.4
c.	Effect on expression of p53, bcl-2 and apoptosis...	12.5
13.	Discussion	13.1
14.	Conclusions	14.1
15.	References	15.1

Declaration

I declare that this thesis is my own unaided work, except where otherwise acknowledged. This thesis is being submitted for the degree of Master of Medicine (Anatomical Pathology) at the University of the Witwatersrand, Johannesburg. This thesis has not been submitted before for any degree or examination at any other university.

Signed this 27th day of January, 1999



MONALISA SUR

Acknowledgments

I would like to acknowledge the following without whom this work could not have been possible:

I would like to express my gratitude for the support and encouragement that I received from Prof. Kum Cooper, who gave me the opportunity to carry on with my research and provided the help, support and encouragement that one looks for in a Head of department. It was my immense pleasure to have worked under his supervision.

I express gratitude for the financial support for the conducting of my research that I received from the South African Institute of Medical Research (SAIMR). I thank the photographic unit of the SAIMR for taking the photomicrographs presented in this thesis.

I express my thanks to Louise Tayler and Zenobia Haffajee who helped me with the material and methods in the research laboratory. I also thank my departmental colleagues and staff for their consistent encouragement and support which enabled me to complete this study.

I would like to thank the Department of Surgery, and Dr. Damon Bizos in particular for making sure that the esophagectomy specimens were sent to the department in my name. I express gratitude to the department of Radiation Oncology for entering patients into this study and for providing the photographs of the procedure involved in HDR ILBT.

I would like to make a mention of the tremendous amount of encouragement, patience and support that I received from my husband, Dr. Ranjan K. Sur. I would like to thank him for initiating the spark to complete writing this report, and for his encouragement and valuable suggestions which enabled me to complete the work in good time.

I would like to thank my father-in-law for his constant support in furthering my career, and his appreciation of my achievements and efforts.

I acknowledge with thanks the efforts of Ms. Katerina Chronias of the department of English, WITS University for going through the manuscript and giving valuable suggestions in the text and language.

I would like to acknowledge the comments that I received from the reviewers of all the peer reviewed refereed journals in which my articles from these studies have been published, which enabled me to write up the discussion with ease.

I would like to dedicate this work to my father, Prof. K.C.Das, and mother, Mrs. M.Das, who have always encouraged me to achieve my ambitions in life.

Monalisa Sur

Johannesburg, South Africa

Preface

The following papers have been published/accepted for publication and are based directly or indirectly on the work presented in this thesis:

1. **Sur M.**, Sur R.K., Cooper K., Levin V., Bizo D., Dubazana N.
Morphologic Alterations in Esophageal Squamous Cell Carcinoma after Preoperative High Dose Rate Intraluminal Brachytherapy.
Cancer 1996; 77: 2200 - 2205
2. **Sur M.**, Taylor L., Cooper K., Sur R.K.
Lack of correlation of P glycoprotein expression with response to MIC chemotherapy in oesophageal cancer.
Journal of Clinical Pathology 1997; 50 : 534
3. **Sur M.**, Cooper K
bcl2, p53 immunophenotypes and apoptosis in squamous cell carcinoma of the esophagus.
Histopathology 1998; 33: 87- 88
4. **Sur M.**, Cooper K., Sur R.K., Taylor L., Bizo D
Effect of Brachytherapy on expression of p53, bcl2 and apoptosis in squamous cell carcinoma of the esophagus
Molecular Pathology 1999: submitted for publication

The following conference presentations are done from work directly based on the thesis presented:

1. Biannual Cardiothoracic Forum, Pilansberg, South Africa, October, 1997:
Morphological changes following brachytherapy in esophageal cancer and effect of brachytherapy on the expression of various oncogenes
2. 37th Annual Congress of the Federation of the South African Societies of Pathologists, Cape Town, September, 1997:
Effect of Brachytherapy on expression of p53 and bcl2 and apoptosis in squamous cell carcinoma of the esophagus
3. Biannual Congress of the South African Society of Radiation Oncology, Durban, March, 1997:
P glycoprotein (MDR 1 gene) is of no value in assessing response to brachytherapy / chemotherapy in squamous cell esophageal cancer
4. Biannual Congress of the South African Society of Radiation Oncology, Th'banchu Sun, South Africa, September 1995:
Morphologic Alterations in esophageal squamous cell carcinoma after preoperative high dose rate intraluminal brachytherapy.

Chapter 6**Abstract**

AIMS

1. To review the radiation changes in total oesophagectomy specimens from 10 patients with Squamous cell carcinoma [SCC], given preoperative High Dose Rate Intraluminal Brachytherapy [HDR ILBT] of 20 Gy in two fractions of 10 Gy each weekly.
2. To evaluate the effect of HDR ILBT on p-glycoprotein, the *MDR-1* gene product in oesophageal SCC.
3. To examine the effect of HDR ILBT in the expression of p53, bcl-2 and apoptosis in oesophageal cancer.

METHODS

Ten consecutive patients with a preoperative diagnosis of moderate to poorly differentiated SCC of the oesophagus were investigated in this study.

1. The post-brachytherapy oesophagectomy specimens were sampled at the resection margins, edge of irradiated length, 1 cm from edge of visible tumour proximally and distally, centre of the tumour and lymph nodes.

Both pre and post HDR ILBT specimens were:

2. examined for the expression of p-glycoprotein, the *MDR-1* gene product using antibody to p-glycoprotein (clone JSB-1, using the modified sandwich technique).
3. examined for the expression of p53, bcl-2 and apoptosis using immunohistochemical markers.

RESULTS

1. Radiation changes in the form of fibrosis was limited to the submucosa at the resection margins, circular muscle layer at the edge of irradiated length and full thickness at 1 cm from the edge of visible tumour and centre of the tumour. Surface epithelium did not show any changes at the resection margins but showed basal cell hyperplasia at the edge of the irradiated length, and ulceration at 1 cm from the edge of visible tumour and centre of the tumour. Endarteritis obliterans in the blood vessels were seen only 1 cm from the edge of visible tumour and at the centre of tumour. Necrosis, intense keratin formation and giant cell reaction were observed at the centre of the tumour. When compared to the pre-radiotherapy biopsies, the amount of keratin in the post-radiotherapy specimens was extensive.
2. p-glycoprotein was not expressed in either the pre-brachytherapy or post-brachytherapy tumour tissue specimens.

3. In one patient there was no expression of p53 in either pre or post-brachytherapy specimens. In 8 patients, p53 staining was strongly positive (3+), with approximately 50% or more cells showing a diffuse pattern in the pre-brachytherapy biopsies. The tumour areas of the post-brachytherapy specimens of these patients showed strong 3+ positivity with p53 (10-50% positive cell count), with the pattern being focal and peripheral in the tumour islands. The centre of the tumour islands showed necrosis and/or keratinization. In one patient, the pre-brachytherapy biopsy showed expression of p53, whilst the post-brachytherapy specimen was negative. bcl-2 expression was equivocal in both pre and post-brachytherapy. Apoptosis was not demonstrated in either the pre or post-brachytherapy tissue sections in the presence of positive control.

CONCLUSION

1. HDR ILBT may induce keratin gene in the irradiated cells to differentiate towards better differentiated cells. Preoperative HDR ILBT may have a role in improving the prognosis of early oesophageal cancer treated with a combination of radiotherapy and surgery.
2. p-glycoprotein expression is of no value in predicting the responsiveness of the tumour to radiotherapy in SCC of the oesophagus.
3. Brachytherapy does not cause cell death by apoptosis but by necrosis and maturation of the cells into better differentiated cells which is caused by OH⁻ free radical and induction of the keratin gene respectively. It is possible that brachytherapy may cause destruction of cells producing wild type p53, while mutant p53 in cells located at the tumour periphery escapes the effect of brachytherapy. This maybe responsible for the high incidence of local recurrence and distant metastasis in oesophageal cancer treated with radiotherapy. Brachytherapy does not affect bcl-2 expression in oesophageal cancer.

Chapter 7**Introduction**

The Problem

Seventy years ago, squamous carcinoma of the oesophagus was a rare disease in black South Africans (Rose, 1978). The incidence has risen since then, and it is currently the most common cancer amongst black men in South Africa, and the second most common cancer amongst black women (Sitas et al., 1997). In the 1950's, a large number of cases were diagnosed in the former Transkei and Ciskei, and in predominantly rural areas of South Africa. The prevalence in the urban population was considerably less (McGlashen, 1988). Cancer of the oesophagus remains the most frequently reported cancer in the Transkei, representing 45.8% of malignant disease (Sitas et al., 1997). The incidence of oesophageal cancer in Soweto, South Africa's largest urban black community, has risen and is estimated to be between four to five times greater than that in the Transkei. In 1985, the age standardised incidence of oesophageal cancer in Soweto was 125/100,000 in men, and 37/100,000 in women (Kneebone & Mannell, 1985).

In South Africa, it is a common perception that squamous cancer of the oesophagus in black South Africans is more advanced by the time of diagnosis, and that the patients are more debilitated, when compared with patients with the same disease in Western countries (Hunt, 1978). In 1989, the national study group for oesophageal cancer in South Africa collected and centralized data on 1926 new cases of squamous carcinoma of the oesophagus, seen in the Ciskei,

and in major provincial centers of South Africa from 1985 to 1988 (Mannell & Murray, 1989). There were 1438 men and 488 women (M: F-3: 1) 55% of whom were between 50 and 69 years of age (mean age, 56 years). 22% of the patients were in their fifth decade, 14.2% were 70 years of age or older, and 8.8% were younger than forty years of age. 62% of patients complained of dysphagia to solid food, 12% had difficulty swallowing liquids, and 24% suffered from total dysphagia. Although 2% of the patients had no dysphagia, they did have evidence of metastatic disease.

The National Cancer Registry, based at the South African Institute of Medical Research, recently reported the incidence of oesophageal cancer in South Africa (Sitas et al., 1997): cancer of the oesophagus is the second most common cancer in all males (8.1% of all cancers). It is the most common cancer in black males (17.9%) (L. R. = 1 in 39). Among white males, by contrast, oesophageal cancer ranked eighth (1.5%) (L. R. 1 in 119), with a risk factor three times lower. Among coloured and Asian males, oesophageal cancer ranked fourth and seventh respectively.

When the South African incidence is compared with that of the rest of Africa, the age standardised rates amongst black males are comparable to rates from Zimbabwe, but are higher when compared to West African countries like Mali or

Gambia. It has been estimated that in Mali, for example, 95% of the patients have advanced stage disease (Sitas et al., 1997).

It has been shown that the length of the primary tumour is inversely related to its curability, and is directly related to its stage of advancement. For tumors smaller than 5 cm in length, 40% are localized, 25% are locally advanced, and 35% have distant metastasis or are unresectable for cure. For tumors larger than 5 cm, only 10% are localized, 15% are locally advanced, and 75% have distant metastasis or are unresectable for cure (Clayton, 1928, Fleming, 1943, Merendiono & Maerk, 1952). The average tumour length seen in South Africa is about 10 cm (Sur et al, 1998). More than 95% of cancers are squamous cell carcinomas (SCC) followed by adenocarcinomas. Rare cancers include undifferentiated carcinoma, carcinoid, malignant melanoma, small cell carcinoma, and adenocarcinoma arising from submucosal glands.

Brachytherapy

Traditionally oesophageal cancer has been treated with external beam radiotherapy (EBRT) wherein the radiation is delivered externally. With the advent of remote afterloading units, it is now possible to treat a tumour with a radioactive isotope placed within, or in close proximity, to the tumour. This form of radiation treatment is called Brachytherapy. In the oesophagus, this is done

by placing a catheter in the oesophageal lumen. A radioactive isotope, such as Iridium-192 or Cobalt-60, is then passed through the catheter by means of a remote after loading system, which delivers a high dose to the luminal aspect of the tumour. This part of the tumour is thought to be more hypoxic and therefore relatively radioresistant. Due to the close proximity of the radiation source, a very high dose of radiation is delivered to the luminal aspect of the tumour, which then undergoes rapid necrosis, thus rapidly restoring lumen patency. This allows the patient to rapidly restore swallowing which is maintained for a prolonged period of time. Further, high specific activity radiation sources, for example, small Ir-192 sources with an activity of 10 Ci (370 GBq) allows a dose rate of $> 2 \text{ Gy / minute}$ to be delivered in a very short time - so called High Dose Rate Intraluminal Brachytherapy (HDR ILBT), are used.

Further advantages include:

1. Short treatment time - the whole treatment including the procedure takes about 20 minutes
2. No anesthesia is required - The procedure is done under Pethidine analgesia
3. The procedure can be performed on an outpatient basis - Therefore no admission is required, which saves hospital beds for optimal utilization of resources.

4. There is minimal patient discomfort and morbidity of the treatment and procedure.
5. Risk of radiation to personnel is minimal.
6. Radiation dose to surrounding normal tissue structures due to rapid dose fall off, is minimal.
7. Many patients can be treated in one day on one HDR unit.
8. It is a cost effective and safe method of treatment.

There are various genes which may show altered expression prior to and after therapeutic procedures implemented in the treatment of oesophageal SCC.

MDR-1 gene

The curative potential of chemotherapy for a number of tumour types has been obscured by the fact that many patients manifest resistance to a wide array of structurally unrelated anti-neoplastic agents. One of the most perplexing problems encountered in chemotherapy is the resistance of certain tumors to all chemotherapeutic regimes, while other tumors which are initially chemosensitive to a particular agent, show resistance to treatment over time and disease progression. Furthermore, tumour cells which are resistant to one drug often show cross resistance to a wide variety of other, structurally unrelated drugs. Resistance may be inherent in a tumour cell or may evolve under the selection pressure of drug administration. This is known as the multidrug drug resistance

(MDR) phenomenon. The MDR phenomenon includes cross resistance amongst anthracyclines, Vinca alkaloids, Taxol, and other compounds. The normal role of MDR is to protect cells from environmental carcinogens, and the tissues that are most at risk, and richly supplied with MDR, will produce drug resistant neoplasms (Spiers A.S., 1994). A number of possible molecular explanations for MDR exist. These include exclusion of the drug from the cells; failure to activate the pro drug to its active form; increased detoxification; alterations in drug target; enhanced repair capability of the cell after injury and failure to engage an appropriate response leading to apoptosis in the damaged cells (Harrison D.J., 1995). The MDR is due to overexpression of p-glycoprotein, the *MDR-1* gene product. The *MDR-1* gene is located on chromosome 13. The family of so-called MDR genes encodes the p-glycoprotein, apparently the only protein encoded by the *MDR-1* gene, which induces the MDR phenotype. Only 2 of the MDR genes are known to be present in man. The *MDR-1* and *MDR-3* genes; but only the *MDR-1* gene product confers the MDR phenotype. The *MDR-1* gene codes for the expression of a cell surface protein (p glycoprotein), which is an integral plasma membrane protein of 170 kd molecular weight. p-glycoprotein contains 12 putative transmembrane regions and 2 ATP binding sites. p-glycoprotein expression serves as an excretory pathway for xenobiotic drugs and toxins. Expression of p-glycoprotein is tissue specific and is found in a number of normal tissues e.g. colon, small intestine, kidney, liver, adrenal gland, capillary of brain and testis (Chin K.V. and Liu B., 1994). p-glycoprotein functions as an ATP-

dependent drug efflux pump with broad specificity for chemically unrelated hydrophobic compounds (Kartner N. and Ling V., 1989; Gottesman M.M. et al., 1991). There is extensive evidence from in-vitro studies, especially with non-human cell lines, that overexpression of p-glycoprotein results in reduced accumulation of the drug within the cell. Recently, mice have been generated with knock out of *MDR-1*. These animals showed abnormalities of transport at the blood brain barrier and were more sensitive to drugs. The *MDR-1* gene has some value in predicting responsiveness to treatment in adenocarcinomas. p-glycoprotein is overexpressed in a number of intrinsically resistant tumors, and appears to vary according to the differentiation of the cell. This has been demonstrated clinically in breast cancer (Glazer R.L and Rohlff C., 1994), renal cell carcinomas (Mickishc G.H., 1994), cancer of the urinary bladder (Scher H.I., 1992), colo-rectal malignancies (Linn S.C. and Glaccone G., 1995), gastric (Wallner J et al., 1993) and ovarian carcinomas (Sekiya S. et al., 1992). Patients who express the gene have poor survival.

p53 protein (Tumor suppresser gene)

The p53 gene is a tumour suppresser gene located on the short arm of chromosome 17 (17p13.1) and is the single most common target for genetic alterations in human cancer (Zenbetti G.P. and Levine A.J., 1993, Chang F., 1993). Homozygous loss of the p53 gene is found in 70% of colon cancers, 30-50% of breast cancers, 50% of lung cancers, oesophageal cancers, stomach

cancers and others. The p53 gene is a nuclear phosphoprotein that regulates DNA replication, cell proliferation and cell death. The p53 protein inhibits DNA replication in response to DNA damage and serves as a checkpoint gene product that induces cell cycle arrest in G1 by activating growth suppresser genes (Harris C.C. and Hollstein M., 1993). Under physiological conditions, p53 has a very short half life measured in minutes, and there is no evidence that this protein is required for normal cell division. A combination of genetic events that affect both alleles of the gene, results in the loss of expression of the wild type gene. Mutations of the p53 leads to the loss of DNA binding and transcriptional regulatory activities of the p53 phosphoprotein, with the corresponding loss of its growth suppressive activity and its role as "the guardian of the genome". The mutated protein has abnormal conformation, impaired DNA binding, and a prolonged or stabilized half life, the latter resulting in immunohistochemically stainable levels within nuclei in nearly all tumors showing p53 gene mutation (Batsakis J.G., L-Nagger A.K., 1995).

When cells are exposed to mutagenic agents such as chemicals or radiation, there are dramatic changes in wild type p53 cycle (Zenbetti G.P. and Levine A.J., 1993). Through post translational modifications, p53 is stabilized, and it accumulates within the nucleus. The accumulated wild type p53 binds to DNA and causes cells to arrest in the G1 phase of the cell. This reversible pause in the cell cycle is welcome, because it allows the cells time to repair the DNA

damage inflicted by the mutagenic agent. If, for some reason, the repair mechanisms fail, p53 triggers cell death by apoptosis. With loss of normal p53 (common in many tumors), cells exposed to mutagenic agents replicate the damaged DNA, and thus mutations become fixed in the genome. Although no single mutation is sufficient to transform cells, the loss of p53 pathway predisposes cells to additional mutations and to ultimately malignant transformation. Radiation and chemotherapy rely in part on their ability to alter the DNA of rapidly dividing cells, leading to their death and elimination by the p53 mechanism. If p53 is absent, the genetic damage goes unnoticed and these mutations may result in the lesion being more aggressive. Inactivation of the p53 gene occurs most commonly in missense mutations. These abnormal proteins fail to bind to DNA but have a remarkably long half life and are detected in tumour cells by immunohistochemical staining (Kaklamanis L. et al., 1993). Loss of wild type p53 activity induces a release from G1-S cell cycle checkpoint control following DNA damage, increasing genomic instability and promoting gene amplification. In addition, certain mutant forms of p53 not only lose their normal function, but also gain the ability to bind and inactivate normal p53 protein.

p53 tumour suppresser gene normally stimulates apoptosis, but, when mutated or absent (as seen in certain cancers), favors cell survival. It appears that p53 is required for the apoptosis following DNA damage by irradiation (Lane D.P., 1993). Immunohistochemical detection of the nuclear p53 protein is based on

the increase in concentration of the protein to detectable levels, secondary to an increased synthesis and a lower degradation with a longer half-life. Although there is good agreement between the frequency of positive immunostaining and the frequency of tumors with mutations, there are discrepancies between these findings and analysis at the protein level. p53 protein can be stabilized by other means, such as sequestration of normal nuclear protein in the cytoplasm with inactivation of its tumour suppressor function or by binding with other cellular proteins. Therefore, positive staining may not always be an indication of an underlying mutation (Hall P.A. and Lane D.P., 1994). The analysis of p53 in neoplastic and pre-neoplastic states and the ability to stain for the abnormal forms of the protein in tissue sections is a powerful tool which provides molecular information on the oncogenic process. Furthermore, there is evidence to suggest that the expression of abnormal p53 may be a prognostic parameter in some neoplasms (Batsakis J.G., L-Naggar A.K., 1995) .

bcl-2 oncogene

The bcl-2 gene was identified more than a decade ago with the discovery and analysis of the t(14; 18) (q32;q21) translocation (Bakhshi A. et al, 1985). This translocation is the most common specific recurrent chromosomal aberration in non-Hodgkins lymphomas, occurring in 70-80% of cases of follicular lymphoma (Cleary M.L. et al. 1986 ; Landanyi M. and Wang S., 1992). As a result of this translocation, bcl-2 sequences are juxtaposed with the immunoglobulin heavy

chain gene on chromosomal 14q32 (Chen-Levy Z. et al., 1989). This results in an overexpression of the translocated *bcl-2* allele induced by enhancers in the immunoglobulin heavy chain region (Pezzella F. et al., 1990). However, this translocation is not a prerequisite for *bcl-2* protein expression and does not result in an interruption of the coding region of *bcl-2*. The *bcl-2* polypeptide is a 26 kD protein that is found on intracellular (mitochondrial and nuclear) membranes and in the cytosol of the smooth endoplasmic reticulum (Krajewski K. et al., 1993). The association of *bcl-2* with lymphomas immediately led to the inference that it was an oncogene. However, *bcl-2* protein was found to have no effect on cell replication (Baer R., 1994). It acts as a survival factor that prevents cells from undergoing apoptosis, and confers a survival advantage on cells harboring the t(14;18) translocation. By extending cell survival, over expression of *bcl-2* allows other mutations that affect proto-oncogene and cancer suppresser genes to supervene. Recent studies suggest that *bcl-2* inhibits apoptosis by regulating an anti-oxidant pathway (Hockenberry D.M., 1993).

Several genes that have sequence homology with the *bcl-2* gene have been identified and are classified as members of the *bcl-2* family of genes (Lu Q.L. et al., 1996). They can be divided into two groups on the basis of their effects on the life span of cells. Those that are anti-apoptotic are *bcl-2*, *bcl-XL* and *MCL1*. Those that promote cell death are *Bax*, *bcl-XS* and *Bak*. *bcl-2* protein is produced early in the process of embryogenesis (Veis-Novack D. and Korsmeyer

S.J., 1994). bcl-2 is present in all three germ layers and once tissues have matured, expression of bcl-2 is restricted. bcl-2 may be required during embryogenesis and development to ensure cell viability and survival so that differentiation and maturation can occur. In normal lymphoid tissue bcl-2 antibody reacts with B-lymphocytes in the mantle zone and with many cells within T-cell areas. In the thymus many cells in the medulla are stained, with weak/negative reaction in the cortex (Chetty R. et al. 1997).

Expression of bcl-2 has been studied in various tumors (Lu Q.L. et al., 1993; Pezzella F. et al., 1993; Colombel M. et al., 1993, McDonald T.J., 1992). In general, bcl-2 positive neoplasms have a better prognosis than negative ones, with certain prostatic cancers being the exception to the rule.

The frequency of bcl-2 positivity in the more common malignancies studied are as follows: Chronic lymphocytic leukemia- almost 100%; chronic myeloid leukemia- almost 0%; chronic myeloid leukemia with blastic crisis- almost 100%; acute myeloid leukemia- almost 100%; follicular lymphoma- 80%; T and B cell non-Hodgkins lymphoma- 65%; Hodgkins disease- 40%; breast cancer- 80%; neuroblastoma- 40%; nasopharyngeal carcinoma- 80%; prostatic cancer 75%; lung SCC- 25%; lung adenocarcinoma 10-15%.

Transcription of *bcl-2* could affect transport across membranes, influence intracellular ion distribution, or initiate pathways that prevent oxidative damage to sub cellular constituents as lipid-containing membranes (Craig R.W., 1995). Both *bcl-2* and calcium are localized in the mitochondrion, so *bcl-2* may affect calcium regulation. *bcl-2* may redistribute or sequester calcium, thereby decreasing calcium dependent nuclease activity and inhibiting apoptosis (Craig R.W., 1995). It is therefore clear that the ability of *bcl-2* to inhibit apoptosis depends on 3 factors: regulation of expression of the *bcl-2* gene, regulation of expression of other members of the family, and the species of dimers then formed.

bcl-2 can block the apoptosis that usually results from treatment with radiation (Strasser A. et al., 1994), or chemotherapeutic agents (Miyashita T. and Reed J.C., 1993). Wild type p53 downregulates *bcl-2* and leads to an increase in Bax, thereby promoting apoptotic death. However, mutant p53 may have the opposite effect and function as a substitute for *bcl-2*, thereby promoting cell survival. These proteins also have an inverse relationship in some malignancies. A reciprocal relationship has been demonstrated between *bcl-2* reactivity and p53 overexpression in 65% of colorectal neoplasia, and in follicular lymphoma (Cooper K and Haffajee Z, 1997). *bcl-2* positive/ p53 negative subgroup shows strong correlation with negative lymph node status, implying a less aggressive pathway of neoplastic transformation (Kaklamani L. et al, 1996). *bcl-2* protein

has been detected in all grades of cervical intraepithelial neoplasia (CIN), with a striking increase in the number of positive cells within increasing severity of CIN, in combination with a mild increase in cytoplasmic staining intensity. Beyond the application of bcl-2 immunostaining, to distinguish a reactive follicular lymphoid hyperplasia from follicular lymphoma, bcl-2 staining offers little diagnostic value (Cooper K and Haffajee Z., 1996). The use of bcl-2 expression in various malignancies has been shown to be of prognostic merit. The results of bcl-2 staining in initial diagnostic biopsies may point to therapeutic options and be of prognostic importance. bcl-2 is an important player in the cell cycle. It has a crucial impact on several other genes and proteins that dictate the path taken by cells, i.e., survival or death.

Apoptosis

It has been shown that a decrease in tumour volume often occurs after treatment with radiation or chemotherapy, due to cell death by necrosis or apoptosis. Necrosis results from direct physical or chemical damage to the plasma membrane, or disturbances in the osmotic balance of the cell. With the entrance of extra cellular fluid into the cell, resultant cell swelling and lysis precedes a subsequent inflammatory response. Furthermore, necrosis affects groups of cells, with consequent disruption of normal tissue architecture. In contrast to necrosis, apoptotic cell death (programmed cell death) is a highly regulated physiological process. The balance between apoptosis and cell

proliferation results in the maintenance of cell homeostasis (Kerr J.F.R. et al., 1972). Apoptotic bodies are rapidly engulfed by the neighboring cells or macrophages without an inflammatory response being elicited. The nuclear structure alteration in apoptotic cells is induced by endonuclease DNA cleavage that results in the generation of large 50-300 Kb fragments. This produces the characteristic DNA "ladders" of apoptosis, as viewed on agarose gel electrophoreses (Oberhammer F. et.al., 1993). The histologic features of apoptosis include cell shrinkage and loss of junctional contact, resulting in a "halo" around the cell. The nucleus shows condensation and margination of the chromatin. This is followed by fragmentation or "pinching" of pieces of nuclear material, which are surrounded by cytoplasm with intact cytoplasmic organelles as shown at ultra structural level. These apoptotic fragments of pyknotic nuclear material and cytoplasm are phagocytosed by adjacent cells or macrophages. Apoptotic cells are also called by other names which include Councilman bodies, Civatte bodies, necrobiotic cells and nuclear dust.

