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Evaluation of the humoral immune response against survivin in patients with lung cancer

Doctoral thesis

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Dedication

To my mother, the source of inspiration, the eternal flame and support, I would like to dedicate this effort and to tell her if it wasn't for you I might have give up before I achieved the goal of my life.

To my late father, the hero who put my feet on the track of education and offered a helping hand whenever I tripped on my first step, but did not live long enough to see me achieve my goals, I say: I'm there father, I love you and I wish you were here to see your dreams come true! God bless you.

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Summary

Autoantibodies have been shown to be present in the circulation of people with various forms of solid tumours before cancer-associated antigens can be detected. Unlike circulating proteins that are shed by bulky tumors, autoantibodies are detectable even when antigen expression is minimal and they can be measured up to 5 years prior to disease detection. They are usually directed against oncoproteins, aberrantly expressed tumour suppression genes, proliferation-associated antigens as well as nuclear antigens.

Existing evidence regarding spontaneous anti-survivin humoral responses in lung cancer is inconclusive. Moreover, despite that cancer cell death elicited by radiotherapy and some chemotherapeutic agents seems to be immunogenic, information about the possible effect of treatment on these responses, is lacking. Serum samples from 33 small cell lung cancer (SCLC) and 117 non-small cell lung cancer (NSCLC) patients upon diagnosis, and from 100 controls, were tested by ELISA for anti-survivin antibodies. Cutoff was set to the mean+2SD of controls. 7.7% of NSCLC, none of the SCLC patients and 2% of the controls appeared with elevated antibody levels (OR 3.6, 95% CI 0.7-17.3 for NSCLC, OR 0.6, 95% CI 0.03-12.6 for SCLC). Measurement of antibodies in 76 NCSLC patients post therapies and during their follow-up, revealed 12 NSCLC patients that increased their antibody levels up to 2-38 times, and 7 others that decreased them by 2-8 times. No significant correlation was uncovered between either the antibody levels upon diagnosis or their changes post therapies and during follow-up, and any clinicopathological parameter, their response to treatment and survival. We conclude that survivin does not induce considerable humoral responses in lung cancer. Potentially, however, strong anti-survivin antibody responses can be elicited during the follow-up of the patients, whose clinical significance remains to be elucidated. These findings, together with our previous data concerning survivin expression and the related cytolytic T cell responses in lung cancer, signify a high tolerogenic potential of this tumor-associated antigen.

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Abbreviations

Ab	Antibody
APC	Antigen Presenting Cell
AIF	Apoptosis inducing factor
B-cell	B lymphocyte
Bcl-2	B cell Lymphoma-2
BIR	Baculovirus IAP Repeat
BSA	Bovine Serum Albumin
°C	Degrees Celsius
CARD	Caspase Activating and Recruitment Domain
CD	Cluster of Differentiation
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
DIABLO	Direct IAP Binding protein with Low PI
EBV	Epstein Barr Virus
ELISA	Enzyme-Linked ImmunoSorbent Assay
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HER-2	Human Epidermal Growth Factor-2
HLA	Human leukocyte Antigen
HPV	Human Papilloma Virus
HRP	Horse radish Peroxidase
HSA	Human Serum Albumin
IAP	Inhibitor of Apoptosis Protein
ICs	Immune Complexes
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
kDa	kilodaltons
MHC	Major Histocompatibility
NK	Natural Killer
NSCLC	Non Small Cell Lung Carcinoma
PBS	Phosphate Buffered Saline
SCLC	Small Cell Lung Carcinoma
STAT	Signal Transducers and Activators of Transcription
Smac	Second mitochondrial activator of caspase
TAA	Tumour Associated Antigen
T-Cell	T lymphocyte
TCR	T cell Receptor
Th	T-helper
TNF	Tumour Necrosis Factor
TMB	tetramethylbenzidine chromogen
TNM	Tumour Nodes Metastases
T20	Tween20
VE	Vascular Endothelial
VEGF	Vascular Endothelial Growth Factor

Curriculum Vitae

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Karanikas V, **Khalil S,** Kerenidi N, Gourgoulianis KI, Germenis AE. Anti-survivin antibodies in patients with lung cancer, Cancer Letters 2009-Apr 18.

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1. INTRODUCTION

Cancer is a public health problem worldwide. Each year, 6 million people die from cancer and 8.1 million new cases are diagnosed. The growth rate of cancer is now 2.1% per year, a rate that exceeds that of the world's population at 1.7 % per year.

Lung cancer was a rare disease at the beginning of the 20th century, but exposures to new etiologic agents and an increasing lifespan, combined to make it a scourge of the 20th century. It tends to be more common in developed countries, particularly in North America and Europe, and less common in developing countries, particularly in Africa and South America [1, 2]. Lung cancer is the leading cause of cancer deaths worldwide; the high mortality associated with this disease is primarily due to the fact that the majority of the lung cancers are diagnosed at advanced stages when the options for treatment are mostly palliative.

Lung cancer is one of the most invasive and devastating lung diseases. Based on histopathological features, it is divided into two main groups; non small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). SCLC and NSCLC cells exhibit differences in mutation spectra and hot spots for loss of heterozygosity, which is consistent with the different biological and clinical features of the two lung cancer types [3]. Of these two types, NSCLC is the most common (80-85%). NSCLC is further subdivided into adenocarcinoma, large cell carcinoma and squamous carcinoma. Adenocarcinoma has glandular features and squamous carcinoma shows characteristics of epithelial cells. SCLC on the other hand normally expresses properties of neuroendocrine cells, characteristics that are not often seen in NSCLC cells and the cells are typically oat-cell like [3].

1.1. Tumour Immunology

1.1.1. Cancer development and its microenvironment

Cancer is characterized by uncontrolled growth and dispersion of cells as a result of abnormal changes within the genetic material contained in those cells. A single cell or group of cells can undergo genetic events such as mutations, influenced by inherited or environmental factors in addition to levels of hormones or growth factors, which may change the cells' behavior. These events, which may take years to arise, represent the process of an oncogenesis.

When cells divide abnormally during their development, they may evolve into immortalized cell population, which may lose the control mechanisms of normal cell division, activity and interactions with neighboring cells. Such immortalized cell populations transform into malignant tumour cell populations, whose behavior can violate the tissue environment, by invading and destroying normal tissues. They also metastasize all over the body by releasing tumour cells into the blood and lymphatic system, where they continue to grow and develop by forming new cancers.

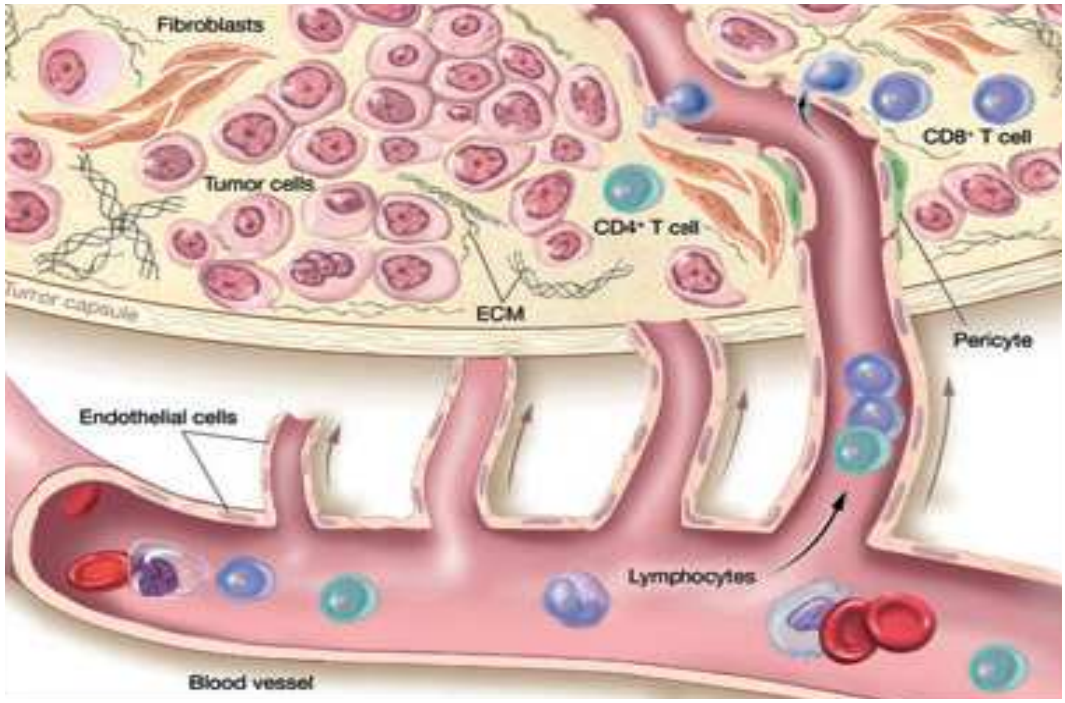

Figure 1: Schematic of the tumour microenvironment, including tumour cells, endothelial cells, pericytes, fibroblasts, CD4+ and CD8+ lymphocytes and extracellular matrix components.

