AN IMMUNOHISTOCHEMICAL STUDY OF SURVIVIN EXPRESSION IN NORMAL AND IN TRANSFORMED CELLS

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AN IMMUNOHISTOCHEMICAL STUDY OF SURVIVIN EXPRESSION IN NORMAL AND IN TRANSFORMED CELLS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF PHOTOS

LIST OF ABBREVIATIONS

PCD	Programmed cell death
TNF	Tumor necrosis factor
IAPs	Inhibitor of apoptosis protein
FADD	Fas-associated death domain
DNA	Deoxyribonucleic acid
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine triphosphate
Tc	The rate of cell division
M	Mitosis
G_1	Growth phase
S	Synthesis phase
G_2	Growth 2 phase
G_o	Quiescent phase
UV	Ultra violet
R	Restriction point
kDa	kilo Dalton
AP14	Apoptosis inhibitor 4
cDNA	Complementary DNA
EPR-1	Effector cell protease receptor 1
BIR	Baculovirus inhibitor
INCEP	Inner centromere proteins
AI	Apoptotic index
CYCLO	Cyclophosphamide
5-FU	5-Fluorouracil
TAM	Tamoxifen
DOXO	Doxorubicin
IL3	Interleukin
PR	Progesterone receptor
3+	+++
2+	++
1+	+
ISS	Intensity specific staining
NSB	Non-specific background staining
ER	Estrogen receptor
N>C	Predominantly nuclear
N/C	Nuclear and cytoplasmic
C>N	Predominantly cytoplasmic
C	Exclusively cytoplasmic
IHC	Immunohistochemistry staining
h	Hour
PBS	Phosphate-buffered saline
OD	Optical density
Mol/L	Molar/ Liter
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

PVDF	Polyvinylidene fluoride
BSA	Bovine serum albumin
IgG	Immunoglobulin G
DAB	3,3'-diaminobenzidine tetrahyrochloride
M	Molar
TBS	Tris-buffered saline
RT	Room temperature
HRP	Horseradish peroxidase
pAb	Polyclonal antibody
mAb	Monoclonal antibody
SUR	Survivin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
V	Voltage
min	Minute
AR	Antigen retrieval
DMSO	Dimethyl sulphoxide
H_2O_2	Hydrogen peroxide
mRNA	Messenger RNA
MW	Molecular weight
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
ABC	Avidin-biotin-complex
df	Degree of freedom
H&E	Heamotoxylin & eaosin staining
kbp	Kilobase pair
MDM-2	Murine double minute
PPC	Peak plasma concentration

AN IMMUNOHISTOCHEMICAL STUDY OF
SURVIVIN EXPRESSION IN NORMAL AND IN TRANSFORMED CELLS

ABSTRACT

Survivin is a new member of the inhibitors of apoptosis protein (IAP) family, selectively over-expressed in common human cancers but not in normal adult tissues. It is also expressed in cancer cell lines. The study was performed generally to investigate the basic and clinical roles of survivin in normal and transformed cells. Rabbits were immunized with two synthetic oligopeptides, MGAPTLPPAWQP and KEFEETAKKVRRAIEQLAAMD amino acids sequences of the survivin molecule. Serum antibodies were purified by ammonium sulphate and caprilic acid and their specificities were confirmed by immunoblotting and pre-absorption tests against survivin positive tissues or synthetic survivin oligopeptides. These antibodies were used to detect survivin in normal and transformed cells by immunohistochemistry in formalin-fixed paraffin embedded tissue sections, evaluated by a standard scoring system and chequerboard analysis. Normal cells were obtained from fetal and adult tissues of mouse and rat whilst the transformed cells were obtained from the human breast cancer cell line MCF-7 and the infiltrating ductal carcinoma (IDC) of the breast patients. In the MCF-7 cell line experiment, the effects of chemotherapeutic drugs namely doxorubicin, 5-fluorouracil, cyclophosphamide, and tamoxifen on the apoptosis index measured by propidium iodide and acridine orange dyes. The relative cell viability was measured by an MTT assay and survivin expression was measured by immunocytochemistry. In IDC patients (n=382), survivin expression in tissues was analyzed for its correlation with clinicalpathological factors, hormonal status, p53, bcl-2 and the survival rate. Patients and their tissue blocks were obtained from three general hospitals in The East Coast of Malaysia. Autoantibodies to survivin were also investigated in the sera of the same IDC patients population (n=57) and were compared

to the control population (n=44). For the immunohistochemistry assay, four rabbit antiserum were produced and tested against survivin. The results of this study indicated that the antigen retrieval buffer, pH 9 was superior than pH 6 and optimization immunohistochemistry was obtained by chequerboard analysis. Furthermore, it was found that survivin is expressed abundantly in normal growing fetal cells but not in normal differentiated adult tissues of mouse and rat. In the MCF-7 cell line, the cell viability was reduced in a dose-dependent pattern when incubated with the drugs. The IC_{50} estimation in MCF-7 cell line for doxorubicin was 6.0 μg/ml, cyclophosphamide 171.1 μg/ml, 5-fluorouracil 0.61μg/ml, and tamoxifen 0.7μg/ml, respectively. It was found that most of the MCF-7 cells expressed survivin, predominantly in the cytoplasm. The percentages of apoptotic cells were increased with the increased concentrations of the drugs. Among the IDC patients, the expression of survivin was 68.1%, p53 29.6%, and bcl-2 43.7%, respectively. There was a significant correlation ($p<0.05$) between survivin expression and lymph node involvement, tumour sizes, p53, bcl-2 expression, and survival rate among the IDC patients. Anti-survivin autoantibodies reactivities were detected in 7% of the sera of IDC patients but not in normal sera. These autoantibodies correlated with the positivity of survivin expression, and with advanced breast cancer. It was concluded that survivin was abundantly and prominently expressed during fetal development of rat and mouse. The polyclonal antibody SUR12A-CFI recognized rat and mouse survivin. It was also concluded that survivin is frequently over-expressed in IDC patients, and in most MCF-7 cells. Survivin expression has a predictive value in predicting the aggressiveness of the tumour cells suggesting that survivin may be a useful tool in assessing a prognosis.

KAJIAN IMUNOHISTOKIMIA TERHADAP EKSPRESI SURVIVIN DI DALAM SEL NORMAL DAN SEL TERTRANSFORMASI

ABSTRAK

Survivin merupakan ahli baru dalam keluarga protin perencat apoptosis, secara terpilih diekspres secara berlebihan dalam kebanyakan kanser tetapi tidak di dalam tisu dewasa normal. Ia juga diekspres di dalam rangkaian sel-sel kanser. Kajian ini dilakukan bagi mengkaji secara asas dan klinikal tentang survivin di dalam sel normal dan sel tertransformasi. Arnab telah diimunkan dengan sintetik oligopeptida, jujukan asid amino, MGAPTLPPAWQP dan KEFEETAKKVRRAIEQLAAMD daripada molekul survivin. Antibodi serum ditulenkan dengan ammonium sulfat dan asid caprilik dan speksifikasinya telah disahkan dengan teknik immunoblot dan ujian penyerapan awal terhadap tisu positif survivin dan sintetik oligopeptida survivin. Antibodi ini telah digunakan untuk mengesan survivin di dalam sel normal dan sel tertransformasi menggunakan kaedah imunohistokimia pada hirisan tisu formalin-paraffin dan diukur menggunakan kaedah sistem pengskoran piawai dan analisis optimasi. Sel normal diperolehi daripada tisu fetus dan tisu dewasa tikus dan mencit manakala sel tertransformasi diperolehi daripada rangkaian sel kanser payu dara MCF-7 dan pesakit karsinoma infiltrasi kalenjar payu dara. Di dalam kajian rangkaian sel MCF-7, kesan dadah kemoterapi iaitu Doxorubicin, 5-Fluorourasil, Cyclophosphamide dan Tamoxifen ke atas indek apoptosis yang diukur dengan kaedah propidium iodida dan akridin oren. Relatif sel viabiliti diukur dengan ujian MTT dan ekspresi survivin diukur dengan kaedah immunositokimia. Korelasi antara faktor klinikopatologi, status hormon, p53, bcl-2 dan kadar hidup di kalangan pesakitr kanser payu dara (n=382) telah dianalisa. Blok-blok tisu daripada pesakit telah diperolehi daripada tiga hospital utama di Pantai