Apoptosis is thought to be responsible for numerous physiological and pathologic events which include the following (Kerr J.F.R. and Harmon B.V., 1991):

1. The programmed destruction of cells during embryogenesis and metamorphosis,
2. Hormone dependent involution in the adults,
3. Cell deletion in a proliferating cell population,

4. Cell death in tumors, most frequently during regression, but also in tumors with active cell growth,
5. Death of (immune cells) both B and T lymphocytes after cytokine depletion,
6. Pathologic atrophy of hormone dependent tissues,
7. Pathologic atrophy in parenchymal organs after duct obstruction,
8. Cell death induced by cytotoxic T cells,
9. Cell injury in certain viral diseases,
10. Cell death produced by a variety of injurious stimuli that are capable of producing necrosis, but when given in low doses—including mild thermal injury, radiation, cytotoxic anticancer drugs and possibly hypoxia- induced apoptosis.

One important feature of apoptosis is its dependence, in many instances, on gene activation and new protein synthesis (Vaux D., 1993). A number of genes can be induced by stimuli causing apoptosis, such as heat shock proteins and proto- oncogenes, but these genes are not directly related to the triggering of apoptosis. Apoptosis specific genes that stimulate (ced-3, 4) or inhibit (Ced- 9) cell death have been identified in the development of the nematode *C. elegans* (Hengartner M.O., 1992), and mammalian homologs have been described. Certain genes involved in growth and in the causation of cancer play a regulatory role in the induction of apoptosis. bcl-2 oncogene inhibits apoptosis. The c-myc

oncogene, whose protein product can stimulate either apoptosis, or, in the presence of bcl-2, cell growth. Alterations in the ratio of bcl-2:Bax are implicated in shifting the fine balance between cell proliferation and apoptotic cell death. Since Bax antagonizes the inhibitory effect of bcl-2 on apoptosis, p53 appears to provide a key regulatory step in the cellular decision to die by apoptosis (Bissonette R.P., 1992). p53 normally stimulates apoptosis but when mutated or absent, favors cell survival. p53 is required for the apoptosis following DNA damage by irradiation. Restoration of wild type p53 protein, which acts upstream of bcl-2 and Bax, is predicted to restore the ability of many tumour cells to respond to genotoxic therapy by undergoing apoptosis. bcl-2 gene protects tumour cells from apoptosis induced by a variety of agents, including ionizing radiation, and is thus related to resistance to DNA damaging therapeutic agents. The p53 tumour suppresser gene, however, has been related with growth arrest, apoptosis, and thus with selective sensitivity to the killing effects of ionizing radiation and DNA damaging drugs. This functional antagonism between the two genes occurs because of reciprocal downregulation, due to the presence of a p53- transcription silencer in the untranslated region of the bcl-2 gene. Growth arrest in the G1 phase of the cell cycle, and induction of apoptosis, are two distinct and dissectable functions of p53. bcl-2 is able to antagonize the induction of apoptosis by p53 but not the growth arrest in G1. However, coexpression of bcl-2 and of the oncogene c-myc efficiently antagonizes effects of p53 on G1 arrest and apoptosis. Therefore, knowledge of

interaction between these three genes involved in human cancer is a fundamental prerequisite for the improvement of knowledge on prognosis, and to design innovative therapeutic approaches (Chiarugi V. and Ruggiero M., 1996).

Radiation induced apoptosis is prevalent in thymocytes, lymphocytes and haemopoietic and embryonal cells, but is expressed at low levels in other mammalian cell types (Allan D.J., 1992). Dewey W.C. et al. (1995) compared published data on the extent of radiation induced apoptosis versus that of clonogenic cell death for a variety of cell systems. At single doses that results in 90% clonogenic cell kill, less than 50% and in most instances, only 10%-25% could be attributed to apoptosis. In fact, several cell types, such as Chinese hamster ovary cells, do not undergo apoptosis after exposure to high radiation doses (Dewey W.C. et al., 1995, Olive P.L. et al., 1993, McKenna W.G., 1996).

To date, the morphological changes that occur in oesophageal cancer following brachytherapy have not been described. Further, no studies have examined the effect of brachytherapy on the *MDR-1* gene or the effect on bcl-2, p53 and apoptosis expression.

In view of the above, the rationale for these studies were formulated:

1. The effect of brachytherapy may extend into all the layers of the oesophagus, causing radiation changes which can be observed histologically.
2. At the tissue level, it is likely that brachytherapy may alter the expression of the *MDR-1* gene, p53 and bcl-2 genes.
3. The above alterations and interaction between the various genes may lead to apoptotic cell death.

Although there are several studies on the effect of chemotherapy on the expression of *MDR-1* gene, p53, bcl-2 and apoptosis in oesophageal cancer, there are no studies in literature that prospectively examine the effect of either EBRT or ILBT on oncogene expression. Furthermore, there are no studies in literature that described morphological changes in the oesophagus after a course of brachytherapy.

In this report, morphological changes following brachytherapy are examined for the first time. The report also examines the effect of brachytherapy on the expression of *MDR-1* gene product, p53, bcl-2 and apoptosis in oesophageal cancer for the first time.

Chapter 8

Aims and Objectives

The following are the aims and objectives of the study:

1. To evaluate the morphological changes that occur in the oesophagus following a course of preoperative brachytherapy.
2. To examine the effect of brachytherapy on p-glycoprotein, the *MDR-1* gene product.
3. To examine the effect of brachytherapy on the protein expression of p53, bcl-2 and apoptosis in oesophageal cancer treated with preoperative ILBT.

Chapter 9

Hypothesis

MORPHOLOGICAL CHANGES

H₁ Brachytherapy causes characteristic changes in the oesophageal mucosa, submucosa, muscularis propria , serosa and in the tumour within the oesophageal wall and lymph nodes in the vicinity.

MDR1 GENE EXPRESSION

H₀ Brachytherapy has no effect on the MDR1 gene expression in SCC oesophageal cancer.

H₁ Brachytherapy has an effect on the MDR1 gene in SCC oesophageal cancer

p53, bcl2 AND APOPTOSIS

H₀ Brachytherapy has no effect on the expression of p53, bcl2 and apoptosis in SCC of the esophagus

H₁ Brachytherapy has an effect on the expression of p53, bcl2 and apoptosis in SCC of the esophagus

Chapter 10

Material and Methods

STAGING AND SELECTION CRITERIA

All patients in this study were staged by the computed tomography (CT) staging system described by Halvorsen and Thompson (1984). Most patients were worked up by the surgical units which referred the patients. They were deemed to be operable by the surgical unit if they fulfilled the following selection criteria :

- a. Good performance score (Karnofsky performance score > 70)
- b. Tumour length < 8 cm length as seen on upper G.I., endoscopy and barium swallow
- c. CT stage II after a CT scan of the chest and abdomen
- d. Good lung function test and pulmonary reserve
- e. Tumour in the thoracic oesophagus
- f. No evidence of distant metastasis on sonar of the abdomen and bone scan
- g. Normal haematological and biochemical parameters including liver function tests.
- h. Initial biopsy of the tumour after upper G.I. endoscopy showing features of infiltrating squamous carcinoma on histopathologic assessment.

A total of 10 patients were entered into the study.

The CT system used was as follows (Halvorsen & Thompson, 1984):

- Stage I Intraluminal Mass
- Stage II Mass and wall thickening**
- Stage III Spread to adjacent structure
- Stage IV Distant Metastasis

** Wall thickening defined as > 5mm s

Karnofsky Performance Scoring System (Karnofsky et al., 1948):

Score	Features
100	Normal; No complaints; No evidence of disease
90	Able to carry on normal activity; minor signs or symptoms of disease
80	Normal activity with effort; some signs or symptoms of disease
70	Cares for self; unable to carry on normal activity or to do active work
60	Requires occasional assistance but is able to care for most needs
50	Requires considerable assistance and frequent medical care
40	Disabled; requires special care and assistance
30	Severely disabled; hospitalization is indicated although death not imminent
20	Very sick; hospitalization necessary; active supportive treatment is necessary
10	Moribund, fatal processes progressing rapidly
0	Dead

BRACHYTHERAPY (as performed by the Radiation Oncologist)

- REQUIREMENT :** The patient must fast at least 12 hours before the procedure
- ANALGESIA :** with Pethidine 50 mg + Buscopan 1 amp given deep I.m.
30-45 minutes before the procedure
- EQUIPMENT :** Flexible fiberoptic upper G.I., Stainless steel guide wire (Figure I), Nucletron 6 and 4 mm intra oesophageal catheters, Marker wire (Figure II), Fluoroscopy control (C-arm) (Figure III), Brachytherapy Treatment Planning system (Figure IV), and afterloading microsource HDR unit (Figure V)
- PROCEDURE :**
1. Oral Gastrograffin is given to the patient, the tumour is localised and tracheo-oesophageal fistula is ruled out prior to the procedure (Figure VI).
 2. Fiberoptic upper g.i. endoscopy is performed in all patients and the tumour is visualised (Figure VII, VIII).
 3. Stainless-steel guide wire is passed across the tumour length under Endoscopic vision (Figure IX, X).
 4. The Nucletron Intra Oesophageal Catheter with an outer diameter of 6 mm is passed across the tumour length over the guide wire (Figure XI).
 5. Positioning of the Catheter is performed under fluoroscopic vision using a C Arm (Phillips BV29) after giving

the patient oral Gastrograffin (Figure XII). A margin of 4 cm is prescribed above and below the visible tumour, including the tumour (Figure XIII).

6. The catheter is connected to a remote after loading HDR unit (Microselectron HDR, Nucletron, The Netherlands) which has an Iridium-192 source of 10 Ci (370GBq) initial activity (Figure XIV, XV).

7. Treatment is given following dose optimization on the treatment planning system for brachytherapy treatment (Plato, Nucletron, The Netherlands) - see Figure XVI, XVII. The dose is prescribed at 10 mm from the centre of the source axis, and includes the tumour and a 4 cm margin proximally and distally to the tumour.

8. Treatment delivery is controlled from the remote console of the HDR unit (Figure XVIII, XIX).

TREATMENT TIME: The entire procedure depending on source activity, takes 20-30 minutes, and is done on an out patient basis

DOSE AND FRACTION: A total of 20 Gy was given in 2 fractions over 2 weeks. 10 Gy was given per fraction at 1 cm from the centre of the source axis. One week interval was given between the 2 fractions.

Figure I
Upper G.I. Fiberoptic scope and stainless steel guide wire

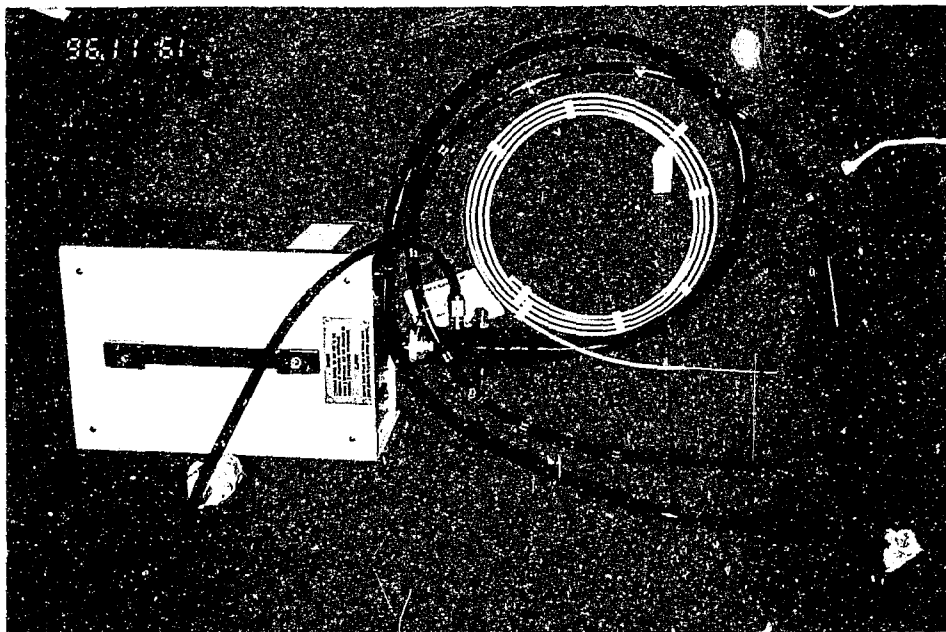


Figure II
Nucletron 4 and 6 mm Oesophageal catheters with marker wire

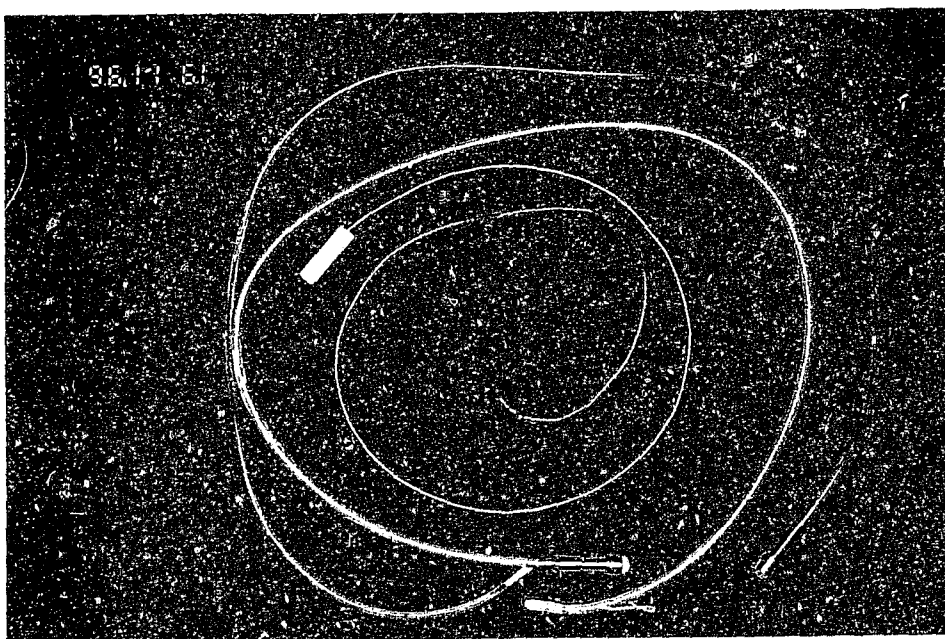


Figure III
Fluoroscopy control - c-arm

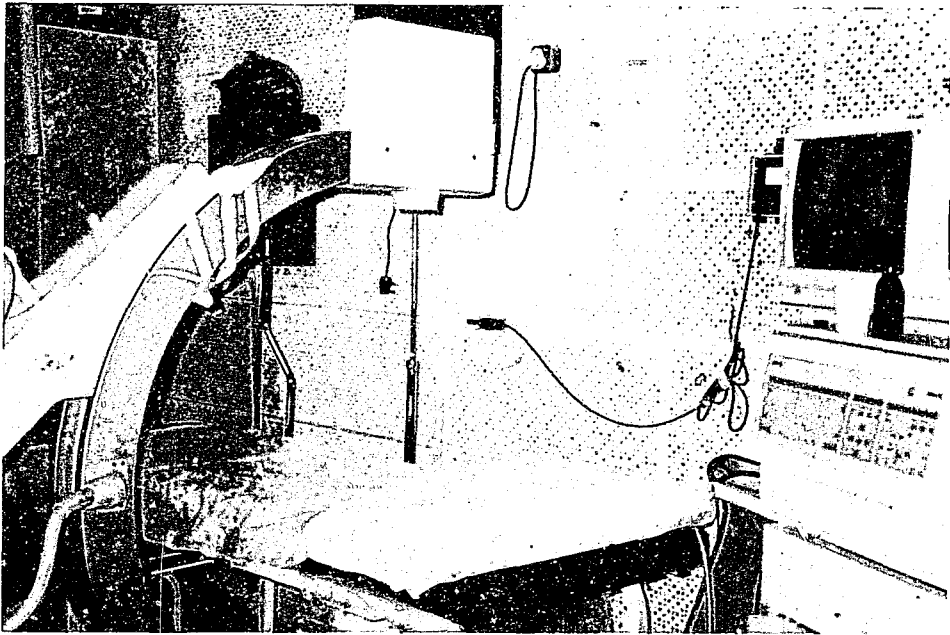


Figure IV
Brachytherapy Treatment Planning System

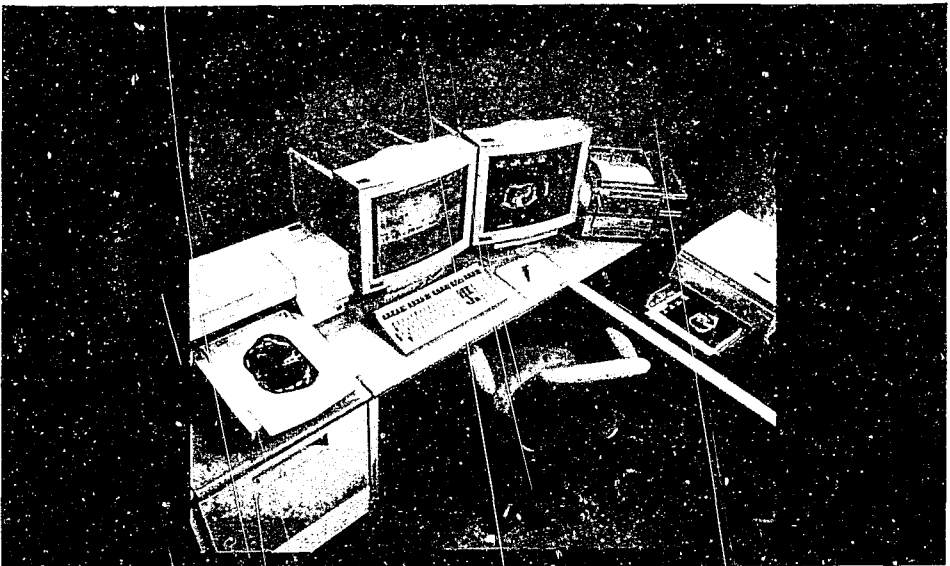


Figure V

Nucletron - Microselectron HDR - Remote After loading HDR unit

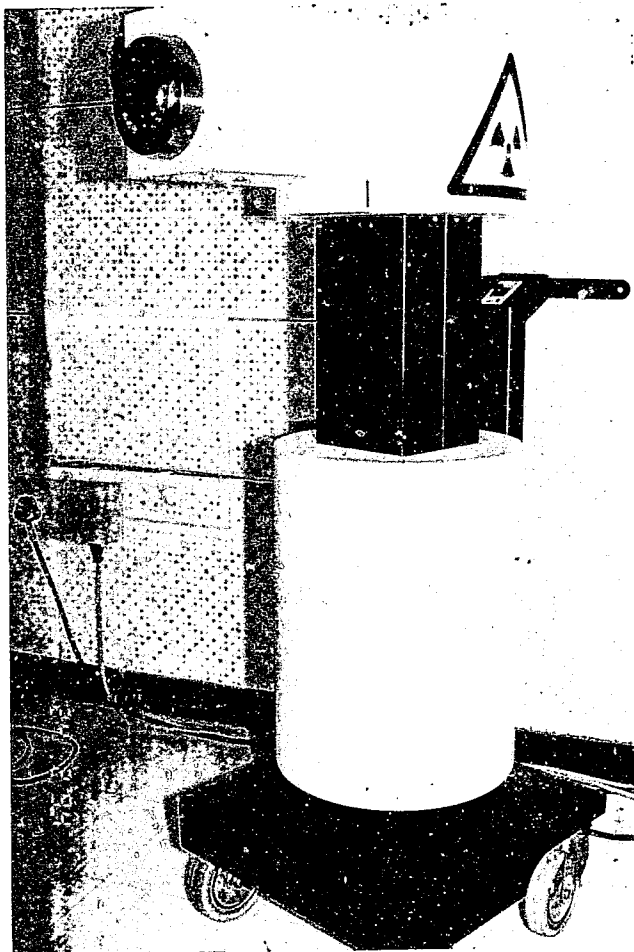


Figure VI

Visualization of the tumor after giving the patient 10 ml of Oral Gastrograffin. This also rules out presence of a tracheo-oesophageal fistula

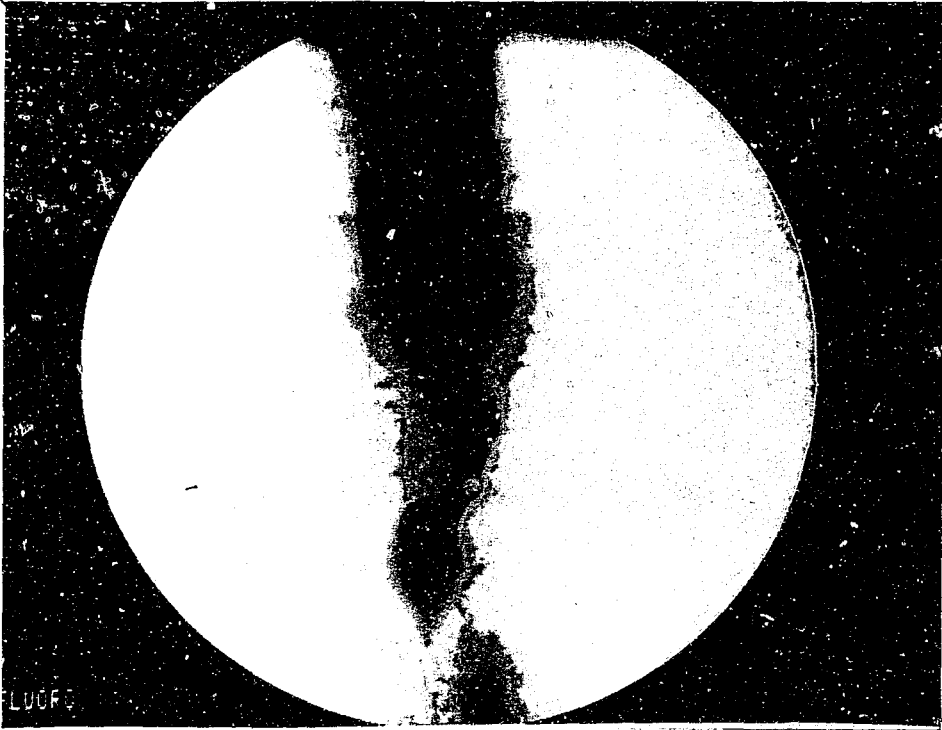


Figure VII

Tumor as visualized on fiberoptic endoscopy



Figure VIII

Tumour as visulised on fluoroscopy with a c-arm



Figure IX

Stainless steel guide wire passed across the tumor length under endoscopic vision

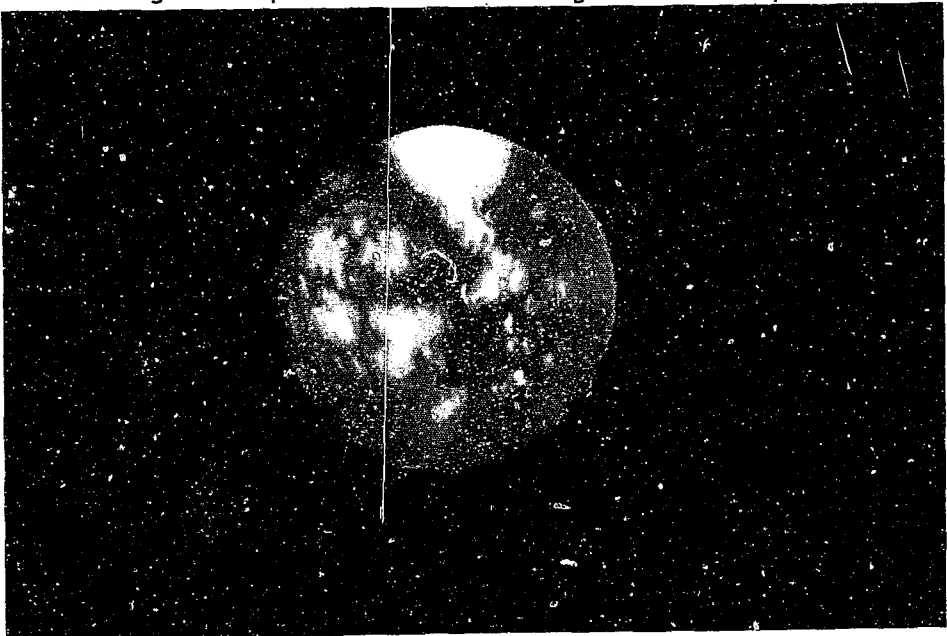


Figure X

Stainless steel guide wire passed across the tumor length as seen on fluoroscopy with a c-arm



Figure XI

Nucletron Intra-oesophageal catheter passes across the tumor length through the guide wire as seen on fluoroscopy with a c-arm

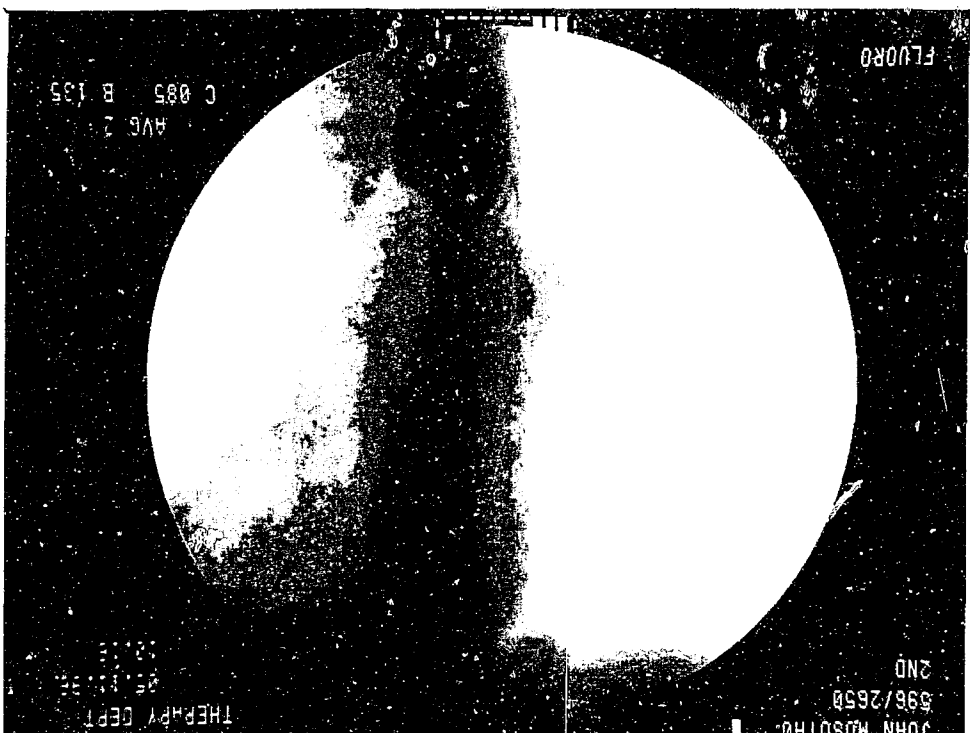


Figure XII

Catheter positioning done under fluoroscopic control after giving the patient 10 ml of oral Gastrografin.

