Chapter 1: Molecular mechanisms of oesophageal cancer

1.1 Prevalence

Cancer of the oesophagus ranks as the ninth most common malignancy worldwide and recent evidence shows that its incidence is rising (Munoz, 1993). Prognosis of this disease is poor with an overall 5-year survival rate of less than 10 %. There are two major types of oesophageal cancer: squamous carcinoma and adenocarcinoma. The incidence of oesophageal carcinoma is increasing, and is most prevalent in the USA (Stoner and Gupta, 2001).

Universally, oesophageal cancer is more common in men than in women, with decreasing sex ratios in higher-risk areas and vice versa for lower-risk areas. The incidence rate for oesophageal cancer increases with age, with the lowest occurring at age 30 and the highest at age 70. The highest mortality rates are found in China accounting for 26.5 % in males and 19.7% in females (Li, 1982). Oesophageal cancer in South Africa is the second most common cancer among all South African men combined and the most common cancer in black males. Regions of South Africa, like the Transkei, have recorded a rise in the incidence of oesophageal cancer from 16 per 100, 000 before 1970 to over 40 per 100, 000 thereafter (Von Zeynek, 1973). To date, high incidence areas (expressed as crude incidence per 100,000) include: China (21 per 100,000), South America (13 per 100,000), Western Europe (11 per 100,000), South Africa (10 per 100,000), Japan (9 per 100,000) and the former Soviet Union (8 per 100,000) (Pickens & Orringer, 2003).

1.2 Aetiology

Oesophageal cancer is a multifactorial disease; no single agent has been identified thus far as the cause of oesophageal cancer. Smoked food has a high content of nitrosoamines and nitrites (Sales and Levin, 1985). Methyl alkyl nitrosamines appear to be specific inducers of carcinoma of the oesophagus, regardless of its route of administration. Smoking is also believed to play a role in oesophageal carcinogenesis. Alcohol is also a major aetiological factor in oesophageal cancer. Fungal toxins and spices are believed to have a positive correlation with oesophageal cancer. It is suspected that home brewed beers and other spirits prepared in African countries cause oesophageal cancer due to contamination with mycotoxins occurring during preparation (Dlamini and Bhoola, 2005). The fungus, *Fusarium moniliforme*, is believed to play a role in the toxicity of maize, and when consumed, these mycotoxins are believed to play a role in the development of cancer. Human papilloma virus (HPV) serotypes 16 &18 have also been found to be associated with the development of the disease.

1.3 Pathology of Oesophageal cancer

The sequential change from normal to malignant oesophageal carcinoma is as follows:

Normal → Metaplasia → Dysplasia (low and high grade) → Carcinoma in situ (CIS) → Invasive Carcinoma

1.3.1 Normal histology of the oesophagus: A schema of the normal oesophageal histology is shown in Figure 1. The lumen of the normal oesophagus is

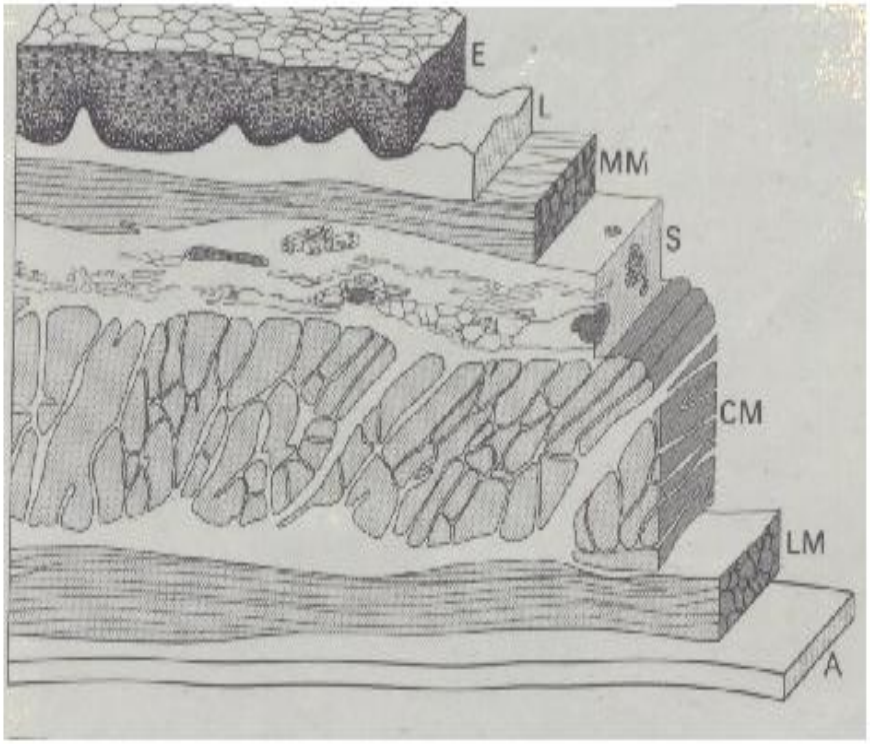
surrounded by the mucosa, submucosa, muscularis propria, and external connective tissue. The mucosa is composed of stratified, non-keratinising squamous epithelium, the lamina propria, superficial submucosal glands, and muscularis mucosa, which produce local movements and folding of the mucosa. The submucosa consists of deep submucosal glands, which aid lubrication, and the muscularis contains inner circular and outer longitudinal layers of smooth muscle, which is the basis of peristaltic contraction (Wheater et.al, 1979). The submucosa and muscularis propria contain lymphatic channels, and this lymphatic drainage accounts for the spread of primary oesophageal carcinomas (Troncoso and Riddell, 1985). The adventitia is the outer connective tissue that conducts the major vessels and nerves (Wheater et.al., 1979).

1.3.2 Squamous carcinoma: Carcinoma in situ of squamous epithelium is characterized by the change in the thickness of the mucosa being replaced by epithelial cells that fail to mature as the surface is approached, and cytologically by pleomorphic nuclei that are usually hyperchromatic and have lost their usual orientation. Mitoses can also be seen at any level and sometimes cells keratinize individually. Dysplasia entails similar changes but the cells mature as the surface is approached. In low-grade or mild dysplasia, only the basal layer of the mucosa may be involved. High-grade dysplasia shows disorganized, irregular, and fast growth that looks different from the normal cells growth pattern and surpasses the mucosal basal layer. Invasive carcinoma may arise from mucosa exhibiting dysplasia or carcinoma in situ, though not always the case.

1.3.3 Adenocarcinoma: Adenocarcinoma can arise from the oesophageal mucosal glands, submucosal glands, heterotopic gastric mucosa or from the columnar-lined Barrett's oesophagus (BE). BE is a metaplastic change of the oesophageal epithelium from squamous to columnar mucosa, which is associated with repeated episodes of chronic gastro-oesophageal reflux (GORD) (Van Der Woude *et al.,* 2002). It has been shown that 86 % of primary oesophageal adenocarcinomas originate from BE (Haggitt, 1978).

The gross appearances of adenocarcinomas are similar to those seen in squamous carcinoma; extensive infiltration of the wall and lymphatic spread are common at the time of diagnosis (Troncoso and Riddell, 1985). Low-grade dysplasia of BE includes gland architecture that is not abnormal, but the epithelial cells have enlarged hyperchromatic nuclei that occupy the lower half of the cells, and the cellular abnormalities extend to the surface of the mucosa. High-grade dysplasia involves glands that are irregular and tightly packed, enlarged and irregular cell nuclei that exhibit abnormal chromatin patterns. Early invasive adenocarcinoma is recognized by the presence of angulated glands, irregular groups of cells or single cells in the lamina propria, and a desmoplastic stromal response (Antonioli & Wang, 1997).

Normal Oesophagus:



E=Epithelium
L=Lamina propria
MM=Muscularis mucosa } Mucosa

S=Submucosa } Submucosa

CM=Circular Muscle Layer
LM=Longitudinal Muscle Layer } Muscularis

A=Tunica adventitia } Adventitia

Figure 1: Explanatory diagram of the longitudinal section of the normal oesophagus. (Wheater et. al., 1979)

1.4.1 Molecular genetics of oesophageal cancer

As oesophageal carcinogenesis is poorly understood, much research is being carried out to understand the precise mechanisms causing the metaplasia-dysplasia-carcinoma in situ sequence of oesophageal carcinoma at a molecular level (Dureja, 2002). It is known that tumour suppressor genes, oncogenes, and apoptotic genes are involved in the initiation and development of oesophageal cancer, but to date no gene directly related to oesophageal cancer has been identified (Cui *et al*, 2003).

Many candidate genes and their role in the development of oesophageal cancer are still to be revealed before a human oesophageal carcinogenesis model can be developed. Key tumour related genes and their specific roles in the development of oesophageal cancer are discussed in more detail.

1.4.1.1 Apoptosis-genetic regulation of oesophageal cancer

Each living cell undergoes cell cycle regulation where an essential assessment mechanism occurs to determine the status of the cell, whether it is healthy enough to proceed to the next stage of cycle or whether it should commit suicide. This willingness to die for the well being of the entire body is known as apoptosis (Reed, 2000).

Apoptosis is generically defined as a programmed cell death that eliminates unwanted cells and is essential for the homeostatic maintenance of an organism. Like cell proliferation, cell death needs to take place in order for normal development to take place. It has been found that elevated levels of apoptosis, as well as low levels of apoptosis, can have a detrimental effect on the organism.

This impaired regulation of apoptosis leads to a variety of pathological conditions, such as neurodegeneration, autoimmunity, chronic inflammation, AIDS, and cancer (Meier et al., 2000).

In the eukaryotic cell cycle there are four stages, the Gap-1 checkpoint (G-1), DNA Synthesis (S-phase), Gap-2 checkpoint (G-2), and the Mitotic phase (M), view figure 2.

Cyclin-cdks regulate the cell through each phase. The G-1 and G-2 checkpoint phases are where the cell assesses whether to proceed to the next stage or commit suicide. The cell cycle results in either of two processes, which is to proceed to the next stage (cell division) or to induce apoptosis (cell suicide), which is determined by the p53 protein (Yuan et al., 2000).

There are two pathways by which a cell commits suicide: (1) the intrinsic or mitochondrial pathway and (2) the extrinsic or death receptor pathway.

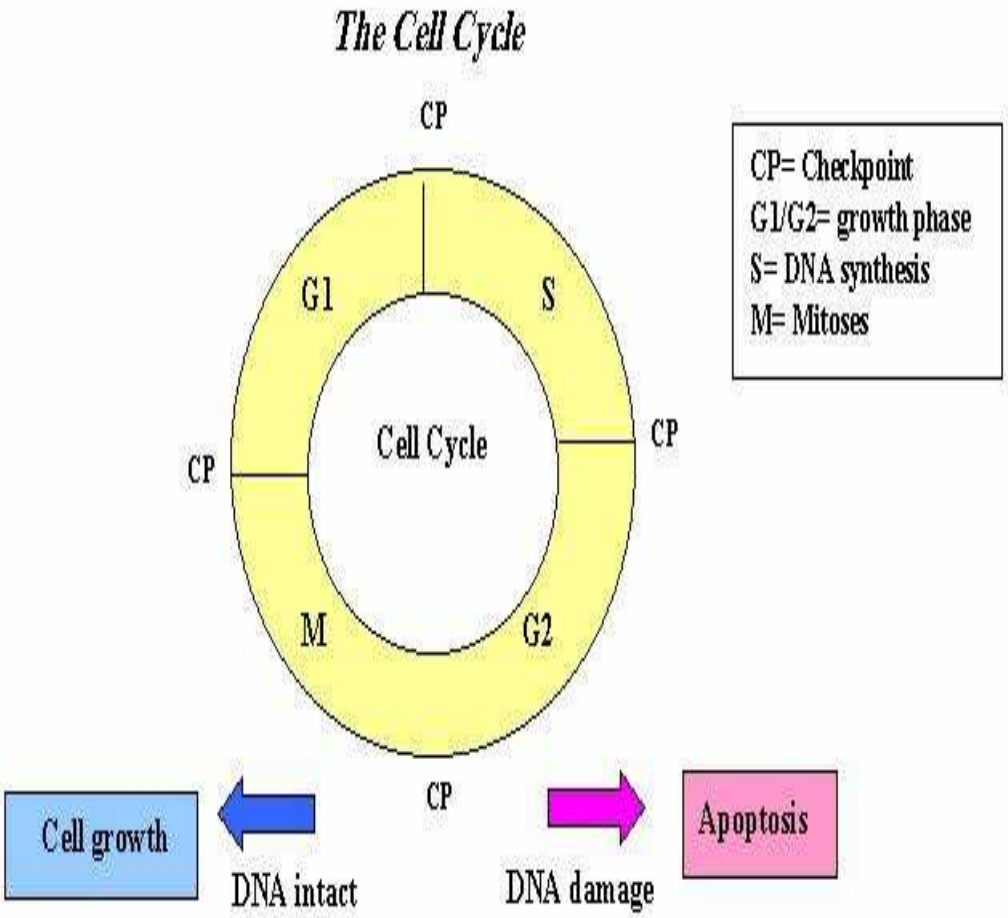


Figure 2: simple schematic diagram of normal cell cycle progression leading to mitosis (M) or cell growth (if DNA is intact) or apoptosis (if DNA damage occurs).