Timur. Autoantibodi terhadap survivin juga telah dikaji di dalam serum pesakit kanser payu dara (n=57) dan dibandingkan dengan kumpulan kawalan (n=44). Keputusan kajian mendapati, empat antiserum telah berjaya dihasilkan dan diuji terhadap survivin secara immunohistokimia. Larutan penampan pemulihan antigen pH 9 adalah lebih baik berbanding dengan pH 6 dan optimasi telah diperolehi dengan kaedah analisis optimasi. Survivin didapati diekspres di dalam sel normal fetus mencit dan tikus yang aktif tetapi tidak pada sel normal yang telah membeza. Nilai IC_{50} bagi rangkaian sel MCF-7 untuk Doxorubixin ialah 6.0 μg/ml, Cyclophosphamide 171.1 μg/ml, 5-Fluorourasil 0.61 μg/ml dan Tamoxifen 0.7 μg/ml. Didapati survivin diekspres kebanyakannya di sitoplasma. Peratus sel apoptotik meningkat dengan peningkatan dos dadah. Di kalangan pesakit kanser payu dara, survivin diekspres sebanyak 68.1%, p53 29.6% dan bcl-2 43.7%. Terdapat korelasi yang bererti ($p<0.05$) di antara ekspresi survivin dengan metastasis nodus limfa, saiz tumor, p53, bcl-2 dan kadar hidup di kalangan pesakit yang dikaji. Sebanyak 7% autoantibodi terhadap survivin dikesan di kalangan pesakit tetapi tidak pada kumpulan kawalan. Autoantibodi didapati berkorelasi dengan ekspresi survivin dan tahap akhir kanser. Kesimpulan kajian ialah survivin banyak diekspres pada sel normal yang aktif membahagi dan sel tertransformasi. Antibodi SUR-12A-CFI dapat mengesan survivin pada tikus dan mencit. Survivin juga secara berlebihan diekspres di kalangan pesakit kanser payu dara dan rangkaian sel MCF-7. Survivin juga mungkin boleh digunakan untuk meramal keagresifan sel tumor dan dicadangkan survivin boleh menjadi alat untuk membuat penilaian prognosis.

CHAPTER I

INTRODUCTION

1.1 The cell cycle

1.1.1a The normal cell cycle

Normal cells of multi-cellular organisms can divide as often as once or twice a day *in vivo*. The rate of cell proliferation within any population of cells depends on three parameters: a) the rate of cell division (Tc), (b) the fraction of cells within the population undergoing cell division (growth fraction), and (c) the rate of cessation of cell division due to terminal differentiation or cell death (Andreeff *et al.*, 2000).

Cellular reproduction is a cyclic process in which daughter cells are produced through nuclear division (mitosis) and cellular division (cytokinesis). Mitosis (M) and cytokinesis are part of the growth-division cycle called the cell cycle (Fig.1.1). Mitosis lasts for about 1 hour, and takes a relatively small part of the total cell cycle. Interphase is the mitosis preparatory stage which is divided into 3 phases, G_1, S, and G_2 (Fig 1.1). The first gap phase, G_1 which lasts for about 6 hours to several days or longer, is a period of growth and metabolic activity following a previous mitosis (Fig. 1.1). The synthesis phase (S phase) follows G_1 and is a period of DNA synthesis, in which the DNA is replicated. Another gap phase, G_2 which lasts about 2 hours, follows DNA synthesis and precedes the next mitotic division. Certain mature cell types do not continue to divide but remain in interphase (in G_0). Cells that are permanently in the G_0

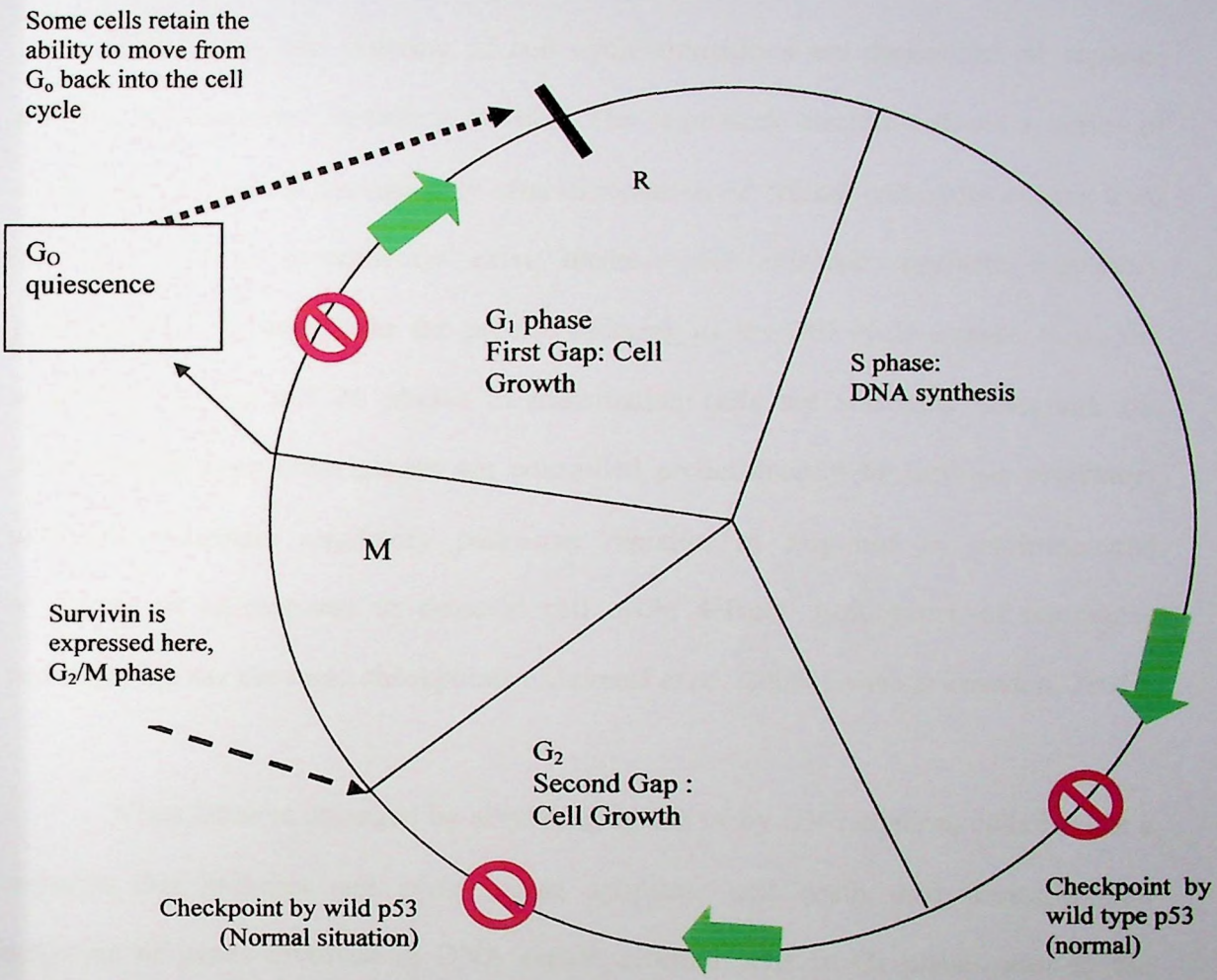

Figure 1.1: Cell cycle pathways showing its check points and regulators of a normal cell. M=Mitosis, G= Gap, S=Synthesis, R=Restriction (Andreef *et al.*, 2000; Li *et al.*, 1998).

phase are in a quiescent state and are called post-mitotic (Andreeff *et al.,* 2000; Levine 1997; Adam *et al.,* 2001; Banks *et al.*, 2000; Bartek & Lukas, 2001;Bursch *et al.,* 2000).