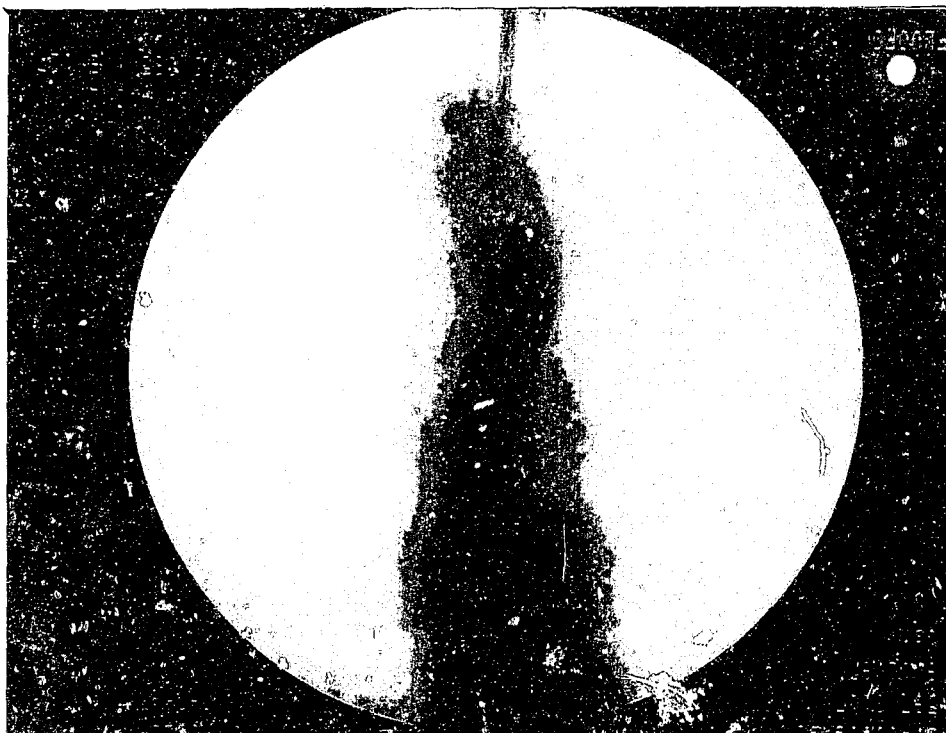


Figure XIII

Catheter in place with at least 2 cm margin proximally and distally to visible tumor

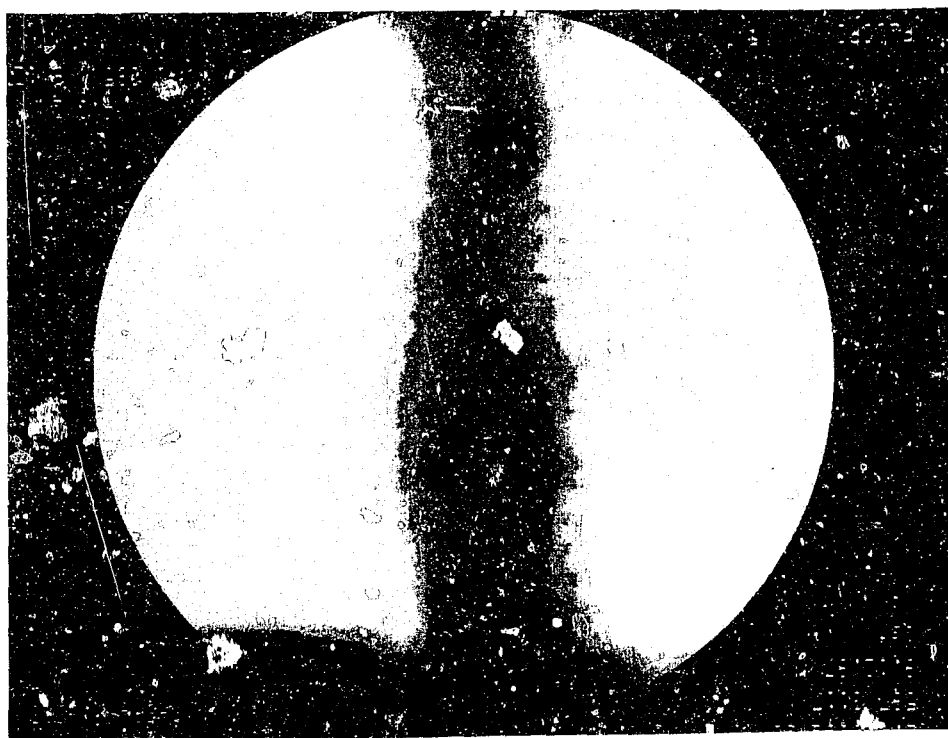


Figure XIV

Microselectron HDR with 18 treatment channels

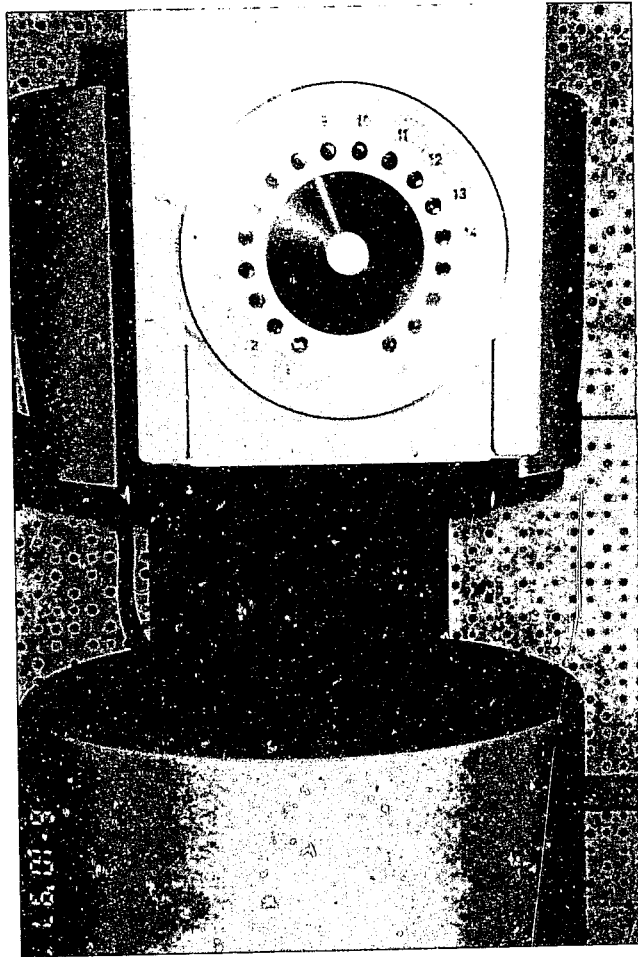


Figure XV

Intra-oesophageal Catheter connected to the HDR Microselectron unit for treatment

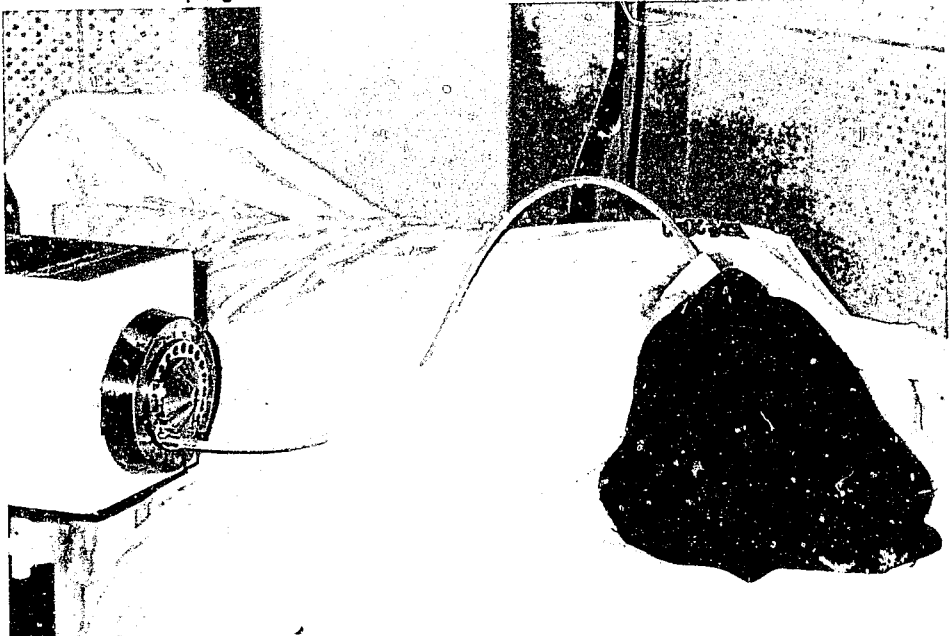


Figure XVI

Isodose curves superimposed on x-rays in AP view to show dose optimization. Dose prescribed at 1 cm from center of the source axis with a 2 cm proximal and distal margin to tumor or as prescribed. Dose fall off is very rapid as can be seen from the x-rays

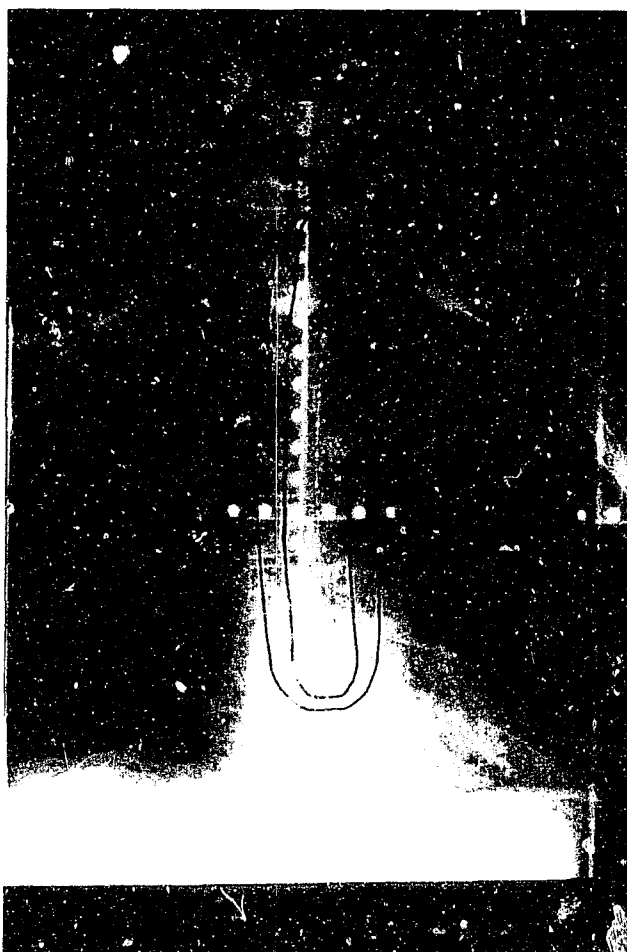


Figure XVII

Isodose curves superimposed on x-rays in lateral view to show dose optimization. Dose prescribed at 1 cm from center of the source axis with a 2 cm proximal and distal margin to tumor or as prescribed.



Figure XVIII
Intra-oesophageal catheter connected to the HDR after dose optimisation

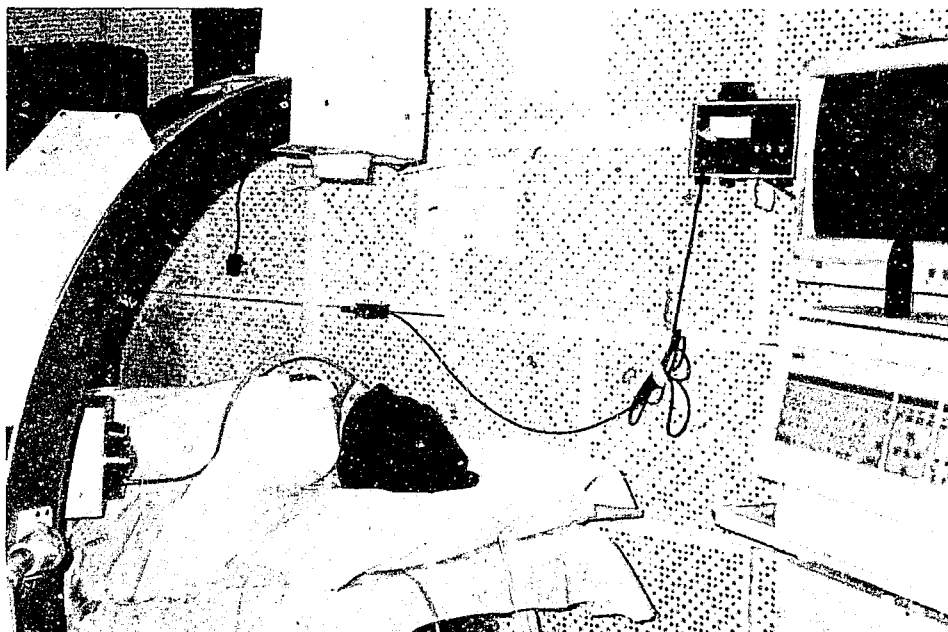
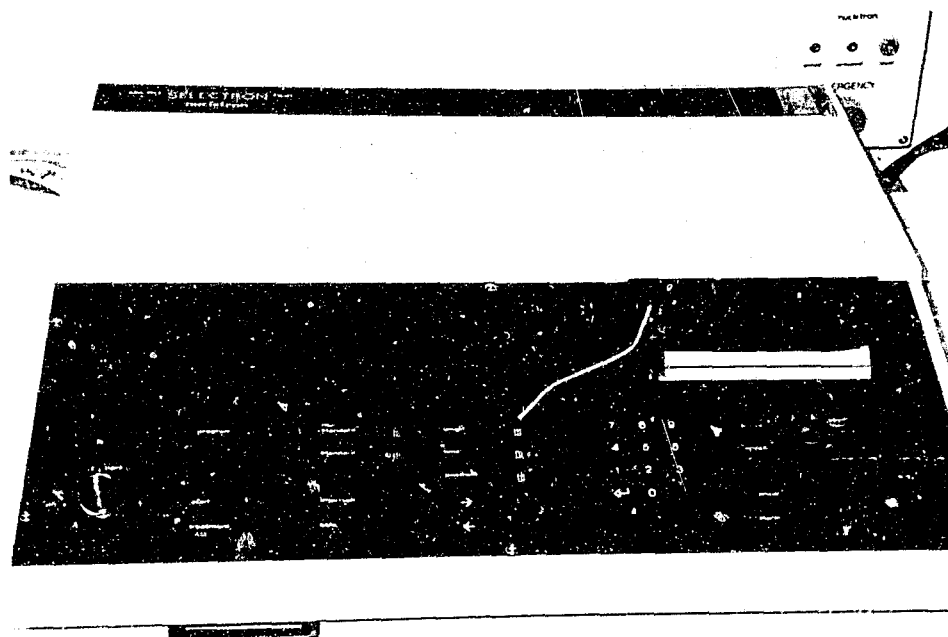


Figure XIX
Remote console of the HDR unit ready for patient treatment



SURGERY

Following completion of brachytherapy, all patients were operated upon within 2-3 weeks. A total oesophagectomy with gastric pull through was done in all patients.

Rationale for Preoperative HDR ILBT in oesophageal cancer

In South Africa, surgical resection is often difficult, even in CT stage II oesophageal cancer, due to the long tumour length at the time of presentation. (Sur R.K. et al., 1998). HDR ILBT allows the delivery of a very high dose to the luminal aspect of the tumour, causing rapid shrinkage of the tumour, making surgical resectability easier. The dose rate in HDR ILBT is high, at over 12 Gy per hour instead of the conventional 2 Gy per hour. Furthermore, the dose per fraction given is also high; 20 Gy in two fractions over 2 weeks, 10 Gy per fraction against the conventional 1.8-2.0 Gy per fraction. Shrinkage of the tumour thus occurs rapidly. The dose fall off with HDR ILBT is rapid. At 1.5 cm from the centre of the source axis, it is around 50% (dose received by lymph nodes which are in proximity to the oesophagus), and at 2 cm from the centre of the source, it falls by over 75% of the prescribed dose (Sur M. et al., 1996). Therefore, there is no significant risk of injury to organs in close proximity, e.g. heart, lung and spinal cord, in contrast to conventional EBRT treatment. It is therefore, the policy of our surgical department to send all operable cases of

oesophageal cancer for preoperative HDR ILBT and operate upon the patients in 2-3 weeks time to allow for rapid shrinkage of the tumour that makes surgical resection easier and more complete.

MORPHOLOGICAL CHANGES IN OESOPHAGECTOMY SPECIMENS

The specimens were fixed in formalin for a period of 12-14 hours. The following sections were then sampled:

1. Proximal and distal resection margins
2. Edge of irradiated length (i.e., 4 cm proximally and distally to the tumour)
3. 1 cm from the margin of the visible tumour proximally and distally
4. Centre of the tumour
5. Palpable lymph nodes in proximity to the oesophagus were also sampled.

Paraffin embedded sections were processed and stained with hematoxylin and eosin (H & E) and examined under light microscopy.

MDR-1 GENE PRODUCT - p-glycoprotein

Paraffin wax embedded tissue blocks of formalin fixed pretreatment biopsies and blocks from the centre of tumour (from surgically resected specimens) following brachytherapy were assessed for expression for p-glycoprotein. All sections were

immunostained for p-glycoprotein using the modified sandwich technique. Formalin fixed paraffin embedded sections were deparaffinized in Xylene and rehydrated with graded Ethanol.

Prior to quenching, and application of primary antibody, sections were subjected to heat induced epitope retrieval (HIER) in citrate buffer (using microwave pretreatment) for antigen retrieval. A three step method for quenching endogenous peroxide was used which is as follows:

1. The slides were treated with 20% methanol + 80% ethanol + 2% Hydrogen peroxide for 10 minutes
2. The slides were then treated with 0.2% periodic acid + 3% Hydrogen peroxide for 20 minutes
3. Lastly, the slides were treated with TBS pH 7.5 for 5 minutes

Normal rabbit serum was used to block nonspecific protein binding. Slides were incubated with primary antibody to p-glycoprotein (clone JSB-1 from Novocastra Labs Ltd., Newcastle upon Tyne, U.K.) at dilution 1:20, overnight at 4 degrees centigrade. The primary complex was amplified using a modified sandwich technique which included incubation in the following:

1. Rabbit anti-mouse IgG 1:20 + peroxide labeled rabbit anti-mouse 1:100 at 4 degrees centigrade for 45 minutes

2. Mouse PAP complex 1:50 dilution at 4 degrees centigrade for 45 minutes
3. Peroxidase labeled rabbit anti-mouse 1:100 dilution at 4 degrees centigrade for 45 minutes

The complex was then detected with diaminobenzidine and counterstained in Mayer's Hematoxylin for 30 seconds. The sections were then dehydrated, cleared and mounted with entellan (Merck, Germany). Both negative and positive controls were included in each run in order to determine if the technique was optimal. Negative controls were processed in a similar manner except that the primary antiserum was omitted. Positive controls comprised normal liver which showed bile canaliculi staining in paraffin wax embedded blocks. All sections were examined under light microscopy.

p53, bcl-2 AND APOPTOSIS

Paraffin wax embedded tissue blocks of formalin fixed pretreatment biopsies and blocks from the centre of the tumour (from surgically resected specimen) following brachytherapy, were assessed for expression of p53, bcl-2 and apoptosis.

p53 and bcl-2 immunodetection : All sections were immunostained for p53 and bcl-2 using the Avidin-Biotin complex (ABC) method. Formalin fixed paraffin

embedded sections were deparaffinized in Xylene and rehydrated with graded ethanol. Prior to quenching and application of primary antibody, sections were subjected to heat induced epitope retrieval (HIER) in citrate buffer (using microwave pretreatment) for antigen retrieval. Endogenous peroxide was blocked with 3% Hydrogen peroxide for 30 minutes. After washing with PBS, slides were incubated with normal goat serum 1:50 in PSA (PBS + 3% BSA) for 30 minutes. Sections were then incubated with p53 primary antiserum DO-7 (Dako, Denmark) 1:50 and with bcl-2 oncoprotein, clone 124 (Dako, Denmark) 1 : 100 for 1 hour at room temperature (antibody DO-7 detects both wild type and mutant p53). This was followed by respective incubation with biotinylated goat antiserum and streptavidin HRP (Duet strep HRP kit, Dako, Denmark) for 30 minutes each. A diffuse brown signal was detected using diamino-benzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St.Louis, MO). Sections were counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted with entellan (Merck, Germany). Negative controls were processed in a similar manner except that the primary anti sera was omitted. Positive controls comprised known p53 protein positive breast carcinoma in paraffin wax embedded blocks. For bcl-2, positive controls comprised known bcl-2 protein positive follicular centre cell lymphoma in paraffin wax embedded blocks.

Apoptosis detection : For detection of apoptosis at single cell level, based on labeling of DNA strand breaks, In Situ Cell Death Detection Kit, POD (Boehringer

Mannheim, Germany) was used. Formalin fixed paraffin embedded parallel sections were deparaffinized in xylene and rehydrated with graded ethanol to water. Sections were quenched in 0.3% H_2O_2 for 30 minutes. Digestion was performed using Proteinase K (Boehringer Mannheim) 20 μg / ml, for 15 minutes at room temperature. Sections were rinsed in PBS and subjected to the TUNEL reaction mixture. The DNA strand breaks can be identified by labeling free 3' - OH termini with modified nucleotides in an enzymatic reaction. In this kit, terminal deoxy nucleotidyl transferase (TdT) which catalyzes polymerization of nucleotides to free 3' - OH DNA ends in a template - independent manner, is used to label DNA strand breaks. Incorporated fluorescein is detected by anti-fluorescein antibody Fab fragments and conjugated with horse radish peroxidase (POD). After substrate reaction, stained cells can be analyzed under light microscopy. Diamino-benzidine tetrahydrochloride DAB, (Sigma) was used as chromagen. Sections were then counterstained with methyl green, cleared and mounted in DPX. Negative and positive controls were used respectively. For the negative control, the step using TdT was omitted and only distilled water was applied. For the positive control, DNA strand breaks were induced using DNase 1=0.001 gm / ml, PBS at room temperature for 10 minutes prior to labeling.

Expression of p53, bcl-2 and apoptosis in pre and post-brachytherapy tumour sections were studied based on :

1. Intensity of brown signals - nuclear (p53) or cytoplasmic (bcl-2) or single cell (apoptosis) was graded as negative, +, ++ or +++
2. Number of positive cells - type A < 10%, type B 10-50% and type C > 50%
3. Pattern of distribution, whether focal or diffuse

All sections were examined under light microscopy.

Chapter 11

Review of literature

This review of literature deals with the morphological changes induced by radiotherapy in oesophageal cancer. It also reviews the effect of radiation on the expression of tumour suppressor genes (p53 and bcl-2) and apoptosis and the effect of radiotherapy on the expression of p-glycoprotein, a product of *MDR-1* gene.

Brachytherapy is a form of radiation treatment in which the radioactive isotope is in and around the tumour. In the oesophagus this is done by placing the catheter in the lumen of the oesophagus and subsequently passing a radioactive isotope such as Iridium - 192 remotely by computers. The methodology involved is described under Material and methods (Sur et al., 1998)

I. MORPHOLOGICAL CHANGES INDUCED BY RADIOTHERAPY IN OESOPHAGEAL CARCINOMA

a. In vivo Studies

Radiation changes induced by brachytherapy have previously been reported. Hishikawa et al (1984) demonstrated that a boost of High dose rate Intraluminal Brachytherapy (HDR ILBT) following External Beam Radiotherapy (EBRT) caused ulceration of the mucosa in all patients. These ulcers developed in the field of ILBT. The ulcers were predominantly circumferential (12/22) and occurred 10-8 months following completion of ILBT. Linear ulcers were also seen (10/22) and occurred 3-12 months following ILBT. All ulcers had a thick crater wall and margins. Hishikawa et al also noted that deep ulceration formation after EBRT, but before ILBT, was usually associated with a tracheo-oesophageal fistula. Based on this observation, Hishikawa et al recommended that ILBT should be avoided in patients who develop deep ulceration following EBRT and that the ILBT dose should not exceed 20 Gy when used with EBRT.

In a subsequent report, Hishikawa and others (1985) treated 53 patients with oesophageal squamous cell carcinoma (SCC) with EBRT and HDR ILBT. EBRT ranged from 40 -70 Gy and HDR ILBT ranged from 6-24 Gy in one to four fractions. Autopsy was performed in 10 cases. Only 2 cases had good microscopic specimens of the irradiated oesophagus. In the first case, microscopic sections of the specimen taken at 1 cm from the superior margin of the tumour showed a difference between the segment treated with EBRT alone and that treated with combined radiation. The histological changes were most serious in the segment that underwent intracavitary radiation. The major epithelial injury was ulceration. Degenerative changes of the small vessels and fibrinous exudates were found in the submucosa. In the second case the mucosa and submucosa were preserved in the portion of external irradiation, however, regenerative epithelium and fibrinous exudates of the sub mucosa were recognized in the area of intraluminal radiation. Capillary vessels of the submucosa had been destroyed, and fibrosis and fibrinous exudates were found in the muscle layers in the portion of ILBT. Fibrosis of the muscle layers was also found. Hishikawa and others therefore concluded that characteristic changes were found in the oesophagus irradiated by ILBT following EBRT.

In a subsequent report, Hishikawa et al. (1988) described the autopsy findings in 35 cases treated with EBRT of various doses- alone, and in combination with EBRT and ILBT. Residual tumour was seen at autopsy in only 7 of 16 patients treated with HDR ILBT following EBRT. In contrast, 13 of 14 patients treated

with EBRT of 50 Gy or more and all 5 patients treated with EBRT of less than 50 Gy had residual tumour. They showed that the addition of ILBT improved local control in treatment of oesophageal cancer with radiation.

The use of ILBT alone in the treatment of small superficial SCC of the oesophagus was subsequently reported by Hishikawa et al. in 1989. Six patients with small, superficial carcinoma of the oesophagus were treated with 18-24 Gy of HDR ILBT alone. Alterations in the oesophagus were examined with an endoscope within one month after therapy, and it was observed that the tumour had disappeared in all 6 patients. Erosion induced by HDR ILBT was seen in five patients. Five of the patients experienced no local recurrence, and endoscopic biopsy showed local recurrence in the remaining patient seven months after radiotherapy. All patients survived 6-16 months. Oesophageal ulceration induced by ILBT occurred in 3 of the 6 patients. The ulcers, however, healed on conservative management. Thus HDR ILBT could be used in the treatment of patients with small, superficial carcinoma of the oesophagus.

Hishikawa's studies therefore demonstrated:

1. Severe radiation changes are induced by the addition of ILBT following EBRT in the radiation treatment of oesophageal cancer. This may improve local control.
2. ILBT alone could be used to effectively to control local disease in superficial carcinoma of the oesophagus.

Berry et al. (1989) reported pathologic findings in 21 oesophagectomy specimens from patients having pre-operative combined ILBT and EBRT. Eleven patients received 15 Gy ILBT and 40 Gy EBRT (group 1) and 10 patients received 15 Gy ILBT and 20-30 Gy EBRT (group 2). Effectiveness of radiotherapy was expressed as the ratio between depth of radiation effect and depth of tumour invasion.

This was expressed as one of four levels:

- Level 1 : not deeper than the muscularis mucosa;
- Level 2 : involving but not deeper than sub mucosa;
- Level 3 : involving but not deeper than muscularis propria;
- Level 4 : involving peri oesophageal soft tissue.

The depth of radiation damage to tumour cells was comparable between the two groups. Light microscopic evaluation included cell type, maximal depth in the oesophageal wall reached by viable tumour cells, predominant depth reached by tumour cells with radiation damage, and the presence and viability of lymph node metastasis. Criteria for diagnosis of radiation damage to tumour included tumour giant cells with pyknotic multilobated nuclei, rare and abnormal mitotic figures and condensation or vacuolization of the cytoplasm. The ratio of depth of radiation damage to depth of identifiable tumour was proportional to the local effectiveness of the pre-operative treatment. When assessing depth of radiation effect (numerator), the level at which all tumour cells showed evidence of

radiation damage was assigned. When assessing depth of recognizable tumour (denominator), the level of the point of deepest invasion was assigned.