1.4.1.1 Intrinsic/ Mitochondrial Pathway

In the mitochondrial pathway, apoptosis is accompanied by an increase in mitochondrial permeabilization (view figure 3). Members of the Bcl-2 family play an important role in the regulation of apoptosis induced by the intrinsic pathway. This family contains proapoptotic members (Bax, Bad and Bak) and antiapoptotic members (Bcl-2 and Bcl-x1), which induce or prevent the release of apoptogenic factors, respectively. Apoptogenic factors include cytochrome *c* or Smac/DIABLO, which is released from the mitochondrial intermembrane space into the cytosol (Schuler *et al.,* 2001). In a healthy cell the outer membranes of the mitochondria express the Bcl-2 protein on its surface and Apaf-1 binds to it. When internal damage occurs in the cell, Apaf-1 is released. This allows cytochrome c to leak out and form a complex with Apaf-1, cytochrome c, caspase-9 and ATP, which is called the apoptosome. The apoptosome then aggregates in the cytosol where Apaf-1 releases the activated caspase-9, which cleaves and activates the effector caspases causing a cascade of proteolytic events. Structural proteins and DNA become degraded and this leads to phagocytosis of the cell.

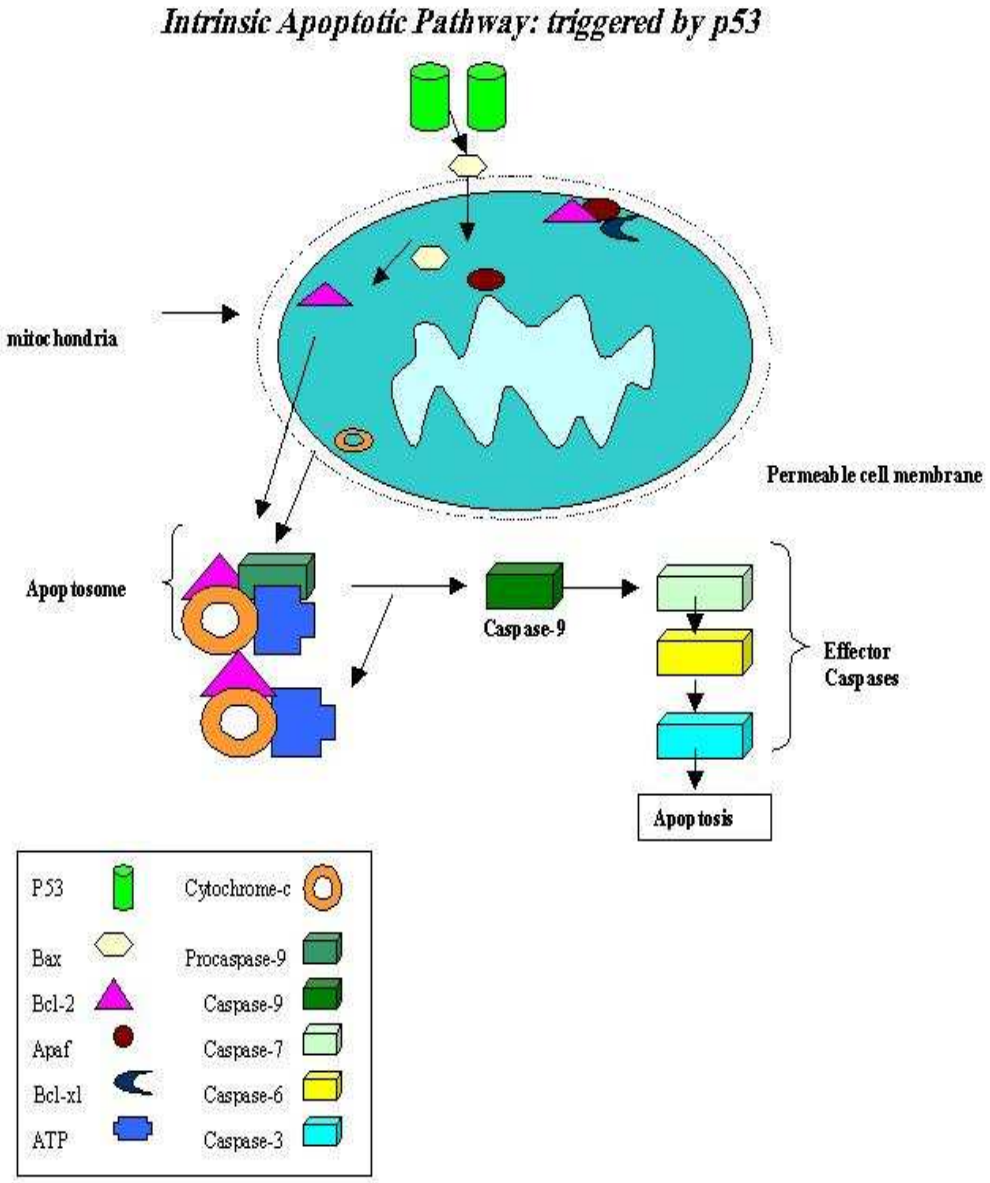


Figure 3: Intrinsic apoptotic pathway, triggered by p53

When internal damage occurs in the cell, p53 signals Bax to induce apoptosis. Bax then tanslocates into the mitochondrial membrane, displaces Apaf-1 and binds to the Bcl-2-Bcl-x complex. The mitochondrial membrane then becomes permeable,and cytochrome-c and Apaf-1 is allowed to translocate out of the mitochondria into the cytosol, where it forms a complex with ATP and procaspase-9. This complex is known as the apoptosome. Procaspase-9 is cleaved and is released from the complex and is now active. Active caspase-9 cleaves the effector caspases 7, 6 and 3 respectively, causing a cascade of proteolytic events, whereby apoptosis is carried out. Degradation of structural proteins and DNA is the result, leading to phagocytosis of the cell.

1.4.1.2 Extrinsic/Death Receptor Pathway

In the death receptor pathway, death activator proteins bind to their respective death receptors on the cell surface. Receptor ligation is followed by the formation of the death inducing signalling complex (DISC), which is composed of the adaptor molecule FADD (FAS Associated Death Domain) and caspase 8. DISC either directly cleaves and activates the effector caspases, or indirectly activates the down–stream caspases through cleavage of the BH3 protein Bid, leading to the engagement of the intrinsic pathway of apoptosis (Schuler *et al.*, 2001).

Death receptors include FAS, TNFR1, CAR1, DR3, DR4 and DR5. Their respective ligands are synthesised and they transmit apoptotic signals across the receptors, through specific pathways. Signals are transmitted from one protein to the next by interactions through certain homology domains including the "death domain" (DD)," death effector domain" (DED) and the "caspase recruitment domain" (CARD).

There are two proposed apoptosis pathways of Fas, one that is activated by the FADD/Caspase-8/-3 pathway and the second pathway activated by Daxx/JNK, view figure 4. FADD/Caspase-8/-3 pathway involves Fas-L bound to the FAS receptor, which results in the clustering of death domains in the receptor, and then FADD (Fas associated death domain) binds to the clustered death domains resulting in an activated Fas receptor. Once activated, the pro-domain of caspase-8 is bound which leads to its oligomerization, which in turn leads to the formation of DISC. Once DISC forms, caspase-8 is able to activate itself by self-cleavage and thereafter activates downstream effector caspases such as caspase-9 and –3.

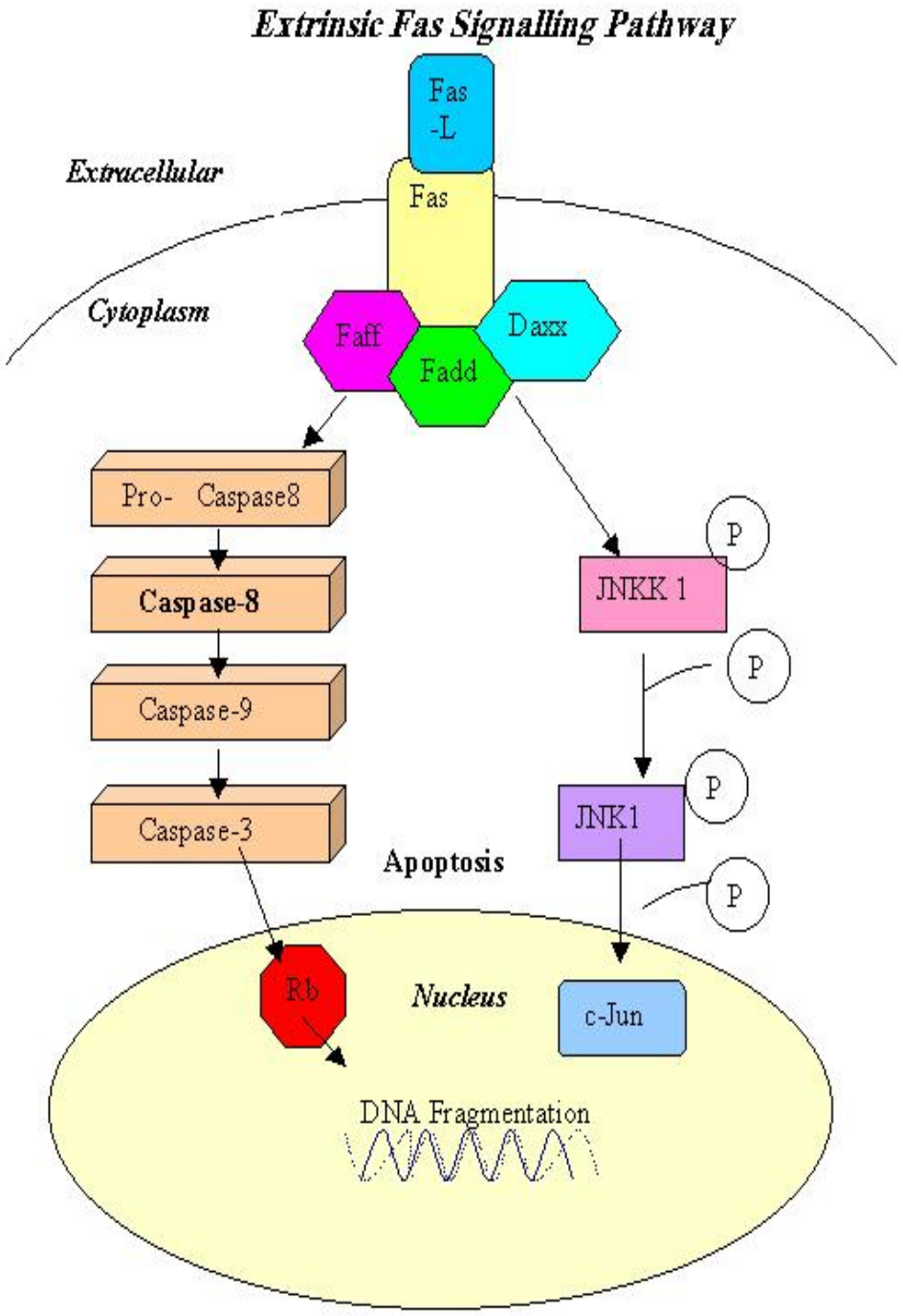


Figure 4: Fas Apoptotic Signalling Pathway

There are two pathways for Fas induced apoptosis, 1- activated by theFadd/caspase-8/9/3 pathway and 2- by the Daxx/JNK pathway.