The timing and ordering of cell cycle transitions are dependent on separate positive and negative regulatory circuits. The regulatory circuits enforce a series of checkpoints, allowing passage only after completion of critical cell cycle events. Two classes of regulatory pathways exist, intrinsic and extrinsic. Intrinsic regulatory pathways are responsible for the precise ordering of the cell cycle events. Since the lengths of S, G_2, and M phases in mammalian cells are relatively invariant, the transitions between these phases are controlled predominantly by intrinsic regulatory pathways. Extrinsic regulatory pathways function in response to environmental conditions or in response to detected cell cycle defects. Both types of regulatory pathways can use the same checkpoints (Andreeff *et al.,* 2000; Evans &Vousden, 2001).

When DNA is damaged by alkylating agents or by UV radiation, cells initiate a response that includes cell cycle arrest, apoptotic cell death, and transcriptional induction of genes involved in DNA repair. Normal cells in G_1 phase prior to the restriction point (R) will arrest in G_1 phase upon sensing DNA damage (Fig. 1.1) (Andreeff *et al.,* 2000; Budiharjo *et al.*, 1999; Fernandez *et al.*, 1998; Fu *et al.,* 2004).

1.1.1b The cell cycle of tumour cell: tumour growth and cell proliferation

Cancer is a disease of accumulation of clonally expanded cells. Tumour cell numbers increase, and the tumour burden accounts for the adverse effects on the host (Andreeff *et al.,* 2000). Thus, cancer is a disease of uncontrolled proliferation. The

mechanisms that underly tumour and normal cell proliferation are very similar (Andreeff *et al.*, 2000). Both bcl-2 and p53 play a role in determining tumour growth by their effects on apoptosis and cell proliferation (Linjawi *et al.*, 2004). Hence, tumour growth is the net result of cell proliferation and cell death (Siziopikou & Schnitt, 2000).

P53 is the guardian or the master brake in the cell cycle. When some cellular mechanism goes wrong, the wild p53 will stop the cell from dividing, but if the wild p53 is altered to a mutant p53, it can no longer stop the cell from dividing. As the situation is not an abnormal control of the cell control but the cell cycle can no longer be controlled if there are genetically altered cells (Levine, 1997; Park *et al.*, 1997; Moreno *et al.*, 2001; Nakahara *et al.*, 1998; Shiratsuchi *et al.*, 2002).

1.1.2 Apoptosis

Apoptosis or programmed cell death (PCD) is a universal and physiological process responsible for removing unwanted, old, damaged, and misplaced cells during embryonic development and tissue homeostasis (Sreedhar & Csermely, 2004; Borner, 2003; Andreeff *et al.*, 2000; Eissa *et al.*, 1999; Strasser *et al.*, 1997). The study of apoptosis has emerged from relative obscurity to become a major focus of research interest in many areas of medicine in the last decade (Rudin *et al.*, 1997).

Apoptosis is derived from Greek and refers to the dropping or falling of leaves from a tree (Sreedhar & Csermely, 2004). The term was introduced by Kerr *et al.*, (1972) to define the morphologic features of the apoptotic process. Some promoter and suppressor genes control this process (Sirvent *et al.*, 2004; Roninson *et al.*, 2001).

1.1.2.1 The major elements of apoptosis

Apoptosis is well characterized by distinct morphological and physiological changes (Sirvent *et al.,* 2004). The p53 protein is also involved in both the extrinsic and the intrinsic pathways of apoptosis by initiating apoptosis through mitochondrial depolarization and sensitizing cells to inducers of apoptosis (Hofseth *et al.,* 2004). Apoptosis is induced by an array of internal and external stimuli or signals and its mechanism has several common elements regardless of the ultimate biochemical pathways utilized (Kiechle & Zhang, 2002). Apoptosis can be divided into three phases. The first phase is the initiation phase (or signalling phase), which involves the activation of surface death receptors (extrinsic pathways), mainly the tumour necrosis factor (TNF) family members, the mitochondrial pathway (intrinsic pathway) or the initiation of apoptosis by other stimuli (e.g., those affecting the endoplasmic reticulum (ER). The second is the signal transduction phase (or preparation phase), where activation of initiator caspases (caspase-8, caspase-9, caspase-10, and caspase-12) and certain kinases/phosphatases takes place. This is followed by the execution phase (or death phase), which involves the activation of effector caspases (caspase-3, caspase-6, and caspase-7) (Fig. 1.2) (Bronchud *et al.,* 2000; Thornberry & Lazebnik, 1998).

Mammals have two distinct apoptosis signalling pathways, extrinsic and intrinsic (Coultas & Strasser, 2003). Signalling through both the extrinsic and intrinsic pathways can be modulated by IAPs (inhibitor of apoptosis proteins) such as bcl-2 and survivin, which are highly conserved polypeptides that selectively inhibit the activation and functional activity of various caspases (Kaufmann & Earnshaw, 2000; Reed, 1999; Sanna *et al.*, 2002; Deveraux & Reed, 1999; Campora *et al.*, 2000; Parton *et al.*, 2001).

1.1.2.2 The extrinsic apoptotic pathway

This is a receptor-linked pathway that requires the binding of a ligand to a death receptor on the cell surface. For example, the cytokine, tumour necrosis factor (TNF), binds to the death receptor, TNF receptor type 1 (TNFR1), which recruits two signal transducing molecules; TNFR 1-associated protein with a death domain, and a Fas-associated polypeptide containing a death domain (FADD). This complex then binds to procaspase 8 to activate caspase 8, which, in turn initiates the protease cascade leading to apoptosis (Fig. 1.2) (Bronchud *et al.,* 2000; Lockshin *et al.,* 2000; Uno *et al.,* 2002).