Within each foci of tumour, a composite of various cell types can be seen histological. Apparent from the malignant cells non cancerous cells are also present these are usually endothelial cells, pericytes, fibroblasts, inflammatory cells, leucocytes and elements of the extracellular matrix (ECM). Collectively, this is known the tumour microenvironment, as can be seen in (Fig 1).

This creates a unique microenvironment, which can modify the neoplastic properties of the tumor cells. The tumor microenvironment is highly dynamic and heterogeneous and neoplastic cells have an active cross-talk with stroma and cellular components of the inflammatory reaction. Myeloid cells in particular, have a pivotal role in coordinating the immune response against a growing cancer [4]. Malignant cells perpetually stimulate host stromal and vascular cells to conduct physiological invasion. Within the same microenvironment, vascular sprouts migrate and invade towards the tumour mass while tumour cells migrate outwards in the opposite direction [5]. Activation of the local invasive environment seems to create a permissive field for the malignant cell [6].

Tumour infiltrating lymphocytes (TILs) and their role in the death of tumors has long being considered as a positive prognostic indicator of disease progression. TILs were defined as "infiltrating lymphocytes" found within the tumor tissues. Ironically, the presence of TILs in the context of tumour growth, usually marked recognition of the disease and failure of immunosurveillance. The most prominent populations of TILs are the $CD4^+$ and $CD8^+$ T cells, the dendritic cell (DC) and macrophages.

$CD8^+$ cytotoxic T cells (CTL) are the critical effector cells of adaptive immunity that actually destroy tumors. They are equipped with specializing cellular mechanisms and molecules that cast cell damage. The phenotypic analysis of T cells in human tumors shows that they are memory lymphocytes, although the CD4/CD8 ratio may be highly variable from one tumor to another [7].

Tumor specific $CD4^+$ and $CD8^+$ T cells act as a "home" to the tumor where they participate in the killing of antigen positive tumor cells. $CD4^+$ T cells produce IL-2 that

not induces production of IL-15 and also helps to maintain the function and viability of the tumor specific $CD8^+$ T cells. Tumor specific $CD8^+$ T cells efficiently recognize their tumor targets and induce tumor cell death by both direct and indirect mechanisms. It is likely that these $CD8^+$ T cells directly kill many of the tumor cells in vivo. However, these cells also produce large amounts of IFN-γ following interaction with their tumor targets and thus might also induce tumor cell cytostasis and killing by the IFN-γ dependent mechanisms of cell cycle inhibition, apoptosis and induction of macrophage tumoricidal activity [8].

Regulatory T cells (Treg) $CD4^+CD25^+$ represent a heterogeneous population and act on maintaining tolerance and exerting immunosuppression, depending on their relative number in the $CD4^+$ T-cell pool. The unique feature of Tregs is represented by the ability to actively suppress immune responses. The characterization of minor features resulted in the classification of Treg into two main subsets: the "naturally arising" Treg that develop in the thymus due to high-affinity TCR and the "adaptive" Treg. The naturally arising Treg triggers and suppresses bystander T-cell proliferation by an unknown mechanism requiring cell-to-cell interaction. The adaptive Treg develops peripherally following antigenic stimulation in the presence of IL-10 (Tr1 subset) or TGF-β (Th3 subset). In humans, it was observed that T lymphocytes infiltrate non-small cell lung cancer and late-stage ovarian cancer expressed CD25 and produced the inhibitory cytokine TGF-β, thus suggesting that Tregs were actively hindering anti-tumor immunity [9]. It was later demonstrated that in patients with pancreatic, breast, hepatocellular and gastric carcinoma, Tregs were expanded not only in the tumor microenvironment but also in the draining lymph nodes, the ascites and the peripheral blood, associated with malignancy progression [10, 11].

Dendritic cells are the most potent antigen presenting cells (APCs) in the body. These cells are efficient at priming naïve T cells because they express high level of costimulatory molecules such as B7.1 (CD80), B7.2 (CD86), and CD40 on their surface. In the absence of high levels of costimulatory molecules on APC, a naïve T cell

becomes energized upon its first encounter with antigen, thus making the host unresponsive to that particular antigen for life. Several studies have reported that patients with large numbers of infiltrating DC within their tumours have better prognosis than those with low levels of DC infiltration [12]. Additionally, patients with limited numbers of DC infiltrating into tumour site also had an increased incidence of metastases [13].

Macrophages (CD45+CD14+) are the major terminally differentiated cells of the mononuclear phagocyte system. They are released from bone marrow as immature monocytes and circulate in the bloodstream before entering tissues. The ones commonly found in human tumors are referred to as tumor-associated macrophages (TAMs). Normal macrophages are phagocytic and antigen-presenting cells, which play an important role in the control of infections. In contrast, TAMs are re-programmed to inhibit lymphocyte functions through release of specific cytokines, prostaglandins or reactive oxygen species (ROS). It is hypothesized that re-programming of macrophages occurs in the tumor microenvironment as a result of tumor driven activation [14].

Anti-tumor antibodies (Abs) are frequently detected in the circulation of cancer patients. It has been assumed that these Abs are made and secreted by plasma cells situated in the tumor draining lymph nodes, spleen or other lymphoid tissues. Although B lymphocytes (CD19+, CD20+) are uncommon components of human solid tumors, plasma cells have been observed in some carcinomas and, occasionally, represent a substantial infiltrating element [15].

Human tumors are sometimes infiltrated by granulocytes, and nests of eosinophils may be seen in association with tumor cells in various squamous cell carcinomas, for example. By far the most frequent cell in tumors has characteristics of the immature myeloid cell (iMC).

1.1.2. Immune surveillance

The immune system has three primary roles in the prevention of tumors. First, it can protect the host from virus-induced tumors by eliminating or suppressing viral infections. Second, the timely elimination of pathogens and prompt resolution of inflammation can prevent the establishment of an inflammatory environment conducive to tumorigenesis. Third, the immune system can specifically identify and eliminate tumor cells on the basis of their expression of tumor-specific antigens or molecules induced by cellular stress. The third process is referred to as tumor immune surveillance, whereby the immune system identifies cancerous and/or precancerous cells and eliminates them before they can cause harm. The idea that the immune system, which so effectively protects the host from microbial pathogens, might also recognize and destroy tumor cells was first discussed over a century ago, experimentally approached 50 years ago and formally demonstrated 10 years ago [16]. Despite tumor immune surveillance, tumors do develop in the presence of a functional immune system, and therefore the updated concept of tumor immunoediting is a more complete explanation for the role of the immune system in tumor development [17].

Immunosurveillance is a multivariable process requiring actions of different immune effectors in a manner dependent on the tumor's cell type of origin, mode of transformation, anatomic localization, stromal response, cytokine and chemokine microenvironment, and the mechanism of immunologic recognition. Thus, it remains critical to assess the effects of a wide range of immunologic components implicated in tumor development in many different models both chemically induced and spontaneous, to determine whether the immunosurveillance of all cancer susceptible tissues of the body is globally similar or locally distinct.

However, despite strong evidence supporting the existence of a functional cancer immunosurveillance process, immunocompetent individuals still develop cancers that are refractory to many treatment approaches. This clinical reality may be explained by the failure of the initial host innate and adaptive immune responses to eradicate all the

transformed cells. This failure in the face of continued immune pressure favors the outgrowth of tumors with reduced immunogenicity. The term ‘‘cancer immunoediting’’ has been used to emphasize the dual roles of immunity in both eliminating and shaping neoplastic disease during periods of equilibrium and escape [18].