The Daxx/JNK induced pathway occurs when Daxx binds to Fas receptor. Daxx does not contain a death domain, but when over-expression of the ligand occurs, JNK (Jun-N-terminal Kinase) becomes activated, and induces Fas apoptosis.

1.4.2 HPV and squamous oesophageal cancer cell transformation

Human papillomavirus (HPV) has been found to be the cause of many types of cancers. There are more than 70 different HPV species that have been identified and divided into two groups, high and low risk HPVs. The high risk HPVs are the cancer causing types (type 16 and 18), and the low risk types give rise to warts and benign lesions (examples include types 6, 11, and 33) (Villa, 1997).

It has been shown that many HPV types, including the low risk types, have been found in oesophageal cancer tissues (Matsha, *et al,* 2002). Particular studies also did not detect the presence of HPV in oesophageal cancer tissues, and the argument is that the detection methods utilized are not sensitive enough. HPV has been associated with oesophageal cancer and its high frequency implicates the possibility of being an aetiological factor in this disease, but definite evidence is still required. (Matsha *et a.l.,* 2002).

Shen *et al.* showed an immortal epithelial cell line from an embryonic oesophagus, transformed to a malignant cell type, by HPV-18 E6 and E7 oncoprotein infection. This infection was shown to induce chromosomal aberrations, telomeric shortening and telomerase activity, and expression of certain genes. It was shown that a crucial event in the process of immortalization may be chromosomal instability and preneoplastic aneuploidy at an early stage, and in the progressive developmental stages, increased telomerase activity and amplification of some genes such as *c-myc, ras, bcl-2 and p53.* HPV genome

inserts and integrates with the chromosomes of the host, causing confusing karyotypes and chromosomal changes and liabilities in genetic characteristics. It has also been shown that HPV E6 and E7 proteins are able to activate telomerase activity. Thereafter oncogenes are activated (c-myc and ras) and tumour suppressor gene expression altered (p53 and Rb), resulting in loss of cell growth control and apoptosis, causing cellular transformation (Shen *et al*., 2001).

Early Stage: ***Metaplasia*** → Chromosomal instability (LOH & Amplification)

Progressive stage: ***Dysplasia*** → 1. Increased Telomerase activity
2. Oncogene activation
3. Tumour suppressor gene alteration

1.4.3 Telomerase activity

Repetitive telomere sequences are at the ends of eukaryotic chromosomes to protect the ends from damage and rearrangement. Progressive shortening of telomeric sequences is associated with cell division and DNA replication (Li *et al*., 2003). In a study carried out by Li et al, it was shown that increased telomerase activity was associated with the progression of squamous oesophageal carcinoma. Grade I (metaplasia) showed a 60% telomerase activity and Grade II (dysplasia) and III (CIS) showed 90 ad 91% telomerase activity. This result supported other reports of telomerase activity being correlated with oesophageal squamous carcinoma differentiation and lymphatic metastasis. The poorer the differentiation, the higher the telomerase activity that occurred, and also patients with lymphatic metastasis showed a higher telomerase activity than those without.

1.4.4 Oncogenes

The oncogenes most frequently activated in oesophageal cancer are cyclin D1, c-erbB1 & 2, c-myc, c-ras, Int-2/hst-1, and EGFR (Wang, *et al*, 2003). Frequent mechanisms activating these oncogenes include point mutations, amplification, rearrangement and over-expression, with amplification and over-expression being the most common (Wang, *et al*, 2003).

1.4.4.1 Cyclin D1

Cyclin D1 binds to and activates CDK4 and CDK6, which can then phosphorylate the tumour suppressor protein, retinoblastoma (pRb) (Kato *et al*, 1993). It was shown that altered expression of the cyclin D1 and Rb genes play a role in human oesophageal cancer. Cyclin D1 gene amplification was found in about 32% of oesophageal tumours analyzed, and 92% of these tumours showed cyclin D1 over-expression, as well as normal pRb expression levels (Jiang *et al*, 1993). In contrast the tumours that did not show cyclin D1 amplification, did not appear to have pRb expression, suggesting that an inhibitory effect of pRb on cell cycle progression can be abrogated during tumour development either by loss of expression of Rb or by elevated expression of the Cyclin D1 (Jiang *et al*, 1993), view figure 7.

1.4.4.2 Frat1

Frat1 is a proto-oncogene and it promotes carcinogenesis through activation of the WNT- ß-catenin – TCF signalling pathway (Saitoh *et a.l.*, 2002). It is known that over-expression of Frat1 leads to the dissociation of GSK-3ß from Axin to inhibit ß-catenin phosphorylation. Unphosphorylated ß-catenin is not recognized by the ubiquitin ligase complex including ßTRCP2, and is stabilized and translocated to the nucleus. ß-catenin-TCF complex activates transcription of WNT target genes, such as c-Myc, WISP1, WISPf2 and cyclin D_1. In one study Frat1 expression was found to be relatively high in human oesophageal cancer cell lines (Saitoh *et al.*, 2002). It is therefore proposed that the up-regulation of Frat1 mRNA, not only in oesophageal cancer but several other malignancies, might promote carcinogenesis through the activation of the WNT- ß-catenin- TCF signalling pathway (Saitoh, 2002).

1.4.5 Tumour Suppressor genes

Tumour suppressor genes are inactivated by genetic or epigenetic changes such as point mutations, deletions (LOH), promoter methylation, abnormal splicing, deregulation of imprinting and haploinsufficiency (Kuroki *et al*, 2002). LOH (loss of heterozygosity) causing inactivation of most candidate tumour suppressor genes have been found on the critical regions of chromosomes 1p, 3p, 4, 5q, 9, 11q, 13q, 17q and 18q. Chromosome region 17q25.2-25.3 carries the autosomal dominant oesophageal disorder, tylosis (Stoner & Gupta, 2001).

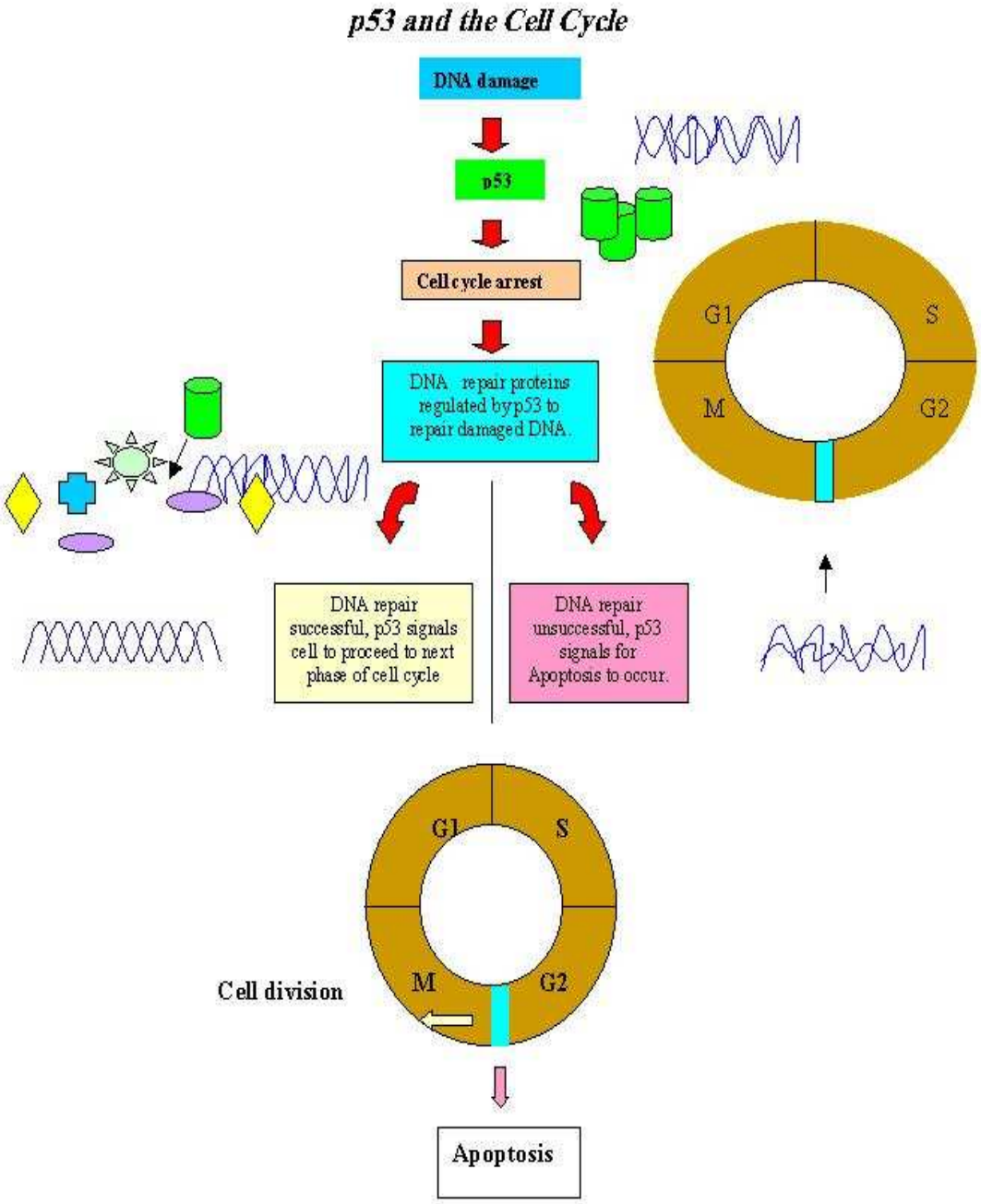


Figure 5: Role of p53 in cell cycle: mediator between cell growth and death

(i) During each checkpoint of the cell cycle, the cell cycle is arrested for p53 (and other tumour suppressor genes) to check if DNA damage has occurred. If DNA damage has occurred p53 halts the cell cycle at the checkpoint and signals appropriate proteins to repair damage. Once damage has been repaired, p53 signals cell to proceed to next phase of cell cycle.
(ii) If DNA damage is irreparable, p53 signals apoptotic inducing factors (AIF), like the Bcl-2 family, to induce apoptosis, rather than proliferate and cause damage to the organism, e.g. cancer.

1.4.5.1 p53

p53 (a protein with molecular weight ~53kD) is a tumour suppressor that halts progression in both the G_1 and G_2 phase of the cell cycle to assess DNA damage, view figure 5. If damage has occurred p53 determines whether damage can be repaired and if so, triggers cell cycle arrest until the damage is repaired. If damage is irreparable, p53 triggers apoptosis, see figures 3 and 5. Cell cycle therefore results in either of two processes, either to proceed to the next stage (DNA synthesis or cell growth) or to induce apoptosis (cell suicide). Mutations in these checkpoint genes result in defective proteins and unsuccessful checkpoints occur. Cells then complete mitoses but aberrant daughter cells arise which lead to disease where uncontrolled cell growth occurs, such as cancer.

Accumulation of p53 in the normal oesophagus, suggested that the loss of suppressor function p53 might be an early event in carcinogenesis of the oesophagus (Stoner and Gupta, 2001), view figure 6.

Mutations in codons 175, 248 and 273 of p53 are considered to have growth advantages which progress to invasive squamous cell carcinoma and occur most frequently (Wang, *et al*, 2003). Whereas codon 158, though considered as being highly sensitive to mutagenesis, does not have the same carcinogenic transformation properties (Hainut *et al*, 1997).

Somatic alterations of p53 abolish its ability to activate p21, Bax, and PIG3 reporter systems, thus altering cell cycle control and apoptosis overall (Robert *et al*, 2000), view figure 7.