1.1.2.3 The intrinsic apoptotic pathway

This pathway is mediated by the mitochondrial release of cytocrome c (Kiechle & Zhang, 2002; Pruschy *et al.,* 2001). It is mainly activated when damaged DNA is not sensed and repaired by checkpoint genes. Initiation of apoptosis may occur immediately or it may be delayed following the DNA damage. The response may or may not be dependent on the presence of the nuclear transcription factor, p53. When p53 is upregulated, it is activated by the phosphorylation of serine 46 by the homeodomain–interacting protein kinase-2, and the two proteins cooperate in the activation of the p53–dependent transcription. (Bronchud *et al.,* 2000; Levine 1997). Proteins induced by p53 include Bax, a bcl-2 homologous protein, which oligomerizes and forms pores in the outer mitochondrial membrane, resulting in either a decrease in the inner mitochondrial transmembrane potential or opening of the voltage–dependant anion channel, releasing cytochrome c from the space between the inner and outer mitochondrial membranes (Heiser *et al.,* 2004). Cytosolic cytochrome c induces the

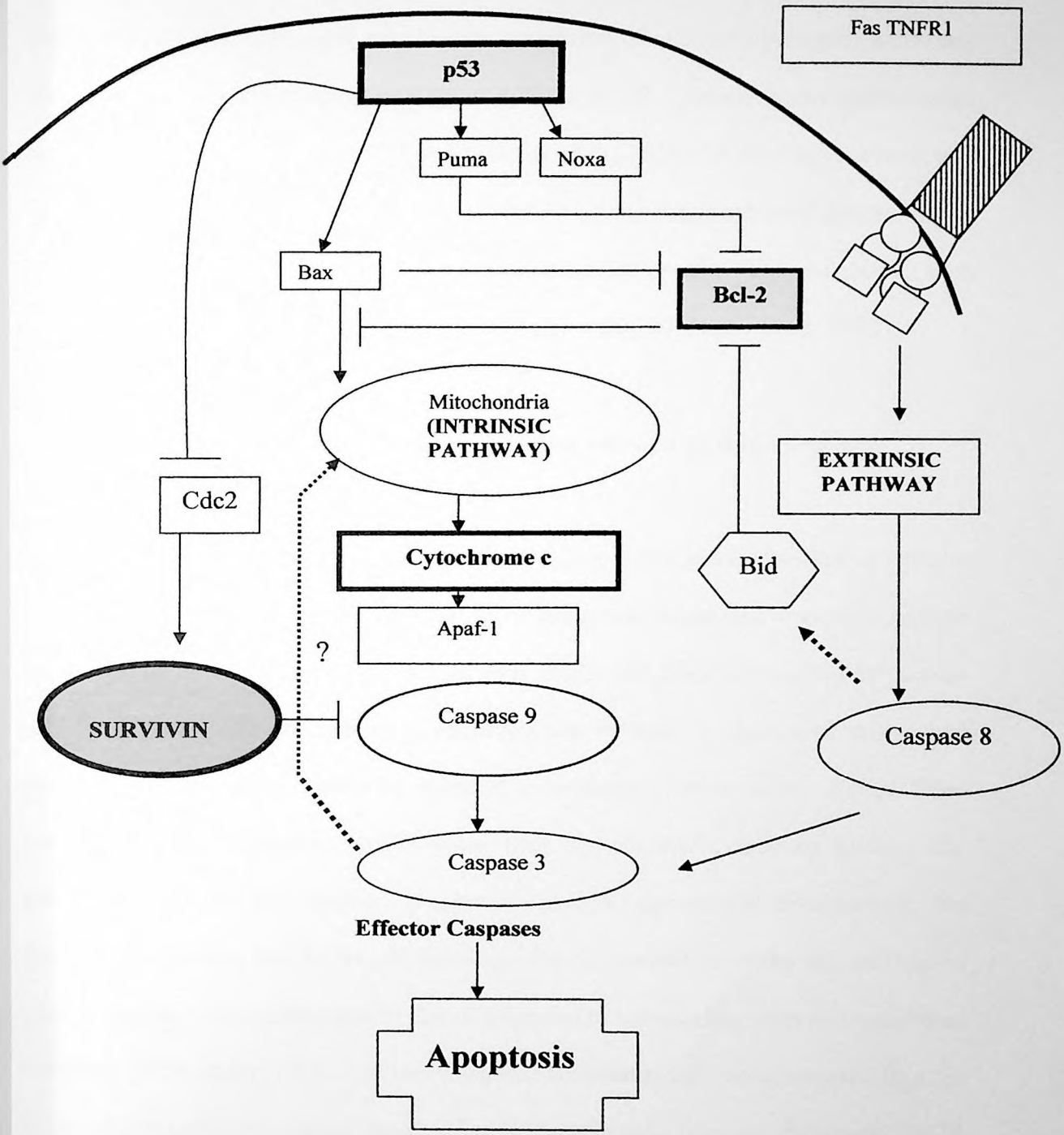

Figure 1.2 : The routes of apoptosis (Reed, 2001; Borner, 2003; Bossy-Wetzel & Green, 1999; Suzuki *et al.,* 2001; Coultas & Strasser, 2003)

formation of the multisubunit apoptosome composed of apoptotic protease activating factor-1 (Apaf-1), procaspase 9 and either ATP or dATP. Caspase 3 then mediates the apoptotic cascade (Heiser *et al.,* 2004; Bronchud *et al.,* 2000). IAPs of both cellular and viral origin have been identified to be intrinsic cellular suppressors of apoptosis that block the apoptotic program in response to viral infection or other forms of stresses such as survivin (Pruschy *et al.,* 2001; Li & Li, 2000; Thomas, 2000; Shu *et al.,* 1997).

1.1.2.4 Cell morphology and physiological changes during apoptosis

The process of apoptosis involves a cell dying in the midst of surviving cells, in contrast to necrosis, which involves clusters of dying cells in an area associated with an inflammatory infiltrate. Apoptosis occurs as a single cell death surrounded by healthy cells. The morphological changes in apoptosis can be seen by light microscopy, and have been characterized further by electron microscopy (Archer *et. al.,* 2000). These include nuclear (chromatin) condensation with the chromatin forming clumps that gather adjacent to the nuclear membrane (nuclear periphery). Furthermore, the cytoplasm condenses leading to cell shrinkage due to contraction of the cell and loss of volume, and to cell rounding due to loss of adhesion to surrounding cells and membrane blebbing. These bodies with condensed nuclear chromatin and, once released into the extra-cellular space, are rapidly ingested by phagocytic cells (Coultas & Strasser, 2003; Robertson *et al.,* 2000; Wylie & Currie, 1980; Gonzalez-Campora *et al.*, 2000).

The major physiological changes comprise fragmentation of nuclear DNA due to activation of specific endonucleases cleaving nuclear DNA into 80-200 oligonucleosomal fragments, and the activation of caspases, resulting in partially

digested proteolytic protein products (Sreedhar & Csermely, 2004; Bronchud *et al.*, 2000). This process produces cell breaking into several fragments of nuclei and cytoplasm or both nuclei and cytoplasm, known as apoptotic bodies (Sirvent *et al.*, 2004). Thus, DNA fragmentation is a characteristic biochemical marker of apoptosis.

1.1.3 Components of the apoptotic pathways

The key elements which execute the apoptotic process have been studied (Borner, 2003). Over the years, many components of the apoptotic pathways have been characterized, revealing apoptosis to be a highly complex process. However, a pattern is emerging with a series of early events that depend on the initial stimulus, followed by a common pathway involving a series of cysteine proteases, the caspases. This common pathway ultimately results in DNA fragmentation and morphological changes associated with apoptosis. Mitochondria have emerged as having a central role in the process and its regulation, with the bcl-2 family of proteins playing a particularly important part (Strasser *et al.*, 1997; Kaufman & Gores, 2000; King & Cidlowski, 1995).

1.1.3.1 The caspase death proteases

Caspase is a nomenclature referring to ICE/CED-3 cysteine proteinase family having a central role during cell death (Suzuki *et al.*, 2001) and in executing the process of apoptosis (Fig. 1.2) (Borner, 2003). In mammals, 14 members of the caspase family have been identified which cleave their substrates after aspartic acid (Asp). Activation of pro-caspases requires two caspases cleaved at the aspartic acid (Asp) residues (Strasser *et al.*, 1997). These cleavages remove the amino-terminal pro-domain and

separate the large and small catalytic subunits. Once activated, caspases can process and activate their own subunits and other pro-caspases (Bossy-Wetzel & Green, 1999). Caspase activation is not reversible and leads to cell apoptosis (Gompel *et al.*, 2004).