The microscopic appearance was one of surface erosion or shallow ulceration with variable degrees of fibrous thickening of the oesophageal wall. There was variable degree of surface epithelial denudation and replacement with cellular granulation tissue. Variable degrees of mucosal atypia in areas adjacent to surface erosion were present. This atypia was characterized by architectural disorder, nuclear enlargement and hyperchromasia, and cellular enlargement. These changes were believed to be radiation induced particularly when involving mucosa of different type from the existing malignancy, although this could not be proven. There was moderate to marked mural fibrosis which was responsible for the gross mural thickening. There was no perforation. The depth of radiation damage to tumour cells was comparable between groups 1 and 2. However, only 1 of 11 patients in group 1 had involvement of peri oesophageal soft tissue as compared to 8 of 10 in group 2. Similarly, although six patients in group 1 had no viable looking residual tumour cells in the oesophageal wall after treatment, this was the case for only one patient in group 2. Lymph node metastasis were identified in 12 patients: 4 in group 1 and 8 in group 2.

A ratio of 1 between radiation effect and depth of tumour invasion was present in 6 patients receiving high dose EBRT and 1 patient receiving lower dose EBRT. The study demonstrated that ILBT combined with EBRT gave good local tumour

control in the majority of patients. Higher doses of EBRT give a better radiation effect in the deeper layers of the oesophageal wall. The ratio between depth of radiation affect and tumour invasion provides a simple and objective approach to the pathologic analysis of oesophagectomy specimens. ILBT is intended to provide high doses to the luminal aspects of the tumour which maybe more hypoxic and relatively radioresistant.

b. In vitro/ animal studies

Freund et al. (1989) examined the tolerance of sound oesophageal mucosa to HDR ILBT with doses of 6Gy and 12Gy in 15 pigs. No macroscopic or microscopic alterations of the mucosa were found after 15 Gy. An application of 12 Gy produced severe side effects in the form of vascular occlusion due to fibrosis of the intima, formation of fistulas, and perforation of oesophageal wall. The authors recommended elaborate schemes for Dose Fractionation in HDR ILBT of oesophageal cancer.

Soejima (1992) investigated the histopathological responses of the rabbit cesophagus to HDR ILBT. Oesophageal ulceration was observed in the specimen that received a dose of 15 Gy, 7-28 days after ILBT. Before the mucosal changes were observed, edema and cell infiltration were found in the lamina propria. Chronic injury such as necrosis and degeneration of epithelium, and degeneration of the wall of blood vessels was seen at six-months. No marked changes were found in specimens that received 5 and 10 Gy HDR ILBT. In

conclusion, it was strongly suggested that a single dose of HDR ILBT should be less than 10 Gy to prevent the oesophagus from severe injury.

The pathologic sequence of events following a single large fraction of radiation have been described by various authors. Phillips et al. (1974) reported the following sequence of events following a single large fraction of radiation. On day 3, vacuolization and absence of mitosis of the basal layer along with the thinning of the keratinized squamous cell layer occurred. Between days 7 and 14, foci of proliferating basal cells and regenerating epithelium with zones of complete denudation occurred simultaneously. The rapidity of repopulation determined the survival or death of the animal. After 21 days, there was usually complete regeneration of the oesophageal lining with increased basal cell layer proliferation and increased thickening of the squamous layer. Reports of acute pathologic response of the oesophagus to radiation in the humans include those of Seaman et al. (1957) and Mascarenhas et al. (1989). The acute pathologic changes described parallel those observed in the mouse.

II. *MDR-1* GENE PRODUCT - P-glycoprotein

Darnton (1995), examined the effect of multidrug drug gene product-p-glycoprotein in biopsy specimens from 27 oesophageal SCC and 10 adenocarcinomas before and after treatment with Mitomycin, Ifosphamide and cis-Platin (MIC). p-glycoprotein was assessed immunohistochemically with

antibody JSB-1. Formalin fixed, paraffin wax embedded tissue sections of the biopsy specimens and post treatment resected tumours were subjected to immunohistochemical stains. Of the SCC, 74% (20/27) responded to MIC but only one expressed p-glycoprotein before and after treatment. Of the adenocarcinomas 30% (3/10) responded. Seven of the 10 adenocarcinomas expressed p-glycoprotein before treatment. But all 10 were p-glycoprotein positive after the chemotherapy. As the difference in prevalence and induction of p-glycoprotein between the histologic types was highly significant, the authors felt that this could correlate with the greater response to MIC chemotherapy seen in squamous carcinomas compared with adenocarcinomas. p-glycoprotein could not be used as the predictive marker of response as tumours expressed it inconsistently. Resistance to MIC chemotherapy could be due to other mechanisms.

Soini et al. (1996) investigated the immunohistochemical expression of the *MDR-1* gene product p-glycoprotein in histological samples in 31 hepatocellular carcinomas. They also examined the correlation of expression of the protein with cell proliferation, p53 expression, disease free interval and cumulative patient survival. C219,CM-1 were used to detect expression of P-glycoprotein, by means of the Avidin-Biotin peroxidase method. Membrane bound positivity for p-glycoprotein was observed in 20/31 (65%) hepatocellular carcinomas. In addition, there were no significant associations between expression of p-glycoprotein and cell proliferation or p53 expression. Patients with

p-glycoprotein positive tumours had a shorter disease free interval and survival time than those with p-glycoprotein negative tumours.

Chou and others (1995) reviewed the results of 29 children treated for medulloblastoma. 13 patients with high grade medulloblastoma characterized by incomplete resection, diploid tumour or subarachnoid dissemination received chemotherapy following radiation therapy. Three patients received post-operative chemotherapy. 8 patients who had been treated with post-operative radiation therapy also received Chemotherapy for recurrent tumours. After a minimum three-year follow up period, 16 were alive but 13 had died from recurrent tumours. In order to evaluate the possible participation of p-glycoprotein mediated multidrug resistance in medulloblastoma therapy and its correlation with prognosis, archival specimens were examined by immunohistochemistry utilizing three monoclonal antibodies against p-glycoprotein and six cases by reverse-transcriptase polymerase chain reaction (RT-PCR) utilizing *MDR-1* specific primers. Sixteen patients (55%) had *MDR-1* expression detected either by one of the three antibodies or RT-PCR. DNA ploidy study was also performed on 18 specimens. Patient outcome was correlated with extent of surgical resection, chemotherapy, DNA ploidy and *MDR-1* expression. A statistically significant association was found between *MDR-1* expression and outcome ($p= 0.007$). Among the patients who received chemotherapy, positive *MDR-1* expression significantly correlated with poor

outcome ($p=0.036$). The results showed that p-glycoprotein mediated intrinsic *MDR-1* in medulloblastomas correlated with an adverse outcome.

Ng I.O. et al. (1998) investigated the effects of radiation on the expression of p-glycoprotein in 56 patients with primary oral cancer. No patient received prior or concurrent Chemotherapy. The patient cohort consisted of three groups.

Group 1: 20 patients with pre-radiation specimens only.

Group 2: 18 patients with both pre and post-radiation specimens.

Group 3 :18 patients with post-radiation specimens only.

p-glycoprotein expression was determined by immunohistochemistry with two monoclonal antibodies, C 219 and C494. Amongst patients in groups 1 and 2, only 1 (2.6%) and 2 (5.3%) patients had p-glycoprotein expression in their tumours before treatment with C219 and C494 respectively. For group 2 patients, 66.7% and 72.2% had tumours that expressed p-glycoprotein with the two antibodies respectively, only after and not prior to radiation. When patients in groups 2 and 3 were combined, 63.9% and 72.2% had p-glycoprotein expression with the two antibodies respectively after radiation. p-glycoprotein expression was significantly induced after radiation compared with expression before treatment ($p<0.001$). The study showed that p-glycoprotein expression was significantly induced by radiation in human oral cancers. This induction of p-glycoprotein expression conferred multi-drug resistance to the cancer cells and could affect the efficacy of subsequent or concurrent chemotherapy. This may also explained the poor rate of response to chemotherapy among patients who

have previously received radiation. This was the first study in literature that looked at the expression of the *MDR-1* gene product after radiotherapy. Prior to this, no studies have confirmed this observation at other sites or the same site.

Drug resistance remains a major problem in gastric carcinomas. To evaluate the mechanisms involved in this resistance, Wallner J and others (1993) examined the expression of the *MDR-1* gene, in primary gastric carcinomas. *MDR-1* RNA levels of gastric carcinoma specimens were determined by Slot Blot analysis. *MDR-1* cDNA (Probe 5 A) was used for the hybridization. *MDR-1* RNA was detected in 41% of the gastric carcinomas, with high levels in 18% of the specimens. *MDR-1* gene expression was independent of patient age, tumour localization, and lymph node involvement. However, *MDR-1* RNA expression was less frequent in locally advanced tumours and was absent in the primary tumours of all six patients who had distant metastasis. The study showed a multi-drug resistant cells were present in primary gastric carcinomas and this suggests that multi-drug resistance might contribute to the clinical drug resistance of these tumours.

The human p-glycoprotein gene family contains the *MDR-1* and the *MDR-3* gene. The *MDR-1* p-glycoprotein is over expressed in multi drug resistant tumour cells and is believed to play a role in the elimination of certain cytotoxic drugs used in the chemotherapy of cancer. The *MDR-3* gene has not been found to be amplified or over expressed in MDR cells. Chao C.C. I et al. (1991) studied the

effect of p-glycoprotein gene expression in human colon cancer cell line. Gene-specific *MDR-1* gene probes were developed for the detection of the gene and the total m-RNA level. Southern and Northern hybridization analysis showed that the MDR gene and the m-RNA levels were increased 30-40 fold in a MDR human colon cancer cell line.

Benson M.C. et al. (1991) used a monoclonal antibody to detect the cell surface P-glycoprotein product of the multi drug resistance gene (*MDR-1*) in the human bladder. A total of 32 patients had 44 different specimens analyzed. The samples consisted of 8 normal bladders, 21 transitional cell carcinomas, 1 mucinous adenocarcinomas, 3 pediatric bladder wall specimens and 10 nonmalignant samples from cystectomies. p-glycoprotein was not detected in the normal adult or pediatric bladders. Transitional cell carcinomas demonstrated low expression at diagnosis (<6%), although 3 patients had enhanced initial expression (11%, 12% and 31% respectively). Three patients treated with chemotherapy demonstrated 56%, 76% and 50% expression of p-glycoprotein. Nonmalignant tissue from cystectomy specimens had low expression of p-glycoprotein. The specificity of this system was confirmed with human bladder cell lines. The study also demonstrated the ability of flow cytometry to detect and quantify the expression of the *MDR-1* gene product. This may allow for the early detection of chemotherapy resistance in patients with transitional cell carcinoma treated with systemic and intravesical therapy.

Sekiya S. et al. (1992) studied the effect of p-glycoprotein expression in multi-drug resistant human ovarian carcinoma cell lines. An increase in p-glycoprotein level, specific to multi-drug resistant tumour cells was not apparently associated with the increase in resistance to Vincristine or Adriamycin. *MDR-1* transcripts (4.5 kilobases) were observed in the RNA preparations obtained from only one cell line (Shin-3) that showed the highest resistance to both drugs in vitro and in vivo. No cell lines showed *MDR-1* DNA amplification. These results suggested that the insensitivity of human ovarian carcinoma to chemotherapy could be partially explained by the expression of *MDR-1* gene.

Glazer R.I. and Rohlff C. (1994) reviewed the development of cross resistance to many natural product anticancer cancer drugs, termed multidrug resistance (MDR) and attributed this to be a major reason for the failure of cancer chemotherapy. This type of MDR is often associated with over expression of the *MDR-1* gene product, p-glycoprotein, a multifunctional drug transporter. The expression of MDR in breast tumours is related to their origin from tissue that constitutively expresses p-glycoprotein as well as to the development of resistance during successive courses of chemotherapy. Therefore understanding the mechanisms that regulate the transcriptional activation of *MDR-1* may afford a means of reducing or eliminating MDR. The authors found that *MDR-1* gene expression could be motivated by type I cAMP dependent protein kinases. This opened up a possibility of modulating MDR by selectively down regulating the

activity of PKA dependent transcriptional factors which up regulate *MDR-1* expression. High levels of type I PKA occurs in primary breast carcinomas and patients exhibiting this phenotype showed decreased survival. This selective type I cAMP dependent protein kinase inhibitors, 8-Cl-cAMP and Rp8-Cl-cAMP (S) may be particularly useful for down regulating PKA dependent MDR associated transcription factors. These compounds down regulate transient expression of reporter genes under control of several *MDR-1* promoter elements. Further studies are needed to understand the mechanisms involved in the expression of the multidrug resistance and also focus on policy intervention by a new class of inhibitors.

III. p53, bcl-2 AND APOPTOSIS

(a) Effect of chemo/radiotherapy on the expression of p53 in oesophageal cancers

Puglisi et al. (1996) investigated the immunohistochemical expression of p53 and bcl-2 proteins in SCC of oesophagus to assess whether expression of these oncoproteins could be used to stratify patients into groups with favorable or unfavorable response to preoperative chemo/radiotherapy. The initial diagnostic biopsy and the corresponding resected samples were obtained from 22 consecutive patients with SCC. All patients underwent preoperative chemo/radiotherapy. Tumour sections were incubated with a monoclonal antibody directed against p53 (DO-7). 24 non-neoplastic oesophageal biopsy

specimens immuno stained for p53 served as controls. 12 randomly chosen sections from the 22 SCC samples were immunostained to test for bcl-2 protein expression. After chemo-radiotherapy, 12 (55%) of the 22 patients had no evidence of tumour in the resected oesophagus. Before chemo-radiotherapy, however, 17 (77%) patients were p53 positive. After treatment, residual carcinoma was detected in 7 (41%) of the 17 p53 positive patients. All non-responsive cases had the same p53 immunopattern as before treatment. bcl-2 immunoexpression was detected in 6 (50%) of 12 patients. Residual tumour was detected in the residual oesophagus in two (33%) of the six bcl-2 positive patients. After treatment, bcl-2 expression was no longer detected in the residual neoplastic cells of a previously bcl-2 positive tumour. Using Fisher's exact test no significant association was found between oncoprotein expression and response to preoperative treatment. The study confirmed the observation that p53 protein is frequently expressed in SCC of the oesophagus probably as a result of a mutation of the TP53 gene. No significant association was found between Onco protein expression and response to chemo/radiotherapy. Anticancer agents did not seem to modify the expression of p53 and bcl-2 proteins.

Resistance to chemotherapy remains a serious problem in the treatment of gastric and oesophageal cancer. DNA-damaging agents alter levels of p53 proteins in several cell types and it has been speculated that regulation of p53 can be involved in the resistance or sensitivity of cancer cells to some

chemotherapeutic drugs, depending on whether cells have mutant /or wild type p53; however, little is known about the relationship of p53 to drug sensitivity in gastric/esophageal cancers. Nabeya Y. et al. (1995) examined human gastric/esophageal adenocarcinoma cell lines for p53 mutational status, chemosensitization to 5 FU, Mitomycin C and cis-Platin, and alteration in p53 levels following exposure of cells to these drugs. The mechanisms involved in regulating p53 levels were also studied. Their results indicated that wild-type p53 protein levels increased after treatment to each of these drugs through either post-translational and/or translational mechanisms. This increase in wild-type p53 appeared to be required for effective chemotherapeutic growth control of gastric/esophageal adenocarcinoma cells. In contrast, gastric/esophageal cancer cells expressing either mutated p53 protein or no p53 protein were resistant to the growth inhibitory effects of these drugs, despite the fact that drug exposure can also increase mutant p53 levels by a translational mechanism. Their data indicated that the mutational status of p53 was predictive of chemosensitivity of gastric and oesophageal adenocarcinomas.

Muro K and others (1996) determined the clinical role of p53 mutations with locally advanced oesophageal carcinoma treated with concurrent chemotherapy by immunohistochemical analysis. 20 patients with previously untreated oesophageal carcinomas with T4 disease and/ or distant node metastasis were selected. Treatment comprised of a protracted 5FU and 2Hr cis-Platin effusions along with radiation treatment with a total radiation dose of 60 Gy. Tumour

specimens from 18 of the 20 patients were analyzed histochemically. Mutant p53 protein expression was analyzed by immunohistochemical staining using a polyclonal antibody, RSP53. Expression of p53 was detected immunohistochemically in 10 of the 18 oesophageal tumours (56%), the cancer cell nuclei of which were diffusely stained. There were no significant differences between the patient backgrounds of the p53- "positive" and "negative" groups. Four of the 10 patients (40%) with p53 expression achieved overall complete remission and 7 of the 10 (70%) achieved complete remission of their primary tumours. In contrast, none of the 8 p53 negative patients achieved overall complete remission (CR) and two (25%) achieved complete remission of their primary tumour. The overall CR rates in the primary tumours tended to be higher in the p53 positive than negative groups, but the differences were not significant. The survival rate for the 10 patients with p53 expression was better than that for the 8 negative ones ($p>0.01$): the median survival times being 12 and 4.5 months respectively. The study concluded that the expression of p53 protein maybe an indicator of a favorable prognosis in patients with locally advanced oesophageal carcinomas treated with concurrent chemoradiotherapy.

The p53 gene product is known to regulate cell growth and proliferation. Whereas the wild type p53 protein suppresses cell growth, mutant p53 protein act as an oncogene. Mutations in the p53 gene usually results in p53 protein stabilization and accumulation; so that the gene product can be detected by immunohistochemistry. Recently, the immunohistochemical detection of the p53

protein was associated with breast, colorectal cancer and other types of cancer. However, its prognostic role in oesophageal cancer remains to be elucidated. Sarbia M. and others (1994) examined p53 expression in formalin fixed, paraffin embedded samples of 204 patients with SCC of the oesophagus, who underwent oesophageal resection. DO-1, a monoclonal antibody that detects wild- type and mutant form of p53 was used. The relationship between p53 immunoreactivity and prognostic factors were determined by the chi-square test, and the prognostic impact of p53 protein expression was analyzed using univariate and multivariate survival analysis. In 204 tumours (67.2 percent), nuclear immunoreactivity for p53 protein was detected. There was no correlation with sex, age, pathologic tumour categories, pathologic lymph node category, metastasis category, residual cancer category, histologic grade, or pre-operative radiation therapy. In addition, p53 expression did not correlate with prognosis using univariate and multivariate survival analysis. The study demonstrated that p53 protein could be detected by immunohistochemistry in a high percentage of SCC of the oesophagus. However, the overexpression of p53 gene product had no impact on the prognosis.

Jung M. et al. (1992) examined alterations of the p53 gene in five of 6 human SCC cell lines characterized as radiation sensitive (SQ-38, SCC-9, SQ-9G) or radiation resistant (SQ-38, SCC-35, JSQ.-3). The point mutations and a deletion were detected by using single stranded conformational polymorphism analysis (SSCP) and polymerase chain reaction (PCR) data sequencing. Three of three

radiation sensitive and two of three radiation resistant cell lines revealed mutations in the p53 gene. Point mutations were located in exons 4, 6, and 8 (at codons 72 and 298 in JSQ-3; 273 in SCC-35; 196 in SQ-38), and deletion consisted of 32 base pairs between codons 274 and 285 in SCC-9 and 1 base pair at codon 271 in SQ-9G. Three mutations resulted in substitutions for an arginine residue. Immunocytochemical analysis, confirmed p53 protein over expression in SCC-35 cells which contain a missense mutation at codon 273. In contrast to previous studies which linked alterations in the ras, myc and raf expression with radiation resistance, this study indicated that mutations in the tumour suppressor gene p53 did not correlate with such resistance.

Wild type p53 protein plays an important role in the cellular response to ionizing radiation and other DNA damaging agents and is mutated in many human tumours. Pomp J. et al. (1998) evaluated the relationship of the immunohistochemically determined p53 protein status and the disease control with radiotherapy alone for carcinoma of the oesophagus. Immunostaining for the p53 protein was performed on paraffin embedded specimens with 69 patients with adenocarcinoma and SCC of the oesophagus. All patients were treated with radiotherapy consisting of combinations of EBRT and ILBT using two different dose levels. 54% (37/69) of the tumours showed over expression of the p53 protein. No difference in pretreatment parameters for p53 positive and p53 negative cases was detected. In multivariate analysis p53 was significantly associated with overall survival next to weight loss, tumour stage

and N stage. For metastasis free survival p53 status proved to be the sole independent prognostic factor. The influence of p53 on local recurrence free survival, however, was not as strong as on overall survival and metastasis free survival. Immunohistochemically detected over-expression of mutated p53 protein in oesophagus carcinoma was an independent prognostic factor in a group of patients treated with radiation therapy alone.

Coggi G. (1997) investigated the occurrence of p53 alterations in oesophageal carcinoma, the correlation between the analysis at the gene and protein level and a prognostic significance. A series of 74 oesophageal carcinomas (46 SCC, 21 adenocarcinomas, 7 undifferentiated carcinomas) were studied by SSCP analysis and immunohistochemistry to detect p53 mutations and accumulation, respectively. p53 mutations in exons 5-8 were detected in 53% of the carcinomas whereas p53 accumulation was observed in 57% of the cases. Comparing SSCP and immunohistochemistry, there were 27 discordant (38%) cases. Overall, only 20 tumours (27%) did not display p53 mutations and/or p53 accumulation. No associations were found between p53 aberrations and clinico pathologic parameters which included patients age and gender, tumour type, stage and grade. p53 protein accumulation and p53 gene mutation were not related to patient survival by univariate or multivariate analysis in oesophageal carcinomas. This study concluded that p53 aberrations were very common in oesophageal carcinomas. However, p53 gene mutation and p53 protein accumulation had a significant discordance suggesting that p53 function maybe

inactivated by mechanisms other than mutations. p53 aberrations did not independently predict prognosis in oesophageal tumours.

(b) Effect of chemo/radiotherapy on the expression of bcl-2 in oesophageal cancers

Sarbia M. et al. (1996) examined bcl-2 expression by immunohistochemistry and correlated it with prognosis in a series of 150 potentially curatively resected SCC of the oesophagus. For comparison, bcl-2 protein expression was analyzed in normal oesophageal mucosa, severe squamous dysplasia, and carcinoma in-situ. bcl-2 immunoreactivity was found in 40 / 150 invasive SCC; the remaining carcinomas were completely negative. bcl-2 protein expression was found more frequently among poorly differentiated than among well differentiated tumours ($p < 0.001$). No correlation was found between bcl-2 protein expression and the parameters tumour size, depth of invasion and nodal status. bcl-2 protein expression had no significant influence on overall survival. Whereas in normal mucosa bcl-2 immunoreactivity was restricted to the basal cell layer, in 9/15 severe squamous dysplasia and 7/14 carcinomas in-situ, bcl-2 staining was detected in all epithelial layers. Therefore bcl-2 protein was found to be frequently expressed in invasive SCC of the oesophagus and precursor lesions of oesophageal cancer, but had no significant impact on the outcome of oesophageal cancer.

Ohbu M. et al. (1996) attempted to determine a correlation between bcl-2 expression and oesophageal SCC histopathology, lymph node metastasis, and clinical variables, and assessed its applicability as a parameter for prognosis. Immunohistochemical staining for bcl-2 (clone 124) was performed on archival material from 105 oesophageal SCC's. bcl-2 was expressed in 58% of oesophageal carcinomas, demonstrating positive correlations with a lack of keratinization and early stage of disease. Lymph node metastasis was significantly less frequently observed in the subset of early stage carcinomas with bcl-2 staining. Univariate analysis revealed significantly longer disease free survival in patients with bcl-2 positive carcinomas than in those which were bcl-2 negative. bcl-2 expression did not have independent prognostic value in a multivariate survival analysis. The study showed that bcl-2 was frequently expressed in oesophageal carcinomas and this expression was positively associated with non-keratinization and an early stage. bcl-2 expression was associated with reduced metastasis to the lymph nodes and may indicate a favorable prognosis.

Goldblum J.R. and Rice T.W.(1995) undertook a study to evaluate the role of bcl-2 in the Barrett's metaplasia- dysplasia- carcinoma sequence. 36 oesophageal resection specimens were studied, using a monoclonal antibody to the bcl-2 protein on fixed paraffin embedded specimens. Barrett's mucosa was present in each specimen: low grade dysplasia in 35, high-grade dysplasia in 34, intra mucosal carcinoma in 23 and submucosal carcinoma in 13. In addition, a section

of the gastric resection margin was evaluated for bcl-2 immunoreactivity in each case. In all cases, the regenerative compartment of the gastric mucosa in the resection margins stained for bcl-2; however, no immunoreactivity was seen in any of the cases of Barrett's mucosa with or without dysplasia or carcinoma. This study shows that although, bcl-2 protein is normally expressed in the regenerative crypt compartment of the colon, small intestine and stomach and has been found to be abnormally over expressed as an early event in the dysplasia - carcinoma sequences of both ulcerative colitis-related and gastric neoplasia, bcl-2 alterations are not an important molecular marker in the neoplastic progression of Barrett's mucosa.

Parenti A.R. et al. (1997) examined 64 cases of SCC of the oesophagus coexisting with squamous intra-epithelial lesion (SIL) in 18 cases for any overexpression of bcl-2 and p53 proteins. Any association of bcl-2 and p53 protein expression with patient survival was also analyzed. bcl-2 and p53 expression was immunohistochemically analyzed using clone 100 monoclonal antibody and Pab 1801 monoclonal antibody respectively. bcl-2 positive immunostaining decreased significantly during the progression of oesophageal carcinogenesis. A decreasing frequency in the expression of bcl-2 in advanced oesophageal SCC coincided with frequent p53 over expression. bcl-2 expression was correlated with patient survival by univariate analysis. This association disappeared after adjusting for tumour stage. p53 over expression showed no association with patient survival by either univariate or multivariate analysis.

bcl-2 positive immunostaining was always detected in the basal layer of the normal epithelium adjacent to the pre-cancerous lesion or oesophageal SCC in the squamous intra-epithelial lesions. bcl-2 positive cells were detected throughout the thickness of the dysplastic squamous epithelium whereas in the infiltrative cancer component, bcl-2 over expression was detected in scattered clusters of neoplastic epithelia located in the submucosa and/ or in the muscularis propria. A bcl-2 positive immuno reaction was detected in 100% of low and high-grade SIL, as well as in the pT1 cancers. pT2, pT3 and pT4 oesophageal SCC showed positive immunostaining 20%, 23% and 20% of cases respectively. A significant inverse correlation was detected between bcl-2 expression and pathological stage of the cancer, the bcl-2 immunoreactivity being detected more frequently in the early (SIL and pT1) than in the advanced (pT2-4) stages of the neoplastic disease (*Spearman's rank correlation*, $p < 0.01$).

p53 positive immunostaining was focally detected in the basal layer of the normal epithelium adjacent to SIL lesions or oesophageal SCC. In the SIL areas, p53 expression was detected throughout the thickness of the dysplastic epithelium. In the infiltrative component p53 over expression was detected in 50%, 50%, 60%, 54% and 80% of SILs, pT1, pT2, pT3 and pT4 neoplastic lesions respectively. No significant correlation was observed between p53 overexpression and the local invasiveness of the tumour. The study showed that down-regulation of bcl-2 and up-regulation of p53 in advanced oesophageal SCC suggested a role for bcl-2 and p53 proteins interaction in the progression of oesophageal SCC.