The elimination phase encompasses the original concept of cancer immunosurveillance since it represents the most complete form of the immunoediting process without progression to the two subsequent phases. It is an ever-ongoing process that must be repeated each time the antigenically distinct neoplastic cells arise. For this reason, it is particularly noteworthy that cancer is more prevalent in aged populations where immune system functions and therefore, cancer immunosurveillance begins to decline.

Although the elimination phase of the cancer immunoediting process can eradicate a significant percentage of transformed cells, there can exist a period of latency extending from the end of the elimination phase to the beginning of the escape phase and the emergence of clinically detectable malignant disease. This potentially protracted period in the course of the immune system/tumor interaction that occurs prior to the detection of clinically apparent tumors constitutes the equilibrium phase. Equilibrium is probably the longest of the three phases and may occur over a period of many years in humans.

In the escape phase, some of the tumor cell variants that emerge from the equilibrium phase develop the capacity to grow in an immunologically intact environment. This breach of the host’s immune defenses most likely occurs either when genetic and epigenetic changes in the tumor cell confer resistance to immune detection and/or elimination, or when the tumor induces a state of immunologic suppression or tolerance in the host allowing the tumors to expand and become clinically detectable. Because both the adaptive and innate compartments of the immune system function in the cancer immunosurveillance network, tumors would most likely have to circumvent either one or both arms of immunity in order to achieve progressive growth.

1.1.3. Tumour Antigens

Proteins contained within a tumour cell are processed and presented to the immune system as short peptide fragments known as epitopes on major MHC class I and MHC class II molecules. The presentation of antigenic epitopes derived from the tumor cells allows the immune system to distinguish between normal and transformed cells and direct the immune attack based on these antigens. Multiple peptides are processed from each protein, allowing several different epitopes or Tumour Associated Antigens (TAAs) from tumour-related proteins to be presented on the cell surface [19]. Tumour-specific antigens are rare, arising from point mutations, post-translational modification or fusion proteins generated by chromosomal translocations [20]. TAAs may also be expressed by other normal tissues, but their differential pattern of expression on tumours allows them to be a target of anti-tumour immunity. Tumor antigens can be classified into five major groups based on their expression patterns (Table1).

Table 1: Tumor antigens based on their expression patterns.

Antigen Class	Antigen	Malignancy
Mutated Antigens	Immunoglobulin Idiotype, TCR, Mutant ras, Mutant p53, P21-/bcr-abl fusion	B & T lymphoma, Colorectal, lung, bladder Head and neck
Cancer Testis Antigens	MAGE-1, MAGE-3, GAGE family, 20 genes on the X chromosome	Melanoma, colorectal, lung Gastric.
Viral Antigens	Human Papilloma Virus, SBV	Cervical, penile, Burkitt's lymphoma, Nasopharyngeal
Tissue specific antigens	Tyrosinase , gp100 Prostatic acid phosphates Thyroglobulin, α-Fetoprotein	Melanoma, Prostate, Thyroid, Liver cancer
Over-expressed self antigens	Her-2/neu, CEA, MUC-1, Survivin, hTERT	Breast, Lung, Colorectal Ovarian, Pancreatic

Mutated Antigens

Mutated antigens are derived from ubiquitous proteins that are mutated in tumor cells. Point mutations, chromosomal translocations, deletions, or gene insertions can lead to the generation of unique tumor antigens distinct for each tumor. The mutated antigens are highly tumor-specific, and some may also be involved in the transformation process, e.g. Bcr-Abl protein, b-actin.

Cancer Testis Antigens

Cancer testis antigens (CTAs), comprise a family of genes that share common characteristics: (1) they are expressed in a wide variety of malignant tumors, but expression in normal tissue is mostly restricted to germ cells of the testis, fetal ovary, and placenta, (2) their expression programs are strictly regulated by epigenetic mechanisms such as DNA methylation; and (3) they are immunogenic [21]. The prototypical germ cell antigen, also the first tumor derived (melanoma) human gene product to be identified as a CD8+ T cell target, is melanoma antigen-1 (MAGE-1) [22]. More than a dozen groups of germ cell antigens have since been identified, including the other members of MAGE, GAGE, and BAGE families and this list is growing to include NY-ESO-1.

Over-expressed antigens

Over-expressed antigens are expressed in a wide variety of normal tissues and over expressed in tumors. Genes encoding widely expressed TAA have been detected in histologically different types of tumors, as well as in many normal tissues, generally with lower expression levels. Among the most interesting TAAs of this group are the antiapoptotic proteins (livin, survivin), human telomerase reverse transcriptase (hTERT), wild type tumor suppressor proteins p53, epidermal growth factor receptor protein HER-2/neu and MUC1 [23].

Tissue-specific differentiation antigens

Differentiation antigens lack the specificity of tumor-specific shared antigens, as they are differentiation markers expressed not just by malignant cells, but also by normal cells of a similar origin. Tyrosinase, for example, is expressed by both normal

melanocytes and most melanoma cells. Other examples include: gp100, prostatic acid, phosphates, Thyroglobulin, α-Fetoprotein.

Oncogenic Viral Products

Viral antigens are foreign and are only found on infected cells, making them ideal targets due to their high specificity. Although viruses have evolved their own set of immune evasion strategies, immunotherapy of virus-associated cancers can be directed against viral-antigens vital for viral replication or growth. The human papilloma virus (HPV) E6 and E7 proteins interfere with normal cell-cycle regulation and are required for the viral life cycle [24 , 25, 26, 27].

1.2. Apoptosis in tumors

In the 1960s, Lockshin and Williams introduced the term "programmed cell death" to refer to a gene-directed form of cell death [28]. The term "apoptosis" was coined in 1972 by Kerr, Wyllie and Currie to describe a form of ischemia induced hepatic cell death. The term comes from the Greek (apo + ptosis) for "falling off" and depicts a distinct morphology of dying cells characterized by cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation [29].

This gene-directed mechanism by which unnecessary or dangerous cells are triggered to undergo self-destruction does not injure neighboring cells nor does it elicit any associated inflammatory response [30]. The core apoptotic pathway was first described through genetic analysis in the nematode Caenorhabditis elegans and subsequently found in species as diverse as Drosophila melanogaster and humans [31]. The dysregulation of apoptosis is intricately involved in the etiology and pathogenesis of many diseases including AIDS, autoimmune disorders, neurodegenerative diseases and cancer. In general, apoptosis can be divided into the initiation phase, the effector phase, and the degradation phase.

1.2.1. Apoptosis pathways

There are two pathways that initiate apoptosis: one is the "extrinsic pathway" mediated by death receptors on the cell surface; the other is the "intrinsic pathway"

involving mitochondria [32]. In a simplified description of the extrinsic pathway, binding of ligands such as FasL, TNF, or TRAIL to their corresponding receptors (i.e. Fas, TNF receptor or TRAIL receptor, respectively) results in the formation of the death induced signaling complex (DISC). The DISC contains the adaptor proteins that allow for the recruitment of pro-caspase-8, which leads to its auto-activation. Active caspase-8, often referred to as an initiator caspase, subsequently cleaves and activates pro-caspase-3. Activation of caspase-3, the effector caspase, leads to the ultimate demise of the cell (Fig.2) [32]. Alternatively, the intrinsic pathway is triggered by various extracellular and intracellular stresses, such as growth factor withdrawal, hypoxia and DNA damage. These stresses induce a series of biochemical events that result in the translocation of a pro-apoptotic Bcl-2 family member into the mitochondria, and the release of cytochrome C from mitochondria into the cytosol.