These enzymes are minimally active in healthy cells and require further activation in response to apoptotic stimuli such as ionizing radiation, chemotherapeutic drugs, and death receptor ligands (Shi, 2002; Alarcon & Ronai *et al.*, 2002; Pruschy *et al.*, 2001). They are divided into two categories; initiator caspases and effector caspases. The former includes caspase-2, caspases 8- to 10, and caspase-12, which are activated in response to a cell death signal, and the latter includes caspase-3, caspase-6 and caspase-7 which transmit the signal activating the cascade that results in DNA fragmentation and cell death (Subsection 1.1.2.4) (Kawamura *et al.*, 2003; Earnshaw *et al.*, 1999).

1.1.3.2 Cytochrome c

Cytochrome c is a protein that is normally stored in the intermembrane space of mitochondria (Scorrano *et al.*, 2003). When the cell receives an apoptotic signal, cytochrome c crosses the outer mitochondrial membrane and accumulates in the cytosol where its functions as a cofactor in the activation of caspases (Fig. 1.2) (Bossy-Wetzel & Green, 1999). Cytochrome c triggers a post-mitochondrial pathway forming an oligomeric complex of cytochrome c/ apoptotic protease activating factor-1 (Apaf-1)/caspase-9, the "apoptosome", which activates the initiator caspase-9 to subsequently cleave the effector caspase-3 and caspase-7 to cause nuclear fragmentation (Scorrano *et al.*, 2003; Kaufmann & Earnshaw, 2000). The treatment of HeLa cells with

staurosporine, a potent pro-apoptotic agent, causes the release of cytochrome c from the mitochondria into the cytosol (Kaufmann & Earnshaw, 2000; Michalides, 1999).

1.2 Survivin

Survivin is a 16.5-kDa protein also known as AP14 or BIRC5. It is an intracellular protein that inhibits apoptosis and regulates cell division and belongs to the inhibitors of apoptosis (IAP) gene family (Verdacia *et al.,* 2000; Altieri, 2001). Members of the IAP family prevent cells from apoptosis, by inhibiting caspases (Fig. 1.2) (Wojcik *et al.,* 2002; Yamamoto & Tanigawa, 2001). Survivin was discovered in 1997 by hybridization screening of a human genomic library with the cDNA of the effector cell protease receptor-1 (EPR-1) (Ambrosini *et al.,* 1997). The survivin gene spans 15 kb, and is located on chromosome 17 t band q25. Survivin has an unusual relationship to EPR-1 in that its sequence is complementary to and in the reverse orientation of EPR-1. The coding strand of survivin contains an open reading frame of 426 nucleotides, and encodes a protein of 142 amino acids (Chiou *et al.,* 2003).

Survivin over-expression *in vivo* increases cell resistance to apoptosis (Chiou *et al.,* 2003). This conclusion has been proven by the study of Grossman *et al.,* (2001a) when transgenic expression of survivin in epidermal keratinocytes significantly reduced the number of apoptotic cells in the epidermis following exposure to ultraviolet (UV) irradiation. Conversely, inhibition of survivin expression *in vitro,* by treatment with antisense survivin oligonucleotide, increased the susceptibility of HeLa cells to receptor-mediated apoptosis, and the human neural tumour cell lines to induced apoptosis, MSN and TC620 (Shankar *et al.,* 2001).

Survivin appears to have an important role in regulating apoptosis at the cell cycle checkpoint(s). Its expression is highly cell cycle-regulated, and is detectable in the nucleus selectively at the G2/M phase (Li *et al.,* 1998). Transcription of survivin has been shown to be directly repressed by wild-type p53, another cell cycle checkpoint-regulating protein that induces apoptosis (Mirza *et al.,* 2002). When acute lymphoblastic leukemia cells are treated with doxorubicin, which causes accumulation of wild type p53, the result is a dramatic down-regulation of survivin, depletion of cells in the G2/M phase of the cell cycle, and increased apoptosis (Zhou *et al.,* 2002).

In addition, survivin appears to be important for cell cycle progression. Disruption of survivin by antisense targeting HeLa cells results in spontaneous apoptosis and aberrant mitosis, as well as an increase in caspase-3 activity at mitosis. Disruption of survivin in cell lines by both antisense targeting and survivin antibodies also induces polyploidy and aneuploidy as a result of cytokinesis failure and the premature onset of anaphase. *In vivo*, survivin is also required for cell division. Homozygous knockout of the survivin gene in mouse embryonic stem cells resulted in disrupted microtubule formation and polyploidy during development, which culminated in early embryonic lethality (Uren *et al.,* 2000).

1.2.1 Structure and function of survivin

The structure of human survivin, as determined by X-ray crystallography, reveals the presence of an amino-terminal globular zinc finger domain, which includes the BIR motif, and a long carboxy-terminal helix separated by a short linker segment, important for dimerization (Rodriguez *et al.,* 2002). The structure of survivin is

intimately linked with its function as an inhibitor of apoptosis. The amino terminal portion of survivin consists of three alpha helices (residues 14-21, 31-41, 68-80) and 3 beta-sheets (residues 43-45, 55-58, 61-64), which closely resemble the BIR domain that is conserved in the IAP family (Fig 1.3). The BIR domains of IAP family members are involved in the function of these proteins as inhibitors of apoptosis (Verdecia *et al.*, 2000). A mutation in the BIR domain, T34A, which inhibits phosphorylation of survivin by p34-cyclin B1, abrogates the ability of survivin to inhibit apoptosis (Chiou *et al.*, 2003).

Three different isoforms of this protein have been identified: survivin (142 aa), survivin-2B (165 aa) and survivin-ΔEx3 (137 aa) (Mahotka *et al.*, 1999). Survivin and survivin-2B are located in the cytoplasm whereas survivin-ΔEx3 is located in the nucleus. Another isoform was reported in 2004 by Badran *et al.* 2004 designated as survivin 3B (120 aa) in human adenocarcinoma cell lines. It is likely that survivin-3B possesses anti-apoptotic activity. Survivin-ΔEx3 has anti-apoptotic properties whilst survivin-2B with markedly reduced anti-apoptotic properties (Badran *et al.*, 2004). It was reported that the localization in distinct cellular compartments of different nuclear-cytoplasmic variants might constitute a regulatory mechanism for the activity of different splice variants of survivin. The different isoforms of survivin is believed to play a distinct role in cancer and therefore that such a role may be partially determined by their differential nuclear-cytoplasmic transport and localization (Rodriguez *et al.*, 2002).

