1. CHAPTER 1: INTRODUCTION

1.1 DWNN

DWNN, an acronym for **D**omain **W**ith **N**o **N**ame, is a novel 76-residue domain. This was first identified by promoter-trap mutagenesis in Chinese Hamster Ovary (CHO) cells, in which the retrovirus is inserted downstream of an active promoter of the host gene thereby disrupting the expression of that gene. If the disrupted gene is involved in the cytotoxic T Lymphocyte (CTL) pathway then the cells should be resistant to cytotoxic killing. Cytotoxic T lymphocytes have two pathways, one of which leads to apoptosis; this raises the possibility that DWNN plays an important role in apoptosis. Apoptosis was also induced in the CHO cells and they did not die, which shows that the cells were resistant to apoptosis and this can also play an important role in apoptosis. In this case, DWNN may be an important candidate in anti-cancer therapy because cancer frequently occurs as a result of defects in the apoptosis cascade. Thus, elimination of this gene in CHO cells makes or renders the cells resistant to cytotoxic T cell killing and to chemically induced apoptosis by staurosporine (Rees *et al*., unpublished data).

Human DWNN gene is located on chromosome 16p21, with 18 exons and it is 36 kb long. It makes two major transcripts, 1.1 and 6.1Kb, encoding 13 kDa and 200 kDa proteins. The 6.1 kb transcript is alternatively spliced at exon 16. The DWNN-13 kDa is made of N-terminal domain of 3 exons (**figure 1.1**). The domain has a highly preserved region of 80 amino acids and a hydrophobic end. The 6.1 kb transcript has two promoters P_0 and P_1, and 6 mRNA transcripts, the significance of this is still undefined. The DWNN-13 kDa domain forms part of the DWNN-200 kDa sequence. This sequence

consists of zinc finger domain on exons 4 to 7, RING finger domain on exons 8 to 10, proline rich region conserved on exons 10 to 15, Serine Rich (SR) and Rb binding domains are found on exon 17 and a p-53 binding domain is conserved on exon 18 (**figure 1.1**).

The fact that the DWNN domain is associated with both the RING Finger and p53-associated domains opens the possibility that DWNN is involved in the p53 dependent apoptotic pathway as a ubiquitin-ligase enzyme. Ubiquitin protein ligases, of which DWNN may be one, are a very important group of enzymes that play a significant role in the pathogenesis of many human diseases through deregulation of targeted proteolysis.

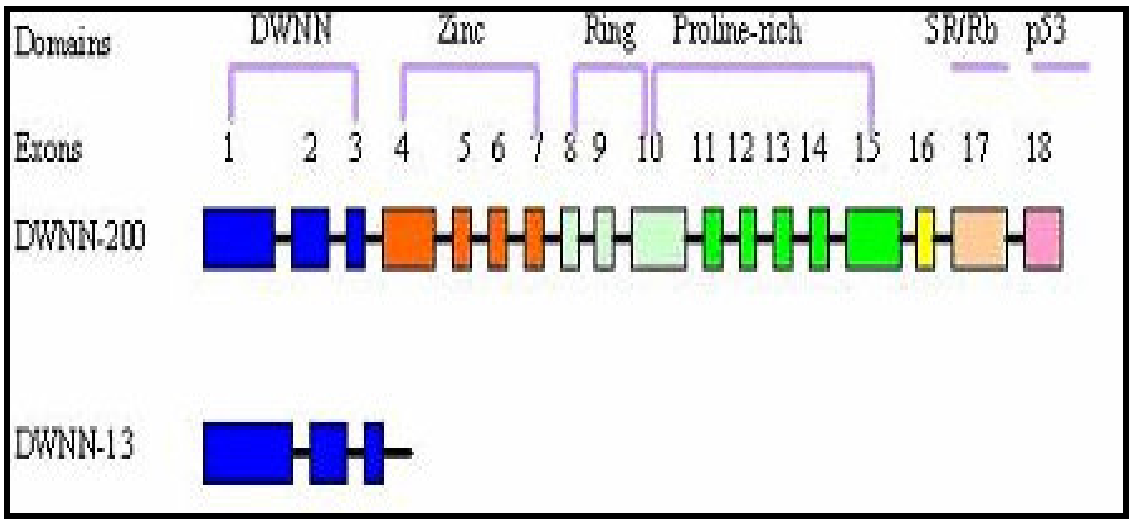

Figure 1.1 Structure of DWNN Gene
(Adapted from Skepu *et al*., unpublished PhD thesis).

It is highly conserved in most eukaryotic kingdoms and in humans, it is found in two forms. It is also associated with other conserved domains, that is, zinc finger, ring finger, proline rich region, SR domain/Rb binding domain and a p-53 binding domain. DWNN gene was found to be highly conserved throughout the eukaryotes (**figure 1.2**) as illustrated on **figure 1.3**.

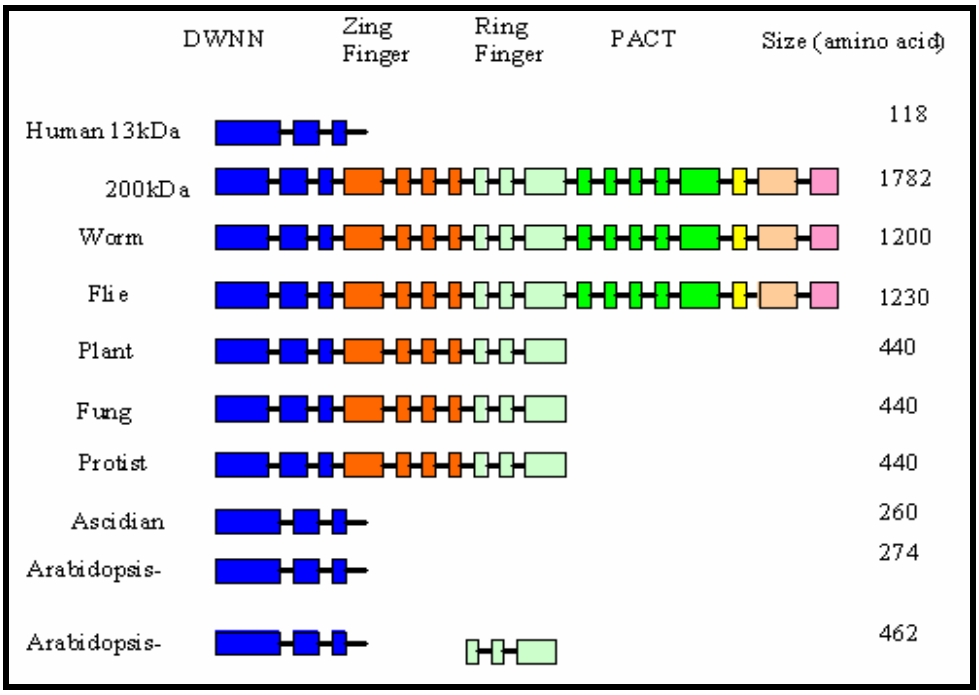

Figure 1.2: DWNN Domain Arrangement.

Schematic illustration of the DWNN-200 kDa protein arrangement from different eukaryotes (Adapted from Skepu *et al*., unpublished PhD thesis).

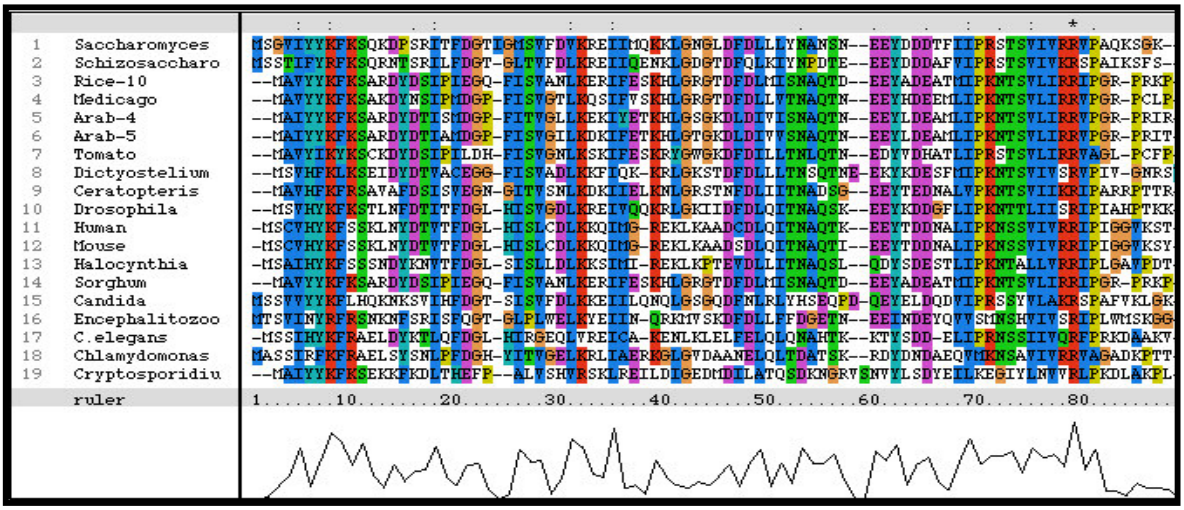

Figure 1.3: Conserved Amino Acids within the DWNN Domain in Various Species.

(Skepu *et al*., unpublished PhD thesis).

1.2 APOPTOSIS

There are two types of cell death, apoptosis and necrosis. Apoptosis has unique morphological features that distinguish it from necrosis (Kiechle and Zhang, 1998). Apoptosis is from a Greek word, which means 'falling leaves'. It is a cell suicide mechanism whereby cells are deliberately eliminated to maintain homeostasis and proper metazoan development (Chinnaiyan and Dixit, 1996). Apoptosis is also referred to as an active process, which removes unnecessary cells whilst necrosis is considered to be a passive degenerative process. Apoptosis is characterised by some morphological features which include chromatin contraction, nuclear fragmentation, cytoplasmic condensation, cell shrinkage, membrane blebbing, and the damage to the cell eventually leads to the cell fragmenting into membrane-bound apoptotic bodies that are phagocytosed by surrounding cells (Kiechle and Zhang, 1998). By contrast, necrosis is characterized by mitochondrial swelling, loss of plasma membrane and cell rupture. The loss of cell membrane integrity and release of cell contents induces an inflammatory response (Israels and Israels, 1999). Regulation of apoptosis by molecular mechanisms is genetically and evolutionary conserved (Tsujimoto, 1997). Apoptosis can be regulated in two pathways, extrinsic and intrinsic (Kiechle and Zhang, 2002).

1.2.1 Extrinsic apoptosis pathway

This is a pathway mediated by receptors, which requires the association of a ligand to a death receptor on the cell surface of target cells to regulate cell proliferation and differentiation (**Figure 1.4**). Fas ligand/CD95L is classified under the tumour necrosis factor (TNF) family and is synthesized as type II membrane protein. Fas/CD95 is activated through trimerization with FasL, thus inducing an apoptotic signal. After

activation Fas-associated death domain (FADD) is recruited to Fas, takes it through a process of interactions with the death domains. FADD carries a death effector domain (DED), which is responsible for downstream signalling that sequentially activates the caspases.

Caspases are cysteine proteases constitutively expressed as zymogens in the cytosol. They activate other caspases in a cascade pattern, and are themselves activated by self-cleavage (see **Figure 1.4**). Caspases are classified as initiators (Caspase-2, 8, 9, 10) and effectors (caspases-3, 6, 7) of apoptosis. Apoptotic stimuli activate the initiator caspases (caspase 8 and 9). Peptide cleavage occurs only at specific aspartic acid residues that results in the cleavage of cytoskeletal proteins, disruption of nuclear membrane and of cell-cell contact and freeing of DNA nuclease. The release of caspase activated deoxyribonuclease from its inhibitor results in DNA cleavage and fragmentation, in the internucleosomal linker regions, giving 180bp fragments observed on the agarose gel electrophoresis. Inhibitors of apoptosis which are overexpressed in malignant cells inhibit effector caspases, thereby blocking the apoptotic process (Israels and Israels, 1999).