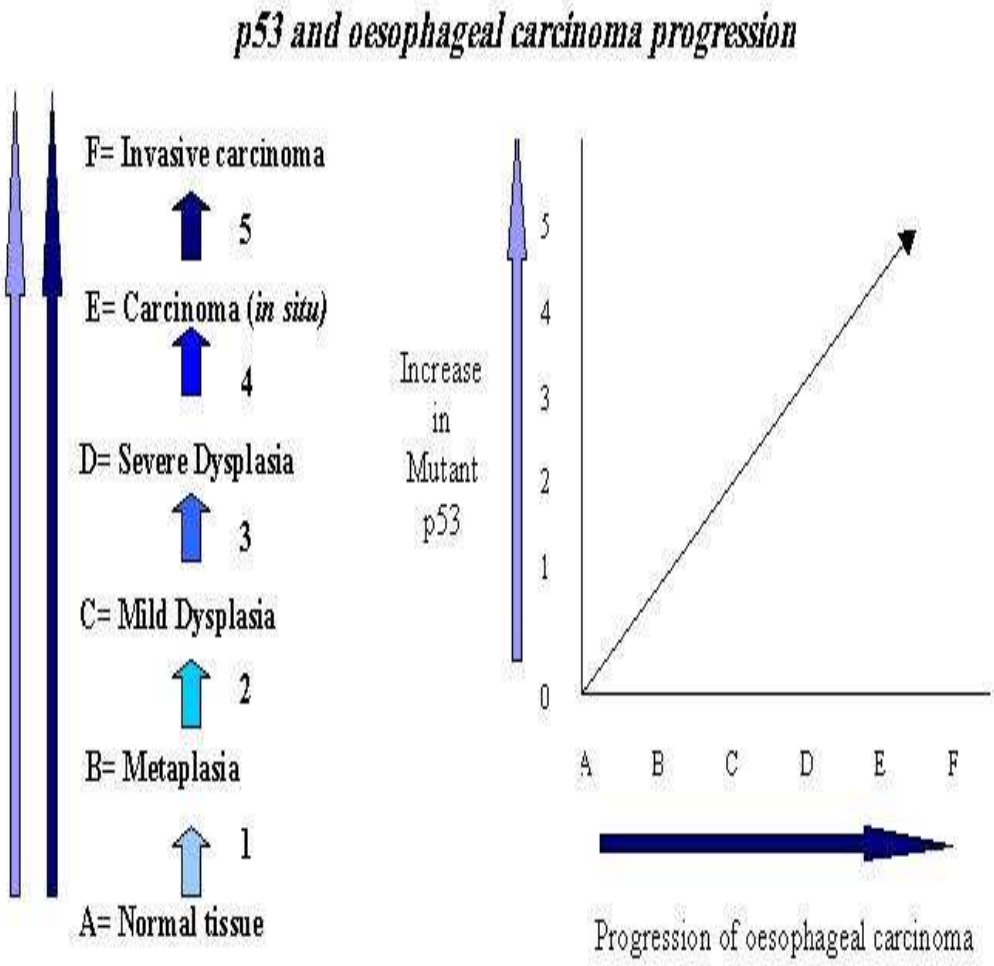


Figure 6: Oesophageal cancer and mutant p53

Progression of oesophageal cancer is associated with an increase in mutant p53. It is believed that mutation in p53 and Rb is the hallmark of oesophageal cancer, and these events are the initiation of an entire cascade of molecular events that leads to the progression of this disease.

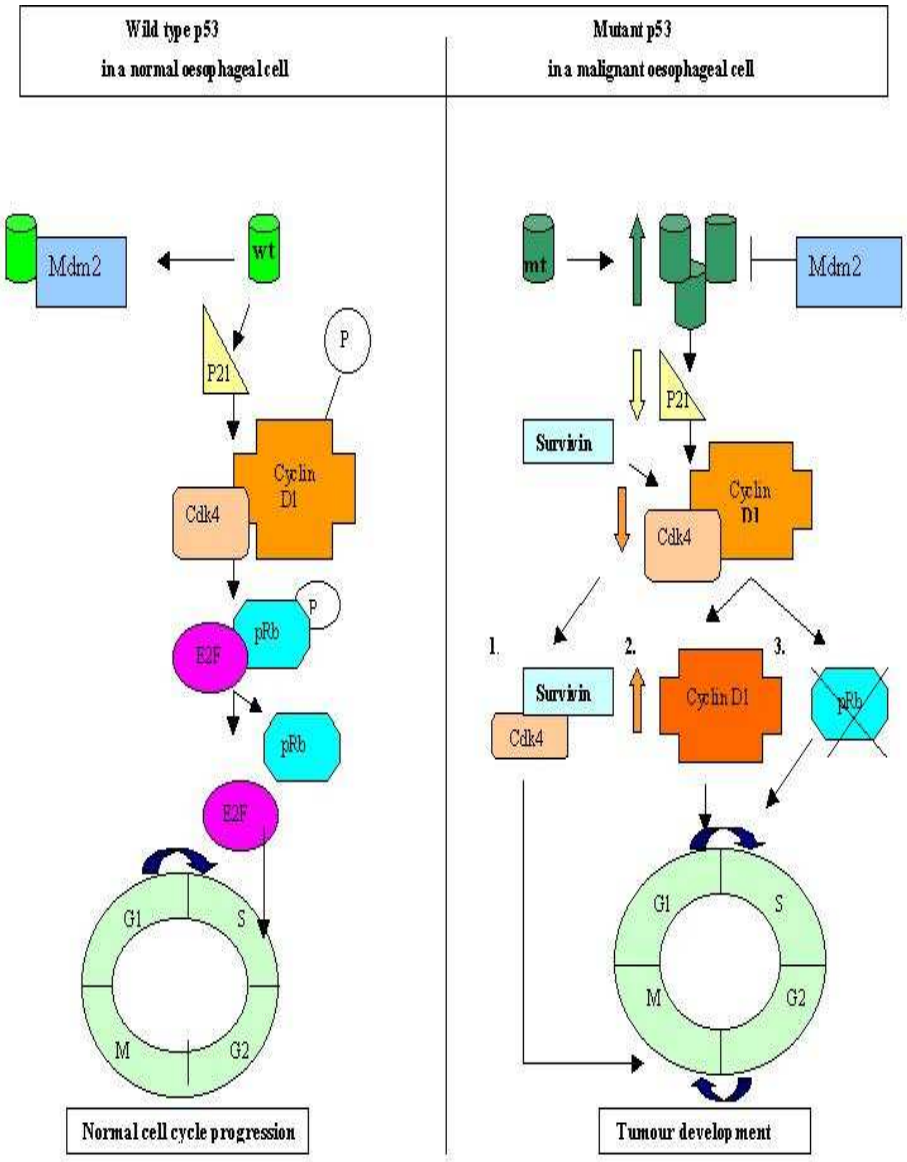


Figure 7: Wildtype vs. mutant p53, and the affect it has on the cell cycle, G1/S phase.

Wild-type p53 activates p21 which inturn activates the cyclinD1/cdk4 complex by hypophosphorylation. Activated cyclinD1/cdk4 then hypophosphorylates the pRb/E2F complex, causing the release of E2F that translocates to the nucleus, leading to transcription of genes responsible for G1 progression to the S phase and also progression from G2 to M phase.

Mutant p53 affects on cell cycle progression leads to abnormal cell growth, like cancer. Mutant p53 regulates p21 and causes it to have negative regulatory affects on cyclins therefore resulting in negative affects on normal cell cycle progression. Once the mutant p53 protein activates p21, p21 interacts negatively with the cyclin/cdk4 complex. Overexpression of cyclin D1 is one result that occurs and has a negative affect on the cell cycle, encouraging cell progression to S phase in oesophageal tumours. In contrast, the tumours that did not show cyclin D1 amplification did not appear to have pRb expression, suggesting an inhibitory effect of pRb on cell cycle progression can be abrogated during tumour development either by loss of expression of the Rb gene or by elevated expression of the Cyclin D1 gene. A protein found to negatively regulate the cell cycle at the G2/M stage was Survivin, by interacting with CDK4 and displacing p21, and ultimately encouraging tumour development.

Furthermore, another protein found to negatively regulate the cell cycle, at the G2/M stage was Survivin. Survivin interacts with CDK4 and displaces p21, and ultimately encourages tumour development.

It was determined that 85% of the p53 mutations in oesophageal adenocarcinoma occurred as GC→AT transitions, with 69% at the CpG dinucleotides (Wang, *et al,* 2003). G→A mutation pattern may have resulted from DNA methylation induced by nitrosamine (Wang, *et al*, 2002).

p53 mutated oesophageal cancer is one of the malignancies that have been recognized as a conventional chemotherapy-resistant disease (Kihara et al, 2000).

1.4.5.2 Retinoblastoma (Rb)

Rb is a nuclear phosphoprotein that plays a role in cell cycle regulation. Hypophosphorylated Rb in the cell prevents cell progression when the cell is being assessed, and upon phosphorylation the Rb protein releases the E2F transcription factor that allows for the expression of important cell-cycle control genes.

LOH of the Rb gene was found to be correlated with the loss of pRb protein expression and associated with p53 alterations in human oesophageal cancer (Xing, *et al*, 1999). It is suggested that associated Rb and p53 inactivation may be the major events in the development and progression of oesophageal cancer, due to the greater selective advantage of the affected cells. It is also believed that other genes in the Rb and p53 pathways contribute to the malignant transformation of the cells; in the majority of cases an alteration of p16, p15 or even both were shown to occur (Xing *et al*, 1999).

1.4.5.3 DLC1

DLC1 (deleted in lung cancer 1) is a putative tumour suppressor gene identified by Daigo *et al*. DCL1 is a commonly deleted region at 3p21.3 as defined by LOH studies in lung cancer, and aberrant splicing of this potential gene was found in a third of oesophageal, lung and renal cancers (Daigo *et al.*, 1999). Normal DCL1 cDNA was introduced into several cancer cell lines and caused significant suppression of growth, indicating that aberrant DCL1 transcripts may play a critical role in the carcinogenesis of those tissues (Daigo *et al*., 1999). The encoding protein (M_r 166) unfortunately has no significant homology to known proteins and so putative functional annotations could not be made. It has been found though that DCL1 protein has 54 phosphorylation sites, 27 of which are casein kinase (CSNK) II phosphorylation sites, and is localized in the cytoplasm (Daigo *et al*., 1999). A ubiquitous, messenger-independent serine/threonine kinase, CSNKII is localized in both the cytoplasm and nucleus and functions as a protease (Voss, *et al.,* 1991), and so it is supposed that DCL1 may act as a downstream gene in the serine/threonine kinase pathway (Daigo *et al*., 1999).

1.4.5.4 p16INK4a and p15INK4b: These are tumour suppressor genes and are localized to 9p21. This region has been shown to undergo hemizygous or homozygous deletion in a variety of tumour types (Xing *et al*, 1999). These two genes encode two cyclin dependent kinase (CDK) inhibitors which negatively regulate the cell from G1-S phase in proliferating cells, contributing to active pRb maintenance (Morgan, 1995). During the G1-S phase p16INK4a binds and inhibits CDK4/6 activity (Xiong *et al*, 1993) and p15INK4b binds to cyclin D-dependent kinase and prevents p27 association (Retnisdottir and Massague 1997). p27 then binds to the E-CDK2 complex, blocking the cell cycle at the G1-S

boundary, risking cells to abnormally proliferate (Retnisdottir and Massague, 1997). Aberrant methylation of p16INK4a has been found to be a key feature in human carcinogenesis and although aberrant methylation of p15INK4b also occurs, it is found to occur less frequently in human oesophageal cancer in Lixian, China (Xing *et al*, 1999). A common feature of p15INK4b is homozygous deletion, which also takes place in p16INK4a.

1.4.5.5 Familial adenomatous polyposis mutation gene (APC) and Mutated gene in colorectal cancer (MCC):

APC is believed to be a tumour suppressor gene (Knudson, 1995), and like MCC it is located to chromosome 5q21 region. APC is mutated in Familial adenomatous polyposis (FAP) and like MCC again, it has been shown to play a role in the pathogenesis of colorectal cancer and also lung cancer (Knudson, 19995) (Ashton-Rickardt *et al*, 1989). It has been shown via linkage analysis that LOH involving the APC and MCC genetic loci occurs in the majority of human oesophageal cancers and is involved in the development and/or progression of the disease (Boynton *et al.*, 1992).