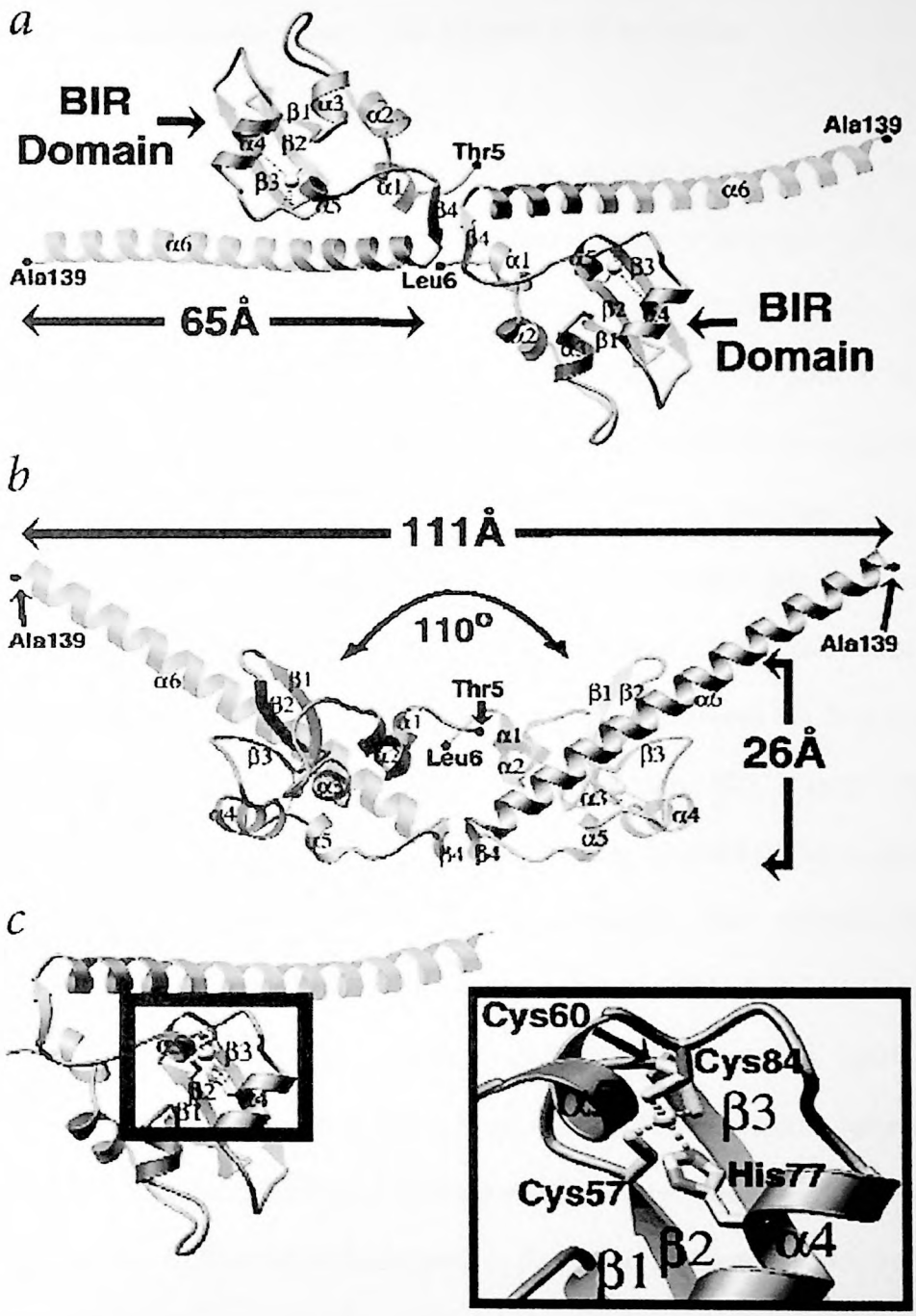

Figure 1.3: The overall architecture of human survivin. *a*, Ribbon representation of the survivin dimer. The Zn^{2+} ion is shown as a shaded sphere. Coordination bonds are shown as dotted orange spheres. One monomer is blue; the other is rose. *b*, Orthogonal view of the ribbon representation shown in (*a*). *c*, Perspective and close up view of the Zn^{2+} binding site on one survivin monomer. The depicted orientation corresponds to that pictured in (*a*). (Verdecia *et al.*, 2000)

1.2.2 The mechanism of survivin inhibition of apoptosis

Survivin is identified as an intrinsic cellular regulator that plays an important role in the suppression of apoptosis by either directly or indirectly inhibiting the activity of caspases (Hikita *et al.,* 2002; Badran *et al.,* 2003; Badran *et al.,* 2004; Bao et al., 2002; Honda *et al.,* 2003; Kuttler *et al.,* 2002; Li *et al.,* 1998; Song *et al.,* 2004; Song *et al.,* 2003). Several IAP family members have been shown to suppress apoptosis by direct inhibition of caspases via the BIR domains. The structure of survivin has been compared to another IAP family member, XIAP, which contains three BIR domains (Tran *et al.,* 1999; Otaki *et al.,* 2000; Shinozawa *et al.*, 2000; Sohn *et al.,* 2003). XIAP inhibits caspase-3 and caspase-7 via a linker region between the first two domains, and also binds to and inhibits caspase-9 through its third BIR domain (BIR3). The BIR domain of survivin appears to be closely related in its three dimensional structure to the BIR3 domain of XIAP, suggesting the possibility that survivin binds caspase-9. Survivin has a capability to bind to caspases and modulate their functions (Kawamura *et al.,* 2003; Sandler *et al.*, 2002; Sarela *et al.,* 2001; Wheatley *et al.,* 2001; Mahotka *et al.*, 2002; Kornacker *et al.,* 2001; Krieg *et al.,* 2002). The interaction between survivin and caspase-9, and the functional implication of this interaction have been studied through mutagenesis. Loss of phosphorylation at threonine 34 on the T34A mutant of survivin results in the dissociation of an immunoprecipitable survivin-caspase-9 complex on the mitotic apparatus, allowing caspase-9 dependent apoptosis to occur (Chiou *et al.,* 2003; Wall *et al.*, 2003; Li *et al.*, 1999; Lu *et al.,* 2004; Fortugno *et al.*, 2003).

1.2.3 The role of survivin in cell division

Recent reports demonstrated how survivin may act in regulating cell division. During the cell cycle, survivin is first detected on the centromere at prophase/metaphase. It is also present in the spindle midzone during anaphase/telophase, but is no longer detected by the end of telophase (Li *et al.*, 1998: Uren *et al.*, 2000). Furthermore, it was indicated that survivin remains localized in kinetochores until metaphase, then in the spindle midzone during anaphase, and in the cleavage plane during telophase. The kinetochore is a DNA-protein complex that assembles on the centromere and its required for attachment of the microtubules during mitosis (Uren *et al.*, 2000). These localization patterns resemble those of the inner centromere proteins (INCENP), TD-60, and Aurora B, which are known as chromosomal passenger proteins. Thus, based on its localization during the cell cycle, it has been postulated that survivin may be an additional chromosomal passenger protein. These four proteins are the only mammalian passenger proteins known to date, which are carried on the chromosomes to the center of the cell at metaphase, in the plane of the future cleavage furrow, and are important for cytokinesis and chromosomal movements during cell division (Chiou *et al.*, 2003). It was concluded that the primary role of survivin is to regulate chromosome segregation and cytokinesis (Uren *et al.*, 2001)

1.2.4 Survivin expression in cell lines, and in embryonic, fetal, and normal adult tissues

The regulation of apoptosis is critical for normal embryonic development and for homeostasis in adult tissues (Grossman *et al.*, 2001b). In animals, survivin is strongly expressed in embryonic and fetal organs, but is undetectable in most terminally

differentiated normal tissues (Mori *et al.,* 2002). Uren *et al.,* (2001) found that survivin is expressed in mouse embryos. In the mouse model, survivin was found to be elevated at the G_2/M phase of the cell cycle during liver regeneration (Deguchi *et al.,* 2002). Kim *et al.,* (2003) reported that survivin is strongly expressed at the bottom of mouse embryonic intestinal crypts. At the protein level, survivin was also detected during all stages of early embryos of mice (Kawamura *et al.,* 2003; Jaskoll *et al.*, 2001).