FLICE is a caspase 8/FADD like protein that binds to FADD domains through interaction with the death effector domain (Nagata, 1997). Oligomerization of caspase 8 leads to autoactivation resulting in downstream activation of effector caspases, which commit the cell to apoptosis (Ashkenazi and Dixit, 1998). Other pathways that mediate apoptosis through their receptors include tumour necrosis factor (TNF) signalling pathway and

tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) signalling pathway (see **Figure 1.4**).

1.2.1.1 TNF signalling pathway

TNF engages with TNF Receptor-1 (TNFR-1) for it to be activated (**Figure 1.4**), and it further activates the transcription factor Nuclear factor kappaB (NF-*k*B) (Ashkenazi and Dixit, 1998). TNF can also initiate apoptosis by recruiting TRADD, which lacks DED but can mediate apoptosis. It was found that TNF R1-associated Death Domain (TRADD) links with FADD through their family of death domains. This indicates that Fas and TNFR-1 use FADD as a signal transducer; therefore, when TRADD recruits FADD it binds caspase 8 that causes self-cleavage and a sequential activation of the caspase cascade that leads to apoptosis.

In another pathway, TRADD recruits Receptor interacting protein (RIP), a serine\ threonine kinase with death domains, which is responsible for inducing the apoptotic signal (**Figure 1.4**). Another death domain RIP-associated Ich-1/ CED 3 homologous protein (RAIDD) binds RIP via its death domain and recruits caspase 2 to RIP. It has been postulated that RAIDD plays a major role in apoptotic signal transduction from one of the receptors. TNFR1 can activate NF-*k*B and Jun amino (N) terminal Kinase (JNK) through TNF-Receptor Associated Factor family (TRAF) and RIP. TRAF2 binds directly to TNR-1 and indirectly to TNFR-2 through TRADD and RIP, which then blocks TNF-induced NF-*k*B activation but not apoptosis; this indicates that NF-*k*B activation results in expression of a protein(s) that inhibits TNF-induced cytotoxicity (Nagata, 1997). TRAF2 and RIP have the ability to activate NF-*k*B-inducing Kinase (NIK), which in turn can also

activate the inhibitor of kB (I-*k*B) kinase complex, IKK (Ashkenazi and Dixit, 1998). IKK phosphorylates I-*k*B, resulting in I-*k*B degradation. NF-*k*B translocates to the nucleus where it activates transcription (**Figure 1.4**). In the Mitogen activated protein kinase (MAPK) pathway, MAPK kinase (MEKKI) phosphorylates and activates MAPK kinase (MKK), which in turn phosphorylates and activates a MAPK subfamily (JNK, p38, Extracellular signal regulated Kinase, ERK). JNK and p38 can be activated by diverse external stimuli, whereas ERK is activated by growth factors. Signal transduction via MAPK results in phosphorylation of inducible transcription factors such as c-Jun (Yue *et al*., 1999).

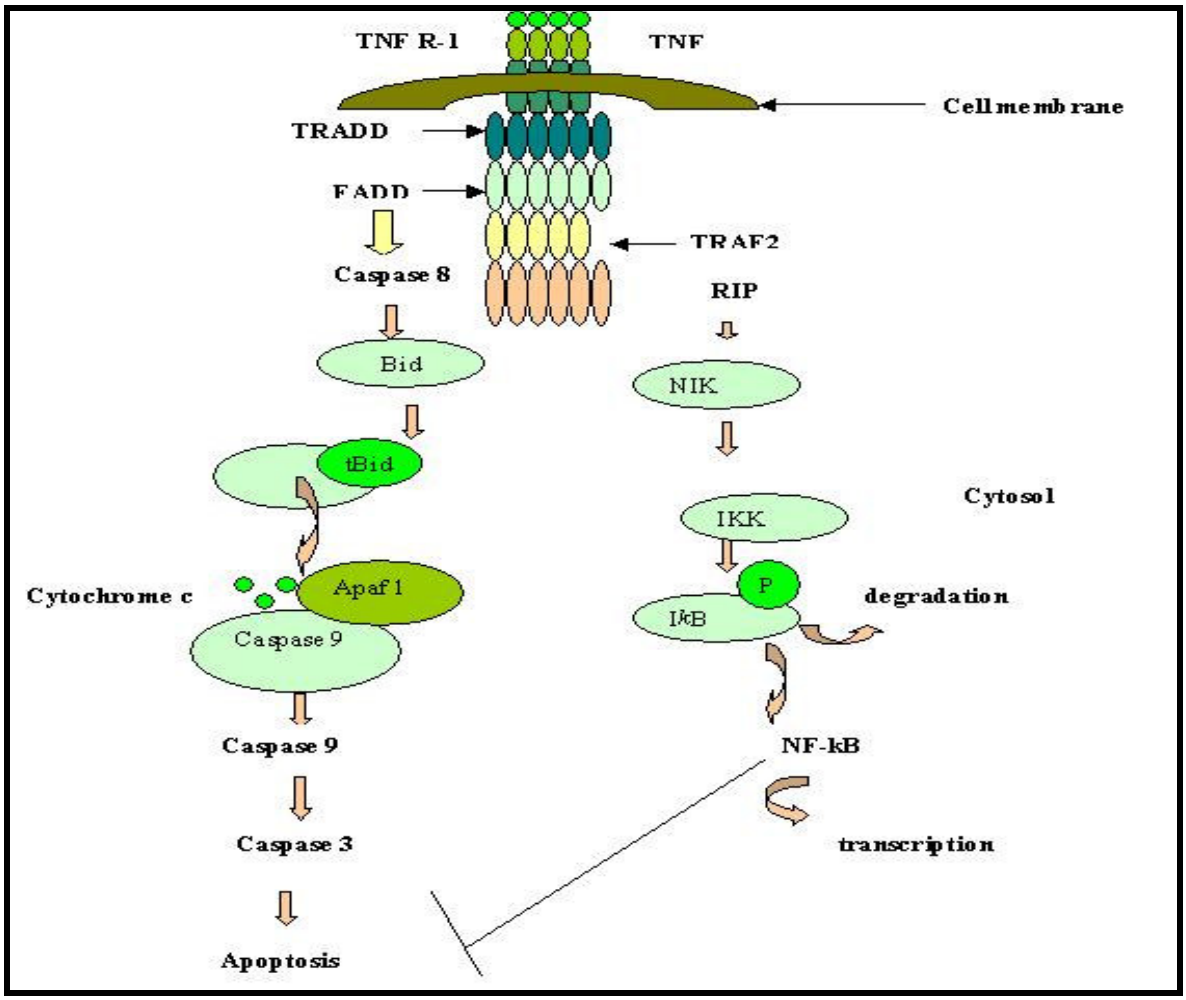

Figure 1.4: TNF signalling pathway

TNF binds TNFR1 and induces trimerization of TNFR1, which recruits TRADD through interaction with death domains. The death domain of TRADD then recruits FADD in order to activate caspase 8. In a separate pathway, RIP together with TRAF2 activates NF-*k*B, which expresses the survival genes (Nagata, 1997).

1.2.1.2 TRAIL signalling pathway

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a proapoptotic cytokine from the TNF superfamily. TRAIL induces apoptosis by attaching to its two proapoptotic receptors DR4 and 5. Trimerization of TRAIL to its receptors forms a death-inducing signal complex. Two antiapoptotic TRAIL decoy receptors DcR1 and 2 mediate activation of caspase 8. DcR 1 and 2 do not have a functional death domain but serve as extracellular links for TRAIL, thus inhibiting TRAIL from binding to DR4 and 5. DcR2 has the potential of inducing antiapoptotic signals through NF-*k*B or other pathways. Expression levels of decoy receptors do not correlate with the observed results that some tumour cell lines can be resistant to TRAIL, DR4 and DR5 and are rarely mutated in human tumours; this suggests that they might be inhibitors of the TRAIL signalling pathway (Burns and El-Deiry, 2001)

Cellular FLICE- inhibitory protein (c-FLIP), which is highly homologous to caspase 8 and 10, is one of the commonly known inhibitors of the TRAIL signalling pathway. It has DED, which allows it to associate with FADD. Elevated levels of c-FLIP inhibit Fas-ligand induced apoptosis or TRAIL treatment by binding to FADD, preventing recruitment of caspase 8. Some of the TRAIL signalling pathway inhibitors includes antiapoptotic proteins such as Bcl-2 and Bcl-X_L; they inhibit apoptosis which requires release of proapoptotic factors from mitochondria such as cytochrome c, apoptosis inducing factor (Apaf) and Smac/ DIABLO (Nagata, 1997 and Burns and El-Deiry, 2001).

1.2.2 Intrinsic apoptotic pathway

Cytochrome *c* released from mitochondria initiates the intrinsic apoptotic pathway if damaged DNA has not been not sensed and repaired by checkpoint genes (**Figure 1.5**). When p53 is upregulated it induces the apoptotic genes, *bax* and *bcl-2*, which may oligomerize and form pores on the outer mitochondrial membrane, thus releasing cytochrome c from the intermembrane space. The cytosolic cytochrome c forms an apoptosome composed of apoptotic inducing factor-1 (Apaf-1), procaspase 9, and either ATP or dATP. In some cases caspase 8 may cleave Bid, a proapoptotic protein, to a truncated form (tBid), which then complexes with another Bax-related protein, Bak to release cytochrome c (Kiechle and Zhang, 2002).

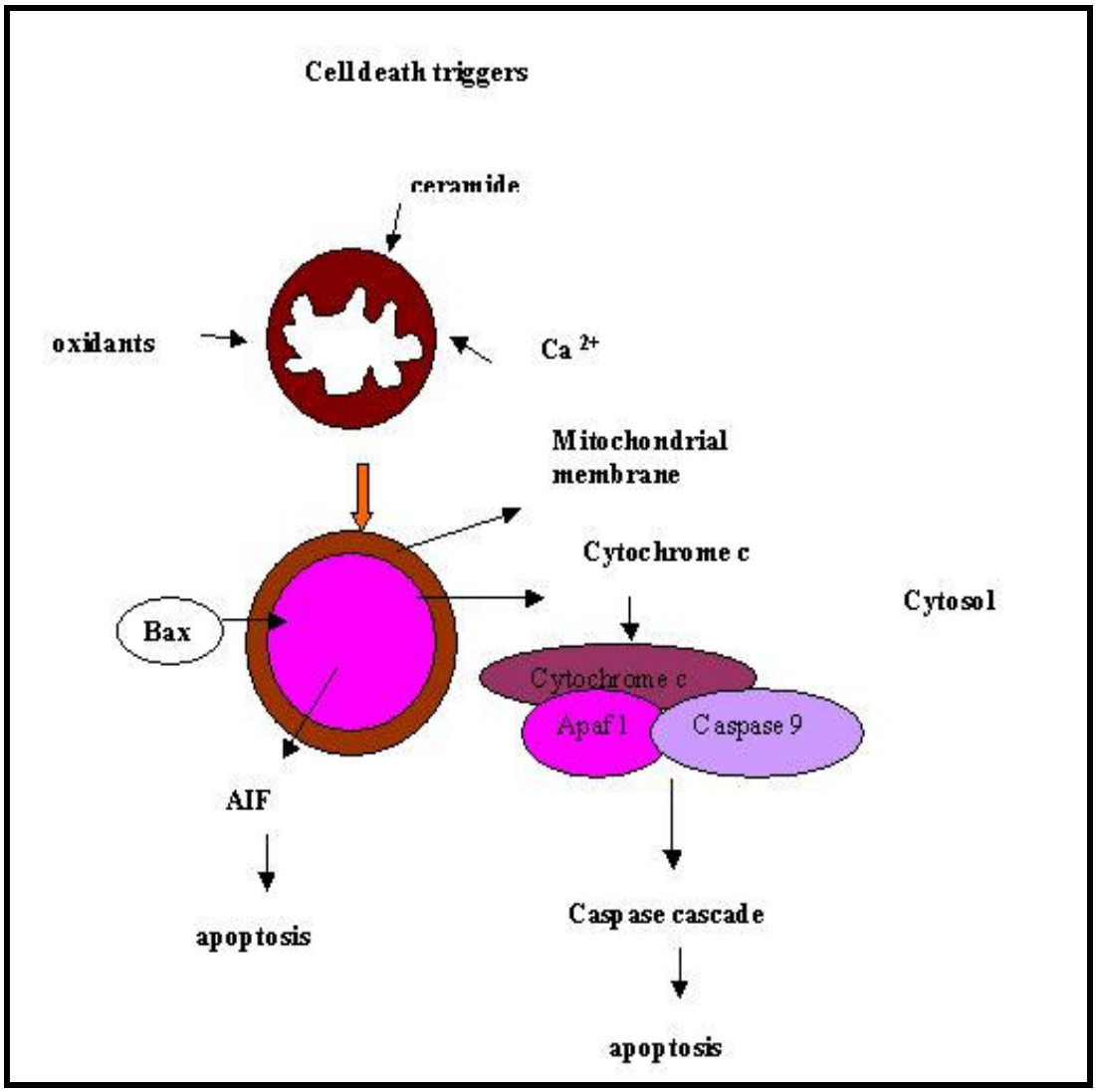

Figure 1.5: Role of mitochondria in apoptosis (Intrinsic Pathway)

Bax translocates from the cytosol to the mitochondrial membrane and then cause changes to the mitochondrial membrane. As a result of these changes mitochondrial membrane proteins are released into the cytosol, including cytochrome c and AIF. AIF moves to the nucleus where it causes DNA fragmentation, and cytochrome c binds to Apaf-1 to self-activate caspase 9, whereby caspase 9 activates caspase 3 (Israels and Israels, 1999).