1.4.5.6 WWOX

The WWOX (WW domain containing oxireductase) gene was recently discovered as a candidate tumour suppressor gene, (Paige *et al,* 2001) at chromosomal region 16q23.3-24.1 (Bednarek *et al*, 2000). It has been demonstrated in a study carried out by Kuroki *et al*, that both alleles of the WWOX gene are inactivated in squamous carcinoma of the oesophagus, as a combination of tumour-specific

mutations and LOH of the WWOX gene locus, which is also referred to as a two-hit mechanism (Knudson *et al*, 1985). The WWOX enzymatic domain is considered to be encoded mostly by the exon 6-8 regions of the gene and mutations in this region have been found to occur in breast cancer (Bednarek *et* al, 2001) as well as in squamous oesophageal carcinoma (Kuroki *et al*, 2002). This data suggests that WWOX could act as a tumour suppressor in squamous oesophageal carcinoma.

1.4.6 Other apoptosis genes

1.4.6.1 Bcl-2 Family

The Bcl-2 family consists of at least 15 proteins with either anti-apoptotic or pro-apoptotic functions. Proteins that have been found to be aberrantly expressed within oesophageal cancer include the anti-apoptotic proteins bcl-2 and bcl-xl (which are both up-regulated) and pro-apoptotic protein bax (down-regulated). (Katada, et al., 1997) View figure 8.

1.4.6.2 FAS*:* The non-functional Fas death receptor pathway is believed to play a role in the development of oesophageal cancer. A study showed increased Fas protein expression during the progression of Barrett's metaplasia to adenocarcinoma (Coppola *et al.*, 1999), suggesting that the Fas receptor could be non-functional and therefore the increase in the Fas ligand occurs. Aberrant Rb proteins were also detected with the progression of metaplasia to dysplasia, and since Rb plays a role in the control of cell cycle regulation and Fas plays a role in programmed cell death, another suggestion could be an attempt by

tumour cells to balance the uncontrolled cell proliferation promoted by non-functional Rb (Coppola *et al.*, 1999). View figure 9

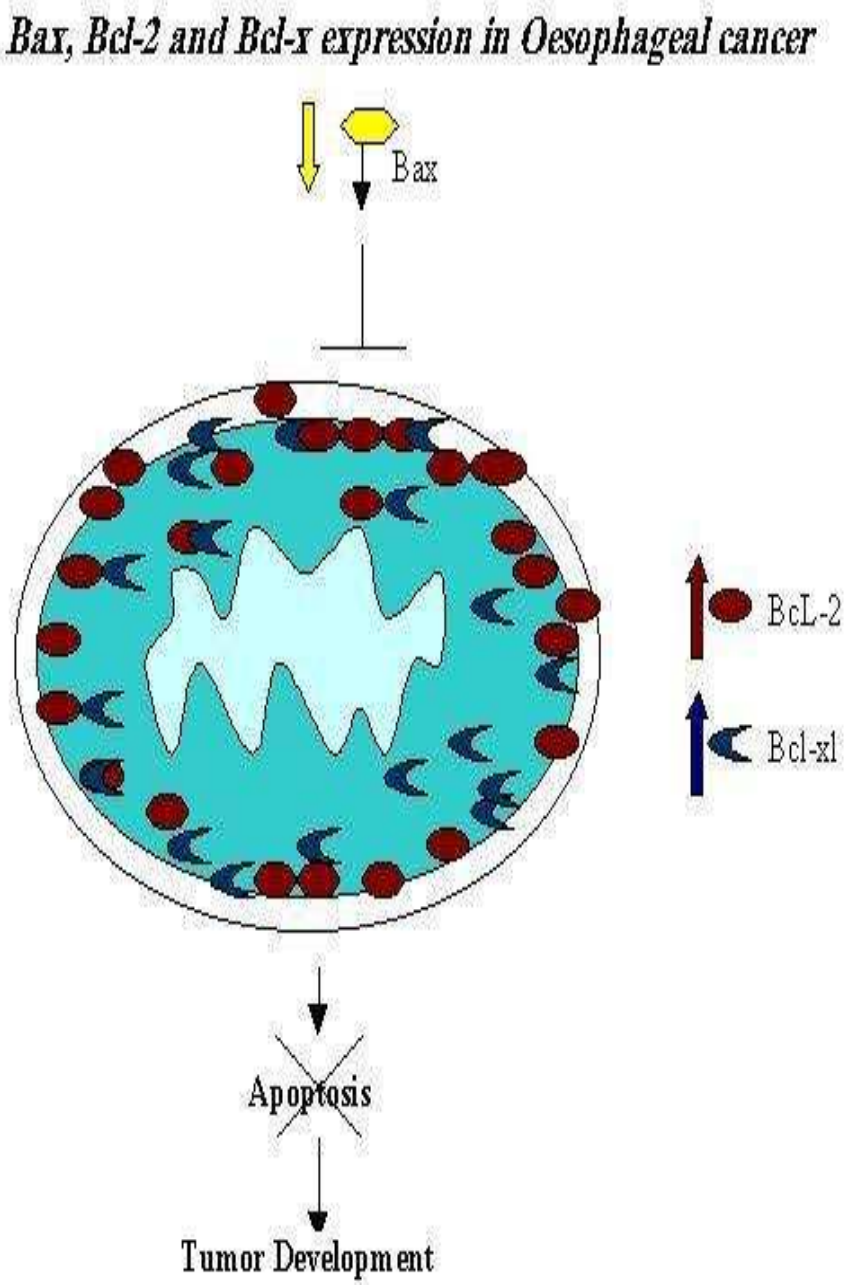


Figure 8: Aberrant Bax, Bcl-2 and Bcl-xl expression in oesophageal cancer cells.

Down regulation of Bax has been found to inhibit apoptosis and lead to the development of tumours in oesophageal cancer. Bcl-2 and Bcl-xl over expression has also been established to prevent the release of cytochrome c from the mitochondria and therefore prevent apoptosis, leading to tumour development.

1.4.6.3 Survivin: Survivin is a unique inhibitor of apoptotic proteins (IAP), and is only expressed in foetal tissue and a variety of human cancers, but is almost undetectable in most normal adult tissue (Ambrosini *et al*, 1997). Survivin inhibits apoptosis by binding to microtubules of the mitotic spindles (Li et al, 1998) and ultimately inactivating caspase-3 and caspase–7 activity (Tamm *et al,* 1998). It has been shown that increased expression of survivin can have a cancerous effect on the cell as it surmounts the G_2/M phase checkpoint proceeding into mitosis and it has been proposed that survivin is only present in the G_2/M phase (Li *et al*, 1998). Rodriguez *et al* also discovered that survivin encouraged cell proliferation by interacting with CDK4 and displacing p21, view figure 7. The nuclear survivin expression in squamous carcinoma of the oesophagus was examined and found to correlate with poor prognosis, but it seems that localization of survivin expression is critical for activity in tumour cells and its negative effect in dysregulating cell cycle definitely plays a role in tumour progression (Grabowski *et al*, 2003). It is clear that survivin expression could be used as a diagnostic tool, and possibly a therapeutic strategy, in the near future.

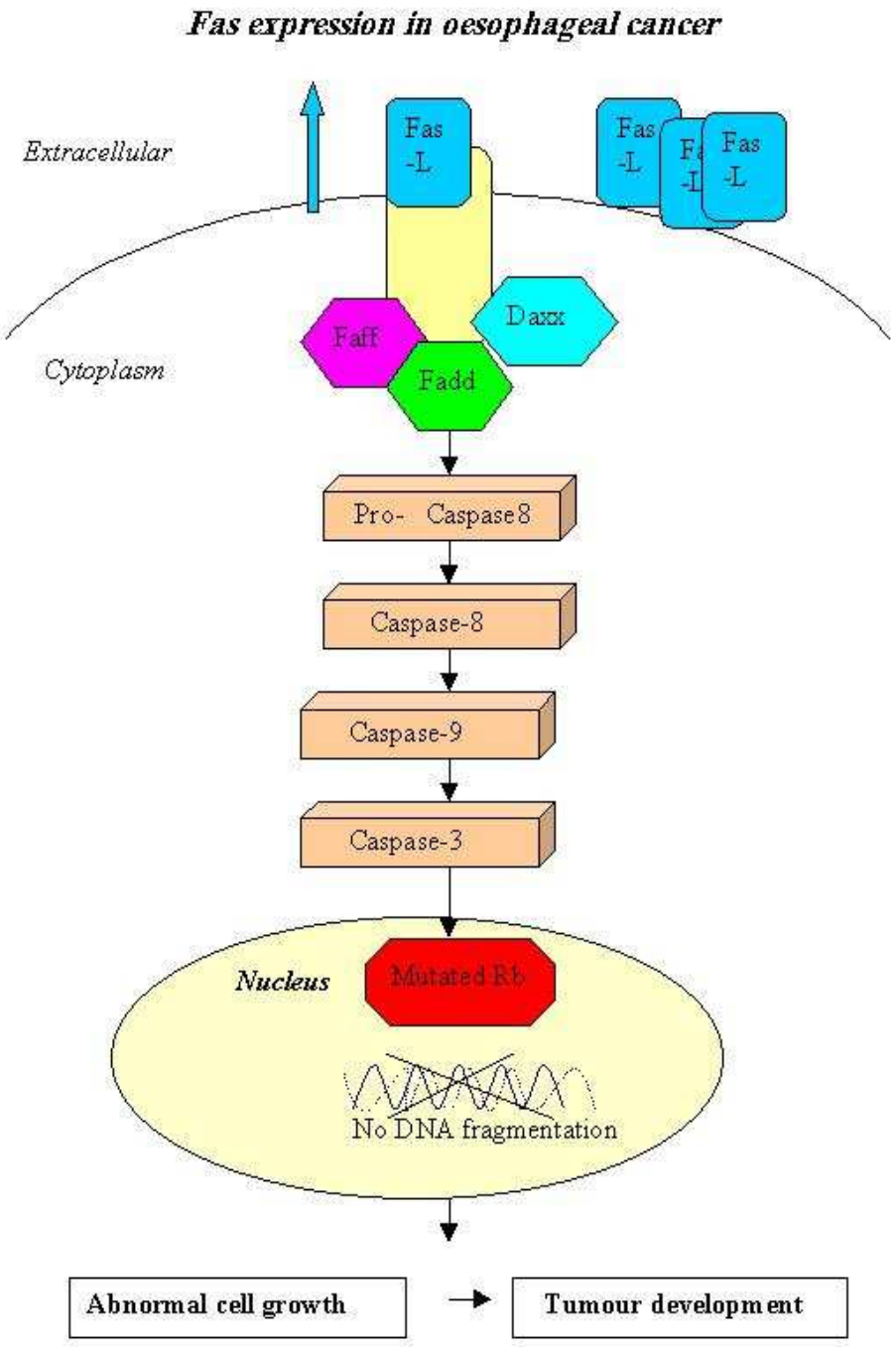


Figure 9: Fas expression in Oesophageal cancer

Increased Fas expression is a characteristic of oesophageal cancer progression and is associated with aberrant Rb expression. It is speculated that increased Fas expression in BAAC represents an attempt by tumour cells to balance the uncontrolled cell proliferation promoted by non-functional Rb (Coppola *et al*, 1999).

1.4..6.4 DcR3 / M68

A secreted decoy receptor (DcR3), member of the tumour necrosis factor (TNF) receptor superfamily, was found to be a negative regulator of Fas-mediated apoptosis by binding to Fas ligand, FasL (Pitti *et al*, 1998). DcR3 shows over-expression in a variety of cancers including gastrointestinal tract tumours, and it is believed the blockade of FasL-induced cell death allows tumour cell growth (Bai *et al,* 1999). It seems that DcR3 over-expression occurs without gene amplification, but it is suggested that DcR3 over-expression precedes gene amplification in tumours (Bai *et al*, 1999).