(c) Role of Apoptosis in treated oesophageal cancers

Graeber T.G. (1996) reported that hypoxia induced apoptosis in oncogenically transformed cells. However, genetic alterations, such as loss of p53 human suppressor gene or over expression of the apoptosis inhibitor protein bcl-2, substantially reduced hypoxia induced cell death. Highly apoptotic regions strongly correlated with hypoxic regions in transplanted tumours expressing wild type p53 whereas little apoptosis occurred in hypoxic regions of p53 deficient tumours. The authors proposed that hypoxia provided a physiological selective pressure in tumours for the expansion of variants that had lost their apoptotic potential, and in particular in cells acquiring p53 mutations. Kinzler K.W. and Vogelstein B (1996) reported that the low levels of oxygen found in those parts of tumours with poor blood supply resulted in p53 dependent programmed cell death. Mutations of p53 reduced the cell death, giving the mutant cells a survival advantage over cells with intact p53. As a tumour grows to a critical size, the central portion suffers from hypoxia. This induces expression of the p53 gene which then leads to growth arrest or apoptosis. Cells that have acquired p53 gene mutation can escape the growth arrest or apoptosis. These clones will eventually outgrow the rest to become the dominant clone. Therefore, emergence of clones with p53 gene mutation may be driven by hypoxia.

Moreira L.F. et al. (1995) examined apoptosis immunohistochemically using the Bm1 Mab in 55 samples oesophageal SCC. Patients not treated (group I, n=12), preoperatively treated by Chemotherapy (group II, n=11), radiation (group III, n

= 13) or both (group IV, n = 8), and 11 additional cases of high grade dysplasia-early cancer were examined. Most of the apoptotic cells were BM1 positive, and when checked by Tunel, also proved to be positive. They accounted for 7 (11%), 19 (29%), 21 (32%), 26 (38%) cells per field in those groups respectively. Chemotherapy and/or radiation significantly increased the number of apoptotic cells as compared to controls ($p = 0.029$ and $p = 0.029$ respectively). Additional sections stained with bcl-2 and p53 were negative for bcl-2 and were positive for p53 in 16 samples (37%). Overall, positive cases for p53 mutations showed a significantly decreased incidence of apoptotic cells ($p=0.03$). These results suggested that in-situ assessment of apoptotic response correlated better to the apoptosis induced by radiation than that by chemotherapy. Further, abnormalities of the p53 protein decreased the apoptotic response in oesophageal carcinoma and the former may help to determine the sensitivity of these anti-cancer agents.

Ohbu M. et al. (1995) assessed apoptotic index, the Ki-67 proliferative index and overexpression of p53 protein in 69 oesophageal SCC surgically resected from Japanese patients. Apoptosis was examined by Gabrieli's method in histological sections, and proved to be significantly related to keratinization and oesophageal SCC progression. Overall labeling indices were 15.68 ± 4.04 (positive/ 1000 nuclei) and 6.79 ± 0.64 respectively, in keratinizing and non-keratinizing types. The apoptosis labeling index increased especially in keratinizing lesions, from 4.50 ± 0.59 with cancer invasion to mucosa through 11.46 ± 2.70 with

involvement of the submucosa up to 21.18 ± 3.72 in cases of penetration to the muscularis propria or adventitia. The relationship between apoptosis, Ki-67 scores and p53 expression was determined in identical cancer nests on serial sections. An inverse correlation was shown between the apoptotic score and the Ki-67 score in both keratinizing and non-keratinizing types. There was no significant correlation between apoptosis score and p53 expression, either overall or separately in keratinizing or non-keratinizing types of oesophageal SCC. The study suggests that a mechanism of induction of apoptosis similar to that operating in normal epidermis may be implicated in keratinizing oesophageal SCC, and that as tumour volume increases, single cell death becomes more frequent.

Hamada M. et al. (1996) examined formalin fixed, paraffin wax embedded sections taken from 25 patients who underwent oesophagectomy for SCC. 14 patients had no preoperative therapy (control group), while the other 11 patients received preoperative radiotherapy (radiation group). There was no difference in pathological TNM classification between the two groups. These sections were examined by immunostaining with monoclonal antibody PAb1801 to determine the accumulation of p53 protein. Apoptotic frequency was determined by Tdt mediated dUTP-biotin nick end- labeling (Tunel). In the control group, well to moderately differentiated cases showed a significantly higher apoptotic index (which was the number of apoptotic cells amongst 1,000 cancer cells), 51.7 ± 83.4 . In the poorly differentiated cases, apoptotic index was 1.3 ± 1.0 .

($p < 0.05$). Similar results were obtained in the radiation group. The group that received no pre-operative therapy (control group) included 4 cases of p53 grade 4 (p53 protein detected in over 70% of the tumour cells). The group that received pre-operative radiotherapy (radiation group) included 2 cases of p53 grade 4. Few apoptotic cells were observed in any of six tumour tissues. Tumour cells with accumulated p53 protein rarely showed apoptosis. In contrast, apoptosis was observed in tumour cells without p53 protein accumulation. Spontaneous apoptosis in oesophageal SCC can be induced more easily in differentiated than in poorly differentiated cases. This tendency may be enhanced by preoperative radiotherapy. Extensive p53 protein may suppress apoptotic induction in oesophageal SCC.

Shears L.L. et al. (1998) examined whether residual tumour cells in oesophagectomy specimens following induction chemo-radiotherapy were viable, in order to provide putative evidence for appropriateness of oesophagectomy. Forty-six patients were entered into an induction chemoradiotherapy trial consisting of 5FU, cisplatin, alpha-interferon and concurrent EBRT followed by oesophagectomy. Response was determined histologically and apoptosis assessed with Tunel method. p53 status was determined by immunohistochemistry and mutational analysis. 38 patients underwent oesophagectomy, 33 of whom had either a complete ($n=10$) or partial ($n=23$) response. None of the 28 patients with residual tumour in the resected specimen had 100% apoptotic cells and a vast majority of specimens had $<10\%$

apoptotic rate. The percentage of apoptotic cells correlated with tumour differentiation but not with the presence of p53 mutations. Tissue sections following upfront chemo-radiotherapy was a necessary component of a multi-modality approach to oesophageal cancer. This would ultimately provide superior local regional control to a non-surgical approach.

Katada N. et al. (1997) evaluated the alteration of apoptosis in the oesophageal epithelium in the oesophagitis- Barrett's oesophagus- adenocarcinoma sequence. Archival tissue samples of 85 lesions in 58 cases were used. The lesions represented seven groups: normal oesophagus (n=10), reflux oesophagitis (n=12), Barrett metaplasia (n=21), Barrett low grade dysplasia (n=17), Barrett high-grade dysplasia (n=5), well or moderately differentiated adenocarcinoma (n=10), and poorly differentiated adenocarcinoma (n=10). Apoptotic cells with fragmented DNA were detected by Tunel method. Monoclonal antibodies against bcl-2 protein were applied using the avidin-biotin complex immuno peroxidase method. The oesophagitis group showed many apoptotic cells on the epithelial surface; whilst few apoptotic cells were seen in the other groups. Weak bcl-2 expression was seen in the basal cells in normal subjects and those with oesophagitis. There was over expression of bcl-2 in 72% of Barrett's metaplasia, 100% of Barrett's low grade dysplasia, 25% of Barrett's high-grade dysplasia, 40% of were well differentiated or moderately differentiated adenocarcinoma, and 20% of poorly differentiated adenocarcinoma. The authors concluded that increased apoptosis in reflux oesophagitis may be a protective mechanism

counteracting increased proliferation. Inhibition of apoptosis by over expression of bcl-2 protein occurred early in the dysplasia- carcinoma sequence of Barrett's oesophagus. The resulting prolongation of cell survival could promote neoplastic progression. Despite the absence of apoptosis, bcl-2 was not widely over expressed in Barrett's high-grade dysplasia and adenocarcinoma suggesting that cells acquire other ways of avoiding apoptosis as malignancy appears. The findings of this study are similar to those previously reported by Goldblum J.R. and Rice T.W.(1995) who also observed that bcl-2 alterations were not an important molecular marker in the neoplastic progression of Barret's mucosa.

Ohashi K. et al. (1997) assessed p53 protein accumulation, apoptosis, and proliferating activity (Ki-67 labeling index) using oesophageal SCC samples of both intramucosa and advanced types. The relationship of these values to progression or differentiation grade of tumours was also analyzed. In terms of proliferating activity and the proportion of positive cases with p53 accumulation, a statistically significant difference was demonstrated between intra epithelial carcinomas and intramucosal carcinomas with stromal invasion (17.2% vs. 31.7% for the Ki-67 labeling index, and 23.5% vs. 67.4% for the proportion of positive cases of p53 accumulation). Values for the latter were almost comparable to those of advanced carcinoma. Immunohistologically, Ki-67 positive, proliferating cells were distributed preferentially in the peripheral fronts of invasion. Apoptotic cells were observed in the areas of the invasion of the intramucosa carcinomas with stromal invasion and in more advanced lesions, but

were rarely observed in the normal epithelium or intraepithelial carcinomas. Apoptotic cells were seen mainly around areas of keratinization, and the apoptotic cell index was higher in well and moderately differentiated types of advanced carcinomas than in poorly differentiated type (2.59% vs. 1.09%). The study showed that an increase in proliferative activity and an accumulation of p53 protein was associated with the onset of early carcinomatous invasion, while apoptosis is closely linked with the differentiation grade of carcinoma cells.

Chapter 12**Results**

MORPHOLOGICAL CHANGES

MACROSCOPIC EXAMINATION :

In all patients, the entire oesophagus was resected. Tumour length ranged from 25-55 mm in length and 10-20 mm in width. Six patients had an ulcerative tumour whilst 4 patients had circumferential tumours. The patient clinical data is shown in Table I.

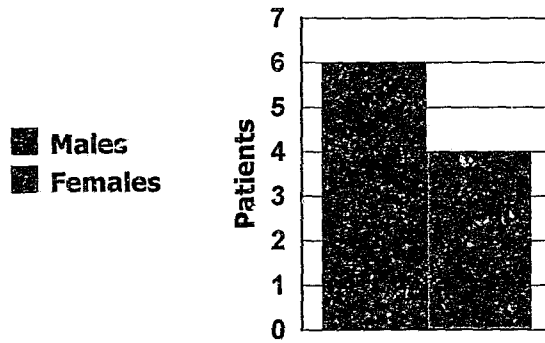
LIGHT MICROSCOPY

Resection margins: In the 7 patients with tumour in the middle-third of the oesophagus, only the proximal resection margin showed characteristic radiation changes. The proximal resection margin was located 5.5, 5.0, 5.5, 5.0, 4.5, 4.5, and 5.5. cm, respectively, from the edge of the visible tumour, in the 7 patients. Similarly, changes were seen in 3 patients with tumour in the lower third, with radiation changes only in the distal resection margin. The distal resection margin was located 5.0, 5.0, and 5.5 cm from the edge of the visible tumour in these 3 patients. Radiation changes were limited to the submucosa with sparing of the muscularis propria and adventitia. There was no ulceration of the squamous mucosa. The lamina propria showed collections of lymphocytes and plasma cells. The muscularis mucosae and the submucosa showed minimal fibrosis. There were no morphological alterations in the blood vessels. These features are demonstrated in Figures XX, XXI.

Table I : PATIENT CHARACTERISTICS

Patient Number	Age	Sex	Site	Tumour characteristics on gross examination
1	53	Male	Middle third	Concentric stricture, 30 mm length x 15 mm width; ulcerating tumour
2	33	Male	Middle third	Ulcerating tumour, 45 mm length x 15 mm width
3	45	Female	Middle third	Concentric stricture, 40 mm length x 10 mm width; ulcerating tumour
4	64	Female	Lower third	Ulcerating tumour, 45 mm length x 15 mm width
5	40	Male	Middle third	Concentric stricture, 55 mm length x 15 mm width; ulcerating tumour
6	58	Female	Lower third	Ulcerating tumour, 30 mm length x 15 mm width
7	61	Female	Lower third	Ulcerating tumour, 50 mm length x 15 mm width
8	67	Male	Middle third	Ulcerating tumour, 25 mm length x 20 mm width
9	63	Male	Middle third	Concentric stricture, 25 mm length x 20 mm width; ulcerating tumour
10	31	Male	Middle third	Ulcerating tumour, 50 mm length x 15 mm width

Sex Distribution



Site Distribution

Oesophageal Cancer

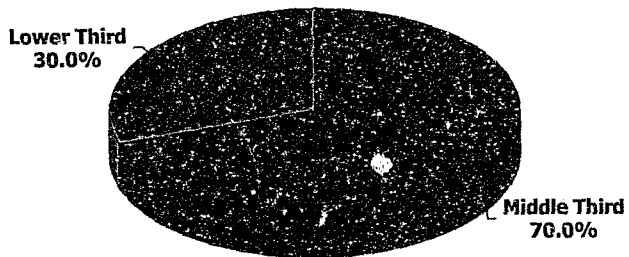


Fig XX

Photomicrograph of a section of the proximal resection margin showing fibrosis involving the submucosa with sparing of the muscularis propria (H&E, X100)



Fig XXI

Photomicrograph of a section of the proximal resection margin showing fibrosis involving the submucosa with sparing of the muscularis propria. Masson's Trichrome stain showing the fibrosis (X100)



Edge of Irradiated length: Radiation changes in the form of fibrosis were limited to the circular muscle layer, with sparing of the longitudinal muscle layer and adventitia. The surface squamous epithelium was mildly acanthotic with minimal basal cell hyperplasia. Although occasional mitotic figures were seen in the basal cell layer, these were not atypical. The lamina propria showed collections of lymphocytes and plasma cells. There were no changes in the blood vessels. The changes as described are shown in Figures XXII, XXIII.

1 cm from the edge of visible tumour proximally and distally: Radiation changes were observed in all the layers. The squamous mucosal lining was markedly attenuated with ulceration. The lamina propria showed large collections of lymphocytes and plasma cells with extensive fibrosis. The muscularis mucosae and the submucosa showed dense fibrosis. The blood vessels within the submucosa demonstrated changes of end arteritis obliterans. Both the longitudinal and circular muscle fibers in the muscularis propria were atrophic with a marked degree of interstitial fibrosis. The adventitia was also fibrotic. These changes are shown in Figures XXIV, XXV.

Centre of the tumour: The lining epithelium showed extensive ulceration with necrosis. The tumour showed necrosis with extensive keratin production by the squamous cells, which was not compatible with the degree of anaplasia observed in the tumour cells morphologically. Foreign body type giant cells were seen as a response to the keratin produced. The intervening stroma was markedly

Fig XXII

Photomicrograph of a section of the edge of irradiated length demonstrating acanthotic epithelium and basal cell hyperplasia with fibrosis limited to the circular muscle layer (H&E, X200)

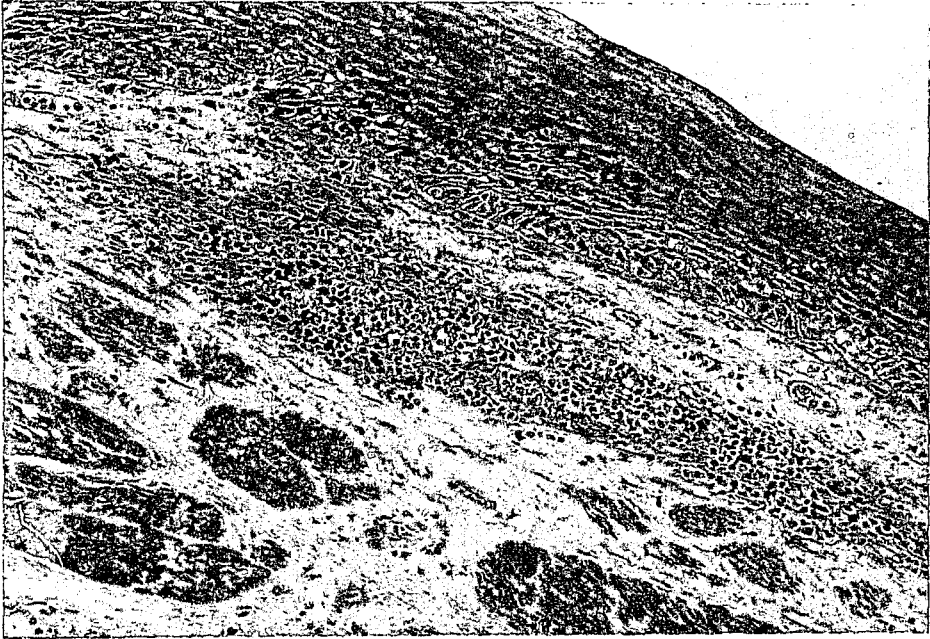


Fig XXIII

Photomicrograph of a section of the edge of irradiated length demonstrating acanthotic epithelium and basal cell hyperplasia (H&E, X400)

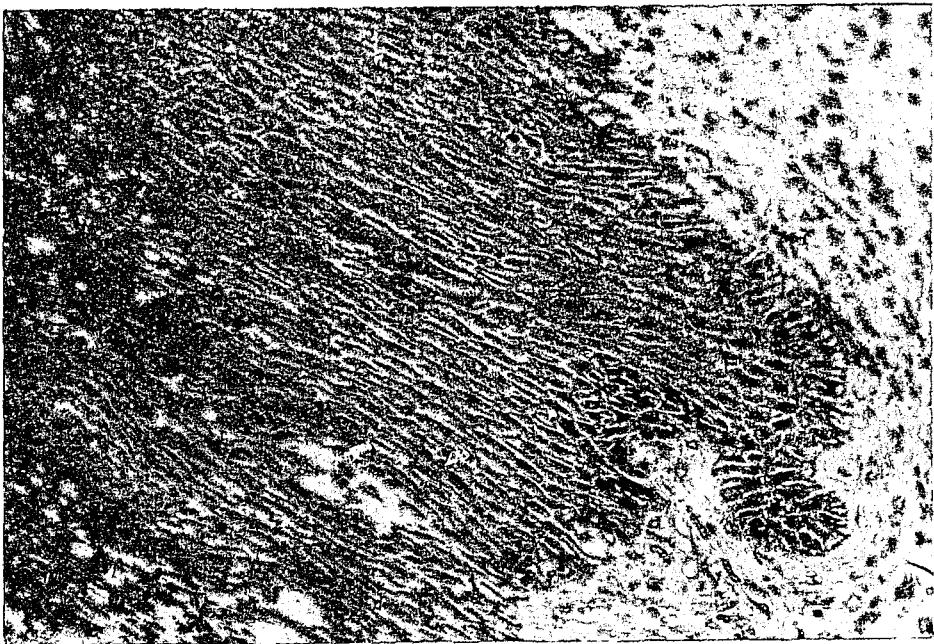


Fig XXIV

Photomicrograph of a section 1 cm from the edge of the tumour showing attenuated epithelium and full thickness dense fibrosis (H&E, X200)

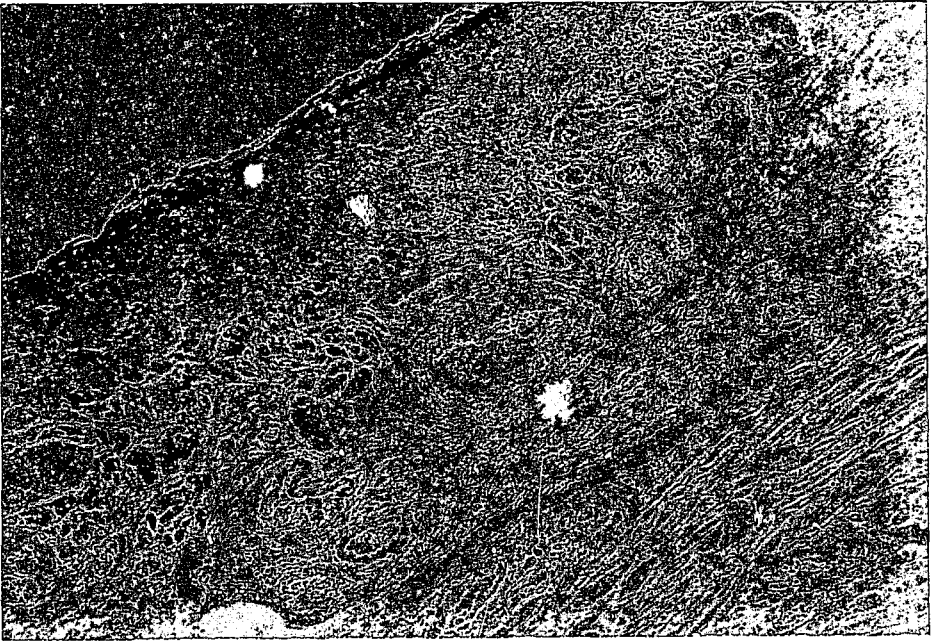
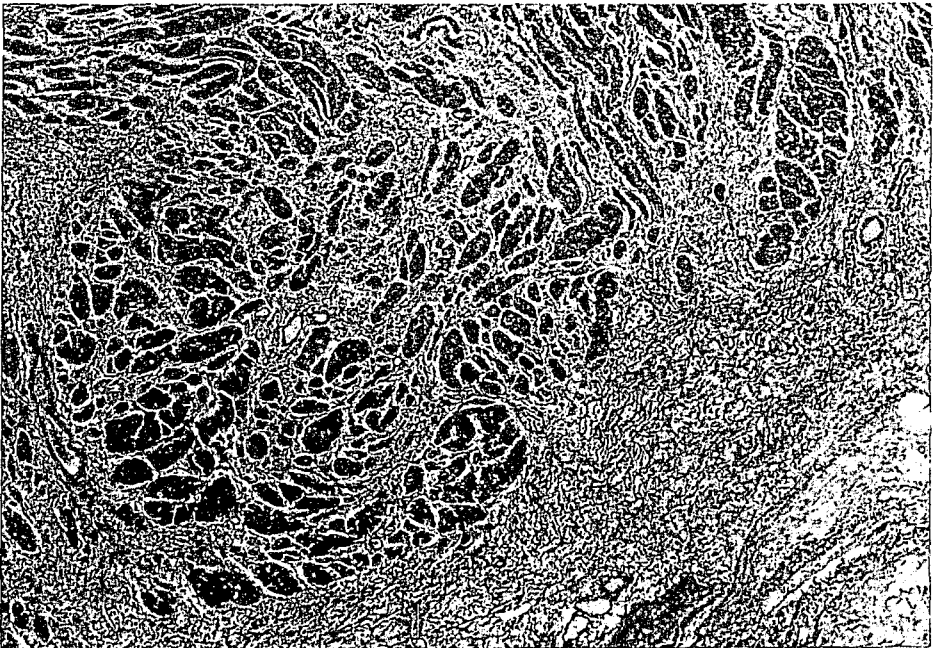


Fig XXV

Photomicrograph of a section 1 cm from the edge of the tumour showing atrophic muscularis propria with intersitial fibrosis (H&E, X200)



desmoplastic, with a keloid type of collagenization that extended to the serosa. The blood vessels in this area also showed changes of endarteritis obliterans. These are shown in Figures XXVI, XXVII, XXVIII, XXIX.

Lymph Nodes: A cluster of lymph nodes with metastatic tumour was present in 3 patients with a tumour in the mid one-third (one patient) and lower third (two patients). The lymph nodes were located in the adventitial tissue around the oesophagus, 2mm away from the oesophageal wall. The entire lymph node parenchyma was replaced by tumour with a rim of normal lymphoid tissue at the periphery. Similar radiation changes present in the tumour in the oesophagus were also noted in the lymph node (Figures XXX, XXXI).

All changes described above have been shown in Table II.

EFFECT ON EXPRESSION OF *MRD-1* GENE PRODUCT: p-glycoprotein:

In all 10 cases, p-glycoprotein was not expressed in either pre-brachtherapy (Figure XXXII) nor post-brachytherapy (Figure XXXIII) tumour tissue specimens (after brachytherapy of 20 Gy). Figure XXXIV shows a positive control of p-glycoprotein staining in normal liver bile canaliculi.