1.2.3 Genetic regulation of apoptosis

1.2.3.1 p53 tumour suppressor gene

p53, a transcription regulator gene, is most commonly mutated in human cancer. It is a transcription factor with a sequence-specific DNA binding domain in the central region, and a transcription activation domain at the N-terminus (Chao *et a*l., 2000). p53 protein is found in low concentrations in the cytosol, and it is negatively regulated by another transcription factor, the Murine Double Minute 2 (Mdm-2) (Chene, 2003; Israels and Israels, 1999).

Mdm2 is a product of a proto-oncogene that is deregulated in 5-10% of human tumours. It binds to the transcription activation domain and inhibits transcriptional activity of p53. This association promotes p53 ubiquitin degradation by tagging it and exporting it from the nucleus to the cytoplasm. The Mdm2-p53 complex creates an autoregulatory loop, in which overproduction of Mdm2 limits the expression of p53 which may occur as a result of external cellular stresses. Decreased or delayed inhibition by Mdm2 leads to activation of p53, and increases transcription of genes involved in cell cycle arrest, as well as Bax which induces apoptosis. Cellular levels of p53 increase from DNA damage and other cellular stresses, thus making it possible to bind specific DNA sequences. p53 protein levels are regulated post-transcriptionally. Phosphorylation of human p53 induces conformational changes in the N-terminus thus disrupting the binding of Mdm2 and leads to its stabilization. The sequence specific DNA binding site is activated by phosphorylation of the C-terminus. Transcriptional activity of p53 is required for p53-dependent cell cycle arrest in G1. p53 has two roles in preventing cancer; cell cycle arrest

in G1, which allows time for the repair of damaged DNA, or, apoptosis, which eliminates cells with damaged genomes (Chao *et al*., 2000). These roles prevent the genome from accumulating mutations and transmitting these to daughter cells. p53 can also regulate several proteins that are involved in apoptosis, Bax and Bcl-2. In the murine leukemia cell line M1, it was found that a p53 mutant decreases bcl-2 expression and increases bax expression. The ratio of bcl-2 to bax can decide the cell's fate. Phosphorylated p53 increases its activity and inhibits binding of Mdm2. p53 promotes cell cycle arrest in late G_1 at a restriction point controlled by Retinoblastoma (Rb) protein (**Figure 1.6**).

pRb protein from the family of Rb proteins protects cells from apoptosis by inducing cell cycle arrest. Phosphorylation of Rb by cyclin D/cyclin dependent kinase 4 (CDK 4) complex allows a cell to pass G_1 into S phase (see **Figure 1.6**). p53 controls cell cycle through upregulation of p21, inhibitor of CDK. Dephosphorylated Rb binds E2F, a transcription factor required for expression of genes necessary for the cell to pass through G1 restriction point. Upon Rb phosphorylation E2F is released and translocates to the nucleus, thus inducing transcription of protein that helps the cell progress to S phase. Maintenance of Rb in its hypophosphorylated state holds the cell in the G1 phase, thereby allowing time for repair. When DNA is severely damaged and non-repairable, p53 induces apoptosis through the Bax/ Bcl-2 pathway (Israels and Israels, 1999).

1.2.3.2 Bcl-2 gene family

Control of the intrinsic apoptotic signalling resides with the *Bcl-2* gene family, which is derived from B-cells. Some are anti-apoptotic (*Bcl-2/Bcl-*X_L) and others are pro-apoptotic (*Bax, Bad* and *Bid*) (**Figure 1.7**). Anti-apoptotic genes share Bcl-2 homology (BH)

sequence from BH1 to BH4, although some may lack BH4, and proapoptotic genes also share sequences in BH1 to BH3 but not BH4**.** Some proapoptotic proteins share sequence homology in BH3 only, example, Bid and Bik. Some of these structural domains allow these proteins to homo- and hetero-dimerize. Heterodimerization results from insertion of BH3 domain into the hydrophobic region of antiapoptotic protein. In addition to BH1 and BH2, BH4 is needed for antiapoptotic activity, whereas BH3 is essential for proapoptotic activity (Adams and Cory, 1998). Elevated levels of *Bax* expression promote apoptosis and those of *Bcl-2* repress apoptosis. Bcl-2 is located on the outer mitochondrial membrane and controls the ion channels in the membrane, whereas Bax resides in the cytosol. When Bax receives apoptotic signals it translocates to the mitochondrial membrane, forms the 'Permeability Transition Pore' and causes loss of selective ion permeability which is followed by the release of cytochrome c and apoptosis inducing factor into the cytosol (**Figure 1.5**). The flavoprotein, AIF, induces nuclear fragmentation in caspase-independent manner, whereas cytochrome c binds to apoptotic protease activating factor via the C-terminal WD-40 repeat domain in the presence of ATP/dATP (Tsujimoto, 1997), which then recruits and activates procaspase-9. The activated caspase 9 next activates downstream caspases (Israels and Israels, 1999).

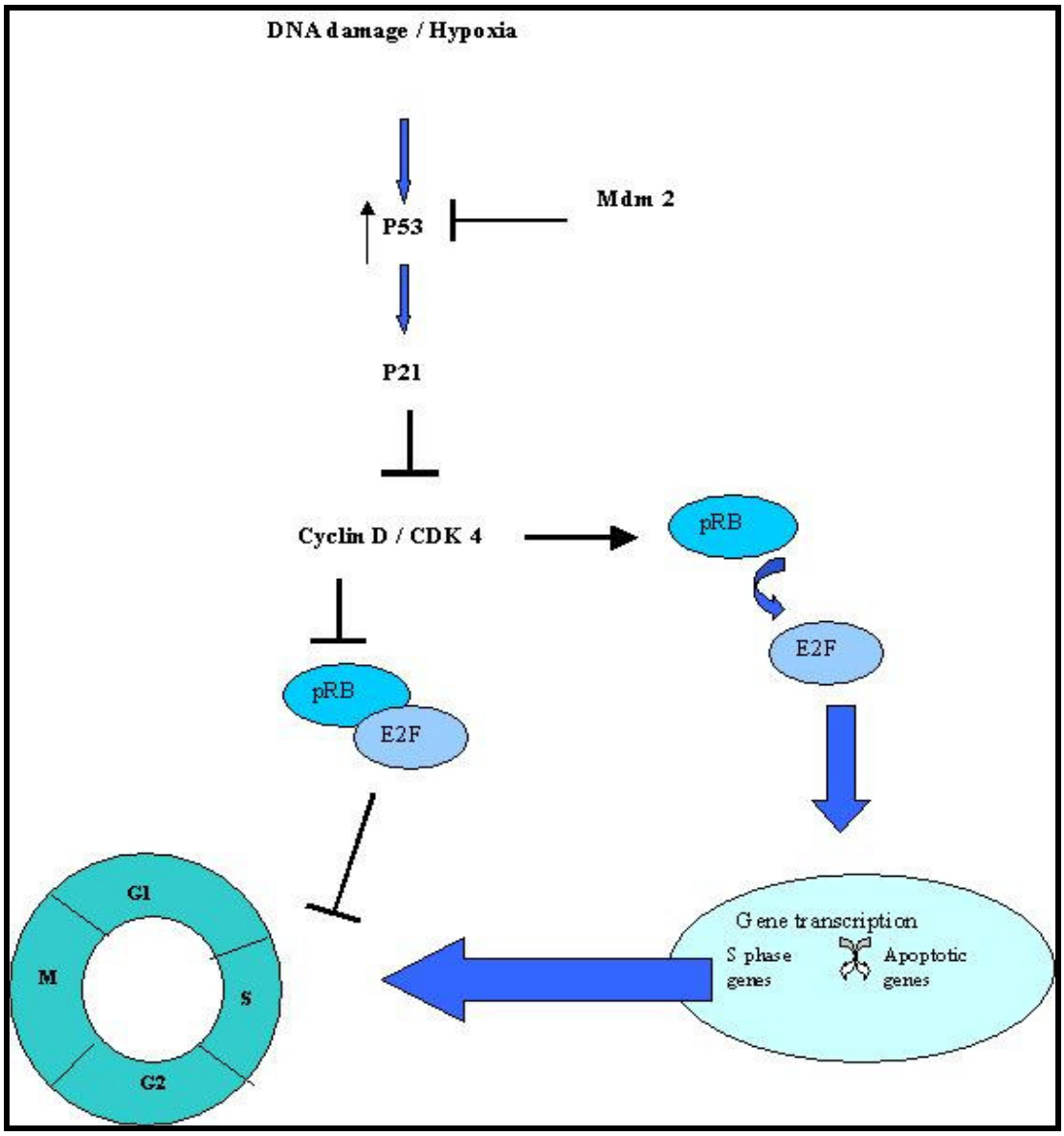

Figure 1.6: p53-Rb pathway.

p53 activates p21 which inhibits CDK thus blocking cell cycle progression. Hyperphosphorylated Rb causes cells to move from G to S phase.