1.4.6.5 E2F-1

Over-expression of E2F-1 has been shown to induce apoptosis in several cancer cell types. Hiang *et al.* studied the effect of adenovirus-mediated E2F-1 over-expression on human oesophageal cancer cell lines, Yes-4 and Yes-6. Over-expression of E2F-1 resulted in cell growth inhibition due to apoptosis induction in the Yes-4 cell lines, but the Yes-6 cells were more resistant to E2F-1 over-expression. The resistance of Yes-6 cells to E2F-1 is believed to be caused by differential expression of cell death inhibitory proteins of the Bcl-2 family. These proteins include Bcl-2, Mcl-1, and Bcl-xl, which decreased after 48hrs in the Yes-4 cells, but remained unchanged in Yes-6 cells. Restinoblastoma gene product (pRB) also declined after 48hrs in Yes-4 cells and remained constant in theYes-6 cells. It is suggested that pRb inhibits apoptosis, as it binds to E2F-1 and negatively regulates its transactivation function (Bagchi, *et al*, 1991). The caspases involved in the E2F-1 mediated pathway of apoptosis in the Yes-4 cells

were demonstrated by caspase-3 and caspase-6, which cleaved caspase3/CPP32 and poly-ADP-ribose polymerase (PARP), as well as fragmentation of the caspase-6 substrate, lamin B. p53 does not seem to play a role in this E2F-1 apoptosis mediated pathway (Hiang *et al*, 2000).

These findings suggest that E2F-1 mediated apoptosis may be related to differential expression of Bcl-2 family member proteins and suggest this may be a promising treatment strategy for this disease (Yang *et al*, 2000).

1.4.6.6 Metallothionein (MT)

MT is a thiol-rich protein that plays a major role in detoxification of toxic metals and in protection against oxidative damage (Kang, 1999). Li *et al*, (2002) studied the relationship of MT and apoptosis in the progression from metaplasia to dysplasia to adenocarcinoma in subjects with BE. It was found that tissue with high activity of apoptosis had high expression of MT and vice versa. It is believed that MT may contribute to cytoprotection, thereby inhibiting apoptosis and increasing the likelihood of BE to progress toward adenocarcinomas. It is hypothesised that MT might act as a zinc donor in favour of tumour proliferation or it is induced by the rapid growth of the tumour (Li *et al.,* 2002). The exact mechanism of MT in the metaplasia-dysplasia-adenocarcinoma sequence has to be clarified.

1.4.7 Other genes believed to play a role in the development of oesophageal cancer

1.4.7.1 FEZ1

Fez1 was identified via genomic analysis of chromosome 8p22 in oesophageal cancer, due to the loss of this region in oesophageal cancer as well as many other types of cancer (Macosaka *et al*, 1995). It was found that Fez1 encodes a leucine-zipper protein. Fez 1 expression was found almost ubiquitously expressed in normal cells, but was undetectable in most of the oesophageal cancer cells (Ishii *et al*, 1999). Three point mutations were detected in Fez1 from oesophageal as well as prostate cancer cell lines. E44 alteration from TTC→CCC at codon 29, results in the substitution of Ser→Pro, which is a predicted cAMP-dependent kinase phosphorylation site (Ishii *et al*, 1999). A second mutation, an E50 alteration of AAG/Lys→ GAG/Glu at codon 119 was found, resulting in the allelic loss of the marker D8S261 (Ishii, *et al*, 1999). The third mutation change of CAG/Gln→TAG/Stop at codon 501 in prostate cancer resulted in coding a putative 166-aa protein lacking the C terminus (Ishii *et al*, 1999). This data suggests the major mechanism for Fez1 inactivation is a "two-hit" mechanism, allelic loss and point mutations, and possibly, allele loss plus failure in transcription (Ishii *et al*, 1999).

1.4.7.2 FzE3

FzE3 is a frizzled gene that forms part of the Frizzled family of seven-transmembrane proteins that acts as receptors for Wnt signalling (Sugimachi *et al*,

1994). The protein contains cystein-rich residues in its extracellular N terminal region to which the Wnt proteins bind (Cadigan and Nusse, 1997). FzE3 was found specifically expressed in oesophageal tumour tissue compared with normal mucosa and is believed to alter the function of the tumour suppressor gene, adenomatous polyposis coli (APC) (Tanaka *et al*, 1998). It has been shown that in normal cells, wild-type adenomatous polyposis coli APC protein bound to the serine-threonine glycogen synthase kinase (GSK)-3β binds to β-catenin within the cytoplasm, resulting in APC degradation (Peifer, 1997). In colon cancer cells, mutated APC was found stabilizing β-catenin to form a complex with transcription factors, Lef (lymphoid enhancer binding factor) and Tcf (T cell specific transcription factor), which then translocates to the nucleus where up-regulation of cell proliferation genes occur.

In oesophageal tumour tissue wild-type APC was found, so it has been suggested that the presence of FzE3 acts as a negative regulator of APC function, allowing β-catenin signal transmission to up-regulate cell proliferation associated genes (Tanaka *et al*, 1998). Figure 10 represents schematic diagrams illustrating the potential role of FzE3 on normal APC in oesophageal carcinoma cells, compared to normal APC function in normal cells and mutated APC in colon cancer cells.

1.4.7.3 ODC

Ornithine Decarboxylase (ODC) has been found to play a critical role in the biosynthesis of polyamines (Heby and Peterson, 1990) that are important in cell proliferation (Steglich *et al*, 1985). Yoshida et al. (1992) and Mafune et al (1999) demonstrated ODC over-expression in oesophageal carcinomas. It has been shown that over-expressed ODC cannot cause tumour progression but increases

the formation of polyamines in premalignant cells (Clifford, *et al.*, 1995). It is believed that the constant overexpression of ODC mRNA in oesophageal tumours, especially in Squamous oesophageal carcinoma, may be evidence that ODC plays a critical role in tumourigenesis of the oesophagus (Mafune *et al*, 1999).

1.4.7.4 Annexin 1

It has recently been discovered by Liu *et al,* (2003) that the protein Annexin 1 translocated from the plasma membrane in normal cells to the nuclear membrane in malignant cells. It was found that Annexin 1 usually formed a consecutive typical trammel net on the plasma membrane of normal oesophageal epithelia, but in oesophageal cancer a great decrease was found on the cell membrane and was highly expressed on the nuclear membrane, which was never found on normal oesophageal epithelia (Liu *et al.*, 2003). This data suggests that Annexin 1 translocation may correlate with the tumourigenesis of oesophageal cancer (Liu *et al*, 2003).

1.4.7.5 Cathepsin B (CTSB)

CTSB is a cysteine protease that also maps to chomosome 8p22 (Wang *et al.*, 1998) and has been found over-expressed or altered in certain tumours of the lung, breast, stomach, colon and prostate (Sloane *et al*., 1990). CTSB was found amplified and over expressed in Adenocarcinoma of the oesophagus, showing genomic alteration involving CTSB (Hughes *et al.*, 1998). Extracellular expression of CTSB protein, correlated with the identification of higher M_r forms

in tumours, was also found (Hughes *et al.*, 1998). It is therefore believed that CTSB plays a critical role in tumour progression or malignant transformation of adenocarcinoma of the oesophagus, and even other types of malignancies (Hughes *et al.*, 1998).

1.4.7.6 GASC1

Gene amplified in squamous cell carcinoma 1 (GASC1) was found to be amplified and over-expressed in several squamous oesophageal cancer cell lines. The GASC1 locus is found on chromosome 9p23-24. It is found that GASC contains one PX domain and two PHD fingers. PHD-finger motifs are found in nuclear proteins that participate in chromatin-mediated transcriptional regulation and are present in a number of proto-oncogenes (Yang *et al*, 2001). It is assumed that GASC1 may be involved in the carcinogenesis or progression of multiple tumours, even though its function is not clear (Yang *et al*, 2001).

1.4.7.7 ECRG4

Oesophageal cancer related gene 4 (ECRG4) is a novel oesophageal cancer related gene and found to be down-regulated in squamous oesophageal carcinoma compared to normal oesophageal tissues. It is located on chromosome 2q14.1-14.3 and it contains 4 axons. ECRG4 down-regulation is believed to play a role in the development of Squamous oesophageal carcinoma and the mechanism inactivating it has been demonstrated to be aberrant methylation of CpG islands in the core promoter of the ECRG4 gene (Yue et al., 2003).

1.4.7.8 Matrix metalloproteinase-7 (matrilysin/ MMP-7)

MMP7 has been implicated in tumour initiation, growth (Rudolph-Owen, et al, 1998), invasion (Senota et al, 1998) and metastasis (Adachi et al., 1999). Matrilysin is a member of the MMP family and it has wide variety substrate specificity, and potency to start an activation cascade of MMPs (Wilson et al., 1998). MMP was found to be over-expressed in a variety of cancer tissues, including oesophageal cancer tissue. MMP was also found to be susceptible to direct therapeutic intervention. Administration of synthetic MMP inhibitor batimastat to *Min* mice suppressed tumour multiplicity (Goss *et al.*, 1998), and antisense technology also demonstrated a suppression of invasive and metastatic phenotypes in *in vitro* and *in vivo* studies respectively (Hasegawa *et al.*, 1998). MMP-7 was found to be up-regulated by the organism Helicobacter pylori in epithelial cells in vivo and in vitro, in a Cag dependent manner and considered to contribute to carcinogenesis (Bebb *et al,* 2003).

1.4..7.9 PCNA

Proliferating cell nuclear antigen (PCNA) expression increases gradually in cell nuclei with the progress of G1 phase and reaches a peak when entering the S phase (Xing *et al*, 2003). Foetal oesophageal epithelia showed a much higher expression pattern compared to normal adult oesophageal epithelia and basal cell hyperplasia (BCH), and a much higher expression pattern was observed in malignant adult oesophageal tissue compared to foetal oesophageal epithelia (Xing *et al*, 2003). It was found that PCNA acts as a good marker for cell proliferation (Rustgi, 1997).

Another study showed that p53 and PCNA are already over-expressed to different extents in normal epithelia and also in precancerous lesions of the oesophagus, but an increase in expression is observed with progressive cancer stages (Chen *et al*, 2003).

It was suggested that since PCNA has been found to play a role in DNA damage repair, it could combine with hMSH6 and hMSH3, the subunits of hMutSulpha and hMutSbeta that act as cofactors in a DNA mismatch repair system (Xing *et al.,* 2003). Malignant tissue is characterized by high frequencies of DNA mismatch, breakages and mutations, and therefore the increase in PCNA expression is thought to occur as a repair response (Xing *et al*, 2003).

1.4.8 Angiogenesis

Angiogenesis is the development of new blood vessels, which provides blood and nutrient supply to tumours for survival. Once the tumour is stable, it can then invade neighbouring cells leading to metastasis.

In oesophageal cancer cells the increased expression of vascular endothelial growth factors (Vegas) stimulate endothelial proliferation and migration. Increased expression of Vegas and Vergers (receptors) were detected in metaplastic tissues of the lower oesophagus but not in normal oesophageal epithelium, indicating sustained endovascular development early in Barrett's carcinogenesis (Morales et al., 2003).

1.4.9 Invasion and metastasis

Invasion and metastasis of oesophageal cancer is poorly understood. The cell-cell adhesion molecules (Cams) hold cells together, and are believed to play an important role in metastasis of the cancer cell. Beta-catenin has been found to play a role in squamous oesophageal cancer cells, by its cell-cell adhesion function and interactions with the cytoskeleton and adhering junctions of cells. Beta-catenin has been implicated in the transcription of oncogenes such as c-myc, c-junk and cyclin D1, which are oncogenes frequently active in oesophageal cancer cells. The APC gene product targets Beta-catenin for degradation and prevents Beta-catenin dependent degradation. Increased Beta-catenin dependent transcription due to beta-catenin binding to Fz receptors, mutations in Beta-catenin, APC, and increased B-catenin expression due to Fz receptor mutations, have all been found in adenocarcinomas and squamous oesophageal carcinomas.

It is therefore believed that down-regulation of B-catenin expression by antisense technology could be an effective treatment for oesophageal cancer (Veeramachaneni, et *al*., 2004).