Fig XXVI

Photomicrograph of a section from the centre of the tumour demonstrating profuse keratin production by anaplastic squamous cells with foreign body giant cell reaction (H&E, X400)

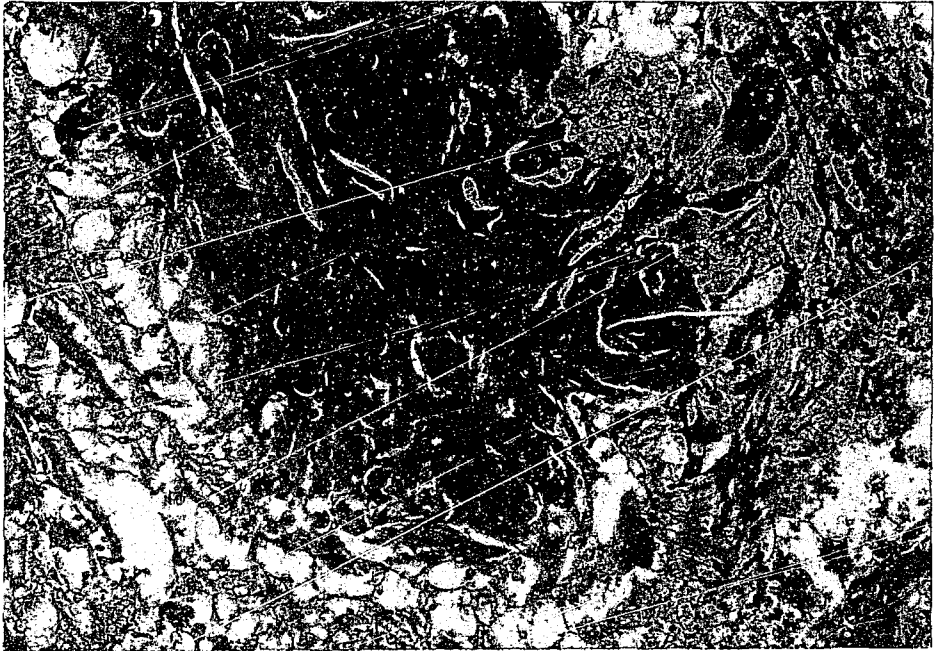


Fig XXVII

Photomicrograph of a section from the centre of the tumour showing a keloid type of desmoplastic fibrosis of the stroma. (H&E, X200)

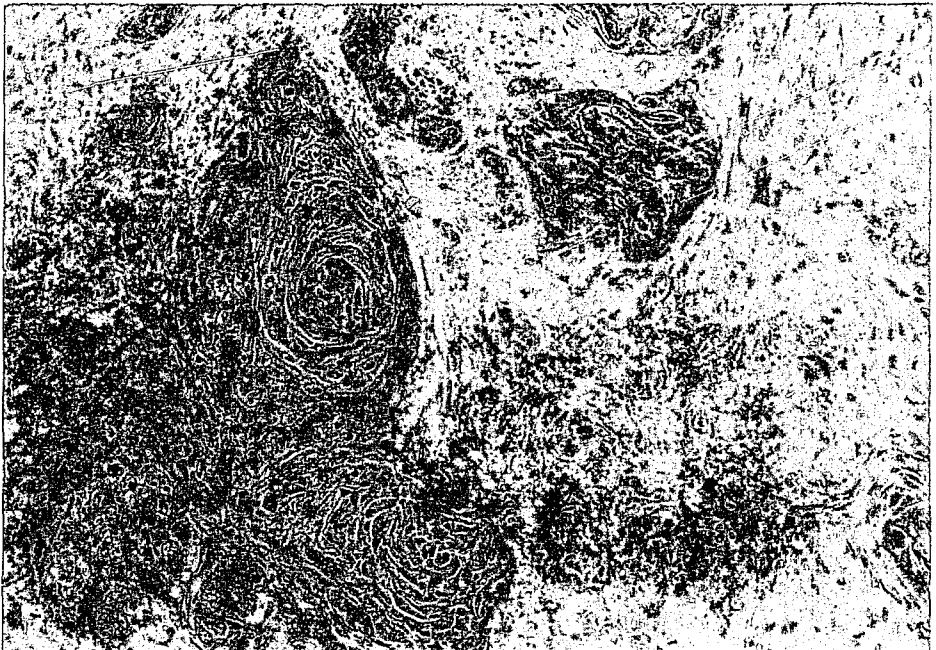


Fig XXVIII

Photomicrograph of a section from the centre of the tumour showing changes of endarteritis obliterans (H&E, X400)

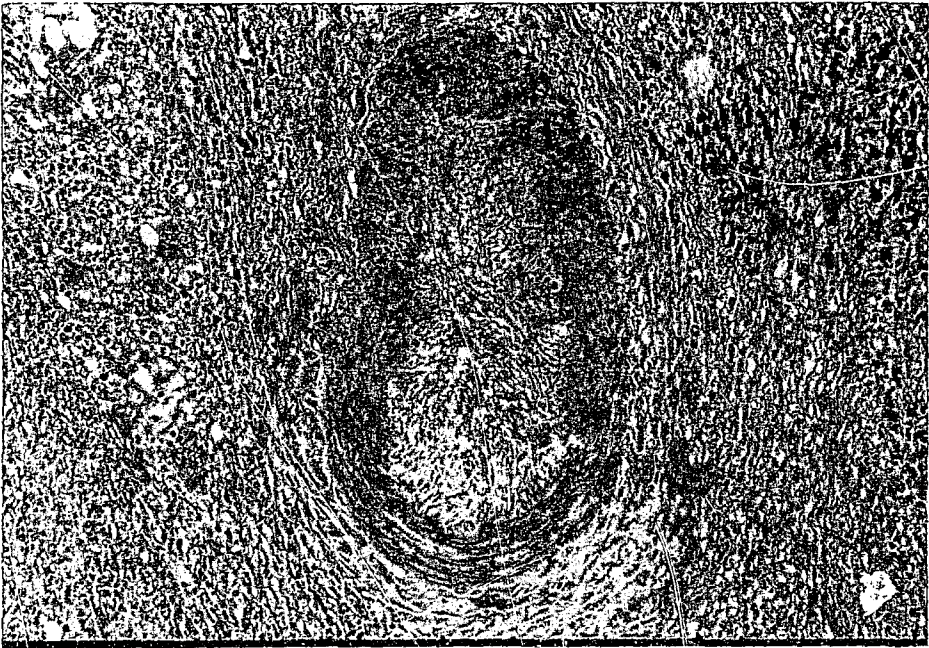


Fig XXIX

Photomicrograph of a 1 cm from the edge of the tumour showing atrophic muscularis [propria with interstitial fibrosis (H&E, X200)

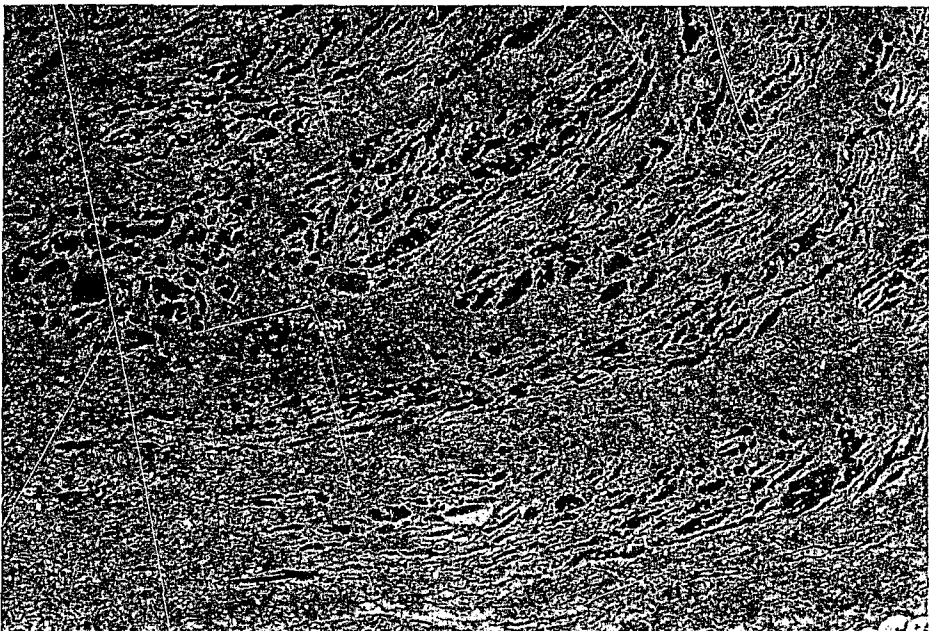


Fig XXX

Photomicrograph of a section from a lymph node with metastatic squamous cell carcinoma showing profuse keratin production and dense desmoplastic fibrosis in the lymph node (H&E, X100)

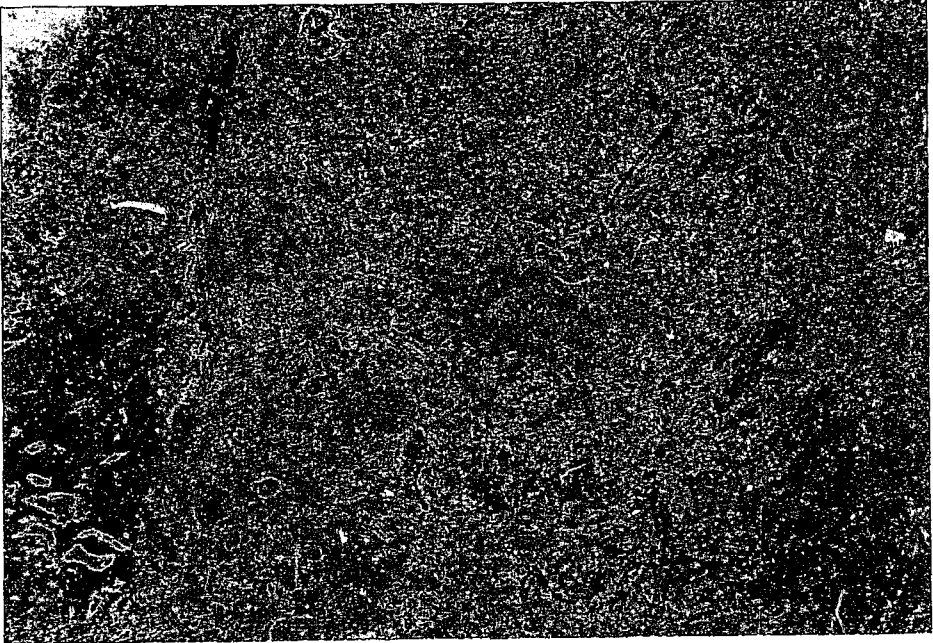


Fig XXXI

Photomicrograph of a section from a lymph node with metastatic squamous cell carcinoma showing profuse keratin production and dense desmoplastic fibrosis in the lymph node. Masson's Trichrome stain showing the fibrosis (X100)

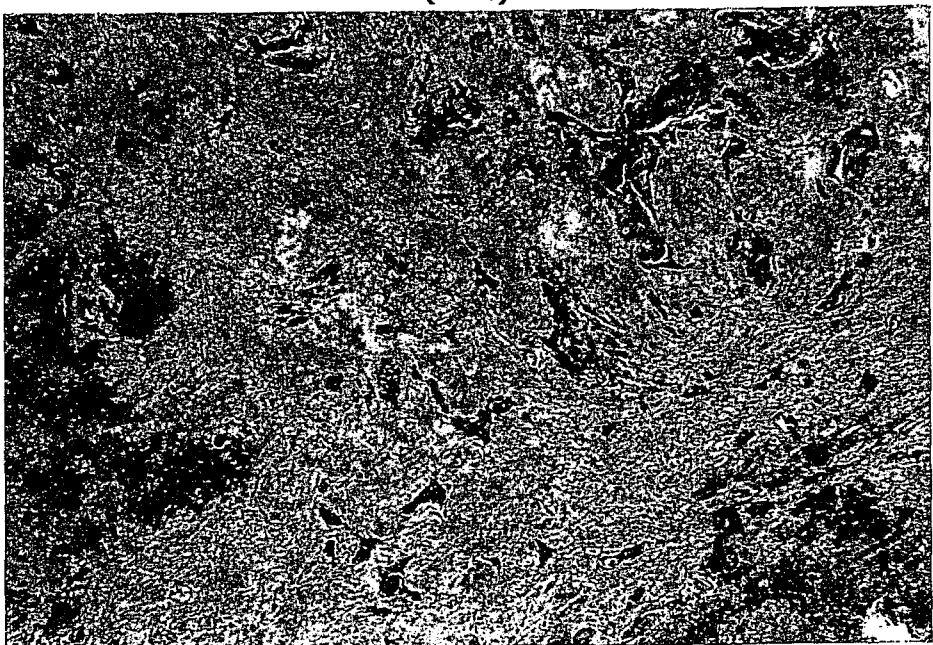


Table II : MORPHOLOGICAL CHANGES AT VARIOUS LEVELS

Site	Depth of Radiation Change	Fibrosis	Surface Epithelium	Blood Vessel	Tumour
Resection Margin	Submucosa	+	-	-	-
Edge of Irradiated Length	Circular Muscle Layer	++	Basal cell Hyperplasia	-	-
1 cm from edge of visible Tumour	Full Thickness	++++	Attenuation and ulceration	Endarteritis obliterans	-
Centre of Tumour	Full Thickness	++++	Ulceration	Endarteritis obliterans	Necrosis Intense Keratinization Giant Cell Reaction

Fig XXXII

Photomicrograph showing preradiation biopsy of oesophageal squamous cell carcinoma. No *MDR-1* gene expression (X200)

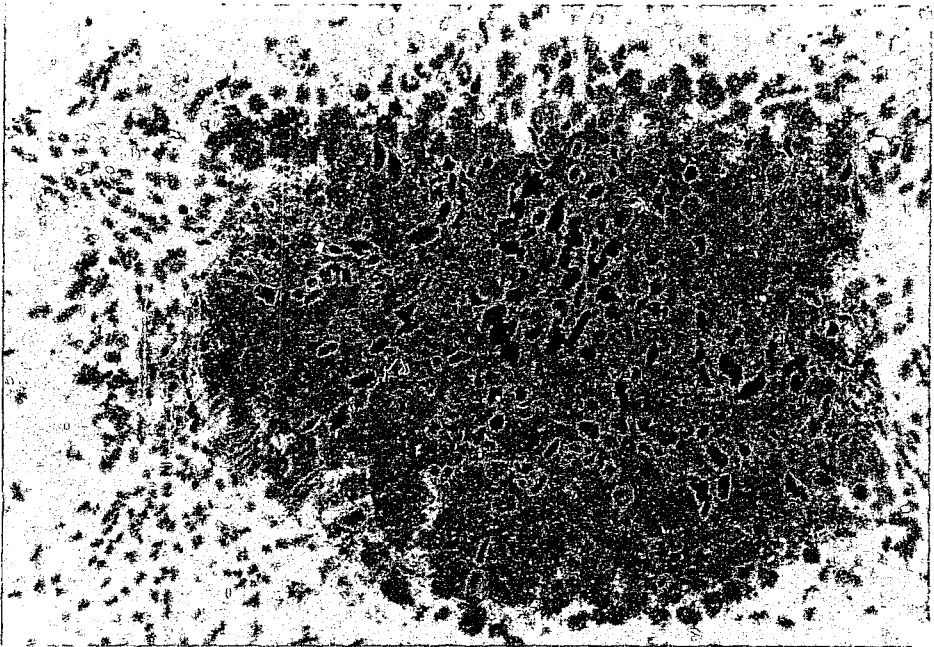


Fig XXXIII

Photomicrograph showing post brachytherapy section of oesophageal squamous cell carcinoma. No *MDR-1* gene expression (X200)

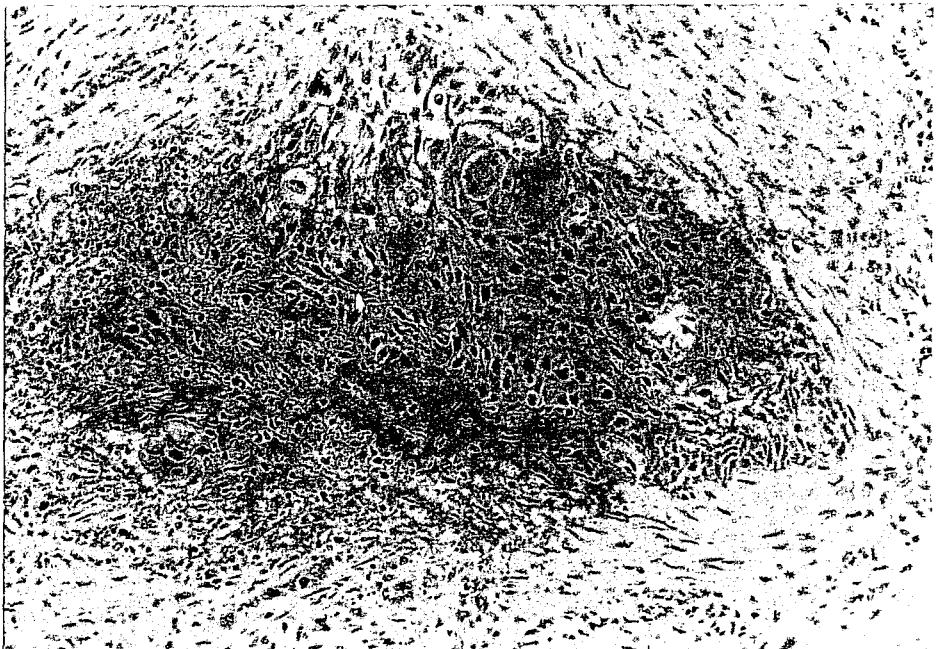
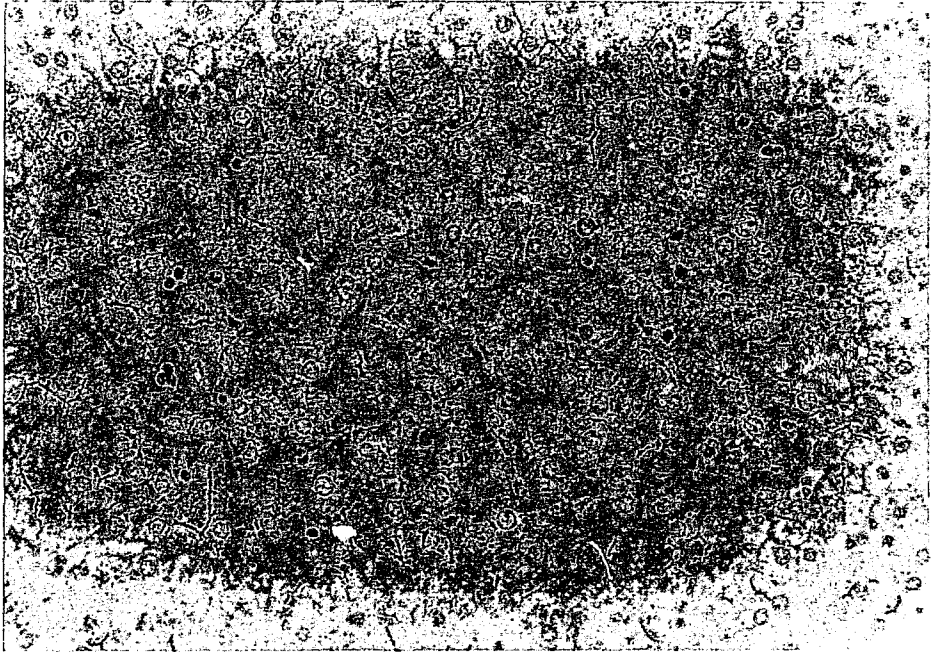


Fig XXXIV
Photomicrograph showing positive control. p-glycoprotein (*MDR-1* gene)
staining in normal liver, bile canaliculi (X200)



EFFECT ON EXPRESSION OF p53, bcl-2 AND APOPTOSIS

The pre and post-brachytherapy findings for expression of p53 are shown in Table III. There was no expression of p53 in one patient (no.1) in both pre and post-brachytherapy specimens (group 1) . In 8 patients (group 2), p53 staining was strongly positive (3+) with approximately 50% or more cells, and with diffuse and no specific pattern in the pre-brachytherapy biopsies (Figure XXXV). The post-brachytherapy specimens of group 2 showed strong 3+ positivity with p53 (10-50% positive cell count) with the pattern being focal and peripheral (Figure XXXVIa) in the tumour islands. The centre of the tumour islands showed necrosis (Figure XXXVIb) and/or keratinization (Figure XXXVIc) (group 2). In one patient (case 3), the pre-brachytherapy biopsy showed expression of p53 (Figure XXXVII), whilst the post-brachytherapy specimen was negative (group 3) (Figure XXXVIII), (Table IV).

bcl-2 expression in both pre and post-brachytherapy is shown in table V. bcl-2 staining was equivocal and inconclusive in both the pre and post-brachytherapy specimens, in that specific patterns of bcl-2 expression could not be established.

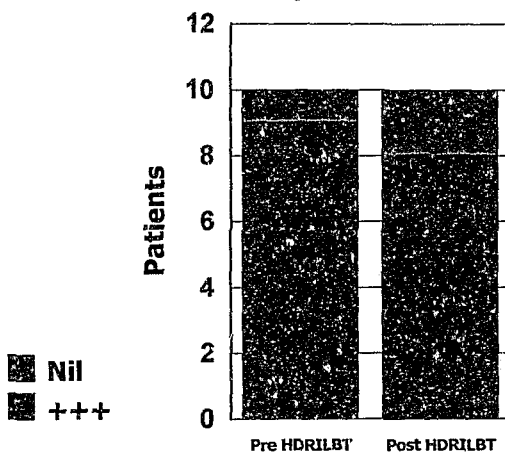
Apoptosis was negative in all the pre (Figure XXXIX) and post-brachytherapy (Figure XXXX) tissue sections in the presence of positive control (Figure XXXXI) .

Table III - p53 Expression

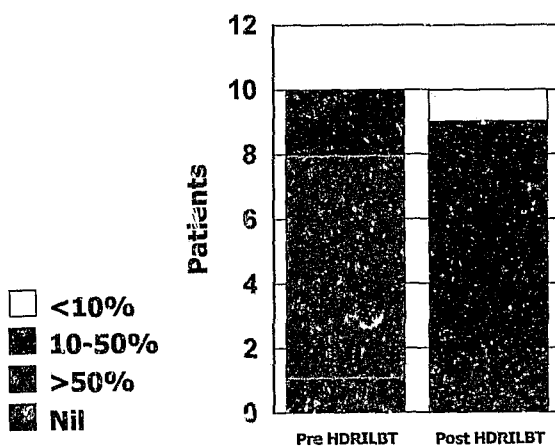
Intensity of Nuclear Staining										
Pt.No	1	2	3	4	5	6	7	8	9	10
Pre HDRILBT	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
Post HDRILBT	-	+++	-	+++	+++	+++	+++	+++	+++	+++
Number of positive cells										
Pt.No	1	2	3	4	5	6	7	8	9	10
Pre HDRILBT	-ve	>50% Type C	10-50% Type B	10-50% Type B	>50% Type C	>50% Type C	>50% Type C	>50% Type C	>50% Type C	>50% Type C
Post HDRILBT	-ve	<10% Type A	-ve	10-50% Type B	10-50% Type B	10-50% Type B	10-50% Type B	10-50% Type B	10-50% Type B	10-50% Type B
Pattern of distribution										
Pt. No	1	2	3	4	5	6	7	8	9	10
Pre HDRILBT	-ve	Diffuse	Focal	Diffuse	Diffuse	Diffuse	Diffuse	Diffuse	Diffuse	Diffuse
Post HDRILBT	-ve	Focal / Peripheral	-ve	Focal / Peripheral	Focal / Peripheral	Focal / Peripheral	Focal / Peripheral	Focal / Peripheral	Focal / peripheral	Focal / peripheral

Number of positive cells : Type A= ~10%; Type B= 10-50%; Type C= > 50% , HDRILBT - High Dose Rate Intraluminal Brachytherapy, Pt. No.= Patient Number, -ve= Negative , Intensity of brown signals - nuclear (p53) : cytoplasmic (bcl-2) or single cell (apoptosis) was graded as negative(-), +, ++ or +++

p53 Expression
Intensity of Nuclear Staining



p53 Expression
Number of Positive Cells



**p53 Expression
Pattern of Distribution**

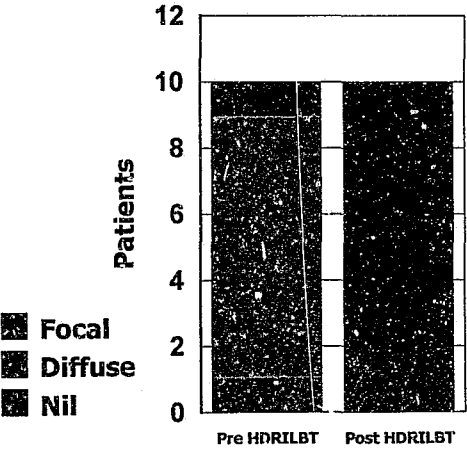


Fig XXXV

Prebrachytherapy tumour showing 3+ positivity with p53 immunostaining (nuclear) in more than 50% of cells with no specific pattern-diffuse. (X200)

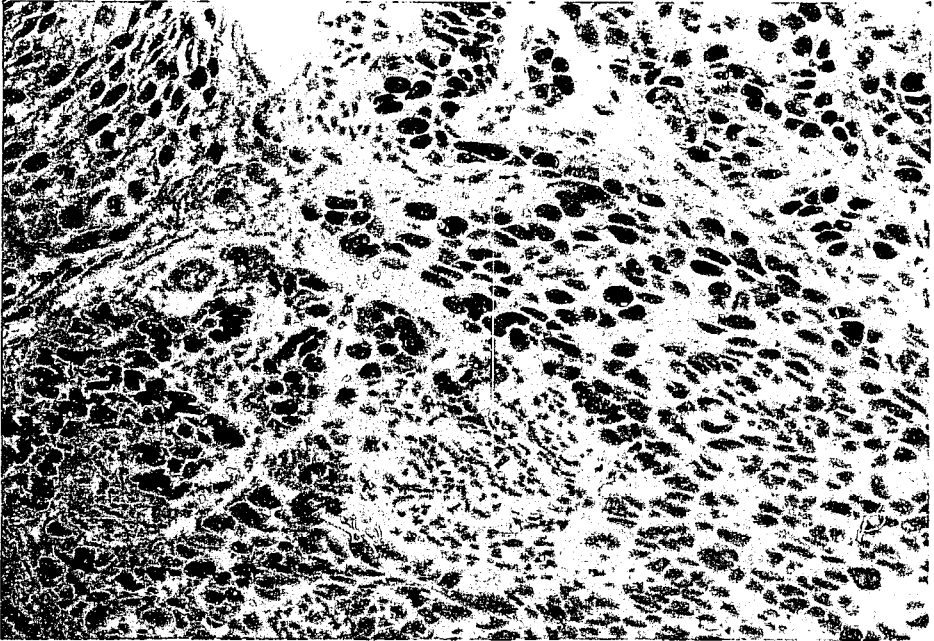


Fig XXXVIA

Postbrachytherapy tumour area from the resection specimen showing strong 3+ positivity with p53 in 10-50% of cells with the pattern being focal and peripheral in the tumour islands (X100)

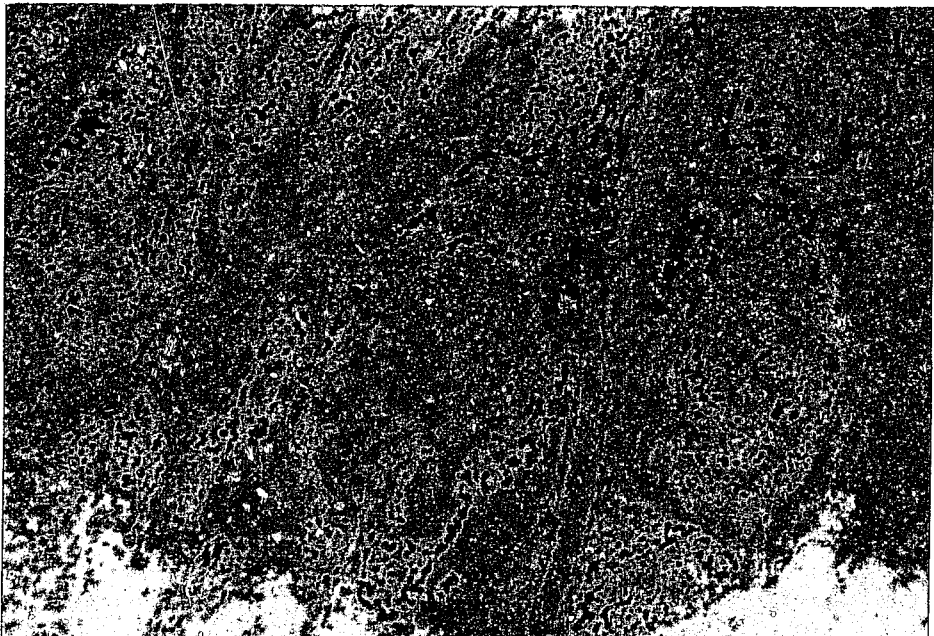


Fig XXXVIB
Postbrachytherapy tumour area from the resection specimen showing
necrosis within the centre of the tumour island (X400)

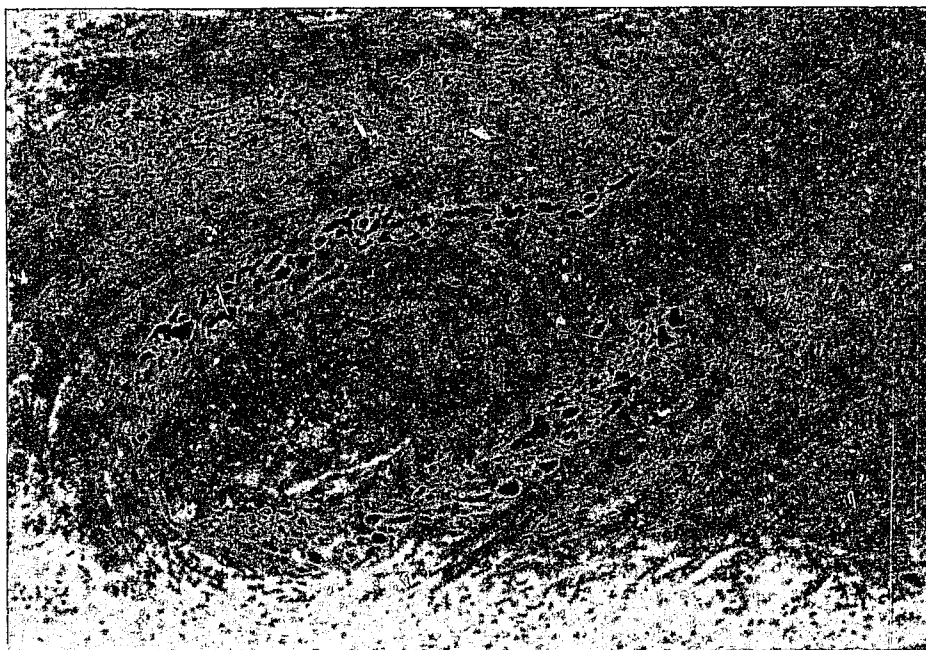


Fig XXXVIC
Postbrachytherapy tumour area from the resection specimen showing
keratinization within the centre of the tumour island (X400)