The expression of survivin in normal human adult tissues, appeared only in a few published reports, contrary to numerous reports examining the role of survivin in cancer (Chiou *et al.,* 2003). However, its expression has been reported in a few normal growing adult human tissues, including thymus (Kobayashi *et al.,* 2002), colonic mucosa (Gianani *et al.,* 2001), placenta (Shiozaki *et al.,* 2003; Lehner *et al.,* 2001), bone marrow (Altieri & Marchisio, 1999), and keratinocytes of the basal layer of the skin (Grossman *et al.,* 2001a; O'Driscoll *et al.,* 2003; Chiodino *et al.,* 1999).

During human development, survivin is expressed in the fetal lung, heart, liver, kidney, and gastrointestinal tract, and in fetal tissues where apoptosis occurs, such as the stem cell layers of stratified epithelia, endocrine glands, pancreas and thymic medulla. In all of these studies, survivin was not found in normal adult tissues. These findings suggest that the regulation of cell division and the anti-apoptosis functions of survivin are important not only during early development, but also during cancer progression (Chiou *et al.,* 2003).

In cell lines, it was reported that survivin was expressed in HeLa cells (Uren *et al.,* 2001), human breast cancer cell line, MCF-7 (Tanaka *et al.,* 2004), melanoma cell

lines (Ambrosini *et al.,* 1998; Grossman *et al.,* 1999), a lung adenocarcinoma cell line (Olie *et al.,* 2000), neuroblastoma and oligodendroglioma cell lines (Shankar *et al.,* 2001), a murine thymic lymphoma cell line (Kanwar *et al.,* 2001) and a colorectal cancer cell line (Mesri *et al.,* 2001), human colon adenocarcinoma cells, HT29 (Yamamoto *et al.,* 2002).

1.2.5 Survivin and cancer

One of the most significant features of survivin is its differential distribution in cancer compared to normal tissue. Over-expression of survivin has been demonstrated in tumours of the lung, breast (Zhang *et al.,* 2004), esophagus, pancreas, bladder, uterus, cervix, ovary (Mei *et al.,* 2001; Tarkowski *et al.,* 2001; Tao *et al.,* 2004), large-cell non-Hodgkin's lymphoma, leukemias (Schlette *et al.,* 2002; Mori *et al.,* 2002; Carter *et al.,* 2001; Carter *et al.,* 2003), neuroblastomas, melanomas, gastric (Li *et al.,* 2004; Lu *et al.,* 1998; Yu *et al.,* 2002), colon (Sarela, *et al.,* 2000; Sarela *et al.,* 2001; Yang *et al.,* 2004), stomach, liver (Chiou *et al.,* 2003), oral (Muzio *et al.*, 2004; Muzio *et al.,* 2003; Muzio *et al.,* 2001), thyroid (Sugawara *et al.,* 2002), and in laryngeal squamous cell (Dong *et al.,* 2002).

Despite its role in mitosis, it is clear that the over-expression of survivin in cancer does not simply reflect the presence of a higher number of proliferating cells. In melanoma, survivin expression was indistinguishable in cases with low or high mitotic index. In addition, the fact that survivin is typically observed in nearly all tumour cells, and not just in the mitotic fraction, suggests that expression of the survivin gene is

Table 1.1 : Meta analysis of survivin expression in cancers in published reports

Authors	Method of detections	Subjects/locations	Findings/conclusions
Li *et. al.*, (2004)	Immunohistochemistry (IHC)	Gastric carcinoma (n=80), China	Survivin (SUR) expression was 76%, no expression in adjacent normal tissues, +ve expression of SUR correlated with worse histological grades and pathological grades, poor prognosis. SUR inhibit apoptosis of tumor cells.
Fields *et al.*,, (2004)	IHC	Hepatocellular carcinoma (n=72)	SUR expression was 43%, SUR correlated with histological grade & stage , nuclear expression correlated with poor prognosis.
Zhang *et al.*,, (2004)	IHC	Breast carcinoma (n=119), China	SUR expression was 72.3%, correlated with poor prognosis.
Kayaselcuk *et al.*,,(2004)	IHC	Breast carcinoma (n=43), Turkey	SUR +ve correlated with Bcl-2 but was not correlated with p53, bax, cerbB-2, estrogen & progestron, clinicopatholohgical factors (grade, stage and mitotic index).
Yao *et al.*,, (2004)	IHC	Gastric adenocarcinoma (n=120), China	SUR expression was 49.17% in cytoplasma of carcinoma cells.
Kennedy *et al.*,,(2003)	IHC	Primary breast carcinoma (n=293), Dublin, Ireland	SUR expression was 60%, 31% nuclear staining, 13% cytoplasmic, 16% both nuclear& cyctoplasmic staining. Nuclear expression is most common and is an independent prognostic indicator of good prognosis.
Kawamura *et al.*,,(2003)	RT-PCR & IHC	9 weeks mouse embryos	SUR protects embryo from apoptosis by inhibiting apoptotic pathway involving caspases.
Kobayashi *et al.*,(2002)	IHC, RT-PCR, flow cytometry	Human tymocytes, Japan.	SUR play an important role in the T-cell development in the human thymus. SUR has a positive correlation with Bcl-2 and CD69.
Ikehara *et al.*,,(2002)	IHC	Small adenocarcinoma of lung (n=79), Japan	SUR expression was 57%, correlated with venous invasion, worse overall survival, poor prognosis.
Mori *et al.*,,(2002)	Reverse PCR	Leukemia (n=31), Japan	SUR expression was 54% in acute myelocytic leukemia, 69% in acute lymphocyctic leukemia, no expression in normal bone marrow, provide prognostic information.
Dong *et al.*,,(2002)	IHC	Laryngeal squamous cell carcinoma(LSCC), (n=102), Japan	SUR expression was 65.7%, associated with tumor site, poor differentiation, tumor size, lymph node metastasis & advanced stage, unfavorable clinicopathological factors.
Hattori *et al.*,, (2001)	RT-PCR	Epithelial ovarian cancer (n=43), Japan	SUR expression was 86%,
Mei *et al.*,,(2001)	IHC	Cervical carcinoma (n=59), China	SUR expression was 69.5%, strongly associated with Bcl-2.
Lehner *et al.*,, (2001)	IHC	Archival formalin-fixed human placenta tissues (n=25)	SUR expression was 68%
Tanaka *et al.*, (2000)	IHC	Breast carcinoma (n=167)	SUR expression was 70.7%
Lu *et al.*,,(1998)	IHC	Gastric carcinoma (n=174), Japan	SUR expression was 34.5%, coexpression with Bcl-2 and segregated with p53 positive.

deregulated in cancer, albeit still retaining cell-cycle periodicity in mitosis (Chiou *et al.*, 2003; Daidone *et al.*, 2001; Endoh *et al.*, 2001; Frost *et al.*, 2002; Gu & Lin, 2004).

In certain cancers, two types of molecular abnormalities have been reported that might contribute to aberrant survivin expression. Gain of chromosome 17q is the most common genetic abnormality in neuroblastoma, where amplification of the survivin locus on 17q25 has been detected by fluorescence *in situ* hybridization (Chiou *et al.*, 2003; Takai *et al.*, 2002). In addition, survivin exon1 sequences are largely silenced by methylation in normal ovaries, but become de-methylated, and thus trancriptionally active, in most ovarian cancers, leading to the over-expression of survivin (Hattori *et al.*, 2001). However, given the widespread survivin over-expression in many types of cancer, it is plausible that multiple oncogenic signalling pathways might converge on the reactivation of the survivin gene in neoplasia (Chiou *et al.*, 2003).