1.5 Potential therapeutic strategies in oesophageal cancer

The management of oesophageal cancer to date remains an unsolved health problem. All over the world diagnostic markers and therapeutic analyses are carried out to generate a solution to this problem. It is believed that the cure to cancer would be a two directional method, one including chemotherapy and radiation, and a key drug that targets a specific molecule present only in the cancer cells and has a low or no toxicity effect on the normal surrounding cells.

Endoscopic mucosal resection (EMR) and photodynamic therapy (PDT) are effective treatment modalities for high-grade dysplasia or even early oesophageal malignancies (Overholt *et al.*, 1999). Photosensitizers currently used in PDT against oesophageal malignancies are hematoporphyrin derivates and profimer sodium, which induces systemic photosensitization for up to two months, causing oesophageal strictures in a third of patients (Gossner *et al.*, 1998). New photosensitizers need to be found and used in PDT of oesophageal malignancies.

Hypericin, a naturally occurring polycyclic aromatic naphthodianthrone isolated from plants of the *Hypericum* genus (Lavie *et al.,* 1995), is a very promising new photosensitizer for innovative photodynamic therapy of oesophageal cancer (Höpfner *et al*, 2002). The nonphotosensitized hypericin has no genotoxicity (Okpanyi et al., 1990) and toxicity in humans (Meruelo *et al.*, 1988) and the photosensitized hypericin produces a high number of photosensitizing effects (Diwu, 1993). Light activated hypericin, utilizing an incoherent light source opposed to the common laser light photosensitization method, proved to be an effective photosensitizer for the treatment of both squamous and adenocarcinomas

of oesophageal cells (Höpfner *et al.*, 2002). It is therefore a promising new approach for innovative photodynamic therapy of intraepithelial oesophageal neoplasia (Höpfner *et al.*, 2002).

Apoptosis inducing nucleosides (AINs) from $CD57^{+}HLA\text{-}DR^{bright}$-natural suppressor (57.DR-NS) cell lines were used to induce apoptosis in human oesophageal cancer cells. This study revealed that AINs induce apoptosis in oesophageal cancer cells through DNA strand breaks and caspase-3 activation (Mori *et al.,* 2001). Further research is currently carried out to develop an ideal anticancer agent, since apoptosis generated in malignant cells lacked toxicity in normal cells, suggesting a possible evasion from side effects in clinical trials (Mori *et al.*, 2000).

A case study was carried out by Morihiro et al., using chemotherapy comprising of 5-fluorouracil (5-FU) with cisplatin (CDDP) and endoscopic resection follow-up as follows: The patient was in his late forties and diagnosed with late oesophageal cancer located in the middle thoracic oesophagus. The patient was treated with four courses of chemo or radio-therapy during a seven-month period resulting in a decrease of the cancer cells, but a small remnant polypoid lesion was found. After an additional two months of therapy, the lesion showed no change and subsequently was resected by endoscopic polypectomy using conventional methods. No complications occurred during or after this procedure and no evidence of disease was found four years after initiation of chemoradiotherapy and three and a half years after endoscopic resection. This treatment suggests that endoscopic resection of remnant lesions after

chemoradiotherapy for advanced oesophageal cancer might offer a small chance of cure and therefore is necessary.

Another case study where multimodal therapy is used to treat extensive lymph node metastases of advanced oesophageal cancer was carried out by Shigemitsu et al. Multimodal therapy comprises of 5-FU, CDDP and methotrextate (MTX) combination chemotherapy followed by radiotherapy after curative resection (Shigemitsu et al., 2002). The patient was a male in his early fifties presenting with advanced oesophageal cancer with histological metastasis of 34 lymph nodes. Four weeks after surgery the patient was treated with 5-FU, CDDP and MTX combination chemotherapy then again two weeks later. From the 67^{th} postoperative day he was treated with radiotherapy. Mild toxicity occurred, which was mild anorexia and leucopaenia, but no serious side effects occurred. After discharge, the patient was treated again but with 5-FU and CDDP combination chemotherapy only. This combination chemotherapy was continued with close follow-up examinations every three to four months without any severe side effects occurring. The patient has remained well five years after surgery without any recurrence. It is therefore inferred that combination therapy with CDDP, 5-FU and irradiation is a promising treatment for the urgently required management of oesophageal cancer (Shigemitsu, et al., 2002).

An antagonist to the anti-apoptotic gene, Survivin, is a promising therapeutic strategy not only for oesophageal cancer but various other types of cancers where Survivin is highly expressed. It is believed that antagonists to Survivin would

increase the effectiveness of chemotherapy by removing the protective role of Survivin on the cancer cell (www.info.md.yale.edu/ycc).

Other drugs of interest would be those targeting angiogenesis. Anti-angiogenesis drugs developed to inhibit blood and nutrient supply to the tumour cells is a potential therapeutic strategy as well. Researchers are currently working on the development of antagonists to the two angiogenesis molecules angiostatin and endostatin, and there is great hope that these drugs in combination with radiation or chemotherapy could be the cure to cancer (www.info.md.yale.edu/ycc)..

1.6 OBJECTIVES OF THE RESEARCH:

The purpose of this study was to establish the expression of three genes; 1-ACBP, B-ACBP and PBR in the development and progression of oesophageal cancer and characterize their roles in the disease. Elucidation of their roles in the development of oesophageal cancer could lead to new therapeutic discoveries.

1.6.1 Aycl-coenzymeA binding protein (ACBP)

Acyl-coenzymeA binding protein (ACBP) has been named after the protein's ability to bind thiol esters long chain fatty acids and coenzyme-A (Krageland et al, 1999). ACBPs are found conserved and are characterised among different eukaryotic species ranging from yeast to animals and plants. ACBP is a so-called house-keeping gene, suggesting its role in the cell to be of a biochemical function (Krageland et al, 1999).

Long chain acyl-coA esters serve as important intermediates in the fatty lipid synthesis and fatty acid degradation and act as important signalling molecules (Faergemann and Knudsen, 1997). It has been found that acyl-coA esters at low levels inhibit a large number of cellular functions and enzymes (Faergeman and Knudsen, 1997). It is therefore important that an intracellular acyl-coA pool former of these molecules exists, and one molecule that fulfils this requirement is ACBP.

ACBP acts as a potent protector of acetyl-coA carboxylase, acetyl sythetase, and adenylate translocase against inhibition by long chain acyl-coA esters. ACBP protects the acyl-coA from being hydrolysed by cellular hydrolases while transporting these molecules across membranes (Faergeman and Knudsen, 1997).

It has been shown that a low dose of ACBP concentration induced swelling of the mitochondria, which lead to mitochondrial transmembrane potential disruption and subsequent apoptosis (Chelli et al., 2001). The study also shows a gradual decrease of mitochondrial transmembrane potential disruption with a dose-dependent increase of ACBP. This infers the increase of ACBP in any system leads to the inhibition of apoptosis and subsequent carcinogenesis.

ACBP is an 86-103 residue protein with a highly conserved amino acid sequence. It has been isolated from a wide range of species including yeasts, plants, reptiles and plants. ACBPs are grouped into at least four goups being, 1-ACBP, B-ACBP, T-ACBP, and M-ACBP.

1-ACBP was first isolated from bovine liver, and it was later found that it is a generally expressed isoform found in nearly all types of tissue. It contains no cysteines and is generally 86-92 residues long (Krageland et al, 1999). B-ACBP is a putative brain specific isoform and has been deduced from duck and frog brain and contains one single cysteine at position number 43 (Krageland et al, 1999).

T-ACBP is a testes specific isoform (also called endozepine-like protein ELP) and has been isolated from three different species and all T-ACBPs contain three cysteines (Krageland et al, 1999).

The final isoform is M-ACBP that is suggested to be a membrane bound isoform. This isoform contains a longer sequence with up to 533 amino acids. Many of the longer forms contain cysteines (Krageland et al, 1999).

The 1-ACBP and B-ACBP isoforms demonstrated to contain the highest number of conserved residues across different species.

The three dimensional structure of bovine ACBP by NMR studies, shows the fold of the peptide backbone, and an up-down-down-up four-α-helix bundle with an overhand loop connecting helices A2 and A3 is demonstrated, see figure 10A. The bundle arrangement is slanted as the A3 helix is disjointed to helices A1 and A4, resulting in only four helix-helix interfaces, and the 'front'-view of the protein takes the shape of a relatively flat disc. Helix A1 (glu4-Leu15) forms strong helix-helix interface contacts with helix A2 (asp-21-Val36), which runs anti-parallel to A1 at an angle of 30 degrees, and with helix A4 (Ser65-Tyr84), which runs parallel to A1 at an angle of 36 degrees. See figure 10B. Helix A2 and A4 runs anti-parallel with a helix-helix angle of 30 degrees. Then the third helix, A3 (Gly51-Lys62), runs parallel to helix A2 as a result of the overhand connection, forming a helix-helix angle of 42 degrees, see figure 10C (Faergemann and Knudsen, 1997).It is apparent that the majority of the residues, making up the interfaces are conserved throughout evolution. The most conserved interface being the one between helix A1 and helix A4 and the least conserved between helix A2 and A4, see figure 10B. The most distinct differences are found at the edges of the interfaces, which are thought to be compensated for by similar changes or by increased dynamic structure (Krageland et al, 1999). The fact that ACBP is highly conserved in all eukaryotes from yeast to man and plants and reptiles, and that it is ubiquitously expressed, suggests that ACBP serves a basic function in all cells (Faergeman and Knudsen, 2002). In a study carried out by Faergeman and Knudsen, 2002, showed the depletion of ACBP levels by short interference studies (siRNA) lead to growth arrest and cell death in a human cell line. The same effects were shown in yeast indicating that ACBP has identical function in both species, making yeast a good model for further ACBP studies *in*

vivo, as depletion of ACBP leads to lethality in human cell lines, making human cell line *in vivo* studies inappropriate (Faergeman and Knudsen, 2002).

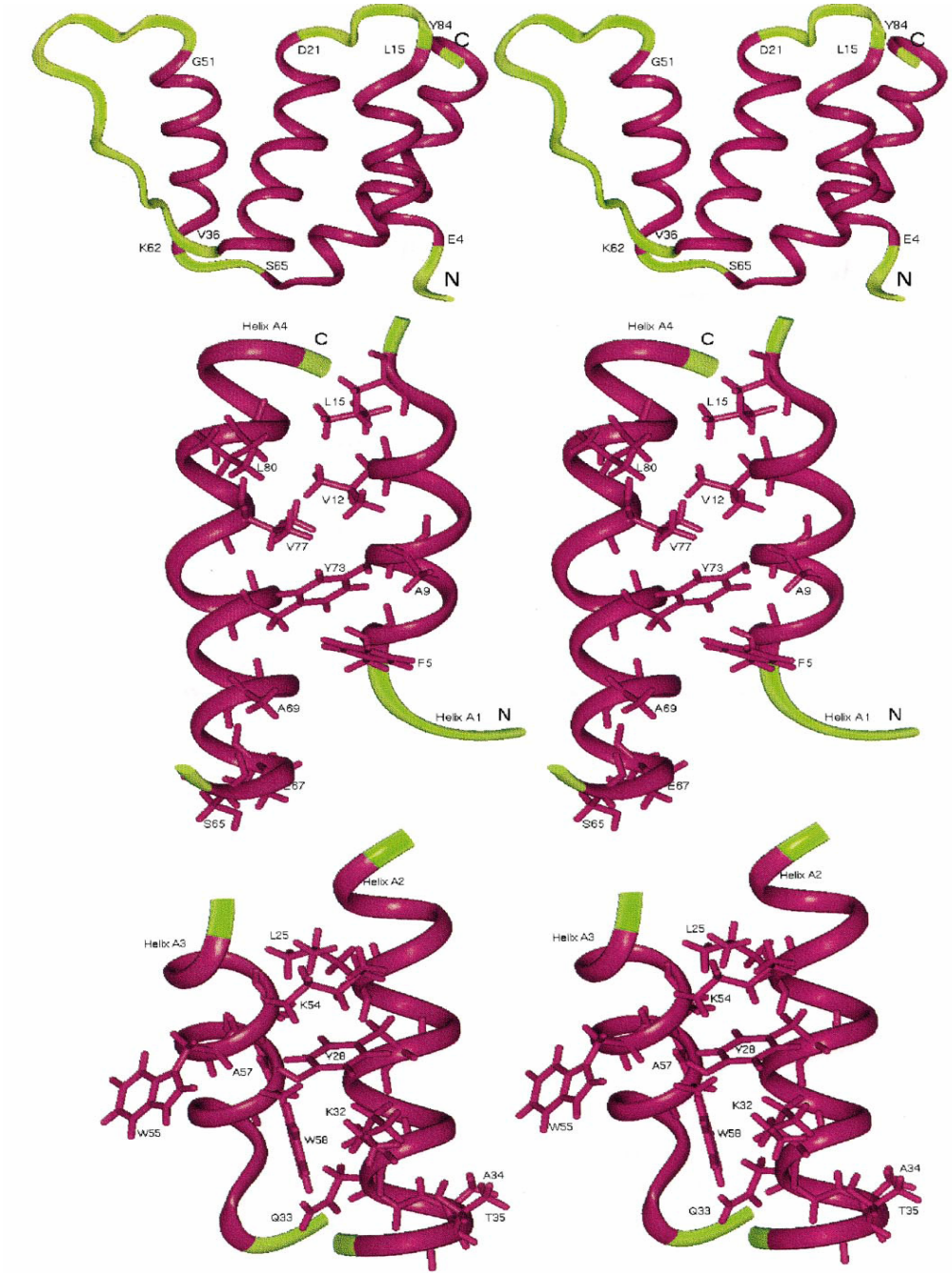


Figure 10: This is the three dimensional structure of bovine ACBP by NMR studies. (Krageland et al, 1999).