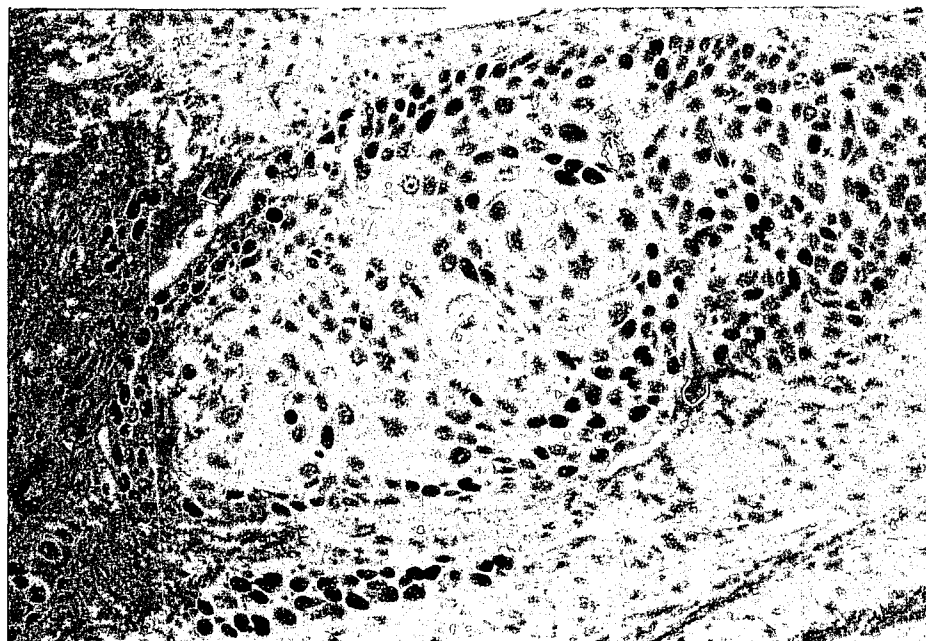


Fig XXXVII

Prebrachytherapy tumour biopsy showing 3+ positivity with p53 immunostaining in >50% cells with no specific pattern - diffuse (X100)

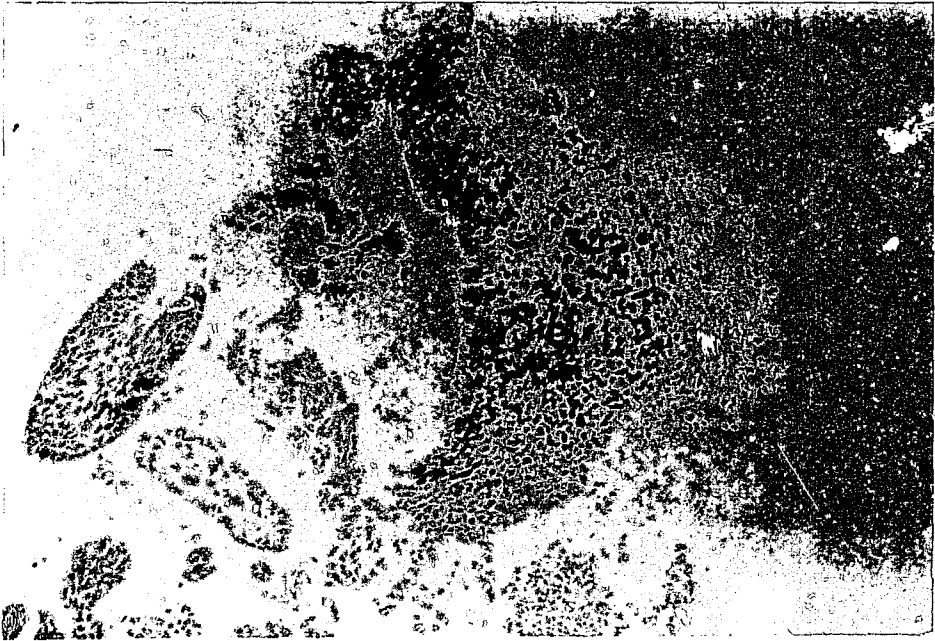


Fig XXXVIII

Postbrachytherapy tumour area from the resection specimen showing no expression of p53 (X100)

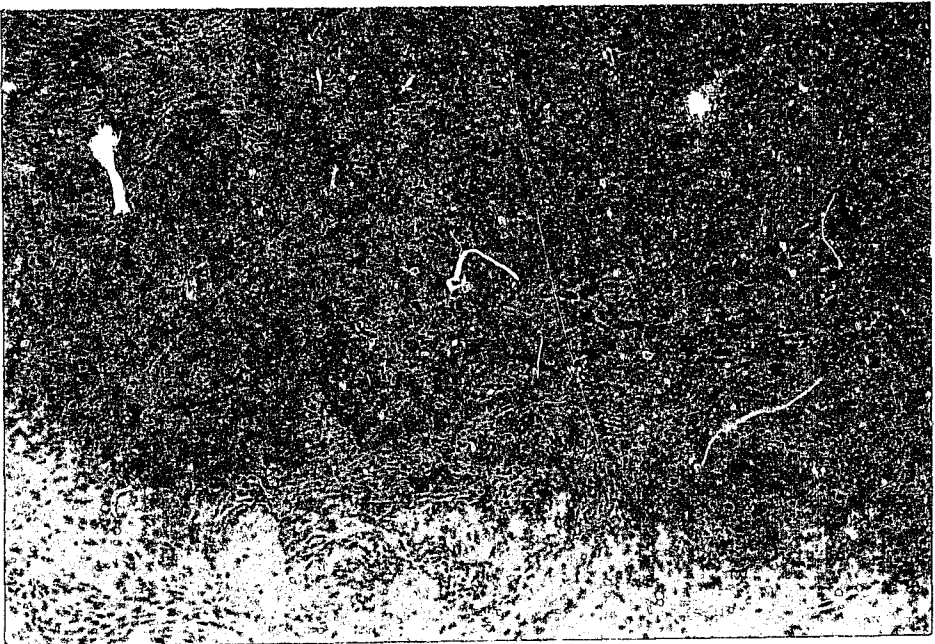


Table IV: Patterns of p53 Expression pre and post brachytherapy

Group	No of patients	Criteria
1	1	Pre and Post brachytherapy - no expression of p53
2	8	Pre brachytherapy - +++ intensity; diffuse staining Post brachytherapy - +++ intensity, diffuse but peripheral staining in tumour islands with central areas showing necrosis with some islands showing central keratinization
3	1	Pre brachytherapy - Expression of p53; Post brachytherapy - No expression

Table V - bcl-2 Expression

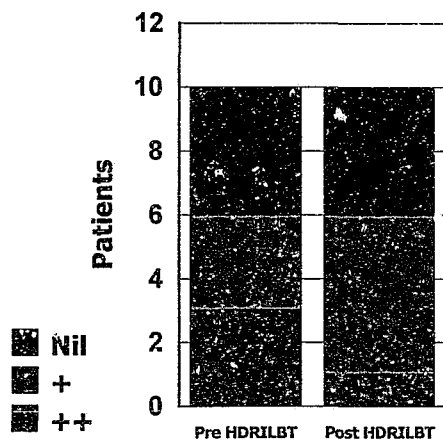
Intensity of Cytoplasmic Staining										
Pt.No	1	2	3	4	5	6	7	8	9	10
Pre HDRILBT	+	+	-	-	-	++	++	++	-	+
Post HDRILBT	-	+	+	-	-	+	+	++	-	+

Number of positive cells										
Pt.No	1	2	3	4	5	6	7	8	9	10
Pre HDRILBT	10-50% Type B	10-50% Type B	-ve	-ve	-ve	>50%	10-50% Type B	10-50% Type B	-ve	10-50% Type B
Post HDRILBT	-ve	10-50% Type B	<10%	-ve	-ve	10-50% Type B	<10% Type A	10-50% Type B	-ve	10-50% Type B

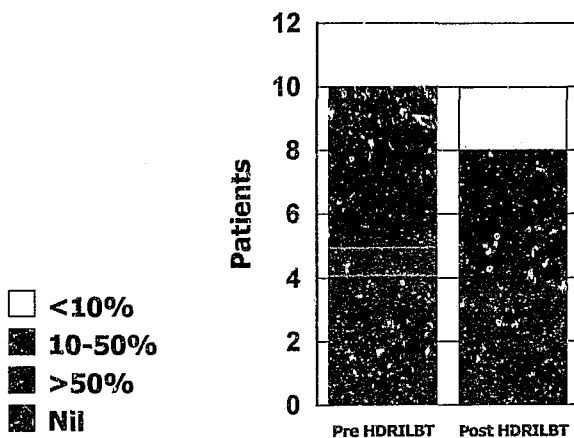
Pattern of distribution										
Pt. No	1	2	3	4	5	6	7	8	9	10
Pre HDRILBT	Focal	Focal	-ve	-ve	-ve	Diffuse	Focal / scattered	Diffuse	-ve	Focal
Post HDRILBT	-ve	Focal	Focal	-ve	-ve	Focal	Focal	Focal	-ve	Focal

Number of positive cells : Type A= <10%; Type B= 10-50%; Type C= > 50%, HDRILBT - High Dose Rate Intraluminal Brachytherapy, Pt. No.= Patient Number, -ve= Negative, Intensity of brown signals - nuclear (p53) or cytoplasmic (bcl-2) or single cell (apoptosis) was graded as negative(-), +, ++ or +++

bcl-2 Expression Intensity of Cytoplasmic Staining



bcl-2 Expression Number of Positive Cells



bcl-2 Expression Pattern of Distribution

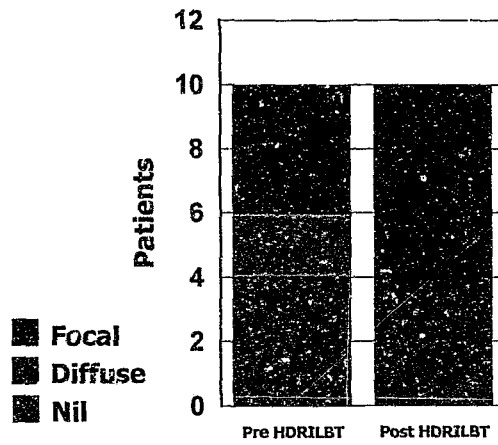


Fig XXXIX
Prebrachytherapy tumour biopsy showing no expression of apoptosis (X100)

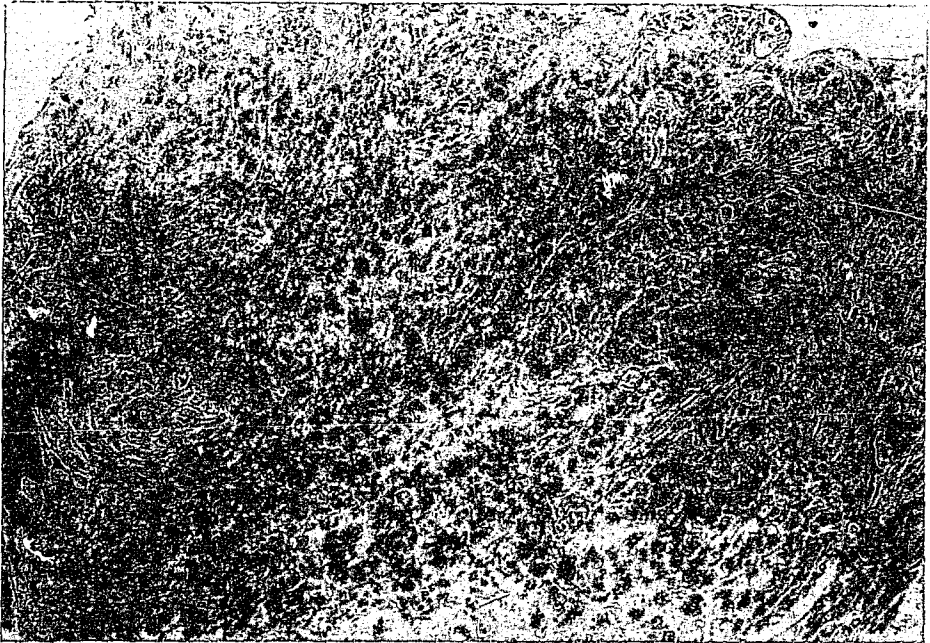
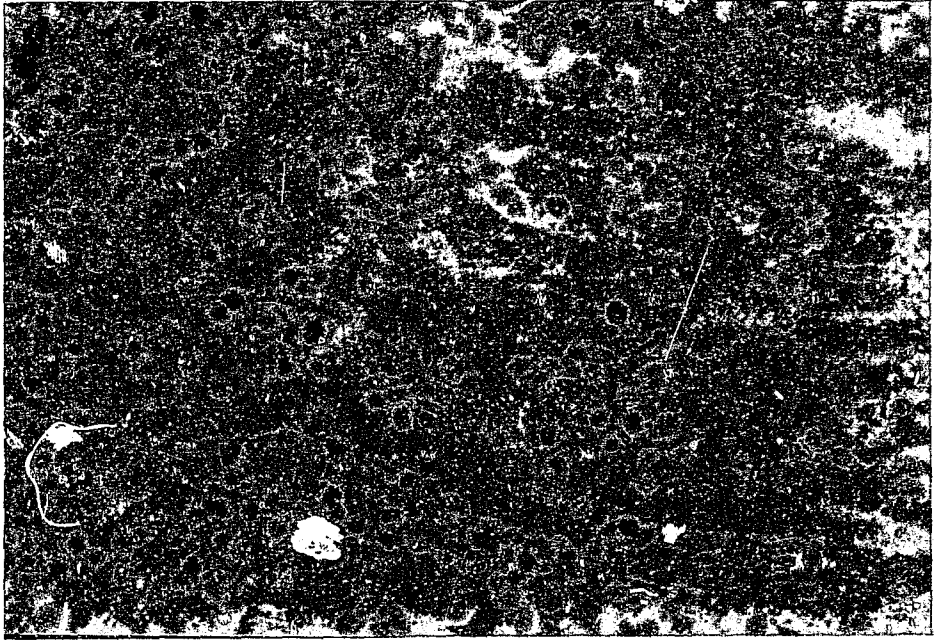


Fig XXXX
Postbrachytherapy tumour area from the resection specimen showing no expression of apoptosis (X200)



Fig XXXXI
Positive control. Photomicrograph of Hodgkin's Lymphoma demonstrating
single cell apoptosis (X400)



Chapter 13**Discussion**

Morphological Changes

The pathological sequence of events occurring in the oesophagus following EBRT have been well described (Berthrong M., 1986, Northway N.G. et al, 1979, Novack J.M. et al., 1979, Phillips T.L. and Margolis L., 1972, Seaman W.B., 1957).

The pathologic sequence of events after a single large EBRT fraction of radiation in mice are as follows (Phillips T.L. and Ross G., 1974):

- Day 3 : Vacuolization and absence of mitosis of the basal cell layer along with thinning of the keratinized squamous cell layer occurs.
- Between day 7 and 14: Foci of proliferating basal cells and regenerating epithelium with zones of complete denudation occur simultaneously.
- After 21 days: There is usually complete regeneration of the oesophageal lining with increased basal cell layer proliferation and increased thickness of the squamous layer.

Similar changes have been reported in humans using EBRT (Coia L.R. et al., 1995). However, there are no reports on radiation changes after ILBT alone, although, this technique has a higher therapeutic effect because there is minimal penetration of radiation beyond the local area (Berry B. et al, 1989).

Hishikawa et al reported radiation changes in autopsy specimens from patients treated with preoperative EBRT and HDR ILBT (1985, 1988). They reported remarkable changes in the tumour, but not in the normal oesophagus following EBRT. However, radiation changes were seen in the normal oesophagus and tumour following EBRT and HDR ILBT. Denudation and regeneration of the epithelium were recognized. Blood vessel alterations and fibrosis with fibrinous exudate of the submucosa were severe. Fibrosis of the muscle layers was also found. Berry et al (1989) correlated the ratio of the depth of radiation damage to the depth of identifiable tumour to the local effectiveness of preoperative treatment after EBRT and ILBT. Surgery was performed 6 weeks after EBRT and HDR ILBT. Ratios above 2:4 (involving but not deeper than submucosa) were considered to indicate a good response. In our study, all patients had radiation effects extending beyond the adventitia all along the irradiated length. Therefore, lymph nodes located in the adventitial tissue around the oesophagus also showed radiation changes, as was seen in our patients. Based on the isodose curves generated from the Treatment Planning System [TPS] (Plato; Nucletron, Amsterdam, Netherlands), approximately 50% of the dose prescribed (10 Gy at 1.0 cm from the source axis) reached the adventitial tissues around the oesophagus (within 0.5 cm from the oesophageal wall or 1.5 cm from the centre of the source axis) per application of HDRILBT. In our patients, the lymph nodes were located 2 mm from the oesophageal wall in the adventitial tissue. Thus, radiation changes were seen as the lymph node received at least 50% of the dose given (20Gy in 2 fraction). Actual measurements can only be performed at

autopsy in which thermoluminescent dosimeters are placed in the adventitial tissue and the oesophagus irradiated. Thus, it is likely, based on the isodose curves generated from the TPS, that lymph nodes that are located beyond the adventitial tissue (more than 0.5 cm from the oesophageal wall) will show minimal changes due to radiation, if the dose is prescribed at 1.0 cm. Although Hishikawa et al. have suggested that the maximum dose to the oesophagus should be limited to 20 Gy by ILBT (1988), this is following a course of radical EBRT of 50 Gy and is therefore not relevant if HDR ILBT is used alone.

The proximal resection margins in 7 patients with tumour in the mid one-third of the oesophagus and the distal resection margins in 3 patients with tumour in the lower one-third of the oesophagus, showed radiation changes limited to the submucosa with minimal fibrosis. This could be explained by the fall off of radiation dose at the resection margins, which were 1-1.5 cm from the edge of the irradiated length and therefore received only 10-25% of the dose prescribed. Thus radiation effects were not observed beyond the submucosa (Table II).

Pathologic analysis of oesophagectomy specimens after EBRT for SCC have been reported. Akakura et al (1970) noted the gross appearance of such cases to usually consist of a small re-epithelialized ulcer with mural fibrosis. Microscopically, approximately 65% of their cases showed a definite cytologic radiation effect, as defined by mitotic incompetence, nuclear pyknosis,

cytoplasmic vacuolation, and/or tumour cell disappearance. We did not find any report in the literature on the acute radiation effects after HDR ILBT.

In all our cases, surgery was performed within 2 - 3 weeks of brachytherapy. Thus, typical acute radiation changes described were seen. Interestingly, the irradiated tumours in all 10 cases showed extensive keratin production as opposed to the previous biopsies, which showed minimal to negligible keratin production due to poor differentiation. This could be attributed to the induction of the keratin gene in tumour cells to differentiate towards better differentiated cells. This has been previously reported in solid tumours with the use of anti-neoplastic drugs (Melguizo G., 1995). In our patients, blood vessel walls were found to be intact with no evidence of vessel wall necrosis. This contrasts to earlier reports with EBRT (Coia L.R. et al, 1995) in which necrosis of the vessel walls is a frequent occurrence. The biological basis is inexplicable, but may possibly lead to increased tumour oxygenation and better radiation effects, as observed in the present study. Radiation effects were also observed in the adjacent peri-oesophageal lymph nodes containing metastatic tumour and even at the resection margins (which were not as extensive). Changes described previously, such as oedema and fibrinous exudate (Coia L.R. et al., 1995), were not seen in any of the patients. HDR ILBT given to our patients in the dose and fractionation of 20 Gy in 2 weekly fractions of 10 Gy each, adequately covered the local disease and peri-oesophageal lymph nodes, all of which showed radiation changes. It is not possible to comment on whether the results achieved with

tumour sterilization because the oesophagectomies were performed within 2 - 3 weeks. Tumour sterilization can only be established from autopsy after long term follow up. Thus, typical radiation changes were observed in the irradiated length of the oesophagus after HDR ILBT that were not observed after EBRT.

Expression of *MDR-1* gene product (p- glycoprotein)

Multidrug resistance phenotype, first described in 1970, is now known to be due to the presence of the transmembrane glycoprotein (p-glycoprotein) product of the *MDR-1* gene. p-glycoprotein has been associated with both intrinsic and acquired resistance to certain chemotherapeutic agents, and cells selected for resistance against one cytotoxic drug simultaneously, acquire cross resistance to a range of other cytotoxins (Yusa K. et al., 1991, Ferry D.R. and Kerr D.J., 1994, Twentyman P.R., 1986). p-glycoprotein appears to be a highly conserved membrane transport protein, and may be a common route for the export of environmentally occurring toxins. Tumours responsive to chemotherapy generally show low levels of p-glycoprotein expression, and solid tumours that are most responsive to systemic chemotherapy, such as seminomas and embryonal carcinomas, rarely display detectable levels of the protein. Tumours from patients previously treated with chemotherapy show frequent elevation of p-glycoprotein, suggesting that the MDR phenotype is induced by exposure to chemotherapy. Tumours that initially respond to chemotherapy maybe resistant on relapse, and commonly show increase in the expression of p-glycoprotein (Yuen A.R and Sikia B.I., 1994).

Darnton et al. (1995) have reported the lack of p-glycoprotein expression with response to MIC (Mitomycin C, Ifosfamide and *Cis*-Platin) chemotherapy in oesophageal SCC. These cancers responded to chemotherapy better than oesophageal adenocarcinomas; the latter showing expression of the *MDR-1* gene. However, p-glycoprotein could not be used as a predictive marker of response as tumours expressed it inconsistently with response to MIC. Saito et al. (1992) studied three cell lines from SCC, and found a pleiotropic mechanism of resistance without expression of p-glycoprotein. Robey-Cafferty et al (1990) suggested that clinical response correlated with expression of p-glycoprotein in adenocarcinomas. These authors did not match pre-treatment biopsy specimens with treated tumours.

There have been no studies on expression of p-glycoprotein in oesophageal SCC prior to and after radiotherapy. In all our 10 cases, p-glycoprotein was not expressed in either pre or post-brachytherapy tissue specimens. These findings together with those of Darnton et al (1995) indicate that p-glycoprotein expression is of no value in predicting the responsiveness of the tumour to radiotherapy and chemotherapy in SCC of the oesophagus. *MDR-1* gene does however have some value in predicting the response to treatment in adenocarcinomas. Clinically this has been demonstrated in breast carcinomas (where the expressors of this gene have been shown to have poor survival), renal cell carcinoma, cancer of the urinary bladder, colo-rectal malignancies, gastric and ovarian carcinomas (Glazer R.I. and Rohlff C., 1994, Mickisch G.H.,

1994, Scher H.I., 1992, Linn S.C. and Glaccone G., 1995, Wallner J. et al., 1993, Sekiya S. et al., 1993). Unlike chemotherapy, radiotherapy effects cell death through the formation of free radicals. This involves interaction with other atoms or molecules, particularly water, to produce free radicals that are able to diffuse and damage the critical targets. These include the DNA in the chromosome (which is the most critical target), and the nuclear membrane. Studies have shown that about 2/3rds of X-ray damage by high voltage ionizing radiation is caused to the DNA by the OH⁻ free radical (Hall E.J., 1988). The p-glycoprotein, on the other hand, acts as an ATP dependent drug efflux pump which transports drugs associated with multidrug resistance out of the cell before cytotoxic effects can occur (Kartner N. and Ling V., 1989, Gottesman M.M. et al., 1991). It is therefore unlikely that the expression of p-glycoprotein could have any bearing on responsiveness of the tumours to radiation. It is likely for this reason that alternate factors maybe associated with resistance in SCC of the oesophagus.

Expression of p53, bcl-2 and apoptosis

The effects of ILBT on oncogene expression and apoptosis have not been previously reported in clinical studies.

In the present study, most patients showed expression of p53 (8/10) immunoreactivity in the pre-operative biopsies. This was not altered following brachytherapy which was evaluated in the resected specimens, with the intensity of p53 nuclear staining being the same (3+). However, the distribution in pre

ILBT biopsies was mostly type C (>50%) whilst the post ILBT specimens showed Type B distribution (10 - 50%). The pattern of distribution was diffuse in the biopsies, and focal and peripheral in the post ILBT specimens. The centre of the tumour islands showed no expression of p53. These areas showed either necrosis or keratinization. It is suggested that ILBT induces destruction of cells expressing wild type p53, which subsequently undergoes necrosis. The surviving p53 positive cells in the periphery of tumour islands, post ILBT, may represent mutant protein, representing cells with p53 gene mutation permitting escape from growth arrest. It is further suggested that these cells may eventually outgrow the tumour population to become dominant clones. It is conceivable that these latter cells may be responsible for the high incidence of local failure and distant metastasis, seen in oesophageal cancer following radiation treatment. In the single patient without p53 expression following brachytherapy, (although the pre ILBT specimen stained p53 positive) it is probable that the tumour may have only expressed wild type of p53 which was obliterated by brachytherapy.

A high degree of p53 mutations is seen in oesophageal squamous cell carcinomas (Coggi G. et al., 1997, Wang L.D. et al., 1996). Muro et al (1996) evaluated 20 patients to determine the clinical role of p53 mutation in locally advanced oesophageal cancer treated with chemoradiotherapy. They observed that patients who did not express p53 protein did not achieve complete remission and response to the tumour. They suggested that expression of p53

may be an indicator for favorable prognosis in patients with locally advanced oesophageal cancer, treated with concurrent chemoradiotherapy. On the other hand, Puglisi et al (1996) investigated (i) the relation between immunohistochemical expression of p53 and bcl-2 protein; (ii) the rate of tumour response after neo-adjuvant chemo-radiotherapy in locally advanced squamous cell carcinoma of the oesophagus ;and (iii) whether antitumour treatment could modify the immunohistochemical expression of these oncoproteins . They reported that p53 positive immunoreaction was detected in biopsy specimens of most patients. Following preoperative chemoradiotherapy there was no difference in p53 expression in the oesophagectomy specimens. These authors suggested that anticancer agents do not seem to modify the expression of p53 and bcl-2 proteins.

Few studies have attempted to establish a relationship between p53 expression and outcome following radiotherapy. Pomp et al. (1998) evaluated the relationship of the immunohistochemically determined p53 protein status and the disease control with radiotherapy alone, for carcinoma of the oesophagus. Immunostaining for p53 protein was performed on paraffin embedded specimens in 69 patients with adenocarcinoma and SCC of the oesophagus. All patients were treated by radiotherapy exclusively, consisting of all combinations of EBRT and ILBT using two different dose levels. 54% (37/69) of the tumours showed overexpression of the p53 protein. No difference in pretreatment parameters for p53 positive and p53 negative cases was detected. In multivariate analysis p53

was significantly associated with overall survival next to weight loss, tumour stage and nodal stage. For metastasis free survival, p53 status proved to be the sole independent prognostic factor. The influence of p53 on local recurrence free survival, however, was not as strong as on overall survival and metastasis free survival. Immunohistochemically detected overexpression of mutated p53 protein in oesophagus carcinoma was thus shown to be an independent prognostic factor in a group of patients treated with radiation therapy alone.