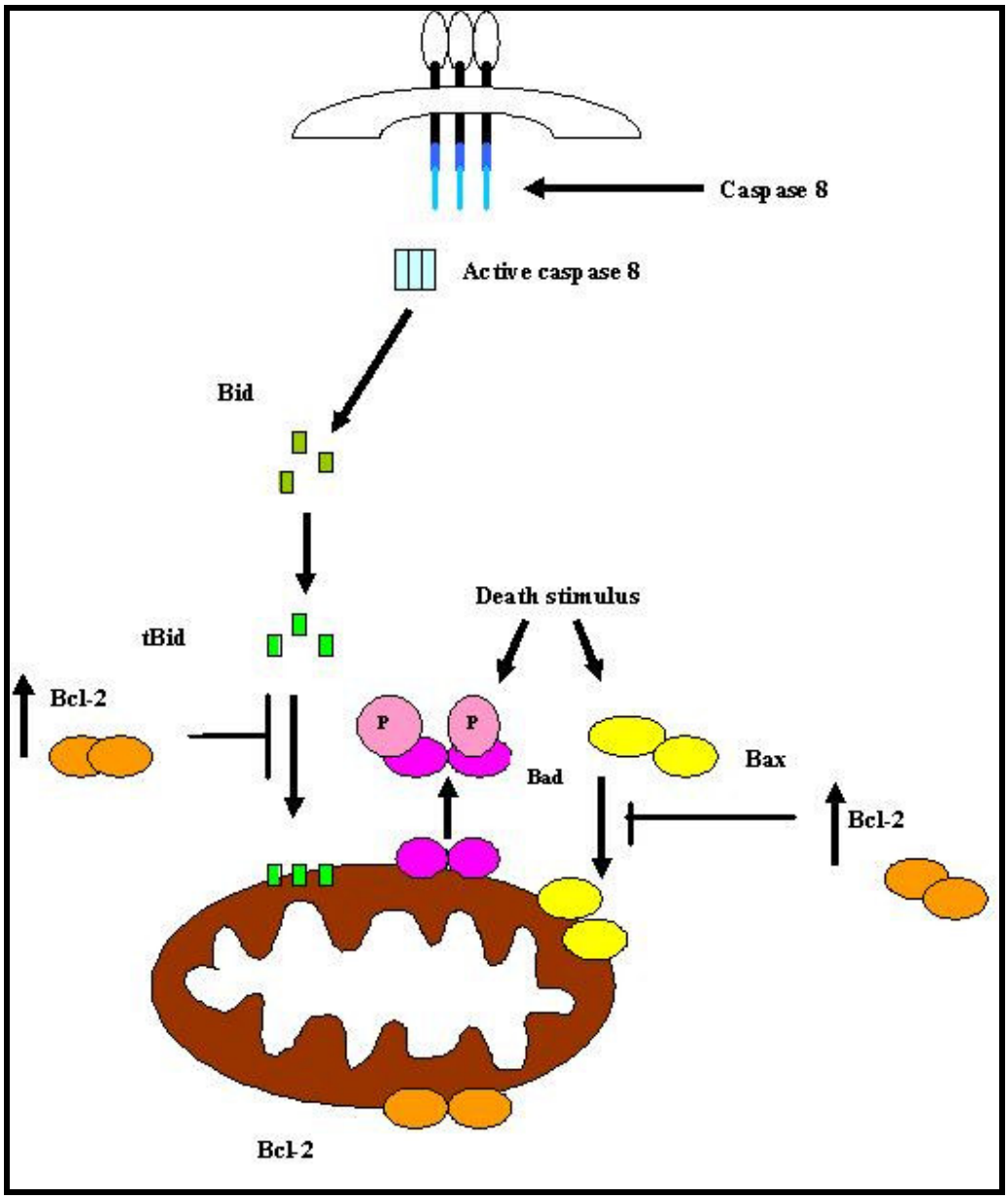

Figure 1.7: Spatial relationship of various members of Bcl-2 family.

Ratio of proapoptotic genes to antiapoptotic genes decides a cell's fate. Upon death stimulus Bax translocates to mitochondrial membrane and induces pores and Bak gets phosphorylated and enters the cytosol. Truncated Bid translocates to the mitochondrial membrane and causes pores. Increased levels of Bcl-2 inhibit apoptosis.

1.2.3.3 c-Myc

c-Myc, a protooncogene whose deregulated expression accelerates cell proliferation and cell transformation, occurs frequently in human tumours. c-Myc has been shown to regulate the cell cycle, and its down regulation may lead to cell death by apoptosis (Brenna *et al*., 2002). c-Myc protein is located in chromosome 8q24, has 3 exons, encodes a 62kDa nuclear protein which is a transcription factor that recognises the CA (C/T) GTG element (Ebox), and is expressed in proliferating cells. Inappropriate over-expression of c-Myc results in bringing cells into a cell cycle arrest phase in the absence of external mitogens, and in addition promotes apoptosis (Evan and Littlewood, 1998). Myc-triggered apoptosis has been observed when an external insult such as growth arrest has been induced. In the absence of such insults, over-expression of c-myc does not initiate apoptosis but causes cell cycle re-entry. Myc can act as an upstream regulator of cyclin-dependent kinases, and functionally antagonises the action of cyclin-dependent kinase inhibitor, p27. Reduction of c-myc expression and its inappropriate expression may lead to cell apoptosis (Brenna *et al*., 2002).

1.3 UBIQUITIN-LIKE PROTEINS

1.3.1 Ubiquitin-proteasome pathway

Protein degradation is a complex enzyme controlled and highly specific process, which is mainly involved in the field of basic cellular processes. Proteins that are to be degraded are tagged with moieties of a small 76 amino acid polypeptide called ubiquitin. Ubiquitin is highly conserved over all eukaryotic cells. Ubiquitination involves the E1-E2-E3 cascade of enzymes. Proteins tagged with ubiquitin are degraded by the proteosome complex, comprising a 20S catalytic core and two 19S which recognize ubiquitinated

proteins, remove the attached ubiquitins and finally degrade them into smaller peptides (Ciechanover, 1998) (**Figure 1.8**).

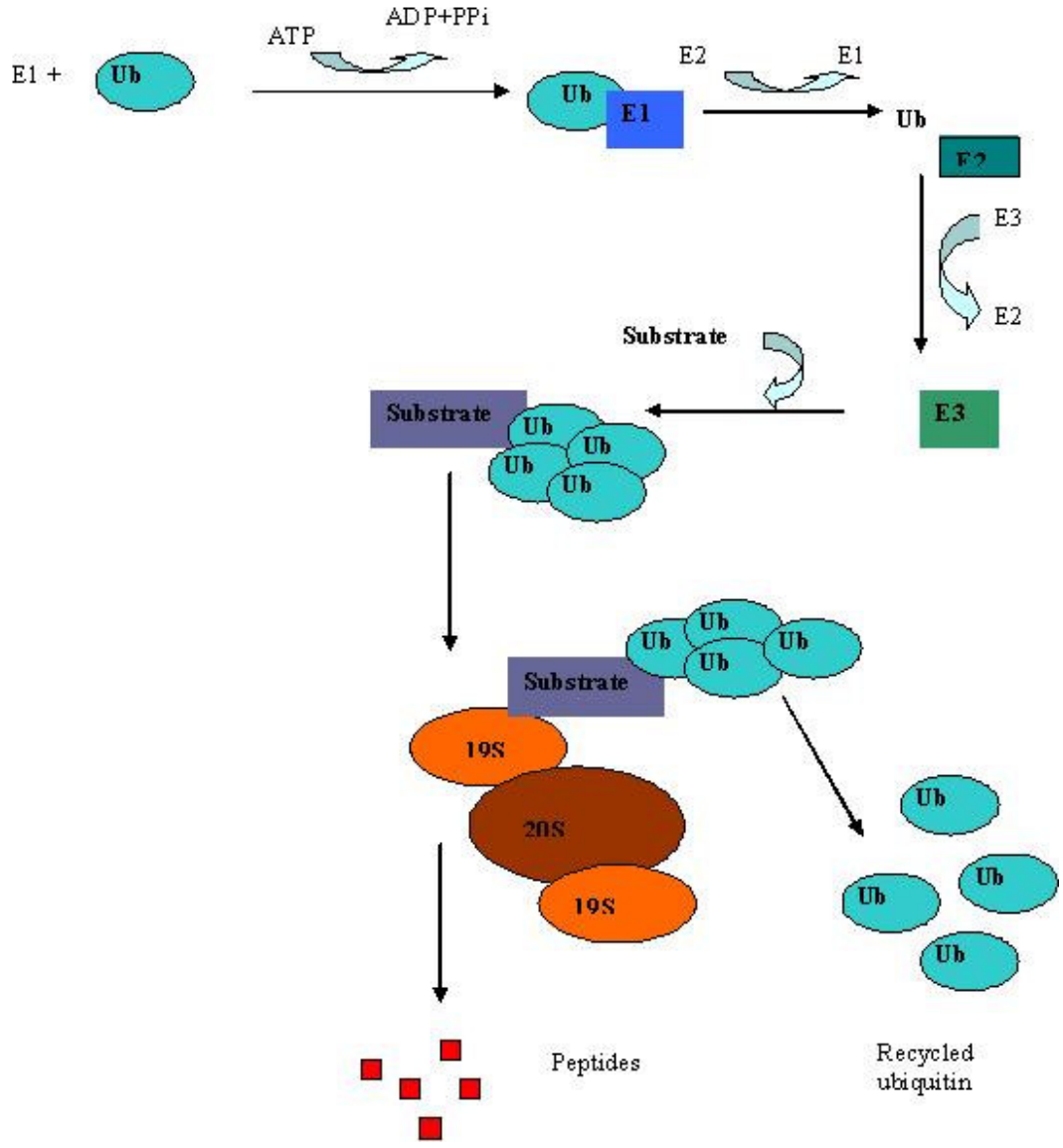

Figure 1.8: Ubiquitin proteosome pathway

This involves the activation of ubiquitin by E1, followed by transfer of ubiquitin to an E2, and finally attached to the protein with the help of an E3. Polyubiquitin protein is recognized by the 26S proteasome and degraded (Adapted from Ciechanover, 1998).

1.3.2 Significance of ubiquitin proteasome pathway in

(i) Cancer.

Ubiquitin proteosome pathway (UPP) has the ability to control the nature of apoptosis by regulating the intracellular level of some proteins involved in apoptosis, including Bcl-2 family proteins, inhibitors of apoptosis and some caspases. Lastly, UPP can regulate DNA repair by degradation of several proteins that take part in nucleotide excision repair (Golab *et al*., 2004). UPP regulates gene expression by degradation of transcription factors that are involved in the development of various malignancies. It can also control the degradation of tumour suppressor gene products and cell cycle regulators and their inhibitors.

The common cyclin-dependent kinase, p27 ^{cip1}, regulated by UPP controls the transitional steps of the cell cycle. It initiates apoptosis, regulates drug resistance in tumour cells and plays a role in the differentiation of many cell types. Levels of p27 ^{cip1} were found to be decreased in human cancers, occurring during the post-translational step, and are mediated by UPP. Inhibitors of the 26S proteosome have been shown to cause apoptosis and cell cycle arrest in tumourigenic cells (Golab *et al*., 2004).

(ii) Apoptosis

Apoptosis signalling and caspase activation results in a conformational change in the normally monomeric Bax. Bax moves into the mitochondria and causes the outward migration of proapoptotic mitochondrial factors such as cytochrome c and second mitochondria-derived activator of caspase (SMAC). This event results in the activation of caspase 9, formation of the apoptosome and molecular signalling that results in the death of the cell.

Proteosome inhibitors can decrease FLIP protein levels in tumours, which results in increased signalling of apoptosis due to increased activation of caspase 8. This step involves ubiquitin ligase TNF receptor activation factor-2 (TRAF 2) which acts indirectly by causing cell cycle arrest whereby there is increased degradation of the FLIP-TRAF2 complex**.** The regulation of TRAIL signalling in the ubiquitin proteasome pathway is mediated by the inhibitor of apoptosis proteins (IAP) E3 ligase. IAP can bind directly to caspases and can act as an ubiquitin ligase for caspases, resulting in the degradation of these caspases. IAP interacting with caspases can be blocked by SMAC, which shows a caspase homology domain. Another mechanism is through the stabilization of the inhibitor of kappaB (I*k*B)/NF-*k*B complex and prevention of nuclear translocation of the antiapoptosis transcription factor NF*k*B. During TRAIL-DR4, DR5 signalling this pathway is activated by interactions of activated Fas-associated death domain with activated receptor- interacting protein (RIP), which in turn activates NF*k*B inducing kinase and phosphorylates I*k*B. Therefore, this inhibition of I*k*B degradation blocks this RIP-mediated antiapoptosis signalling event. P53 protein levels and susceptibility to apoptosis can be deregulated by Mdm2 E3 ligase. This process can be inhibited by p53 phosphorylation and by appropriation of Mdm2 by ARF (Zhang *et al*., 2004).

Summary

Can't live with it, can't live without it; such is the paradox of apoptosis, the tightly regulated process of programmed cell death. Apoptosis is essential in maintaining homeostatic balance between cell proliferation and cell death during development of an organism. On some occasions, high or low levels of apoptosis might give rise to a variety

of diseases including cancer and immune directed disorders. There are three different mechanisms of apoptosis by which a cell commits suicide and these are, 1) generated by signals arising within the cell, 2) triggered by death activators binding to receptors at the cell surface and, 3) by dangerous reactive oxygen species. DWNN deficient Chinese Hamster Ovary cells have been found to be resistant to staurosporine-induced apoptosis and this raises an interesting notion that DWNN may be involved in apoptosis. Ubiquitin protein ligases, of which DWNN may be one, are defined as proteins required for the recognition and ubiquitination of specific substrates, and in so doing, marking them for degradation. These proteins are a very important group of enzymes that take part in pathogenesis of many human diseases through deregulation of targeted proteolysis.