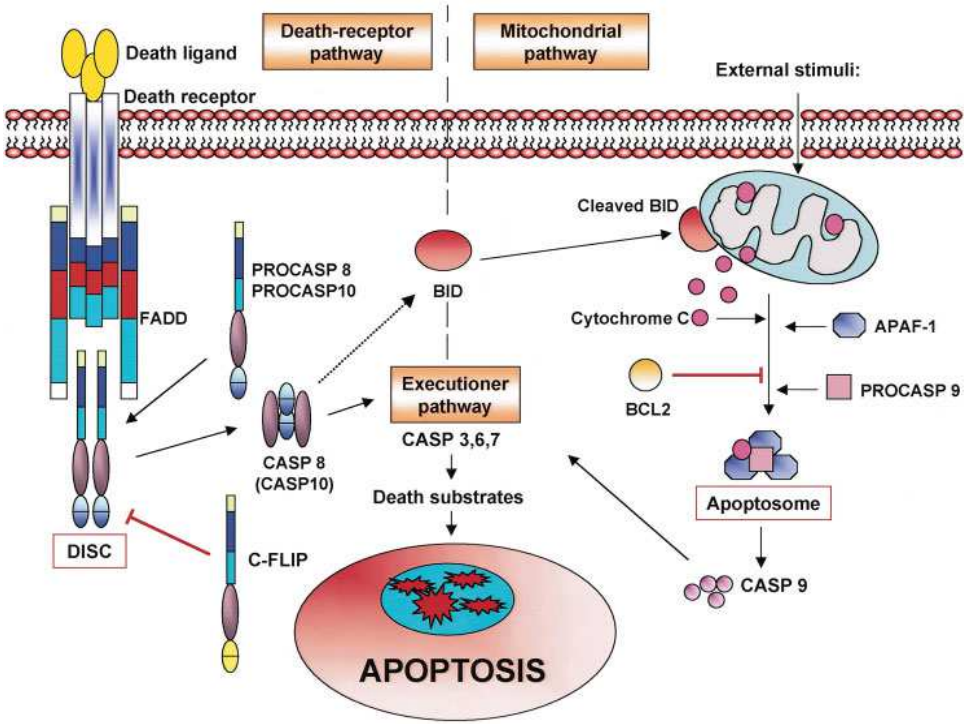

Figure 2: The major pathways to apoptosis. Two alternative, but overlapping, pathways lead to apoptotic death; the extrinsic pathway acts via death receptors while the intrinsic pathway acts via release of mitochondrial proteins. Activation of either pathway leads to activation of a common executioner pathway. In both pathways, the extrinsic and intrinsic pathways, initiator caspases are activated, CASP 8 (and probably 10) for the extrinsic pathway and CASP 9 for the intrinsic pathway. The initiated caspases active executioner caspases which cleave death substrates, leading to cell death. The two major initiator pathways interact via the Bcl-2 family member.

1.2.2. Inhibitors of apoptosis proteins (IAPs)

"Inhibitor of apoptosis proteins" (IAPs), a family of endogenous caspase inhibitors, are highly conserved throughout evolution and comprise the human analogues XIAP (X-linked inhibitor of apoptosis), cIAP1 (cellular IAP1), cIAP2, survivin, apollon and livin [33 , 34]. The IAP family of proteins contains three conserved structural motifs, the Baculoviral IAP repeat (BIR), RING (RING zinc-finger) and the Caspase Activating and Recruitment Domain (CARD) (Fig.3). Classification as an IAP protein requires the 70-80 amino acid long BIR domain, referring to the original discovery of these protein, XIAP-associated factor-1 (XAF-1) has been identified to negatively control IAP activity [35]. The second structural motif found within many IAPs, the RING zinc-finger, functions as an E3 ubiquitin ligase. Moreover, the CARD is found within c-IAP1 and c-IAP2, located between the three N-terminal BIR domains and the C-terminal RING-finger domain. CARD domains are structurally related to death and death effector domains and typically mediate oligomerization with other CARD-containing proteins.

IAPs are negatively regulated by caspase-mediated cleavage, processing by the serine protease Omi/HtrA2 or by proteasomal degradation, for example through RING domain-mediated auto- or heteroubiquitination. In addition, mitochondrial proteins, e.g. Smac/DIABLO or Omi/HtrA2, translocate into the cytosol upon induction of apoptosis and promote apoptosis through binding and antagonizing. Human IAPs contain from one to three copies of the BIR domain and usually, also harbor one or more of the other functional domains. Of the mammalian IAP proteins known to be involved in apoptosis, XIAP has been characterized most extensively. XIAP contains three BIR domains and a C-terminal RING motif. The second and third BIR domains of XIAP are potent inhibitors of caspase-3/-7 and caspase-9 respectively, via distinct mechanisms [36]. The linker segment preceding the BIR2 domain binds to the active site of effector caspase-3 or -7, thereby preventing substrate binding and subsequent catalysis [37 , 38].

1.3. Survivin

Survivin is the smallest member of the inhibitor of apoptosis family of proteins (IAPs). It is implicated in the preservation of cell viability, directly inhibiting caspase-3 and -7 activity and regulating the cell cycle in G2/M phase [39 , 40]. During oncogenesis survivin gene undergoes alternative splicing resulting in the appearance of four as yet identified variants [41 , 42]. Amongst them, the function of the survivin-3B and -2α is not yet elucidated, while survivin-2B seems to be pro-apoptotic, and survivin-ΔEx3 appears to function similarly with survivin [43 , 44]. Survivin and its variants are rarely expressed in terminally differentiated adult tissues but they appear to be highly upregulated in most cancers [40]. Especially in lung cancer, expression levels of survivin appear to relate with disease prognosis [45 , 46].

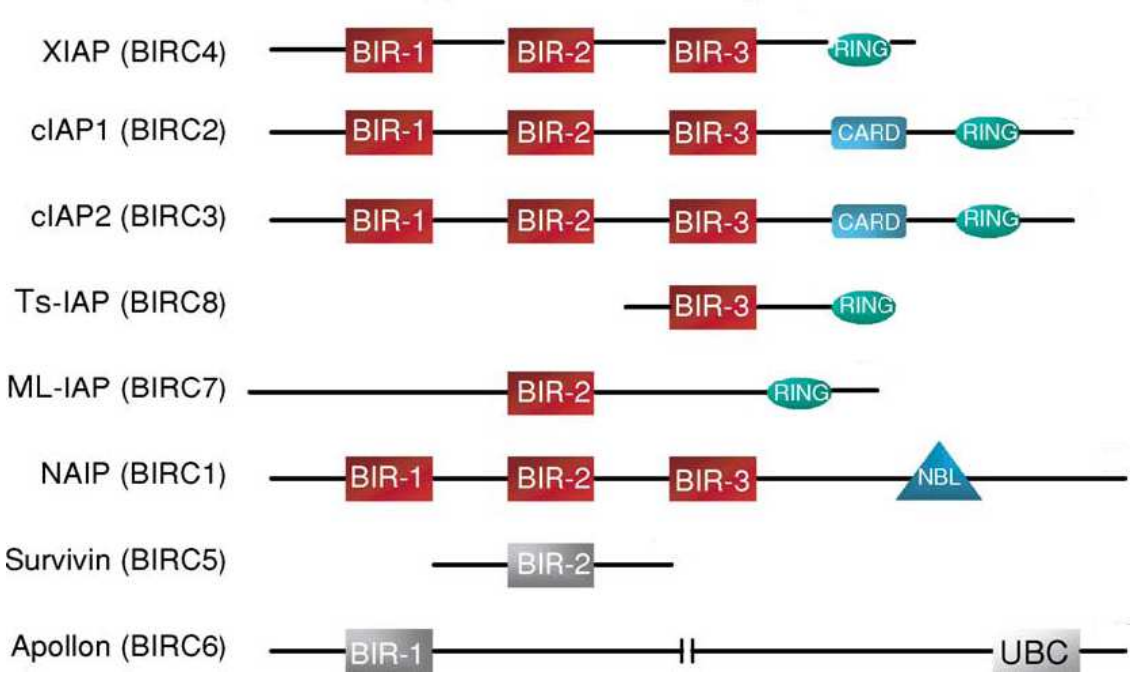

Figure 3: Schematic representation of the human inhibitor of apoptosis protein family (IAP). Eight human BIR containing proteins, Abbreviations as follows: BIR: Baculovirus IAP repeat; XIAP: X-linked IAP, CARD, caspase activation and recruitment domain, RING: Ring zinc-finger, NBL: nucleotide-binding locus, UBC: ubiquitin-binding complex, NAIP: Neuronal apoptosis-inhibitory protein.

1.3.1 The biology of Survivin

At 16.5 kDa, survivin is the smallest mammalian member of the IAP gene family [47]. Structurally, it contains a single BIR, an approximate 70-amino acid zinc finger fold that is the hallmark of all IAPs, and an extended –COOH terminus α-helical coiled-

coil (Fig.4), but no other identifiable domain typically found in IAPs [48]. Based on X-ray crystallography of the human or mouse protein, survivin is a stable homodimer in solution with the –COOH terminus α -helices protruding from the core dimer [49, 50, 51]. A single copy of survivin gene located on chromosome 17q25 (human), or 11E2 (mouse), gives rise to four alternatively spliced survivin transcripts (Fig.5). In addition to wild-type survivin (142 amino acids), four survivin isoforms are generated by insertion of an alternative exon 2 (survivin-2B, 165 amino acids), (survivin-3B, 120 amino acid) contains 5 exons including an extra novel exon3B, removal of exon 3 (survivin-ΔEx-3, 137 amino acids) or acquisition of an in-frame stop codon in intron 2 (survivin-2a) [52, 53]. In survivin- ΔEx-3, the splicing event introduces a frame shift that generates a unique –COOH terminus of potential functional significance [54].