Nabeya et al. (1995) reported on the mutational status of the p53 protein in gastric and oesophageal adenocarcinoma cell lines following chemotherapy. They observed that the wild type p53 increases after treatment with chemotherapy. They suggested that wild type p53 appears to be essential for effective chemotherapeutic response. Cell lines exhibiting mutated p53 were more resistant to the effect of chemotherapeutic drugs. A similar situation may exist in the clinical setting with brachytherapy.

In the present study, one patient did not express p53 protein after brachytherapy, although, there was p53 positive staining in the pre ILBT biopsy specimen. It is likely that the p53 may have been of the wild type, and had therefore responded to brachytherapy. The other 8 patients may have expressed mutant p53 that may have escaped the effect of brachytherapy, especially at the periphery of the tumour islands. The single patient who did not express any p53

before or after brachytherapy did not show any response and has subsequently developed recurrent disease.

Our initial findings are in concurrence with Pomp et al (1998), who observed that p53 expression was related to the outcome of the disease to treatment. However, long term followup is required before a definite association can be established on p53 expression and outcome. This is beyond the scope of this study which examines the expression of p53 following HDR ILBT.

The recent emergence of apoptosis as a mechanism of cell death has been reviewed by various authors (Haimovitz F.A. et al., 1996). Apoptosis is a death pathway of sequential biochemical events that can be induced and is constitutively expressed in many cells, even in an inactive form. The apoptosis cascade can be triggered by a variety of physiological and stress stimuli. Graeber T.G. (1996) reported that hypoxia induced apoptosis in oncogenically transformed cells and that further genetic alterations, such as loss of p53 tumour suppressor gene or overexpression of the apoptosis inhibitor protein bcl-2, substantially reduced hypoxia induced cell death. Hypoxia also selectively affected cells with defects in apoptosis, because small numbers of transformed cells lacking p53 overtook similar cells expressing wild type p53 when treated with hypoxia. Highly apoptotic regions strongly correlated with hypoxic regions in transplanted tumours expressing wild type p53, whereas little apoptosis occurred in hypoxic regions of p53 deficient tumours. Hypoxia therefore provided a

physiological selective pressure in tumours for the expansion of variants that had lost their apoptotic potential, and in particular in cells acquiring p53 mutations. Kinzler K.W. and Vogelstein B (1996) reported that the low levels of oxygen found in those parts of tumours with poor blood supply resulted in p53 dependent programmed cell death. Mutations of p53 reduced the cell death, giving the mutant cells a survival advantage over cells with intact p53. As a tumour grows to a critical size, the central portion suffers from hypoxia. This induces expression of the p53 gene which then leads to growth arrest or apoptosis. Cells that have acquired p53 gene mutation can escape the growth arrest or apoptosis. These clones will eventually outgrow the rest to become the dominant clone. Therefore, emergence of clones with p53 gene mutation may be driven by hypoxia. The molecular events that regulate the process of apoptosis have been closely linked to the identification of oncogenes and tumour suppressor genes in various neoplasias.

Expression of bcl-2 oncoprotein blocks many instances of apoptotic cell death in developing neural (Allsop T.E. et al., 1993) and lymphoid tissues (Vaux D.L. et al., 1988). It has been reported that bcl-2 can block apoptosis that usually results from treatment with ionizing radiation (Strasser A. et al., 1994) or chemotherapeutic agents (Miyashita T. and Reed J.C., 1993). Expression of p53 gene has been shown to be a positive regulator of apoptosis (Puglisi F. et al., 1996, Kernohana N.M. and Cox L.S., 1996). It has been suggested that loss of

function of p53 gene and gain in function of bcl-2 gene can produce tumours resistant to chemo- radiotherapy.

bcl-2 expression was equivocal in this series. Brachytherapy does not appear to alter the bcl-2 expression in oesophageal squamous cell carcinomas. Further, apoptosis was not present in the pre and post-brachytherapy tissue specimens. This study therefore demonstrates that oesophageal squamous cell carcinomas do not undergo apoptosis following brachytherapy. Following brachytherapy, the decrease in tumour volume is due to necrosis and maturation of the cells by induction of the keratin gene (Sur M. et al., 1996).

This, brachytherapy does not cause cell death by apoptosis, but by necrosis and maturation of the cells into better differentiated cells, which is caused by OH⁻ free radical, and the induction of the keratin gene respectively. It is possible that brachytherapy may cause destruction of cells containing wild type p53, while mutant p53 in cells located at the tumour periphery escapes the effect of brachytherapy. This maybe responsible for the high incidence of local recurrence and distant metastasis in oesophageal cancer treated with radiotherapy. There is no effect of brachytherapy on bcl-2 expression in oesophageal cancer.

Chapter 14**Conclusions**

Morphological Changes

1. Typical acute radiation changes were observed in the irradiated length of the oesophagus after preoperative HDR ILBT. These are not observed following EBRT.
2. Changes were seen at various levels of the oesophagus, with full thickness changes observed at the center of the tumour, and 1 cm from the edge of visible tumour.
3. HDR ILBT may cause the keratin gene in the irradiated cells to induce differentiation towards better differentiated cells.

Expression of *MDR-1* gene product (p-glycoprotein)

1. p-glycoprotein was not expressed in either pre or post- brachytherapy tissue specimens in this study.
2. These findings indicate that p-glycoprotein expression is of no value in predicting the responsiveness of the tumour to radiotherapy in SCC of the oesophagus.

Expression of p53, bcl-2 and apoptosis

1. Prior to HDR ILBT, there is expression of both wild and mutant p53 protein in oesophageal cancers. (antibody DO-7 detects both wild type and mutant p53)
2. Following HDR ILBT, there is destruction of cells. It is postulated that this may involve destruction of cells expressing wild type p53, whilst residual

surviving cells contain mutant p53. These cells are located at the tumour periphery and escape the effect of brachytherapy. These cells survive and may eventually become the dominant clone, responsible for the high incidence of local recurrence and distant metastasis in oesophageal cancer treated with radiotherapy.

3. There is no effect of brachytherapy on bcl-2 expression in oesophageal SCC.
4. Oesophageal SCC, do not undergo apoptosis following brachytherapy.
5. Following brachytherapy, the decrease in tumour volume is due to necrosis and maturation of the cells by induction of the keratin gene.

Chapter 15**References**

Allan D.J. Radiation induced apoptosis: its role in a MADCaT (mitosis-apoptosis-differentiation-calcium toxicity) scheme of cytotoxicity mechanisms. *Int J Radiat Oncol Biol Phys* 1992; 62: 145-52.

Allsopp T.E., Wyatt S., Paterson H.F., Davis A.M. The proto- oncogene bcl2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. *Cell* 1993; 73: 295-307

Baer R. bcl2 breathes life into embryogenesis. *Am J Pathol* 1994; 145: 7- 10.

Bakhshi A., Jensen J.P., Goldman P. Cloning the chromosomal breakpoint of t (14; 18) human lymphomas: clustering around J_H on chromosome 14 near a transcriptional unit on chromosome 18. *Cell* 1985; 41: 899- 906

Batsakis J.G., L-Nagger A.K. p53: 15 years after discovery. *Advances Anatomic Pathol* 1995; 2: 71- 88

Benson M.C., Giella J., Whang I.S., Buttyan R., Hensle T.W., Karp F., Olsson C.A. Flow cytometric determination of the multidrug resistant phenotype in transitional cell cancer of the bladder: Implications and applications. *J Urol* 1991; 146: 982- 6

Berry B., Miller R., Luoma A., Nelems B., Hay J., Flores A.D. Pathologic findings in total esophagectomy specimens after intracavitary and external beam radiotherapy. *Cancer* 1989; 64: 1833- 7

Bissonette R.P. Apoptotic cell death induced by c-myc is inhibited by bcl2. *Nature* 1992; 359: 552

Chang F. The p53 tumour supressor gene as a common cellular target in human carcinogenesis. *Am J Gastroenterol* 1993; 88: 174

Chao C.C. the, Ma C.M., Lin- Chao S. Co-amplification and over expression of two MDR genes in a multidrug resistant human colon carcinoma cell line. *Febs Lett* (Netherlands) 1991; 291: 214-8

Chen- Levy Z., Nourse J., Cleary M.L. The bcl2 candidate proto-oncogene is a 24 kD integral- membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14; 18) translocation. *Mol Cell Biol* 1989; 9: 701- 10.

Chetty R., Dada M.A., Gatter K.C. bcl2: longevity personified. *Advances Anatomic Pathol* 1997; 4: 134- 8.

Chiarugi V., Ruggiero M. Role of three cancer "mastergenes" p53, bcl2 and c-myc on the apoptotic process. *Tumori* 1996; 82: 205- 9

Chin K.V., Liu B. Regulation of the multidrug resistance (MDR1) gene expression. *In vivo* 1994; 8: 835- 41

Chou P.M., Reyes-Mugica M., Barquin N., Yasuda T., Tan X., Tomita T. Multidrug resistance gene expression in childhood medulloblastoma: correlation with clinical outcome and DNA ploidy in 29 patients. *Pediatr Neurosurg* 1995; 23: 283-91

Clayton E.S. Carcinoma of the Oesophagus. *Surg Gynecol Obstet* 1928; 46: 52

Cleary M.L., Smith S.D., Sklar J. Cloning and structural analysis of c-DNAs for bcl2 and a hybrid bcl-2 immunoglobulin transcript resulting from the t(14; 18) translocation. *Cell* 1986; 47: 19- 28.

Coggi G., Bosari S., Roncalli M., Graziani D., Bossi P., Viale G., Buffa R., Ferrero S., Piazza M., Blandamura S., Segalin A., Bonavina L., Peracchia A. p53 protein accumulation and p53 gene mutation in esophageal carcinoma. *Cancer* 1997; 79: 425-32

Colombel M., Symmans F., Gill S. Detection of the apoptosis suppressing oncoprotein bcl2 in hormone refractory human prostate cancer. *Am J Pathol* 1993; 143: 390- 400.

Cooper K., Haffajee Z. bcl2 immunohistochemistry distinguishes follicular lymphoma from follicular hyperplasia in formalin-fixed tissue with microwave antigen retrieval. J Cellular Pathol 1996; 1: 52- 6

Cooper K., Haffajee Z. bcl-2 and p53 protein expression in follicular lymphoma. J Pathol 1997; 182: 307-10

Craig R.W. The bcl2 gene family. Semin Cancer Biol 1995; 6: 85- 43.

Darnton S.J., Jenner K., Steyn R.S., Ferry D.R., Mathews H.R. Lack of correlation of p-glycoprotein expression with response to MIC chemotherapy in esophageal cancer. J Clin Pathol 1995; 48: 1064-6

Dewey W.C., Ling C.C., Meyn R.E. Radiation induced apoptosis: Relevance to radiotherapy. Int J Radiat Oncol Biol Phys 1995; 33: 781-96.

Ferry D.R., Kerr D.J. Multidrug resistance in cancer. BMJ 1994; 308: 148- 9

Flumeing J.A.C. Carcinoma of thoracic esophagus: Some notes on its pathology and spread in relation to treatment. Br J Radiol 1943; 16: 212

Freund U., Brambs H.J., Laaff H., Roth R., Bruggmoser G., Wannenmacher M. Intracavitary High Dose Rate afterloading radiotherapy in the esophagus of the pig. *Strahlenther Onkol* 1989; 165: 852- 4.

Glazer R.I., Rohlff C. Transcription regulation of multidrug resistance in breast cancer. *Breast Res Treat* 1994; 31: 263- 71

Goldblum J.R., Rice T.W. bcl2 protein expression in the Barretts metaplasia-dysplasia- carcinoma sequence. *Mod Pathol* 1995; 8 : 866-9

Gottesman M.M., Goldstein L.J., Fojo A. Expression of the multidrug resistance gene in human cancer. in : Ronison I.B. (ed). *Molecular Cellular Biology of Multidrug Resistance in Tumour cell*. New York; Plenum Press, 1991; 291- 301

Graeber T.G., Osmanian C., Jacks T., Houseman D.E. , Cameron J.K. Scott W.L., Amato J.G. Hypoxia mediated selection of cells with diminished apoptotic potential in solid tumors. *Nature* 1996; 379: 88- 91

Hall E.J. The Oxygen Effect and re-oxygenation. in *Radiobiology for the Radiologist*. 3rd ed. J.B.Lippincott Co. Philadelphia, 1988: 137- 59

Hall P.A. and Lane D.P. p53 in tumor pathology: Can we trust immunohistochemistry. *J Pathol* 1994; 172: 1-5

Hamada M., Naomoto Y., Fujiwara T., Kamikawa Y., Tanaka N. Suppressed apoptotic induction in esophageal squamous cell carcinomas expressing extensive p53 protein. *Jpn J Clin Oncol* 1996; 6: 398-404.

Harmsel B.T., Smedts F. Kruijpers J., Jeunink M., Trimbos B., Ramaekers F. bcl2 immunoreactivity increases with severity of CIN: A study of normal cervical epithelium, CIN, and cervical carcinoma. *J Pathol* 1996; 179: 26- 30.

Harris C.C., Hollstein M. Clinical implications of the p53 tumour supressor gene. *New Eng J Med* 1993; 329: 1318

Harrison D.J. Molecular mechanism of drug resistance in tumours. *J Pathol* 1995; 175: 7- 12

Hengartner M.O., Horvitz H.R. *C. elegans* gene *ced-9* protects cells from programmed cell death. *Nature* 1992; 356: 494

Hishikawa Y., Tanaka S., Miura T. Esophageal Ulceration induced by intracavitary irradiation for esophageal carcinoma. *AJR* 1984; 143: 269-73

Hishikawa Y., Mitsunobu M., Uematsu K., Miura T. Histological findings of esophageal injury induced by intracavitary irradiation. *Radiat Med* 1985; 3: 112-7

Hishikawa Y., Taniguchi M., Kamikonya N., Tanaka S., Miura T. External Beam Radiotherapy Alone or combined with High Dose Rate Intracavitary irradiation in the treatment of cancer of the esophagus: Autopsy findings in 35 cases. *Radiother Oncol* 1988; 11: 223-7.

Hishikawa Y., Kurisu K., Taniguchi M., Kanikonya N., Miura T. Small superficial esophageal carcinoma treated with high dose rate intracavitary irradiation only. *Radiology* 1989; 172: 267-70

Hockenberry D.M. bcl2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 1993; 75: 241

Hunt J.A. Squamous cancer of the oesophagus in urban South African blacks: A preliminary report of baseline studies. in: Silber W., ed. *Carcinoma of the Oesophagus*. Cape Town, South Africa: AA Balkema, 1978: 287- 311

Jung M., Notario V., Dritschilo A. Mutations in the p53 gene in radiation sensitive and radiation resistant human squamous carcinoma cells. *Cancer Res* 1992; 52: 6690-3.

Kaklamanis L. p53 expression in colorectal adenomas. *Am J Pathol* 1993; 142:

Kaklamanis L., Savage A., Mortensen N. Early expression of bcl2 protein in the adenoma- carcinoma sequence of colorectal neoplasia. *J Pathol* 1996; 179: 10- 4

Karnofsky D.A., Abelmann W.H., Craver L.F. The use of nitrogen mustards in the palliative treatment of carcinoma with particular reference to bronchogenic cancer. *Cancer* 1948; 1: 634- 69

Kartner N., Ling V. Multidrug resistance in cancer. *Science* 1989; 260: 44- 51

Kerr J.F.R., Wyllie A.H., Currie A.R. Apoptosis: A basic biological phenomenon with wider ranging implications in tissue kinetics. *Br J Cancer* 1972; 26: 239- 257

Katada N., Hinder R.A., Smyrk T.C., Hirabayashi N., Perdakis G., Lund R.J., Woodward T., Klingler P.J. Apoptosis is inhibited early in the dysplasia-carcinoma sequence of Barrett esophagus. *Arch Surg* 1997; 132: 728-33.

Kerr J.F.R., Harmon B.V. Definition and incidence of apoptosis: A historical perspective. in: Tomey L.D. and Cope F.O. (eds): *Apoptosis: The Molecular basis of cell death*. Coldspring Harbor, NY, Coldspring Harbor Laboratory Press, 1991

Kinzler K.W., Vogelstein B. Life and death in a malignant tumor. *Nature* 1996; 379: 19-20

Kneebone R.L., Mannell A. Cancer of the Oesophagus in Soweto. S. Afr Med J 1985; 67: 839- 42

Krajewski K., Tanaka S., Takayama S, Schibler M.J., Fenton W., Reed J.C. Investigation of the subcellular distribution of the bcl2 oncoprotein : residence in the nuclear envelope, endoplasmic reticulum and outer mitochondrial membranes. Cancer Res 1993; 53: 4701- 14

Landanyi M., Wang S. Detection of rearrangements of the bcl2 major breakpoint region in follicular lymphomas. Correlation of polymerase chain reaction results with Southern blot analysis. Diagn Mol Pathol 1992; 1: 31- 5.

Lane D.P. A death in the life of p53. Nature 1993; 362: 786

Linn S.C., Glaccone G. MDR 1 / p glycoprotein expression in colorectal malignancies. Eur J Cancer 1995; 31: 1291-4

Lu Q-L, Abel P., Foster C.S., Lalani E.N. bcl2: role in epithelial differentiation and oncogenesis. Human Pathol 1996; 27: 102- 10.

Mannell A., Murray W. Oesophageal cancer in South Africa- A Review of 1926 cases. Cancer 1989; 64: 2604- 8

Mascarenhas F., Silvestre M.E., Small da Costa M., Grima N., Campost C., Chaves P. Acute secondary effects in the esophagus in patients undergoing radiotherapy for carcinoma of the lung. *Am J Clin Oncol* 1989; 12: 34- 40.

McDonnel T.J., Troncoso P., Brisbay S.M. Expression of the protooncogene bcl2 in the prostate and its association with emergence of androgen independent prostate cancer. *Cancer Res* 1992; 52: 6940- 4.

McKenna W.G., Bernhard E.J., Markiewicz D.A. Regulation of radiation induced apoptosis in oncogene-transfected fibroblasts: Influence of H-ras on the G2 delay. *Oncogene* 1996; 12: 237- 45

Merendiono K.A., Maerck V.J. An analysis of 100 cases of squamous cell carcinoma II with special references to its theoretical curability. *Surg Gynecol Obstet* 1952; 94: 110

Mickisch G.H. Chemoresistance of renal cell carcinoma. *World J Urol* 1994; 12: 214- 23.

Miyashita T., Reed J.C. bcl2 oncoprotein blocks Chemotherapy-induced apoptosis in a human leukemia cell line. *Blood* 19993; 81: 151-7

Moreira L.F., Naomoto Y., Hamada M., Kamikawa Y., Orita K. Assessment of apoptosis in esophageal carcinoma preoperatively treated by Chemotherapy and radiotherapy. *Anticancer Res* 1995; 15: 639-44

Muro K., Ohtsu A., Baku N., Chin K., Oda Y., Fujii T., Hosokawa K., Yoshida S., Hasebe T. Association of p53 protein expression with responses and survival of patients with locally advanced esophageal carcinoma treated with chemo radiotherapy. *Jpn J Clin Oncol* 1996; 26: 65-9

Nabeya Y., Loganzo F., Maslak P., Lai L., Oliveria A.R., Schwartz G.K., Blundell M.L., Altorki N.K., Kelsen D.P., Albino A.P. The mutational status of p53 protein in gastric and esophageal adenocarcinoma cell lines predicts sensitivity to chemotherapeutic agents. *Int. J Cancer* 1995; 64: 37-46

Ng. I.O., Lam K.Y., Ng M., Kwong D.L., Sham J.S. Expression of P glycoprotein, a multi-drug resistance gene product, these induced by radio therapy in patients with oral squamous cell carcinoma. *Cancer* 1998; 83: 851-7

Oberhammer F., Wilson J.W., Dive C. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/ or kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J* 1993; 12: 679- 84

Ohashi K., Nemoto T., Eishi Y., Matsuno A., Nakamura K., Hirokawa K. Proliferative activity and p53 protein accumulation correlate with early invasive trend, and apoptosis correlates with differentiation grade in esophageal squamous cell carcinomas *Virchows Arch* 1997; 430: 107-15

Ohbu M., Saegusa M., Okayasu I. Apoptosis and cellular proliferation in esophageal squamous cell carcinomas: differences between keratinizing and non-keratinizing types. *Virchows Arch* 1995; 427: 271-6

Ohbu M., Saegusa M., Kobayashi N., Tsukamoto H., Mieno H., Kakita A., Okayasu I. Expression of bcl2 protein in esophageal squamous cell carcinomas and its association with lymph node metastasis. *Cancer* 1997; 79: 1287-93

Olive P.L., Frazer G., Banath J.P. Radiation induced apoptosis measured in TK6 human B lymphoblast cells using the comet assay. *Radiat Res* 1993; 136: 130- 6.

Parenti A.R., Rugge M., Horng Shiao Y., Ruol A., Bozzola L., Ninfo V. bcl2 and p53 immunophenotypes in pre-invasive, early and advanced esophageal squamous cancer. *Histopathology* 1997; 31: 430-5

Pezzeila F., Ralfkiaer E., Gatter K.C., Mason D.Y. The t(14:18) translocation in European cases of follicular lymphoma: Comparison of Southern blotting and polymerase chain reaction. *Br. J Haematol* 1990; 76: 58- 64.

Pezzella F., Turley H., Kuzu I. bcl2 protein expression in non- small cell lung carcinoma. Immunohistochemical evidence for abnormal expression and correlation with survival. New Eng J Med 1993; 329: 690 - 4

Pezzella F., Gatter K. What is the value of bcl2 protein for histopathologists. Histopathol 1995; 26: 89- 93.

Phillips T.L., Ross G. Time dose relationships in the mouse esophagus. Radiology 1974; 113: 435-40

Pomp J., Davellar J., Blom J., van Krimpen C., Zwinderman A., Quint W., Immerzeel J. Radiotherapy for esophagus carcinoma: the impact of p53 on treatment outcome. Radiother Oncol 1998; 46: 179-84

Puglisi F., Di Loreto C., Panizzo R., Avellini C., Fongione S., Cacitti V., Beltrami C.A. Expression of p53 and bcl2 and response to preoperative Chemotherapy and radiotherapy for locally advanced squamous cell carcinoma of the esophagus .J Clin Pathol 1996; 49: 456-9.

Robey-Cafferty S.S., Rutledge M.L., Bruner J.M. Expression of a multidrug resistance gene in oesophageal adenocarcinoma. Correlation with response to chemotherapy and comparison with gastric adenocarcinoma. Am J Clin Pathol 1990; 93: 1-7

Rose E.F: Cancer of Oesophagus. S Afr J Hosp Med 1978; 4: 110- 2

Saito T., Hikita M., Kohno K., Sato S., Takano H., Kobayashi M. Different sensitivities of human oesophageal cancer cells to multiple anti- cancer agents and related mechanisms. Cancer 1992; 70: 2402- 9.

Sarbia M., Porschen R., Borchard F., Horstmann O., Willers R., Gabbert H.E. p53 protein expression and prognosis in squamous cell carcinoma of the esophagus. Cancer 1994; 74: 2218-23

Sarbia M., Bittinger F., Porschen R., Verreut P., Dutkowski P., Willers R., Gabbert H.E. bcl2 expression and prognosis in squamous cell carcinomas of the esophagus. Int J Cancer 1996; 69: 324-8

Scher H.I. Systemic chemotherapy in regionally advanced bladder cancer. Theoretical considerations and results. Urol Clin North Am 1992; 19: 747- 59.

Seaman W.B. The effect of radiation of the esophagus. Radiology 1957; 68: 534-

Sekiya S., Nunoyama T., Shirasawa H., Kimura H., Kawata M., Iijima N., Sugimoto Y., Tsuruo T., Takamizawa H. Expression of a human multi drug resistance gene in human ovarian carcinoma cell lines. Arch Gynecol Obstet 1992; 251: 79- 86

Shears L.L., Ribeiro U., Kane J., Safatle- Ribeiro A. Watkins S., Posner N. Apoptosis in esophageal cancer following induction chemo radiotherapy. J Surg Res 1998; 79: 20-4

Sitas F. Blaauw D., Terblanche M., Madhoo J., Carrara H. Incidence of histologically diagnosed cancer in South Africa. 1992. National Cancer Registry of South Africa pb., South African Institute of Medical Research, Johannesburg, 1997: 22

Soejima T. Histopathological study of the esophageal injury induced by high dose rate intracavitary irradiation. Experimental study on the rabbits. Nippon Igaku Hoshasen Gakkai Zasshi 1992; 52: 1168-76

Soini Y., Virkajarvi N., Raunio H., Paakko P. Expression of p-glycoprotein in hepatocellular carcinoma: a potential marker of prognosis. J Clin Pathol 1996; 49: 470-3

Spiers A.S. Multidrug resistance, the MDR gene, and the law of maximum perversity as it applies to Oncology: an Hypothesis. *Hematol Oncol* 1994; 12: 155- 61

Strasser A., Harris A.W., Jacks T., Cory S. DNA damage can induce apoptosis in proliferating lymphoid cells via p53- independent mechanisms inhibitable by bcl2. *Cell* 1994; 79: 329-39

Sur M., Sur R.K., Cooper K., Levin V., Bizon D., Dubazana N. Morphologic alterations in oesophageal squamous cell carcinoma after preoperative high dose rate intraluminal brachytherapy. *Cancer* 1996; 77: 2200-5

Sur R.K., Donde B., Levin C.V., Mannell A. Fractionated High Dose rate intraluminal brachytherapy in palliation of advanced oesophageal cancer. *Int J Radiat Oncol Biol Phys* 1998; 40: 447-53

Twentyman P.R., Fox N.E., Wright K.A., Bleehen N.M. Derivation and preliminary characterization of Adriamycin resistant lines of human lung cancer cells. *Br. J Cancer* 1986; 53: 529- 37

Vaux D. Towards and understanding of the molecular mechanisms of physiological cell death. *Proc Nat Acad Sci.* 1993; 90: 786

Vaux D.L., Cory S., Adams J.M. bcl2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre- B cells. *Nature* 1988;335: 440-2

Wallner J., Depisch D., Gsur A., Gotzl M., Haider K., Pirker R. MDR1 gene expression and its clinical relevance in primary gastric carcinomas. *Cancer* 1993; 71: 667- 71

Weis- Novack D., Korsmeyer F.J. bcl2 protein expression during murine development. *Am J Pathol* 1994; 145: 61- 73.

Yuen E.R., Sikia B.I. Multidrug resistance in lymphomas. *J Clin Oncol* 1994; 12: 2453- 9.

Yusa K., Sato W., Yamazaki A., Tsukahara S., Tsuruo T. Cross- resistance of human multidrug- resistant cells to mitomycin-c. *Anticancer Res* 1991; 11: 1301-4

Zenbetti G.P., Levine A.J. A comparison of the biologic activities of wild type and mutant p53. *Faseb J* 1993; 7: 855

Author Sur M

Name of thesis Morphological Alterations And The Effect On Tumour Suppressor Gene Expression Induced By High Dose Rate Intraluminal Brachytherapy In Oesophageal Carcinoma Sur M 1999

PUBLISHER:

University of the Witwatersrand, Johannesburg

©2013

LEGAL NOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed, or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page) for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.