1.4 CERVICAL CANCER

1.4.1 Prevalence

Cancer of the cervix is the second most common, serious malignant tumour in women. South Africa is reported to have the highest incidence of cervical cancer in the world. Of the approximately 30/100,000 women with cervical cancer, it is the most common cancer in black (31.2%) and colored (22.9%) women, second most common in Asian women (8.9%) and fourth most common in white women (2.7%) (Department of Health, Statistical Notes, 2001). The majority of women who present with advanced stages of the disease have either no or very limited knowledge about risk factors linked to cervical cancer and the need for PAP smears. The incidence of this cancer has been declining in developed countries, partly as a result of improved socio-economic circumstances, better access to medical facilities and PAP smear screening (http://www.cansa.org.za/Research/registry 1997_cervix.asp).

1.4.2 Risk factors

Cervical cancer is considered primarily to be a sexually transmitted disease. Inadequate screening for human papillomavirus (HPV), multiple sexual partners, young age at intercourse and high parity all emerge as significant risk factors. The choice of contraceptive methods appears to be important: whereas barrier mechanisms have been associated with reduced risk, the use of oral contraceptives has been associated with increased risk (Kim *et al*., 2003). Early onset of sexual activity is thought to be associated with greater risk, because during puberty cervical tissues undergo a variety of changes that may make the stratified epithelium more vulnerable to damage and invasion by the virus. Not undergoing regular PAP smear testing is the single major risk factor, and is linked to poor outcome in women who develop cervical cancer (Huh *et al*., 2003).

1.4.3 Morphology

The cervix is a tube-like organ at the lower end of the uterus. Its central canal opens at its upper end into the uterus and at the lower end into the vagina. The wall of the cervix comprises three layers, namely mucosa (endothelium), myometrium and the perimetrium.

The mucosal layer that lines the cervical glands comprises of columnar epithelium and is called the endocervix. The glands secrete mucus, and undergo hormone directed changes during the menstrual cycle. The portion of the cervix that projects into the vagina is covered by stratified squamous epithelium that may or may not be keratinized. The columnar lining abruptly changes to the stratified squamous at the external opening of the cervical canal. This segment, which is sited just inside the distal end of the cervical canal, is referred to as the exocervix (Fletcher, 1993).

1.4.4 Histopathology

Micro-trauma enhances the entry of HPV into the host cells. The virus usually enters the epithelial cells of the basal layer that contains reserve (basal) and stem cells. The virus generally infects cells that originate from the stratified epithelium, causing benign genital warts and benign tumours, and these generally take the form of condylomas that are characterised by thickening of the squamous epithelium. Precursor lesions commence with mild dysplasia that progresses to moderate and then to severe dysplasia, eventuating in carcinoma-in-situ. Following an incubation period in the squamous cell layers that varies from 3 to 12 months, the viral oncogenes E6 and E7 produce abnormal proliferation and cell cycle deregulation of the basal and stem cells. Cytological changes also begin to appear in the completely differentiated cells. The carcinoma advances from the precursor lesions to intra-epithelial neoplasia. Invasive carcinoma occurs when malignant cells break through the basement membranes and enter the cervical stroma. Squamous cell carcinoma is the most common malignant tumour (about 80%) of the cervix. The lesions commence at the epithelial squamo-columnar junction, and may be keratinizing. Adenocarcinomas arise from the endocervical columnar cells, account for about 14% of cervical cancer, show greater malignancy than the squamous cell variety and have a poor prognosis. The glassy cell tumour is a poorly differentiated form of adenosquamous carcinoma (Carr and Gyorfi, 2000).

1.4.5 Stem cells and stem-cancer cells (carcinogenesis)

The stem/progenitor cell is a reserve cell which has the capacity to replicate into self, as well as differentiate into any cell type under the guidance of specific cell cycle regulators. For self-replication a stem cell has to control the cell cycle in such a manner so as to produce two daughter cells which renew and maintain the integrity of their genome and their original identity. For a stem cell to remain undifferentiated in a niche that is made up of a set of tissue cells and extracellular biomolecules, depends on the expression of intracellular transcription factors (Preston *et al*., 2003). The basal and the stem cells of the cervical epithelial layers have the capacity to differentiate into multiple phenotypes. In fact, adult stem cells are able to produce and maintain the required number of differentiated stem cells by slow cell cycling that protects them from accumulating errors in the process of DNA synthesis. The question arises therefore as to the mechanisms by which HPV alters cell cycle regulation to drive stem cells into becoming carcinogenic. Stem cells do not possess a molecular cascade that avoids replication errors, and therefore they are highly susceptible to dysplasia and carcinogenesis (Tsai, 2004).

Whereas cancer cells have the ability to divide continuously, the self-renewal of stem cells is highly ordered, and responds to a feedback mechanism that senses the number of mature cells and regulates the rate of cell division; in contrast cancer cells lack this feedback. Secondly, malignant cells show dysplastic changes, varying states of differentiation and are susceptible to accumulating replication errors whereas somatic stem cells develop a mechanism by which the cells are chaperoned and prevented from making such errors (Tsai, 2004). Stem cells multiply over the lifetime of an individual

and have the ability to renew themselves; however amplifying cells in transit are susceptible to mutation that may lead to dysregulation of self-renewal, dysplasia and carcinogenesis. Stem cell homeostasis is maintained through programmed cell death by increased expression of Bcl-2 (see section on Bcl-2 family and **Figure 1.5**). Stem cells, like cancer cells are considered to be derived from endogenous stem cells. This is because their differentiated progeny may have reactivated some of the stem cell programs and undergone de-differentiation. The underlying mechanism may be because both stem cells and cancer cells have high and unlimited ability to proliferate. Stem cells are frequent targets for transformation; it is easier for tumour cells to capture the cell cycle regulatory and genomic machinery of stem cells because stem cells continuously divide and are prone to errors (Tsai, 2004).

With time the life span of stem cells and their capacity for self renewal becomes restrained. As the restorative capacity of tissue stem cells declines, they become susceptible to oncogenic mutations and together with the failure of tumour suppressor systems [p16 (INK4a)-Rb, ARF-p53 and telomere] to protect against carcinogenesis, they transform into cancer cells (Sharpless and DePinho, 2004). Some cancers contain rare cells with the potential to support the formation and growth of the neighbouring tumour population. These unusual cells have the same functional and phenotypic features as stem cells, and are therefore called cancer-stem cells. For a stem cell to evade capture by cancer cells, it has to avoid senescence (cell rest). Environmental and hyper-proliferative stresses are initiators of senescence. There are two pathways that contribute to stress-induced senescence, and these involve the p19-p53 and p16-Rb signalling cascades that

are activated by DNA damage arising from telomere and other dysfunctions which lead to apoptosis (Pelicci, 2004).

Bmi-1 is a transcriptional repressor that functions as an inhibitor of repressor genes which initiate cell senescence and death (Park *et al*., 2004). Bmi-1 regulates senescence through the p16-Rb pathway, by repressing p16 expression. Bmi-1 may contribute to carcinogenesis by inhibiting p16-mediated senescence specifically in stem cells (Pelicci, 2004).

1.5 HUMAN PAPILLOMAVIRUS

1.5.1 Structure

Papillomaviruses are classified under the family of papovaviridae. The human papillomavirus (HPV), of which there are 120 subtypes, is a small and non-enveloped icosahedral particle ~ 55-60 nm diameter. It is composed of 72 capsomers, which are arranged in a star-shaped manner attached on T7 lattice (60 hexameric and 12 pentameric; **Figure 1.9**) (Hagensee *et al*., 1994). Its genome comprises a circular and double stranded DNA of ~7 900 base pairs in size, complexed with histones (Carr and Gyorfi, 2000) (see **Fig. 1.10**), and can be reconstituted into 3 regions (**Figure 1.10**): (1) upstream regulatory, (2) early and (3) late. The early region genes are E1 to E7, which encode for proteins that are responsible for viral replication. The E6 and E7 regions possess the oncogenic properties of HPV. The late region genes (L1 and L2) encode the viral structural proteins, L1 major capsid protein of ~55kDa and L2 minor capsid protein of ~74kDa, both of which are required late in the viral life cycle to encapsulate the virus (see **Figure 1.10**), (Carr and Gyorfi, 2000; Chen *et al*; 2000).

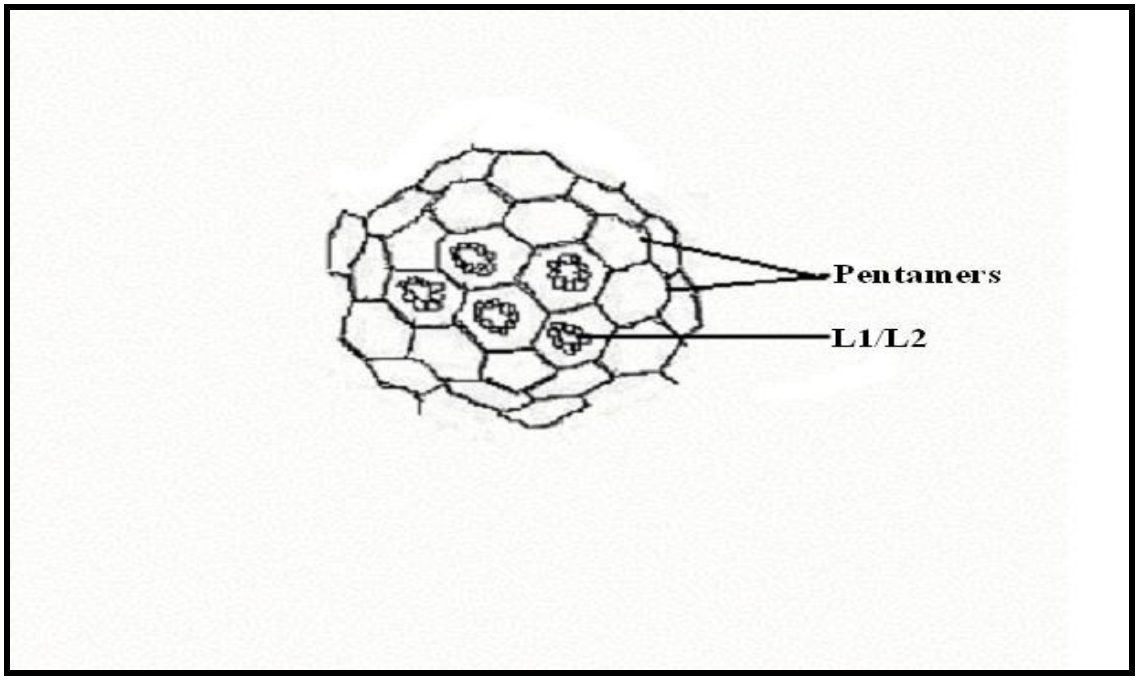

Figure 1.9: Arrangement of L1/L2 on the virus like particles

Each pentamer has associated L1/L2 subunits and makes a tightly linked ring (Adapted from Hangensee *et al*., 1994).