A unique property of survivin is a sharp cell cycle-dependent expression at mitosis. This is largely, but not exclusively, controlled at the level of gene transcription and involves canonical CDE/CHR boxes in the survivin promoter acting as potential G1-repressor elements [55 , 56]. Among the post-translational modifications that affect survivin expression levels, interest has recently focused on the control of protein stability. Survivin is a relatively short lived protein (t1/2 = 30 min), and polyubiquitylation followed by proteasomal destruction has been shown to contribute to cell-cycle periodicity by keeping survivin levels low at interphase [57]. In addition, mitotic phosphorylation of survivin on Thr34 by p34cdc2– cyclin B1 has been associated with increased survivin stability at metaphase [58].

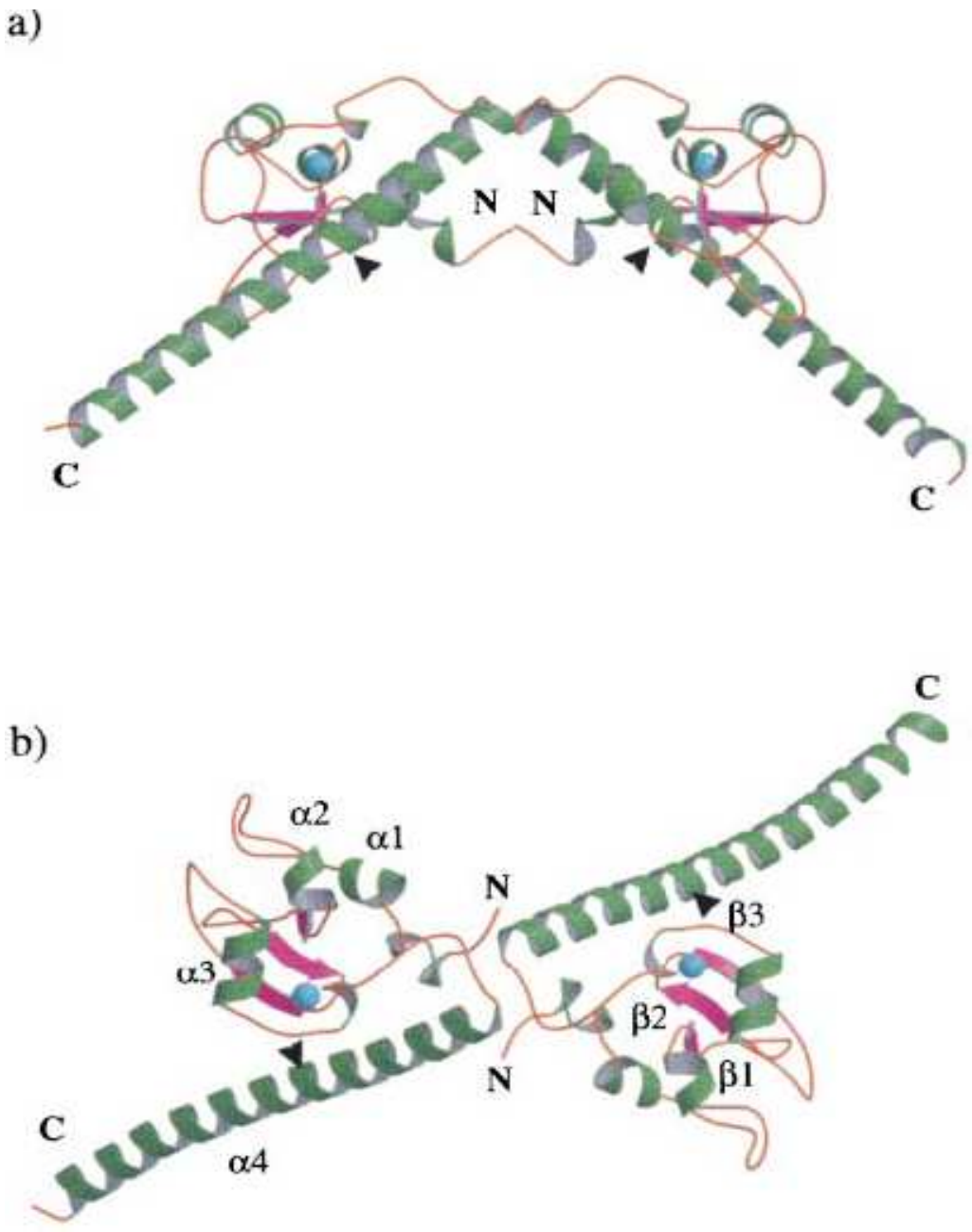

Figure 4: Ribbon Representation of the Survivin Dimer. The β strands, α- helices, and loops are represented as violet arrows, green coils, and brown lines, respectively. The zinc atoms are shown as blue spheres, and black arrows indicate the trypsin cleavage sites [59]. (a) The dimer 2-fold axis is in the plane. (b) The view in (a) rotated by 90° around the horizontal axis; the 2-fold axis is perpendicular to the plane.

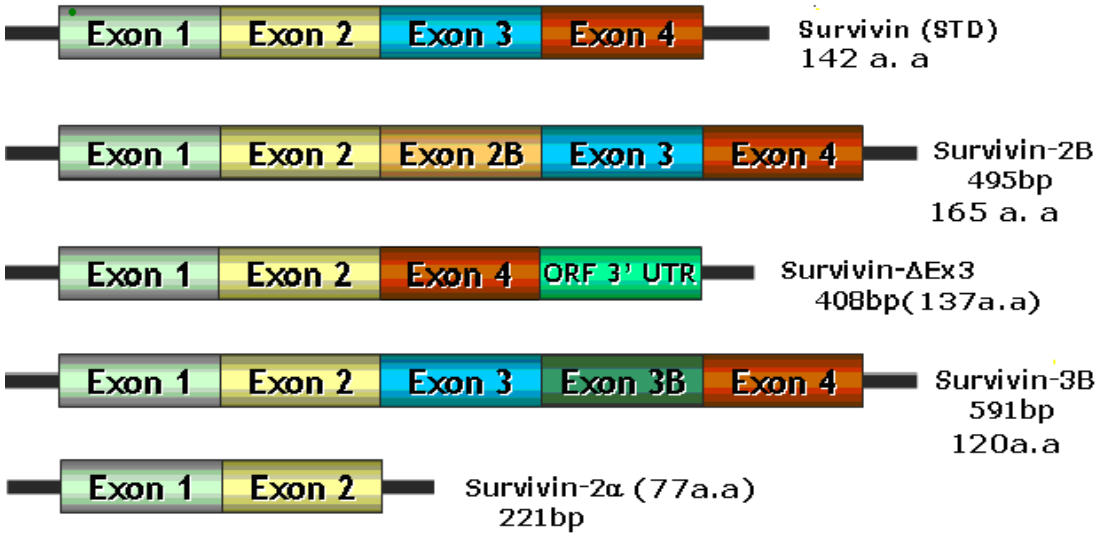

Figure 5: Survivin splice variants: The survivin gene is composed of exons1–4. All identified survivin isoforms contain exons1 and 2. Survivin-2B has an extra exon2B; survivin-deltaEx3 is missing exon 3; survivin-3B contains 5 exons including an extra novel exon3B derived from a 165 bp long portion of intron3; and survivin-2a consists of 2 exons(exons1 and 2) as well as a 30 197 bp region of intron 2.