1.6.2 PBR

Peripheral-type benzodiazepine receptor (PBR), first characterized by Braestrup and Squires, 1977, was originally found as an alternative binding site for the benzodiazepine diazepam. Despite the name, PBRs are not only located in peripheral organs but also present in the central nervous system (CNS) (Gandolfo *et al.*, 2000). PBR is an 18k Da protein receptor consisting of 169 amino acids, and has a structure of five α-helices spanning the membrane lipid bilayer (Bernassau et al., 1993; Joseph-Liauzun et al., 1998). PBR also forms part of a trimeric complex with the 32 k Da voltage-dependant anion channel (VDAC) protein and the 30 kDa adenine nucleotide carrier (ANC) in the outer mitochondrial membrane (Anholt et al., 1986). The 18 kDa PBR subunit is assumed to be responsible for the binding of isoquinolines, whereas both the 18 kDa PBR and the 32k Da VDAC subunits are required for the binding of benzodiazepines.

Expression of PBR has been found in every tissue examined, however it is most abundant in steroid-producing tissues such as the adrenals, testes, ovaries, placenta, and brain, where it is primary localized to the outer mitochondrial membrane. For nearly 30 years, numerous studies have been carried out in elucidating the function of the peripheral benzodiazepine receptor. This protein awakened great interest because of its association with numerous biological functions including the regulation of cell proliferation, immunomodulation, porphyrin transport and heme biosynthesis, anion transport, regulation of steroidogenesis and apoptosis (Strohmeier et al, 2002).

Insights into the function of the PBR were initially provided by the receptor binding studies, which showed PBR density could be modulated under a variety of physiological and pathological conditions (Cassellas et al., 2002). PBR is highly expressed in endocrine tissue and its density can be modulated by hormonal fluctuations, in the ovary, PBR expression varies with the ovarian cycle increasing following ovulation. Interleukin-1 also modulates the expression of PBR (Moynagh et al., 1993) and also dopamine, serotonin and norepinephrine (Itzhak and Norenberg, 1994a).

PBR is believed to play a role in steroidogenesis, by transporting cholesterol from the cytoplasm across the mitochondria from the outer to inner membrane. This function was inferred by two observations of PBR primarily always found on the outer mitochondrial membranes of cells in steroidogenic cells. Other studies also showed that a spectrum of PBR ligands with various affinities for the receptor stimulate steroid biosynthesis in various cell systems (Mukhin et al., 1989) (Papadopoulos et al., 1990). Hormone stimulated steroidogenesis, occurs in a biosynthetic pathway and the first step involves where choleterol is converted into pregnolene by the cholesterol side-chain cleavage cytochrome P450 enzyme (P450scc) and auxillary electron transferring proteins, localized on the inner mitochondrial membrane. This hormone dependent transport mechanism is mediated by cyclic adenosine monophosphate (cAMP), to be regulated by a cytoplasmic protein, and to be localized in the mitochondria where it regulates the intramitochondrial transport of cholesterol (Jefcoate et al., 1992).

PBR and its role in apoptosis have become an interest to many researchers. Apoptosis is almost consistently accompanied by an increase in mitochondrial membrane permeability. Mitochondrial membrane permeability is established by the activation of the mitochondrial permeable transition pore (MPTP). The exact MPTP composition is still not exactly determined, but a study carried out by Casellas et al (2002) found that a number of six proteins were implicated in either pore formation or its regulation: hexokinase which is located in the cytosol; VDAC in the outer membrane, creatine kinase in the intermembrane space, ANC in the innermembrane space, cyclophilidin D in the matrix, where it interacts with the ANC, and PBR (Casellas et al., 2002).

The exact mechanism accounting for membrane permeabilization is still a matter of debate. PBR ligands have been found to be able to induce the MPTP. Most activators of the MPTP require the accumulation of excessive amounts of calcium in the matrix, whereas PBR ligands have been found to induce MPTP even in low calcium levels. PBR ligands include ROS-4864 (7-chloro-5-(4-chlorophenyl)-11,3-dihydro-1-methyl-2H-1,4-Benzodiazepine-2-one); PK11195 (1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline carboxamide) and Diazepam ligands. ROS-4864 and PK11195 have been demonstrated to enhance inhibition of cell proliferation in micromolar concentrations *in vivo* and *in vitro* studies on different tissues. In contrast nanomolar concentrations of these proteins induced seemed to stimulate cell proliferation (Harwick *et al.,* 1999). Overexpression of Bcl2 has been shown to block MPTP opening, but this inhibition can be abolished by PK11195.

1.6.3 PBR and ACBP interactions

PBR and ACBPs are common elements of the steroidogenic machinery in all steroidogenic tissues, in all eukaryotes examined (Papadopoulos and Brown, 1995). ACBP molecules bind to PBR, and the cholesterol bound is transported across from the outer to inner mitochondrial membrane within the contact sites. The cholesterol is catalysed to pregnolone by the enzyme P450scc, and the greater the amount of cholesterol the greater the rate of conversion also becomes. Once pregnolene becomes available it moves out of the mitochondria to the smooth endoplasmic reticulum where steroid products are produced and used by the cell as an energy source (Papadopoulos and Brown, 1995).

1.6.4 PBR and ACBP in diseases

PBR and ACBP expression have been found upregulated in various types of diseased tissues. PBR and 1-ACBP (also known as DBI) were found upregulated in liver tumours, breast cancer (Hardwick wt al., 1999) (Strohmeier et al., 2002). PBR has also been found upregulated in colon cancer (Maaser et al, 2004). B-ACBP and PBR have been found upregulated and believed to control glial cell proliferation in the CNS (Swinnen et al., 1998; Casellas et al., 2002). The mechanism of this activation and cellular proliferation is still unknown. Altered characteristics of PBR were also found in epileptics, schizophrenic and anxious patients (Maeda et al., 1998).

DBI is also a gene of interest in oesophageal cancer, as the cellular localization of DBI has been found in the gastrointestinal tract of mice (Yanase *et al.,* 2001). Research has also revealed the cellular expression of DBI mRNA throughout the

gastrointestinal tract of mice to be intensely expressed in the spinous layer in the stratified squamous epithelium of the oral cavity, oesophagus and forestomach, in surface mucous cells in the glandular stomach, and in columnar (absorptive) cells of the intestinal villi (Yanase *et al.,* 2001).

1.6.5 PBR and ACBP in Blood Cells

PBR site density in human blood cell populations was found highest in monocytes and neutrophils, with an intermediate density in lymphocytes and low levels in platelets and erythrocytes (Canat et al., 1992). Canat et al (1992) concluded that PBR must be located on the plasma membrane of erythrocytes and neutrophils, because the mitochondria of erythrocytes are extruded before leaving the bone marrow, and in neutrophils mitochondrial content decreases along the granulocyte differentiation pathway (Woods and Williams, 1996). It is also suggested that different subtypes of lymphocytes may express PBR to various degrees on their membranes (Maeda et al., 1998) and also within the intact lymphocyte (Berchovich et al., 1993).

1.6.6 PBR and ACBP in immunomodulation

Benzodiazepines, including diazepam, are extensively used in therapeutic drugs that alleviate anxiety, convulsions, and insomnia due to their interactions with the central-type benzodiazepine binding site associated with the $GABA_A$ receptor. Diazepam although it binds potently to the non-neuronal, PBR site, the exact pharmacological role has not been established yet (Woods and Williams, 1996).

The PBR site localizing to lymphoid cells and macrophages indicates a potential role in immune function. It has been determined that benzodiazepines have stimulatory and inhibitory effects on cell growth at low and high concentrations respectively. Lymphoid cell growth is stimulated at nanomolar and inhibited and micromolar concentrations of certain benzodiazepines. A study showed picomolar concentrations of Ro5 4864 and diazepam induced human monocyte chemotaxis (Pawlikowski, 1993), whereas nanomolar concentrations of PBR ligands stimulated oxidative bursts in neutrophils and macrophages *in vitro* (Zavala and Lenfant, 1987). *In vivo* studies also demonstrated a reduced oxidative response in macrophages and decreased interleukins 1 and 6 secretion and tumour necrosis factor following injection of Ro5 4864 in mice (Zavala et al., 1990). It is clear that too much of certain benzodiazepine ligands cause an inhibitory effect on lymphoid cells and macrophages, and needs to be modulated to keep the immune system functional and effective to eradicate diseases such as cancer.

In lymphocytes, two locations for PBR sites have been identified, with different specificities for two types of endogenous ligands (Berchovich et al., 1993). Essentially DBI/ACBP fragments recognize the PBR site on the plasma membrane, whereas the intracellular form of PBR, which has been reported in only 22% of lymphocyte mitochondria, binds both DBI fragments and protoporphyrin with low affinity (Cahard et al., 1994). An interaction of extracellular ACBP with the plasma membrane form of PBR may be of functional importance for immunomodulatory effects of peripheral-type ligands. It seems ACBP's functional association with plasma membrane PBR is to account for effects on Leydig cell growth (Garnier, et al., 1993).

1.6.7 Aim:

The purpose of this study was to establish the expression of three genes; 1-ACBP, B-ACBP and PBR in the development and progression of oesophageal cancer and characterize their roles in the disease.

Colorimetric and fluorescent *in situ* hybridization was carried out to determine the mRNA expression patterns of these genes in oesophageal carcinoma tissue sections. Since there are five isoforms of ACBPs, 5' and 3' probes were synthesized for the two genes in this study, to increase specificity of the genes and to correlate expression patterns, ensuring that only the specific two genes of interest were being focused on.

The ORFs of the three genes were also synthesized for the generation of proteins for antibody production, and also for future RNAi studies.

Absolute Quantification was carried out to verify expression levels determined by *in situ* hybridization and also to compare the 5' and 3' mRNA levels to the open reading frame mRNAs of the genes of 1-ACBP, B-ACBP and PBR.

1.7 Summary

Oesophageal cancer is a disease that urgently needs a consistent diagnostic tool for early diagnosis, and also an effective therapeutic strategy that ensures non-recurrence, best quality of life and an increased lifespan. Many genes have been found that are believed to play a role in the development of oesophageal cancer but the underlying mechanism by which this disease develops is still not clear. A few genes with significant correlation to this disease have been found, and are currently being analyzed as potential candidates for determining prognosis and therapeutic strategies. A human oesophageal tumour model would be of interest, as it would help other researchers to focus on the genes of interest, and diagnostic and therapeutic tools could be developed at a much quicker rate. This study focuses on the expression analysis of three genes in oesophageal cancer, to determine their potential roles in the development and progression of oesophageal cancer.