The role of survivin in many cancers has been reported in numerous publications, but little has been published about the role of survivin in breast cancer. A few reports have shown the expression of survivin in breast cancer ranging from 60% to 72.% (Zhang *et al.*, 2004; Kennedy *et al.*, 2003; Tanaka *et al.*, 2000).

1.2.6 Clinical significance of survivin

Recently, survivin has emerged as a diagnostic and prognostic marker and a potential drug target because it is predominantly overexpressed in most cancer types. The role of survivin in the inhibition of apoptosis in breast cancer has not been clearly established. Furthermore, only a few reports have highlighted the clinical significance of

survivin. Further investigations of survivin during tumour growth and progression may yield important insights into its functional role(s) in carcinogenesis and allow the development of important therapeutic strategies for combating cancer.

1.2.6.1 Prognostic value of survivin in cancer

Assessment of prognosis is important in patients with malignancies because its results serve to separate a large heterogeneous population into smaller populations with more concisely predictable outcome (Hayes, 2000). In the majority of neoplasms investigated for survivin expression including breast, lung, colorectal, gastric, liver, bladder and kidney cancers, neuroblastoma, gliomas, soft tissue sarcomas and hematological malignancies, high levels of the IAP proteins were predictive of tumour progression, either in terms of disease-free survival or overall survival, thus providing prognostically relevant information (Zaffaroni & Daidone 2002; Altieri 2001; Altieri & Marchisio 1999).

1.2.6.2 Survivin as a therapeutic target in cancer

Two general considerations make survivin an attractive therapeutic target in cancer: it is selectively expressed in tumour cells and it is required for their viability (Chiou *et al.*, 2003; Altieri, 2003b; Ueda *et al.*, 2002; Sasaki *et al.*, 2002; Poetker, 2002; Pizem *et al.*, 2004; Iurlaro *et al.*, 2004; Guan *et al.*, 2004). Results obtained by different studies aimed at targeting survivin by means of different approaches demonstrated that inhibition of this cell survival factor promotes some favourable biological effects (Zaffaroni & Daidone 2002; Altieri, 2003a; Altieri 2004; Tsurama *et al.*, 2004).

There are several therapeutic strategies targeted to survivin including the use of antisense oligonucleotides; natural antisense (EPR-1 cDNA), oligonucleotide 4003, and oligonucleotides 903 and 904 (Ambrosini *et al.,* 1998; Grossman *et al.,* 1999; Olie *et al.,* 2000; Shankar *et al.,* 2001), dominant negative mutants: Cys 84 → Ala, Thr 34 → Ala, and Cys 84 → Ala (Grosman *et al.,* 1999; Grossman *et al.,* 2001a; Mesri *et al.,* 2001b; Kanwar *et al.,* 2001), and ribozymes (hammerhead ribozymes) (Pennati *et al.,* 2002). The biological effects *in vitro* and *in vivo* using these therapeutic strategies demonstrated as decrease in cell proliferation, an increased spontaneous apoptosis and an enhanced efficacy of several types of conventional treatments including chemotherapy, radiotherapy and immunotherapy (Zaffaroni & Daidone 2002).

1.3 Bcl-2

1.3.1 Structure and biological functions of bcl-2

B-cell lymphoma-2 protein (Bcl-2) is the first known regulator of cell death (Heiser *et al.,* 2004). The bcl-2 family proteins are important regulators of apoptosis in mammalian cells (Schinzel *et al.,* 2004). Bcl-2 is an anti-apoptotic protein (Townsend *et al.,* 2002; Giatromanolaki *et al.,* 2001) and is a proto-oncogone (Formby *et al.,* 1999; Strasser *et al.,* 1997) that resides on the cytoplasmic face of the mitochondrial outer membrane, in the endoplasmic reticulum and in the nuclear envelope (Mullauer *et al.,* 2001; Robertson *et al.,* 2000; Schinzel *et al.,* 2004). Bcl-2 is an acronym for the B-cell lymphoma/leukemia-2 gene which was identified at the site of the t(14;18) chromosomal translocation, occurring in 85% of diffused B-cell lymphomas (Joosens *et al.,* 1998; Strasser *et al.,* 1997).

The bcl-2 inhibits apoptosis (Eissa *et al.,* 1999) by blocking the release of cytochrome c from mitochondria, thereby preventing Apaf-1 (apoptotic protease-activating factor-1) and consecutive caspase activation (Fig. 1.2). The bcl-2 may also inhibit apoptosis by binding to the pro-apoptotic molecules Bax and bcl-x_s (Mullauer *et al.,* 2001).

A number of proteins that are structurally related to bcl-2 (Bcl-2 protein family) have been discovered in mammals, birds, frogs, nematodes and viruses and can be sub-divided into two groups. The first group includes those that inhibit apoptosis (mammalian $BcLx_L$, Bcl-w, A1, Mcl-1, nematode Ced-9, adenovirus E1B19kD, Epstein Barr Virus (EBV) BHRF1, African Swine Fever Virus LMW5-HL, Human Herpes Virus 8 KSBcl-2 and Herpes Virus Saimiri ORF16). The second group includes those that enhance apoptosis (Bax, Bcl-x_S the product of alternative splice variant of the bcl-x gene, Bad, Bak, Bik/Nbk, Bid and Harakiri (Strasser *et al.,* 1997). The bcl-2 family of proteins appears to be involved in either enhancing or opposing the apoptotic process.

In most situations, the mitochondria act as focal points in the apoptotic pathway and provide convenient positions for regulatory molecules to intervene. In cell free systems, nuclear condensation and DNA fragmentation were found to be dependent on the presence of mitochondria and inhibited by bcl-2 (Heiser *et al.,* 2004).

Bcl-2 plays a crucial role in maintaining the viability of cells that are meant to be long-lived such as peripheral lymphocytes (Strasser *et al.,* 1997). The involvement of bcl-2 in apoptosis was first seen indirectly when it was noted to prolong cell survival. Immature pre-B cells dependent on IL-3 for survival in culture were noted to persist

despite IL-3 withdrawal when the cells were transfected with bcl-2, an effect that seemed to occur without cell proliferation. This persistence was later shown to be due to the ability of bcl-2 to block apoptosis (Bossy-Wetzel & Green, 1999).

The bcl-2 family of proteins is also involved in embryogenesis where it controls developmentally programmed cell death during tissue differentiation. After birth, bcl-2 and its relatives play critical roles in regulating programmed cell death in the haematopoietic system, tissue homeostasis and mammary gland involution (Coultas & Strasser, 2003).

1.3.2 Bcl-2 and its role in breast cancer

In normal breast, bcl-2 is expressed in the non-pregnant and non-involuting mammary epithelium. Bcl-2 expression has previously been reported in normal breast ductal cells where it is supposed to be involved in the hormonal regulation of hyperplasia and involution (Vetrani *et al.,* 1995). Bcl-2 is thought to be expressed through hormone-dependent pathways (Ioachim *et al.,* 2000; Vetrani *et al.,* 1995) such as estrogen and progesterone (Park *et al.,* 2002).

The exact mechanism and the effect of the down regulation of bcl-2 expression on breast cancer cells are not clearly defined (Park *et al.,* 2002). In breast cancer, bcl-2 expression appears to be inversely correlated with the presence of p53 mutations in its pathways (Joosens *et al.,* 1998; El-Ahmady *et al.,* 2002; Takei *et al.,* 1999; Van-Slooten *et al.,* 1998).