The search for signaling intermediates that control survivin expression has generated interesting findings. One common denominator of this pathway is the requirement for PI3-kinase/Akt activation. This is a general antiapoptotic signal that has been implicated in up-regulation of survivin induced by granulocyte macrophage-colony stimulating factor (GM-CSF), CSF, Ang-1, and fibronectin-dependent cell adhesion [60, 61 , 62]. In addition, modulation of survivin expression has been observed in endothelial cells stimulated with non-mitogenic concentrations of interleukin (IL)-11 engagement of vascular endothelial (VE)-cadherin expression, and function or angiotensin II stimulation [63, 64]. Another critical transcriptional regulator of survivin expression in pleural effusion lymphoma cell sustained by vascular endothelial growth factor (VEGF), IL-6, or IL-10, was identified as activated signal transducers and activators of transcription (STAT3) [65]. This is potentially important for the known role of

STAT3 in cytoprotection, which may have direct consequences for oncogenic transformation [66, 67]

1.3.2. The role of survivin in cell division

Survivin is essential for proper execution of mitosis and cell division [68]. Its specific expression in G2/M is transcriptionally controlled as being typical of mitotic genes [69]. During mitosis, survivin binds to the micro-tubules of the mitotic spindle through its carboxy terminal alpha helices. Interference of survivin-microtubule interactions, by means of an antisense-mediated reduction in the expression of survivin, results in a failure of the antiapoptotic function of survivin and an increase in caspase-3 activity with subsequent apoptosis [70]. Disruption of survivin function has likewise been linked with cell division defects exemplified by supernumerary centrosomes, formation of multipolar mitotic spindles, and failure of cytokinesis, with cells becoming polyploid and multinucleated. Additional evidence for the crucial role of survivin in mitosis has been demonstrated in knockout mice. Homozygous disruption of the survivin gene resulted in embryonic death at 4-5 days, with the null embryos revealing failed cytokinesis and disrupted microtubule formation [71].

1.3.3. Role of survivin in inhibition of apoptosis

The question as to whether survivin had also a distinct and separable role in apoptosis inhibition similar to other mammalian IAPs or whether its function is limited to cell division, as with IAPs in yeast and C. elegans, has been amply investigated in recent years [47, 72 , 73] . With the expansion of experimental work on survivin, three lines of experimental evidence clearly support the notion that survivin has an independent and separable role in apoptosis inhibition, in vitro and in vivo. First, overexpression of survivin has been associated with inhibition of cell death initiated via the extrinsic or intrinsic apoptotic pathways. Second, transgenic expression of survivin resulted in apoptosis inhibition in vivo and livers isolated from heterozygous survivin +/− animals exhibited exaggerated apoptosis in response to suboptimal ligation of Fas [74 , 75]. Third, molecular antagonists of survivin, including antisense, ribozymes, siRNA

sequences, or dominant-negative mutants resulted in caspase dependent cell death, enhancement of apoptotic stimuli, and anticancer activity, in vivo [76 , 77 , 78 , 79]. However the precise mechanism by which survivin suppresses apoptosis is still not well understood. Several mechanisms are under consideration (Fig. 6). Direct suppression of caspase-3 by survivin has been speculated by some investigators; yet, survivin lacks structural components present in other IAPs that allow their direct binding to caspase-3. There is also speculation that survivin binds to caspase-9 but there are likewise problems with this theory. Phosphorylation of survivin on the threonine at position 34 (Thr34) is critical for a functional survivin molecule. However, phosphorylation of survivin fails to explain why this would promote interaction with caspase-9.

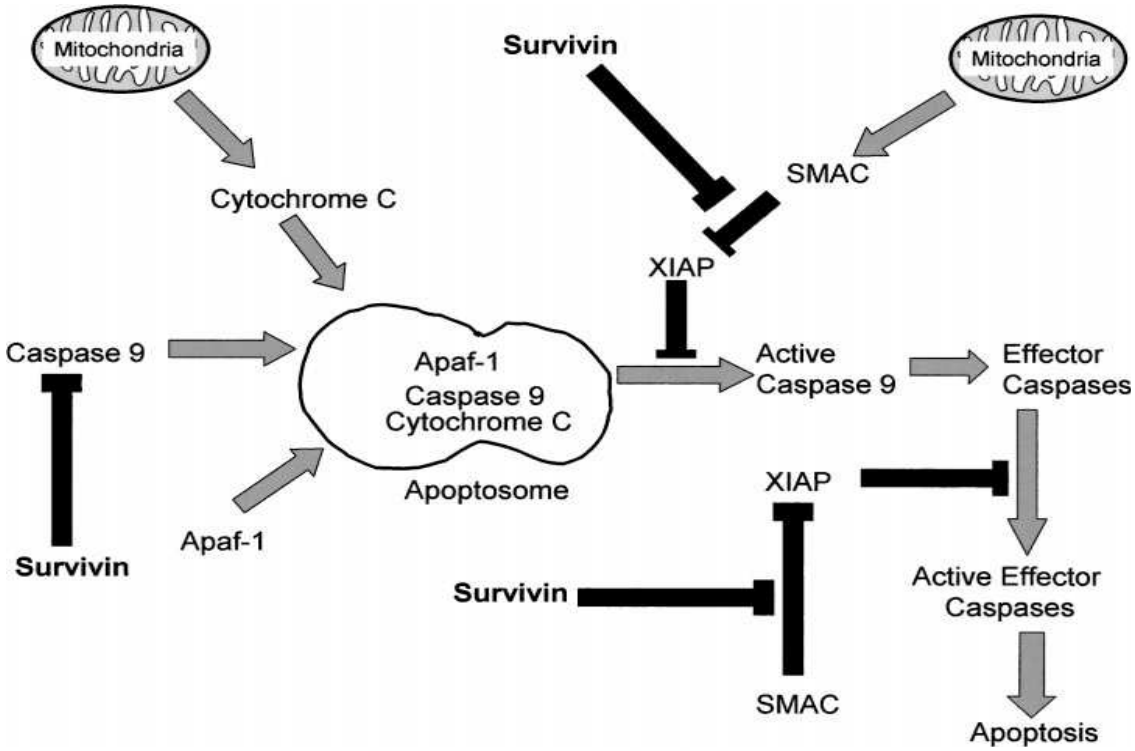

Figure 6: Potential mechanism by which survivin inhibits apoptosis: survivin may inhibit apoptosis by binding caspase-9 or it may block Smac, thus preventing this proapoptotic protein from blocking IAP protein.

1.3.4. Survivin and cancer

Strong survivin expression is observed in the vast majority of cancers [80]. These include esophageal, lung, ovarian, central nervous system, breast, colorectal, bladder, gastric, prostate, pancreatic, laryngeal, uterine, hepatocellular, and renal cancers, as well as melanoma and soft tissue sarcomas. Survivin is also highly expressed in patients with hematologic malignancies including lymphomas, acute leukemias, and myelodysplastic syndromes, which progress to overt leukemia [81]. In cancer cells, elevated survivin is commonly associated with enhanced proliferative index, reduced levels of apoptosis, resistance to chemotherapy , and increased rate of tumor recurrence [82 , 83, 84 ,85]. Retrospective studies evaluated the correlation between survivin, disease variables, and clinical outcomes. Elevated survivin expression is associated with clinicopathologic variables of aggressive disease and shows a strong correlation with shorter disease-free or overall survival in most studies, identifying it as a significant independent prognostic indicator of poor outcome in patients with most tumor types.

1.3.5. Survivin as a target for new anticancer strategies

Overall, the results obtained by different studies aimed at targeting survivin by means of different approaches, demonstrated that inhibition of this cell survival factor: (a) promotes spontaneous apoptosis in tumour cells (although this effect was not observed in all studies) and (b) enhances the efficacy of several types of conventional treatments including chemotherapy, radiotherapy and immunotherapy. Accordingly considerable efforts have been made to validate survivin as a new target in cancer therapy. Phase I/II clinical trials of survivin-directed immunotherapy have been conducted in patients with advanced melanoma, pancreatic, colon, and cervical cancer, and have shown promising results without significant adverse effects [86, 87].

Also several experiments targeting survivin expression are currently under investigation. For example, a replication-deficient adenovirus encoding a survivin Thr34-Ala

(pAd-T34A) mutant to target tumor cells of breast, cervical, prostate, lung, and colorectal cancers causes the tumor cells to undergo apoptosis without affecting proliferation of normal fibroblasts, endothelium, or smooth muscle cells. The combination of pAd-T34A with the chemotherapeutic agent, taxol, results in enhanced tumor cell death. Intratumor administration of pAd-T34A in mice in vivo also inhibits tumor growth and induces apoptosis.