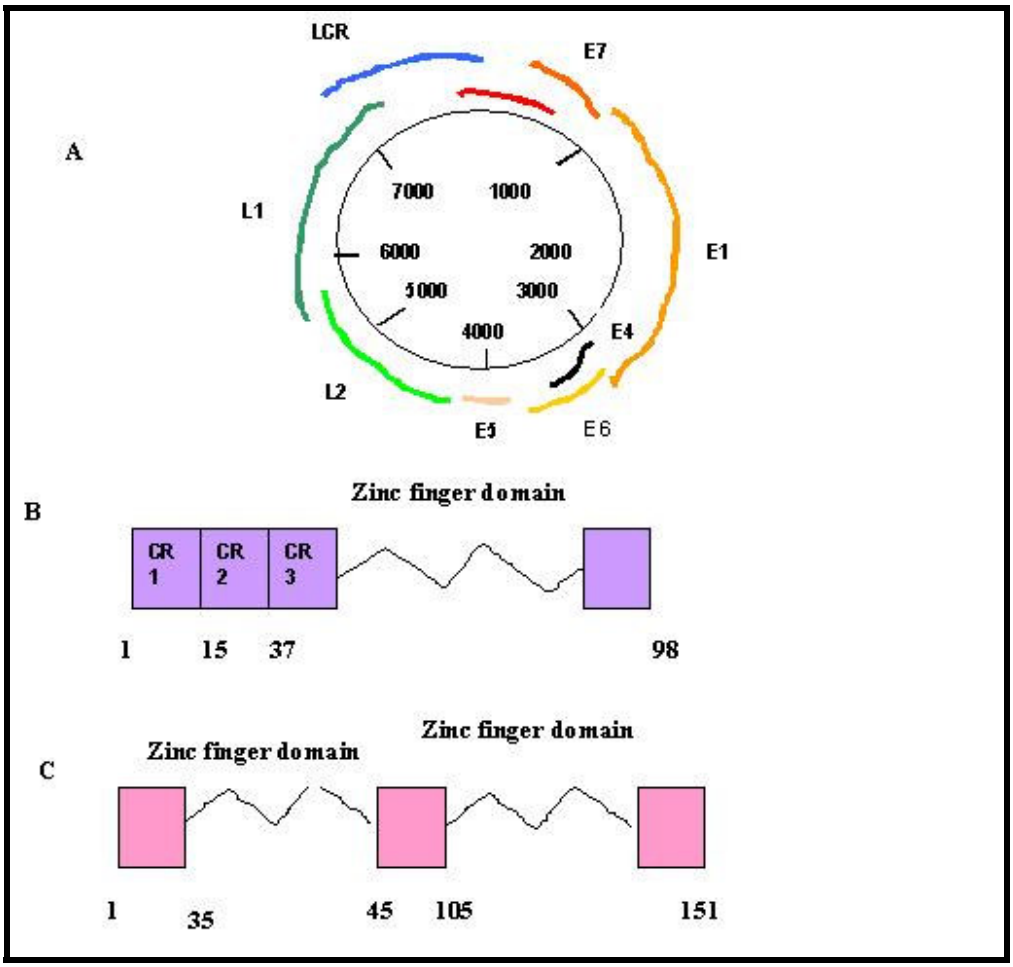

Figure 1.10: (A) Schematic representation of the genomic organization of Human papillomavirus (HPV)

Circular double-stranded DNA with early region genes E1-E7 and late region genes L1 and L2. (**B**) **HPV E7 oncoprotein** and (**C**) **HPV E6 oncoprotein**.

(Chakrabati and Krishna, 2003; Finzer *et al*., 2002).

1.5.2 Viral Risk

The human papillomavirus is collated into two groups: low risk that includes HPV 6, 11, 31, 33, 35, 42, 43 and 44 and high risk that includes HPV 16, 18, 45 and 46. Precursor cell lesions of the less aggressive squamous tumours have been associated with the HPV 31, 33 and 35. The high risk viruses (16, 18, 45 and 56) cause lesions which more frequently progress to malignancy, and are present in 70% of invasive squamous cell carcinomas. However, 90% of squamous cell dysplastic lesions are caused by HPV 16, and more than 50% of the invasive squamous intraepithelial tumours contain HPV 16. In contrast, HPV 18 is implicated in small cell undifferentiated- and adeno- carcinomas (Chen *et al*., 2000; Finzer *et al*., 2002).

1.6 GENOME AND GENOMICS

The high risk oncogenes E6 and 7 inhibit tumour suppressor proteins, thus causing immortalisation and transformation of human cells (Kehmeier *et al*., 2002; Mantovani and Banks, 1999). The oncogenic potential of high risk viruses is centred in two small open reading frames encoding the proteins HPV E6 and 7. The HPV E7 protein, is a nuclear protein of 98 amino acids that possesses casein kinase II phosphorylation sites at serine residues 31 and 32. It has 3 domains, CR1, CR2 and CR3. It can dimerize in the CR3 domain through the zinc finger motif (see **Figure 1.10**) (Chakrabarti and Krishna, 2003). The E7 expression inactivates Rb protein (and related proteins). In addition to that effect it inhibits p21 (an inhibitor of cyclin-dependent kinase) genes responsible for the G1/S transition and proliferating cell nuclear antigen-dependent DNA replication. The HPV E6 protein associates with the cellular p53 protein for proteosome-dependent degradation, and

has the ability to induce a telomerase activity in the normal telomerase activity-negative mucosal keratinocytes (Kehmeier *et al*., 2002).

1.7 GENOMICS OF CERVICAL CANCER

In human papillomavirus immortalized cells, the structural changes are commonly detected in chromosomes of 1, 3 and 5, and less frequent aberrations occur in chromosomes 7, 8, 12, 13, 16 and 22. Cervical carcinoma has some loss of heterozygosity (LOH)**,** and 3p, 6p and 18q are the most commonly deleted chromosomal regions in the genome. Chromosome 3p LOH has been detected in cervical carcinoma, and 3q gain is the one with most frequent aberrations, which mark the cellular transition of the cervical cells from severe dysplasia to become invasive squamous cell carcinoma of the cervix (Matthews *et al*., 2000). But isochromosome 5p and 5p gain have also been identified as aberrations characteristic of cervical carcinomas. Matthews *et al*., (2000) found chromosome 3q gain to be the most common chromosomal aberration detected in the primary cervical tumour samples, but 3p loss was also detected, but only in squamous cell carcinoma. Gain of chromosome 3q may represent the presence of an oncogene at this locus; chromosomal loss might represent location of a putative tumour repressor gene (Dasgupta *et al*., 2003). High frequencies of LOH were found in 3p26.1, 3p22.3, 3p21.2 and 3p13, suggesting the location of putative tumour suppressor genes in these regions, which may have an inhibitory effect on tumour progression. Deletions in 3p21.2 appear to occur early during the development of cervical carcinoma (Dasgupta *et al*., 2003). Amplification of chromosome arm 3q is the most common unusual event whereby dysplastic premalignant cervical squamous lesions progress to invasive cancer (Ma *et al*., 2000).

The phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*) gene is located at 3q26, and encodes the catalytic subunit p110α of a class I_A PI3K, one component of a lipid-signalling pathway, which plays a role in regulating cell growth and apoptosis (Singh *et al*., 2002). Ma and colleagues (2000) have shown that cervical cancer cell lines of epithelial origin containing amplified *PIK3CA* demonstrated elevated levels p110α (gene product of *PIK3CA*) expression. Physical properties of transformation in these cell lines, including increased cell growth and decreased apoptosis, are in a way affected by the treatment of specific PI-3 kinase inhibitor. Therefore, over-expression of *PIK3CA* in cervical cancer may result from enhanced cell proliferation and reduced apoptosis. *PIK3CA* is considered to be an oncogene in cervical cancer, and *PIK3CA* amplification may be linked to cervical carcinogenesis (Ma *et al*., 2000 and Singh *et al*., 2002).

1.8 ONCOGENES

The two proteins (E6 and E7) of the human papillomavirus oncogenes interfere with cellular functions of tumour suppressor proteins, thereby causing immortalisation and transformation of human cells (Kehmeier *et al*., 2002). This effect is produced by one mechanism, by which there is integration of viral DNA into the host genome, and integration of the viral genome into cells of cervical cancer (Finzer *et al*., 2002). c-Myc, a protooncogene whose deregulated expression accelerates cell proliferation and cell transformation, occurs frequently in human tumours. c-Myc has shown to regulate cell cycle, and its down regulation may lead to cell death by apoptosis (Brenna *et al*., 2002). c-Myc protein is located in chromosome 8q24, has 3 exons, encodes a 62kDa nuclear protein which is a transcription factor that recognises the CA (C/T) GTG element (Ebox), and is expressed in proliferating cells. Inappropriate over expression of c-Myc results in bringing

cells into a cell cycle arrest phase in the absence of external mitogens, and in addition promotes apoptosis (Evan and Littlewood, 1998). Myc-triggered apoptosis has been observed when an external insult such as growth arrest has been induced. In the absence of such insults, over expression of c-myc does not induce apoptosis but causes cell cycle re-entry. Myc can act as an upstream regulator of cyclin-dependent kinases, and functionally antagonises the action of cyclin-dependent kinase inhibitor, p27. Reduction of c-myc expression and its inappropriate expression can lead to cell apoptosis (Brenna *et al*., 2002). Continuous expression of E6 and E7 is another reason for immortalisation and transformation of cells; it is also believed that they maintain transformed phenotypes. E6 and E7 change pathways are involved in cell cycle control by interacting with two important tumour suppressor proteins, p53 and Rb (Chakrabarti and Krishna, 2003).

1.8.1 HPV E7 and Rb protein

The pRb family members play a crucial role in regulating cell cycle progression through G1 to S phase. The pRb protein does not only disturb E2F transactivation properties but has the ability to change E2F into a transcription repressor (Scheffner and Whitaker, 2003). E2F and pRb interaction is regulated by pRb phosphorylation. HPV infected cells maintain a strong virus progression as they move into S phase to allow viral DNA replication. The E7 expression inactivates retinoblastoma protein (Rb) and related proteins (**Figure 1.6**). Additionally, it inhibits p21 (an inhibitor of cyclin-dependent kinase) genes responsible for the G1/S transition and proliferating cell nuclear antigen (PCNA) - dependent DNA replication.

Recent studies have suggested that the binding of HPV E7 to pRb is related to pRb phosphorylation, in the sense that binding of E7 to pRb releases E2F from the pRb protein, and then E7 binds to hypophosphorylated Rb and induces the cells into premature S phase, thereby disrupting Rb-E2F complexes (see **Figure 1.6**)(Chakrabarti and Krishna, 2003). It has been observed that any association with high risk HPV E7 causes pRb proteasome-mediated degradation to occur (Scheffner and Whitaker, 2003). A possible mechanism devised was similar to the E6/p53 interaction (to be discussed), in that E7 recruits an ubiquitin-protein ligase to the target protein (pRb) with subsequent degradation, but this concept has yet to be proven. Scheffner and Whitaker (2003) have reported that E7 interacts with S4 ATPase subunit of 19S of the 26S proteasome, through the carboxyl-terminal zinc motif. It has been suggested that E7 may act as a link between pRb and proteasome, thereby targeting pRb directly to the proteasome for degradation without tagging the pRb with any ubiquitin. This interaction is considered to be similar to the association of pRb with Gankyrin, a protein that is over expressed in hepatocellular carcinomas. Gankyrin binds pRb leading to the release of E2F, and this increases the rate of pRb proteosome-mediated degradation. In addition, Gankyrin binds the S6 ATPase subunit of 19S regulatory complex. It has therefore been concluded that Gankyrin is a cellular homologue of E7, with regard to pRb degradation (Scheffner and Whitaker, 2003). Expression of E7 protein increases protein levels of p21 and depending upon the degree of E7 expression and differentiation state of the cell type, since increased p21 levels either induce DNA synthesis and apoptosis or growth arrest (Park *et al*., 2000a). Basile *et al*., 2001 found that E7 binds and stabilises p21, and thereby interferes with its function. E7 expressing cells are able to resist tumour necrosis factor (TNF)-mediated growth arrest,

not only through the ability of HPV 16 E7 to degrade pRB but also through blockage of p21 E7-mediated p21 inactivation, by assisting the cell to evade TNF-mediated growth arrest and by enhancing apoptosis (Basile *et al*., 2001).

1.8.2 HPV-mediated immune evasion mechanism

Interferon Regulatory Factor (IRF-1) is a novel target of E7 for transcriptional repression, and it requires both the domains of E7. This inactivation might be due to the recruitment of histone deacetylase (HDAC) by conserved region (CR3) zinc finger domain of E7 to IRF-1. This suggests that both domains are important for the complete transforming activity of human papillomavirus (HPV) E7 in vivo. Recent studies showed that HPV-positive cervical cancer cells might be associated with the evasion of host immune surveillance. In HPV positive cervical cancer tissues, there is markedly down regulated expression of major histocompatibility complex class molecules, one such being transporter 1, ATP-binding cassette, subfamily B. Moreover, monocyte chemoattractant protein is also less expressed in the stroma surrounding carcinoma cells. These immune modulators are transcriptionally induced either directly by interferon-activated signal transducer activators of transcription or indirectly by interferon-induced regulatory factor-1. Therefore, the inactivation/disturbance of signal transducer activators of transcription or regulatory factor-1 could be a way in which HPV escapes host immune surveillance (Park *et al*., 2000b).