Evidence that dysregulation of the survivin pathway leads to apoptosis in cancer is also seen in melanoma cell lines transfected with T34A, which interferes with phosphorylation of endogenous survivin. This results in spontaneous apoptosis of the melanoma cell line and enhanced in vitro cell death by the chemotherapeutic agent cisplatin [88]. Finally, down regulation of survivin expression using an antisense oligonucleotide within a lung carcinoma cell line induces apoptosis and inhibits growth of the neoplastic cells [89]. Furthermore, a synergistic effect was noted when this cell line was treated with antisense oligonucleotides and the chemotherapeutic agent, etopside. This finding demonstrates that targeting of the survivin pathway in cancer, alone or in conjunction with chemotherapeutic agents, has potential as a novel therapeutic regimen.

Immunotherapy appears to be a plausible approach to treating survivin-positive tumors; autoantibodies against survivin have been detected in humans with lung and colorectal cancer, making this a potential diagnostic tool in certain neoplasms [90]. In some of the lung cancer patients, anti-survivin reactivity was discovered before the clinical findings. Thus, antibodies against survivin may prove to be an early predictive marker of cancer. Spontaneous cytotoxic T lymphocyte responses to survivin, in a major histocompatibility complex class I restricted manner, have been detected in patients with chronic lymphocytic leukemia, melanoma, and breast cancer [91, 92]. In addition, in vitro cytolytic T-cell induction against a survivin epitope results in lysis of a wide variety of human tumors, including renal cell carcinomas, breast cancer, colon cancer, multiple myeloma, and leukemias [93]. Hence, survivin appears to be a uni-

versal tumor antigen and immunotherapy emerges as a conceivable approach to treating survivin positive tumors.

1.3.6. Survivin as a therapeutic target for radiation sensitization

Since survivin is preferentially expressed in malignant cells and is prognostically important, it acts as an attractive therapeutic target. Efforts are under way to develop survivin inhibitors for clinical use with the dual aim, to inhibit tumor growth through an increase in spontaneous apoptosis and to enhance tumor cell response to apoptosis-inducing agents [93].

Different kinds of survivin molecular antagonists, including antisense oligonucleotides, ribozymes, small interfering RNAs (siRNAs), as well as cancer vaccines, have been used. The results obtained in a variety of studies indicate survivin to be an important factor in determining the radiation response of human tumor cells. Due to its differential expression in cancerous tissues and its potential requirement for maintaining cancer-cell viability survivin displays a suitable molecular target for radiosensitization [94]. (Fig. 7).

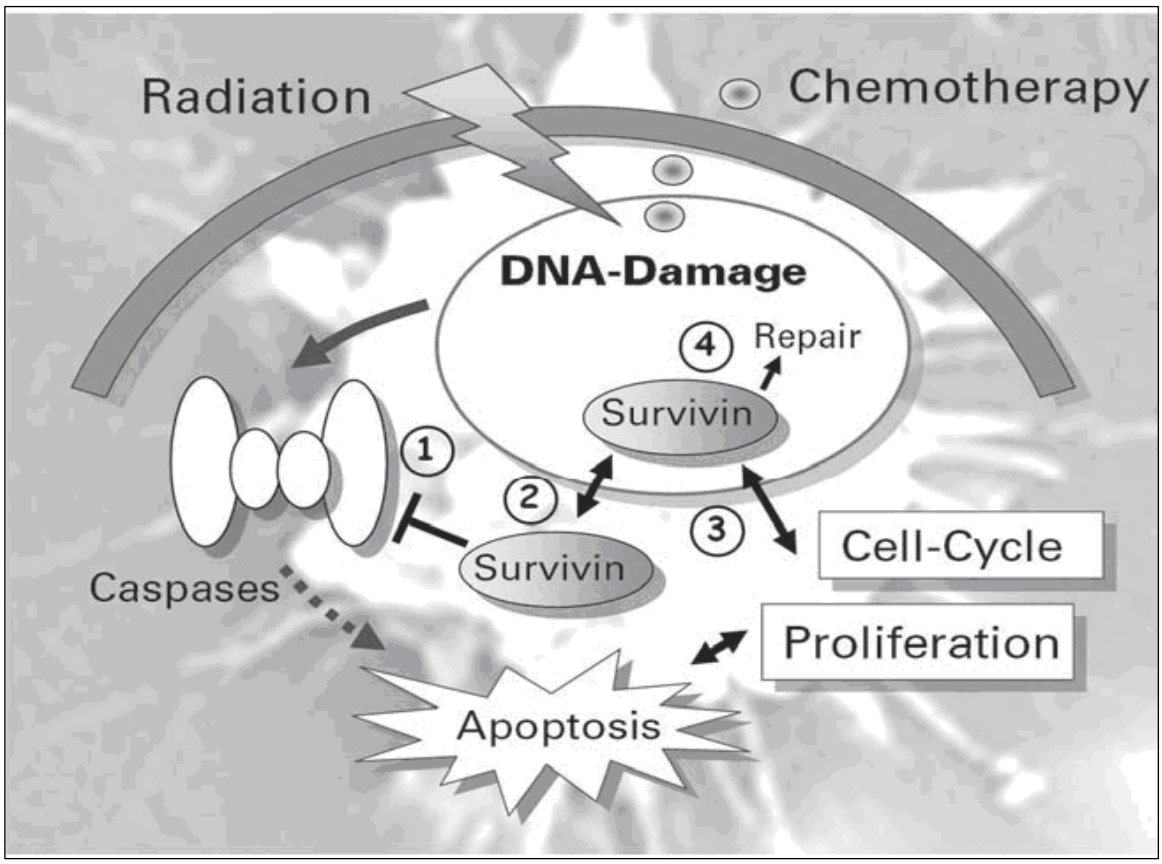

Figure 7: Schematic presentation of the role of survivin as a radioresistance factor and molecular target for radiosensitization of tumor cells. (1) Enhanced expression of survivin mediates radiation resistance of tumor cells through suppression of apoptosis by interfering with caspase activity(2) The antiapoptotic activity of survivin is dependent on a CRM1-mediated pathway of nuclear export, as export-deficient survivin mutants failed to protect tumor cells against radiation-induced apoptosis . (3) Besides its role as an inhibitor of apoptosis, survivin also acts as a cell cycle regulatory protein, enabling coordinated cellular division. Accordingly, depletion of survivin alters cell cycle distribution, resulting in a G2 and mitotic arrest. (4) In addition, survivin appears to be involved in the regulation of DNA repair, thereby enhancing tumor cell survival upon radiation exposure.

Different strategies to counteract survivin's expression or activation in tumor cells have demonstrated that its inhibition results in an increase in spontaneous and radiation-induced apoptosis and thus enhances the response towards cancer treatments including chemotherapy and radiotherapy [95 , 96]. These strategies comprise antisense oligonucleotides, ribozymes, siRNA and dominant-negative mutation [97 , 98 , 99]. Due to the fact that CDC2 phosphorylation of survivin is required for cancer- cell viability, the use of kinase inhibitors, including CDC2 antagonists, is another promising

approach to enhance the response towards cancer treatments. The radiosensitizing activity of survivin inhibition seems to be multifaceted and involves caspase-dependent and caspase-independent mechanisms like impaired DNA repair as well as an altered cell-cycle distribution, formation of multinucleated cells and mitotic arrest and subsequent cell death [100].

Overall, the results obtained in the different studies indicate survivin to be a cellular factor potentially involved in the chemo-resistant and radio-resistant phenotypes of human tumours cells and suggest that approaches designed to inhibit survivin expression may lead to human tumour sensitization to chemical and physical agents.

1.4. Measurement of antibodies against tumour proteins

It is well demonstrated that cancer is immunogenic, and multiple tumour antigens have been identified in cancer patients. It is now possible to potentially harness the immune response elicited by cancer growth as a potential diagnostic tool. Humoral immunity, or the development of autoantibodies against tumor-associated proteins, may be used as a marker for cancer exposure. Unlike circulating proteins that are shed by bulky tumors, serum autoantibodies are detectable even when antigen expression is minimal.