1.8.3 Interaction of HPV E6 with transcription regulators

E6 of human papillomavirus (HPV) 16 interacts with the three regions of CREB-binding protein (CBP) and p300 that leads to disturbances of co-activation functions (Patel *et al*., 1999). These two co-activators play a role in normal cell differentiation and cell cycle transition, and they are the best candidates for vital proteins that deregulate these cellular processes. Interaction between E6 and CBP/p300 may inhibit differentiation of epithelial cells surrounding the virus and down-regulate the immune recognition machinery for continuous viral infection to occur (Patel *et al*., 1999).

1.8.4 Interaction of HPV E6: small molecules-protein kinase, p21 and proliferating cell nuclear antigen (PCNA) bind E6.

A novel interaction has been reported between E6 and protein kinase, a fatty acid and Rho-small G protein-activated serine/threonine kinase. However, the question as to how protein kinase plays a role in the cellular transformation activity of E6 is still undefined (Gao *et al*., 2000). P21 targets p53 during transcription, and is an important regulator of G1 arrest resulting from DNA damage. There is a second cyclin binding site located near the carboxyl terminus. This site partly overlaps the domain then binds to PCNA, which is an assistant to DNA polymerases δ and ε; it helps in loading of the polymerases onto DNA templates and elevates their activity in both DNA replication and repair. When PCNA binds p21 it inhibits PCNA-dependent DNA replication. The E7 protein removes p21 from PCNA-dependent DNA replication, and it ultimately allows PCNA to function in viral DNA replication (Funk *et al*., 1997).

1.8.5 HPV E6 and PDZ domain-containing protein

The PDZ domains exhibit protein-protein interactions that form part of the molecular scaffolds that hold multi subunits firmly together. The PDZ domains play an important role in mediating the correct spatial arrangement of ion channels, of receptors or other molecules involved in signalling pathways, of interaction of adhesion molecules in relation to one another and in relation to different regions of polarized cells (Scheffner and Whitaker, 2003). The high risk human papillomavirus E6 type has a PDZ-binding motif at its C-terminus. It is a short motif (xT/SxV) found in proteins that specifically interact with PDZ-domains. High risk E6 binds to the homologue of the Drosophila disc large tumour suppressor, the human homologue of the Drosophila scribble, the membrane-associated guanylate kinase and the multi-PDZ domain protein-1 through the C-terminus. In addition, with oncogenesis, the C-terminal region of E6 is not wanted for the binding of E6 with E6-associated protein (E6-AP) and p53; but C-terminal deletion mutants of E6 are unable to transform, in certain cell culture systems. This implies that the binding of, and possibly degradation of, PDZ domain proteins contributes to the oncogenic potential of E6 (Scheffner and Whitaker, 2003).

1.9 GENETIC REGULATION OF CERVICAL CANCER THROUGH APOPTOSIS

1.9.1 Degradation of HPV E6 oncogene by p53.

There are two roles played by p53 in preventing cancer: a) cell cycle arrest in G1 which allows time for the repair of damaged DNA, b) apoptosis which eliminates cells with damaged genomes. Most human tumours possess mutations of p53, but in cervical cancer p53 mutations are uncommon even though p53 is present in more than 95% of cervical carcinomas (Hietanen *et al*., 2003). The E6 protein associates with the cellular p53 protein to cause proteasome-dependent degradation (Kehmeier *et al*., 2002). Furthermore, E6 has the ability to induce telomerase activity in mucosal keratinocytes which normally do not possess such activity. E6 protein uses an ubiquitin-dependent proteasome pathway to target p53 tumour suppressor for degradation (Mantovani and Banks, 1999). This pathway requires a sequential action of the ubiquitin activating enzyme E1, E2 and E3. When human papillomavirus causes degradation of p53, both the E6-associated protein (E6AP) as well as E6 act as E3 ligases. E6 associates with E6AP and p53 simultaneously, transferring the ligase to p53. E6AP alone cannot bind p53; firstly, E6 interacts with E6AP (a property not found in low risk types), then the E6/ E6AP complex binds to p53, thereby contributing to the E6AP mediated ubiquitination of p53. Finally, poly ubiquitinated p53 is sensitized to degradation by the proteasome.

In cells not infected by HPV, E6AP plays, if any, a minor role in the degradation of p53; this view is supported by the fact that Murine Double Minute 2 (MDM2) is the main pathway for p53 degradation (Scheffner and Whitaker, 2003). E6* (an alternatively spliced E6 which lacks the C-terminal portion of E6) binds to the full length E6 and E6AP, and is able to block the proteasome-mediated proteolysis and apoptosis (Mantovani and Banks,

1999). Furthermore, E6 has also an alternative pathway to function as an anti-apoptotic protein. It does not reduce the level of tumour necrosis factor (TNF)-receptor, but inhibits apoptosis in its presence by altering the binding of tumour necrosis factor receptor 1 (TNFR1) intracellular death domain to Fas-association with death domain, FADD (Thompson *et al*., 2001).

Some *in vitro* studies on p53 polymorphism at codon 72, which gave rise to either Arg or Pro, were shown to affect E6-mediated degradation of p53. The arginine variant was found to interfere with one of the p53 polyproline regions that play a role in the induction of apoptosis (Mantovani and Banks, 1999). Evidence suggests that p53 Pro shows an elevated level of transcriptional activity; in contrast to p53 Arg which prevents immortalization of primary rodent cells, an activity that is related to the induction of apoptosis. It has been reported that individuals who possess the Arg allele at codon 72 of p53 are susceptible to the development of HPV-associated cervical cancer, when compared to those who have the Pro allele. This finding supports the view that not only the presence of HPV infection, but also structural differences in the p53 locus may be important in the development of cervical cancer (Cho *et al*., 2003). P53 degradation results in the loss of G1 checkpoint in transformed cells expressing HPV 16/18 E6. These cells are also resistant to p53- induced growth arrest and apoptosis from DNA damage. HPV 16 E6 alters the G2 checkpoint, and this shows a relation to the disruption of p53 regulated G2/M checkpoint (Cho *et al*., 2003).

1.9.2 HPV E7 interaction with Bak

Human papillomavirus E6 has been postulated to evade apoptosis in both p53 dependent and p53 independent pathways (Charkrabarti and Krishna, 2003). Research based on the HPV E6 oncogenic property found that E6 has the ability to interact with Bak (proapoptotic member of Bcl-2 family) in a p53-independent manner, and targets it for degradation by reducing Bak-induced apoptosis; an effect that is dependent on E6AP (Chakrabarti and Krishna, 2003). In fact, Bak binds to E6-associated protein (E6AP) in the absence of E6, unlike p53 (Chakrabarti and Krishna, 2003). There appears to be a difference in capacity of HPV oncogenic and nononcogenic HPV types to circumvent both p53 and Bak-induced apoptosis, because the non-oncogenic HPV types replicate in the lower levels of the stratified epithelium, and this is where DNA replication is switched off (Chakrabarti and Krishna, 2003).

1.9.3 HPV E6 and telomerase activity

The normal somatic cells undergo cell division and the process eventually reaches senescence, which may suppress the development of cancer cells in humans. Senescence arises mainly from shortened telomeres, an important structure at the chromosomal end and made of repetitive DNA sequences (Horikawa and Barrett, 2003). Cervical carcinoma cells show high telomerase activity, and express mainly integrated copies of the HPV genome (Baege *et al*., 2002). Telomerase activity is present in immortalized and cancer cells but not in normal somatic cells, indicating that telomerase activation is a useful step in the process of immortalization and malignant transformation. Loss of telomerase activity in normal cells results in gradual decrease in telomere length with successive rounds of cell cycle/cell division, comprising unfinished end replication of

linear DNA (Chakrabarti and Krishna, 2003; Veldman *et al*., 2001) that leads to a scenario whereby there is instability in the chromosome and in cellular senescence. It therefore implies that telomere shortening acts as the "mitotic clock" that determines normal cellular life span (Veldman *et al*., 2001). Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase; it regulates the enzyme. It has been shown that overexpression of c-myc induces the telomerase activity by binding to the hTERT promoter (Horikawa and Barrett, 2003). E6 is believed to activate telomerase activity during progression to malignancy, but the underlying mechanism remains unclear. Studies have found that c-myc and sp1 binding sites on the hTERT promoter work together to promote the E6- mediated hTERT induction (Oh *et al*., 2001); the mechanism is still in question.

1.9.4 HPV E2 and telomerase

Human papillomavirus E2 regulates transcription by binding the 12bp repeats of the HPV regulatory region (Desaintes *et al*., 1997). The E2 protein, both low and high risk, downregulates telomerase by affecting the expression of the human telomerase reverse transcriptase (hTERT) (Lee *et al*., 2002). E2 targets sp-1 binding sites to induce downregulation of the hTERT promoter. The c-myc binding sites do not play a role in E2-mediated downregulation of the hTERT promoter, as evidenced from the association of E2 with cis elements of hTERT promoter. Repression of the E6/E7 by E2 transcriptional regulatory protein results in reactivation of the dormant p53 and Rb related protein (p105) tumour suppressor proteins, repression of telomerase and growth arrest (Goodwin and DiMaio, 2001).

The human papillomavirus E2 plays a major role in controlling viral replication and transcription of the viral genome (Desaintes *et al*., 1997). E2 expression activates the transcription of p53 in two ways; the first involves binding of E2 to its recognition elements located in the integrated viral P_{105} promoter. E2 binding represses transcription of HPV E6 oncogene, thereby promoting p53 degradation. The second pathway does not involve E2 binding. E2 expression influences apoptosis and G_1 arrest. E2 protein contains two domains of relatively high amino acid conservation. The amino terminal part is responsible for the dimerization of the protein and its specific binding to DNA (Desaintes *et al*., 1997).

In HPV 18, the viral oncogenes E6 and E7 are expressed from a single promoter, P_{105}, which is regulated by an upstream 800bp long control region that contains a keratinocyte-specific enhancer, upstream of promoter elements (TATA and Sp1) crucial for transcription. E2 has four binding sites located in the long control region, two of which are within the promoter tightly flanked 5' by Sp1 and 3' by TATA motifs. The E2 function is almost lost during carcinogenic progression as a result of viral DNA integration into the cellular genome, and is followed by the disruption of the E2. Loss of E2 increases the levels of E6 and E7, whose continuous expression maintains the transformed state of the cells. In conclusion, E2 induces apoptosis and G1 growth arrest by two different pathways. The major one involves interaction between E2 and p53. The other pathway is triggered when E2 represses the P_{105} promoter transcription of E6 gene, inhibiting the degradation of p53. The transcriptionally active p53 will induce G1 growth arrest, while the transactivation-deficient p53 will lead to apoptosis (Desaintes *et al*., 1997).

1.10 INTERACTION OF HPV WITH CELL SURFACE RECEPTORS

Human papillomavirus has established a new strategic way of changing an apoptotic stimulus after having infected primary human keratinocytes. Upon HPV infection, E5 has the ability to decrease the expression of cell surface receptors (Kabsch and Alonso 2002). The viral oncoprotein E5 is believed to be a transmembrane protein (83 amino acids) that interacts with the golgi apparatus. It rearranges the actin cytoskeleton, blocks endocytic trafficking and enhances signal transduction pathways. The elevated expression of E5 in human keratinocytes increases activation of the signalling cascade originating from the epidermal growth factor receptor, and also activates *c-jun* gene expression via the *ras* – dependent pathway (Kabsch and Alonso, 2002). HPV E5 has an antiapoptotic effect which allows the host cell to stay alive long enough for the virus to replicate, an effect mediated by tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and FasL-mediated apoptosis (**Figure 1.11**). (Kabsch and Alonso, 2002).

E5 blocks the DISC formation that is upstream in the death-signalling pathway. Association of TRAIL to decoy receptors (DR) 4 and 5 induces receptor trimerization, generates interactive surfaces for death domain, and contains adaptor proteins such as Fas-association with death domain (FADD). FADD then recruits procaspase 8 zymogen in the DISC induced self-cleavage, which then leads to activation of downstream effector caspases such as caspase 3 that execute the apoptotic death sequence (see **Figure 1.11**). One of the substrates of caspase 3 is the DNA repair enzyme poly (ADP-ribose) polymerase (PARP). Apoptotic stimuli are inhibited by c-FLIP (a structural homolog of caspase 8), which binds to FADD with higher affinity than caspase 8. The binding of c-

FLIP inhibits further recruitment of caspase 8. E5 does not downregulate DR4 and DR5, but it is accepted that it reduces the functionality of the receptors. It has been reported that DR4 and DR5 associate with the Golgi apparatus. The hydrophobic E5 molecule, which is located in the endoplasmic reticulum and Golgi, has the ability to bind to surface receptors during their post-transcriptional processing and their transport to the cell surface. The receptor is therefore not able to bind to TRAIL, or to send an apoptotic signal via the FADD protein. Alternately, E5 may be responsible by causing overexpression of apoptosis inhibitors, but this effect is still undefined (Kabsch and Alonso, 2002).

Most cancer cells express FasL. This helps tumours to evade immune surveillance by inducing apoptosis in the host's cytotoxic lymphocytes and natural killer cells that respond to the tumours (Nagata, 1997). Invasive adenocarcinoma of the cervix occurs when cancer cells spread beyond the uterine cervix. FasL is expressed with high frequency on the surface membrane of cervical carcinoma cells, and may be an important site of colonization of the cancer cells which have metastasized to the lymph nodes, where local antitumour immunity is conferred on them by the lymphocytes (Kase *et al*., 2003). During tumour progression a limited number of FasL expressed cells, from heterogeneous primary tumours survive and proliferate. Therefore one may concluded that FasL plays a major role in escaping immunity, and the progression and metastasis of cervical carcinoma.

Cells expressing E6 are protected from tumour necrosis factor (TNF). When HPV 16 E6 binds to TNF R1, transmission of proapoptotic stimuli initiated by TNF are affected (Filippova *et al.*, 2002). When E6 interacts with TNF R1 it participates in a series of sequential interactions to form the DISC. These interactions have in turn been predicted to inhibit activation of initiator caspase (e.g. caspase 8), leading to repression of effector caspases (e.g. caspase 3), and the blocking of apoptosis. Hence, E6 prevents both TNF R1/TRADD binding and pro-apoptotic signal transduction. The finding that apoptotic elimination of HPV 16 positive cancer cells may be inhibited by peptide aptamers, and that E6 acts as an anti-apoptotic factor within HPV transformed tumour cells, showed that it was possible to remove virus-positive cancer cells by molecules specifically targeting a viral oncogene product (Butz *et al*., 2000). c-myc protein is located in chromosome 8q24, the locus within which the HPV 16 sequence is integrated, suggesting that HPV integration in the fragile site of c-myc may be the one where HPV-induced oncogenesis occurs (Butz *et al*., 2000).

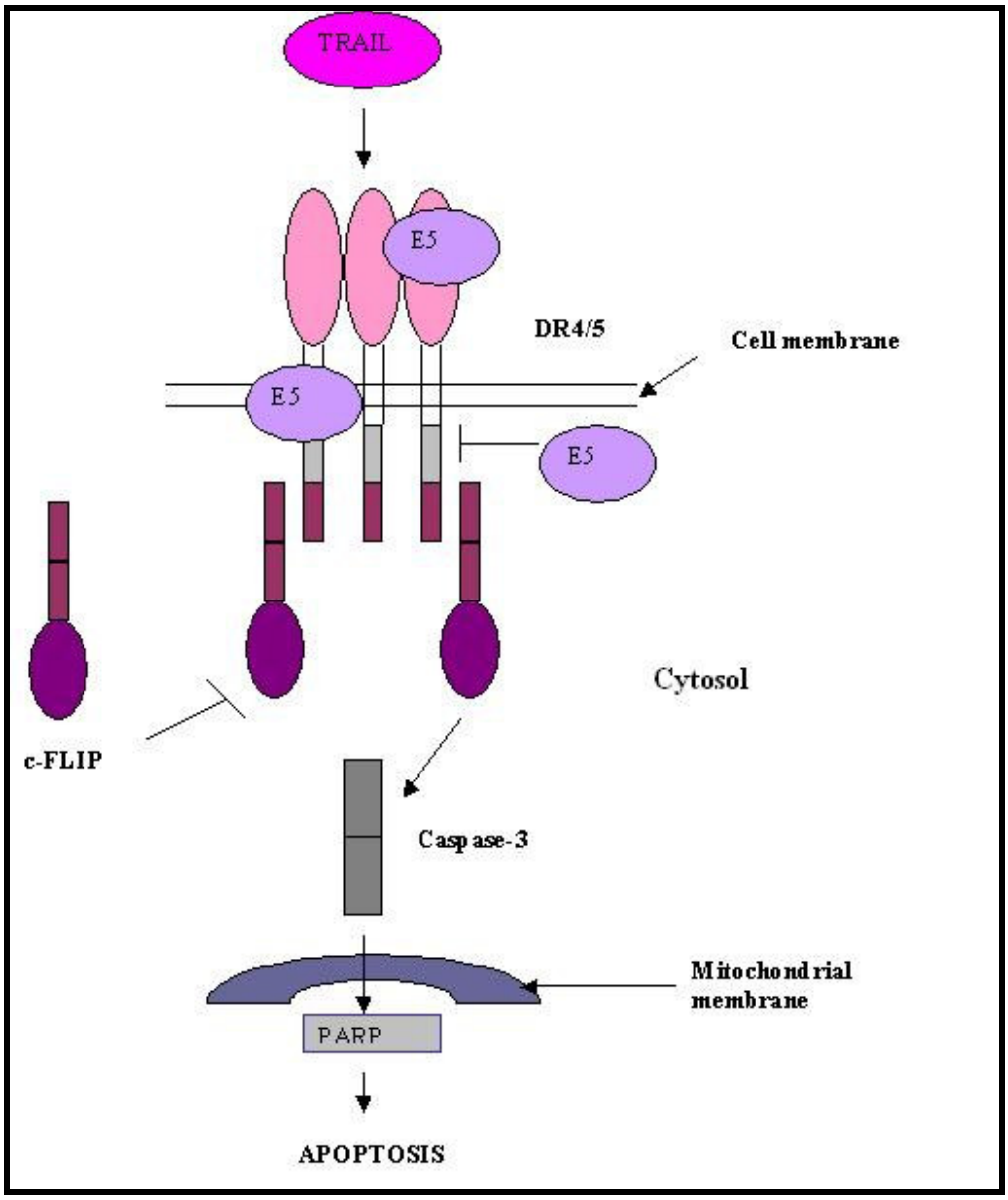

Figure 1.11: Model for the mechanism by which human papillomavirus (HPV) 16 E5 impairs death-inducing signal complex (DISC) formation (Kabsch and Alonso, 2002).

1.11 THERAPEUTIC STRATEGIES FOR CERVICAL CANCER.

Cervical cancer is largely a preventable disease with a known causative agent, the human papillomavirus. This ubiquitous virus is the key to understanding the natural history of cervical carcinoma and its relationship to the immune system. The known viral trigger (at the molecular level), and the well-described stages of disease progression make cervical carcinoma an ideal model for investigating potential immune therapies. Chemotherapy alone is generally ineffective against this relatively slow-growing carcinoma. Vaccines and immunotherapy are the promising strategies for the current therapeutic inventory (Makin and Dive, 2001; Janiceck and Averet, 2001).

1.11.1 Vaccination

Since the vast majority of cervical cancers result form viral infection, a vaccine would be ideal for its prevention. Women with cervical cancer do not trigger an immune response. One vaccine strategy currently being used in clinical trials engulfs virus-like particles (VLP). VLP consists of L1 or L2 (see **Fig. 1.9**) capsid proteins and is aimed at inducing neutralizing antibodies. Ideally, it is aimed at adolescent girls before they are sexually active and at risk of human papillomavirus infection. Another type of vaccine (chimeric) contains some of the early proteins of HPV in a non infective state, in order that therapeutic cell-mediated immunity may be elicited so as to stimulate viral clearance (Shiffman and Castle, 2003). Chimeric VLPs have E7 protein conjugated to either L1 or L2 and have been developed for immunotherapeutic vaccine strategies against cervical cancer (Fausch *et al*., 2002).

In the mouse, HPV vaccine produces cytotoxic T-cell responses, which not only remove HPV-positive tumours but also protect against subsequent challenges by the tumour. Those vaccines that target the outer surface of the virus may both offer protection against infection, and also have an effect on established HPV-positive lesions. Recent studies show that the L1 capsid protein has been targeted for neutralizing-antibody formation using a DNA or polynucleotide vaccine in the cotton-tailed Rabbit Papilloma virus model.

It was previously reported that human dendritic cells are capable of binding, internalizing and becoming activated by HPV 16-L1L2 VLP. Dendritic cells incubated with chimeric HPV16-L1L2-E7VLP are able to induce an E7 epitope-specific human T cell response *in vitro*. When HPV VLP is given as a vaccine, it is injected below the basal layers of the skin, and therefore interacts with dendritic cells, initiating an immune response (Fausch *et al*., 2002). Langerhans cells form a 3D network in the epidermis of the skin and the epithelial layers of vaginal and cervical mucosa. They are able to bind and internalize HPV virus-like particle VLP, similar to dendritic cells, but they are not activated. These cells lack the ability to initiate E7-specific responses *in vitro* after incubation with chimeric HPV16 L1L2-E7 VLP, whereas dendritic cells initiate human leukocyte antigen (HLA)-restricted E7-specific T cell response.

An oral vaccine using VLP induces IgG and IgA antibodies in mice. This indicates a potential antigenic stability of virus-like particles in the gastrointestinal tract, and raises the possibility of developing a large-scale vaccination programme for females at risk. In 2000, human clinical trials using HPV 6b VLP were begun, with VLP acting as a potent immune response to the L1 capsid protein. Complete regression of genital warts was observed in 25 of 33 patients who were vaccinated with HPV 6b VLP. Another HPV

vaccine approach has focused on targeting the oncogenic E6/E7 proteins as therapeutic vaccines. The E6/E7-based vaccine strategy induces T-cell immunity. These peptide vaccines are designed to induce cytotoxic T-lymphocytes against specific E7 epitopes that were found to be conserved and continually expressed in cervical cancers. The trials were grouped in two, one based on lipidated E7 and peptides with immune adjuvant. Both groups are focusing on HPV 16 in HLA - A2 positive patients. Thus, HPV type and HLA restriction limit the immediate applicability to all cervical cancer patients.

Vaccinia virus constructs with mutated E6 or E7 may be used to vaccinate without restrictions imposed by HLA. Heat shock protein (HSP) vaccines may be considered as an alternative strategy in order to circumvent HLA restriction by peptides. It is advantageous to use HSP because they can chaperon a vast majority of peptides into patient's antigen-presenting cells for potent immune representation in the context of proper HLA type, which makes them potentially useful "messenger" vaccines. Another advantage is that multiple epitopes for HPV can be bound to the HSP. Since immune evasion by tumours often involves 'antigen escape' by mutation, a vaccine based on multiple epitopes for large-scale use, may prevent individual antigens from escaping immune surveillance and neutralization (Janicek *et al*., 2003).

1.11.2 Immunotherapy

The T-cell vaccine approach can be both protective and therapeutic, and has been developed to induce HPV-specific cytotoxic T lymphocytes (CTL) in patients. Adaptive immunotherapy reconstitutes the vaccination process in the laboratory to stimulate and grow CTLs on a large-scale. This production of CTLs in the laboratory can be controlled by the addition of cytokines, and the ability to remove these T cells from the host, who

may have immuno-suppressive factors produced by cervical cancer, and has advantages over the vaccine approach. The CTLs produced can be introduced into the patient in huge amounts to overcome the tumour with tumour specific killer cells. Such an approach can produce more than 10-fold greater production of T-cells than may be obtained by vaccinating the patient. T-cells go through programmed cell death, namely apoptosis after it has been triggered, and genetic engineering of CTLs is important for circumventing the immune system's natural regulatory mechanisms. Therefore, CTL therapy has to develop as an "autoimmune" phenomenon that targets only human papillomavirus infected cells in the body (Janicek *et al*., 2001).

1.12 AIM AND OBJECTIVES OF THE STUDY

The purpose of the study is to elucidate:

i) The role of DWNN gene in cervical cancer and apoptosis

ii) To establish the expression patterns and determine the expression levels of the DWNN gene at protein and mRNA level in cervical cancer.