1.4.1. Methods

An optimal method for detecting cancer-specific antibodies must be defined. The two major methods for detection of tumor-specific antibodies are immunoblotting (Western blot) and enzyme linked immunosorbent assay (ELISA) systems. Western blot analysis involves separating tumor proteins by gel electrophoresis, transferring to nitrocellulose, and then probing with patient serum samples. While Western blotting is useful for identifying new immuno-genic tumor proteins, it is not a quantitative assay. By contrast, ELISA methodology allows a quantitative evaluation of the antibody response and is capable of allowing the analysis of a great number of patients with little effort. Use of ELISA requires a source of reasonably pure protein for detection of anti-

bodies. The type of ELISA used to detect antibody responses, such as "sandwich" or "indirect" ELISA, may affect the sensitivity of the test. A "sandwich" ELISA allows an impure source of protein to be used. Briefly, the method entails the binding of a protein-specific monoclonal or polyclonal antibody to the surface of a plate followed by incubation with the protein of interest in solution with protein binding to the antibody, impurities are washed away, and the antibody-bound protein can then be probed with patient sera. An "indirect" ELISA requires a purified source of protein, such as recombinant protein or chemically synthesized fragments of protein termed "peptides." The pure antigen can be directly bound to the surface of a plate and probed with patient sera.

Besides these two major methods there are other ones like; Immunofluoresence assay (IFA) in this test serum is incubated with antigen immobilized on a 96-well plate or microscope slide, secondary antibodies labeled with fluorescence are then added. After washing, any bound secondary antibodies can be detected by UV light on the slide. Moreover, a high-throughput approach to autoantibody discovery is protein array as robotic microarray spotters. This allows the grouping of thousands of proteins in replicate onto a single glass slide and makes it possible to evaluate the presence of serum antibody to hundreds of proteins simultaneously.

The sensitivity of each approach may differ depending upon experimental conditions, and the ability to develop a successful diagnostic assay. This includes several factors such as the ability to detect the autoantibody in the premalignant state, the prevalence of the autoantibody in a specific population, or even the specificity of the autoantibody for a specific tissue type. Population-based studies of individual autoantibodies can give some indication of whether the detection of humoral immunity may aid in discriminating cancer patients from non cancer bearing individuals. It is important to explore several strategies with a series of candidate antigens to determine the most optimal method to be further developed for use in screening for the presence of cancer in a high-risk or asymptomatic population.

1.4.2. Antibody response to oncogenic proteins

The humoral immune (HI) response is mediated by B lymphocytes through the production of antibodies. Antibodies can kill tumor cells by different mechanisms:

- IgG or IgM antibodies fix complement and can destroy soft tumors.
- Antibodies directed against antigens expressed on tumor cell surfaces may interfere with adhesion molecules that some tumor cells need to survive.
- IgG antibodies, can mediate tumor cell lysis through antibody dependent cell–mediated cytotoxicity (ADCC), involving effector cells such as macrophages, natural killer (NK) cells, $CD8^+$ T cells and perhaps blood neutrophils.

Oncogenic proteins are encoded by oncogenes or by tumour suppressor genes, or both, and play a part in the control of cell growth and differentiation. Antibody immunity to a number of oncogenic proteins has been identified over the last several years. However, production of these antibodies does not confer protection, but, paradoxically, correlates with poor prognosis and decreased survival for several human cancer types [101]. Increased levels of immunoglobulins (Ig) in neoplastic microenvironments also result in accumulation of immune complexes (ICs) that engender tumor-promoting inflammatory responses [102]. Ig-IC formation is a significant feature of cancer development. High circulating levels of ICs are associated with increased tumor burden and poor prognosis in patients with breast, genitourinary, and head and neck malignancies, Ig deposition in neoplastic stroma has been reported in pre-malignant and malignant human breast and prostate tissues [103, 104, 105]. While investigators are using the presence of antibody immunity as an indicator for a potential T-cell response, and, thus, identification of T-cell antigens that may be exploited for therapy, antibody immunity on itself may have great utility in the diagnosis and management of human malignancy.

Antibody immunity to a number of oncogenic proteins has been identified over the last several years. The representative ones are those against P53, HER-2/neu, ras, c-myc, bcr-abl and recently, Survivin.

Antibody response to P53

The antibody immune response to p53 has received the most attention. This is basically because mutations in the p53 gene are common, and occurring in approximately 50% of all human cancers. P53 is a tumor suppressor gene, and mutations that occur in the gene cause inactivation of encoded p53 protein. Antibody immunity to the p53 protein has been reported for nearly every human tumor with which p53 mutations are associated: colon, breast, ovarian, lung and gastric cancer to name a few [106]. The incidence of these antibodies in the serum of patients with cancer ranges from 10% to 50% of patients evaluated in an each individual study. A p53 specific antibody response may eventually be developed as a diagnostic or prognostic tool in monitoring the therapy of many types of cancers.

It has been shown that patients with various types of neoplasias have p53 antibodies in their sera. ELISA was used to detect anti-p53 antibodies in the sera of 167 patients with lung cancer. Among these, 32 individuals (16 positive for p53 antibodies and 16 negative) were monitored over a period of 30 months for p53 antibodies. Twelve of 16 antibody positive patients had reduced titers during chemotherapy more than 50% compared to the initial titer. Among these patients, eight underwent a complete response to therapy, whereas four obtained only a partial response, that led to partial or complete remissions of disease and 4 had no change in the level of p53-Ab, or else had a decrease of less than 50%. The specificity of these antibodies was confirmed by two different ELISA procedures and by immunoprecipitation. The very rapid, specific decrease in these antibodies during therapy suggests that a constant level of tumoral cells with nuclear accumulating p53 protein is necessary for a detectable humoral anti-p53 response. The good correlation found between the specific evolution of the p53 antibody titer and the response to therapy suggests that p53 antibodies could represent a useful tool for checking the response to therapy and for monitoring some relapses before they are clinically detectable [107].

Antibody response to HER-2/neu

The HER-2/neu protein is a member of the epidermal growth factor receptor family and it's a self-protein that is expressed by some normal tissues but is often overexpressed by a variety of cancer cells. The presence of endogenous HER-2/neu-specific antibodies has been identified in breast (11%), prostate (15.5%) and colorectal cancer patients [108 , 109].

Antibody response to ras protein

Three ras genes, H-ras, K-ras and N-ras, have been defined, and these genes encode a 21 kDa protein designated as p21 ras. Antibodies to p21 ras proteins have been detected in patients with colon cancer, by using ELISA and purified ras proteins. Sera were examined from 160 colon cancer patients and 60 normal controls to determine whether antibodies to mutated p21 ras protein were present. Antibodies of IgA subtype against mutated p21 ras protein were detected in 51 of 160 (32%) colon cancer [110].

Antibody response to c-myc protein

The c-myc protein belongs to the myc oncoprotein family which also includes N-myc and L-myc. Circulating antibodies to the myc protein have been detected in cancer patients. Serum antibodies to myc were first described in 4 of 6 (67%) of patients with colon cancer, 12 of 125 (10%) of breast cancer patients, 1 of 2 (50%) osteosarcoma patients, 1 of 9 (11%) ovarian cancer patients, and 3 of 3 (100%) of patients with cancer of unknown origin [111]. In a study the serum c-myc antigens and antibodies against c-myc were examined in 68 lung cancer patients and 30 healthy volunteers. Anti-c-myc antibodies were detected in 9 of 68 (13.2%) patients with lung cancer and 1 of 30 (3.3%) of normal controls [112].

Antibody response to bcr-abl

The molecular hallmark of chronic myelogenous leukemia (CML) is the Philadelphia (Ph) chromosome, which results from a reciprocal translocation of human c-abl proto-oncogene from chromosome 9 to the bcr region on chromosome 22. A study was performed to demonstrate the presence of antibodies against p210 Bcr-Ab in both, Ph-

positive and Ph-negative leukemia patients, and in healthy volunteers. Plasma from 18 of 31 (58%) individuals was able to immunoprecipitate p210 Bcr-Abl including 14 of 20 patients (70%) with Ph-positive CML. Plasma of 2 of 7 (29%) normal subjects also contained p210 specific antibodies [113].

1.4.3. Antibody response to survivin

Previous reports show that survivin is expressed at high levels in a number of human tumours [80]. Concurrently, it has been demonstrated that survivin epitopes are present on a broad variety of malignancies prompting the development of both, cellular and humoral immune responses [93]. It has been suggested that survivin is associated with tumor progression and poor prognosis of patients with brain tumors. Survivin-specific antibodies were detected by using ELISA in patients with brain tumours , and it were detected in 5 of 42 (11.9%) patients with meningiomas and 3 of 35 (8.6%) patients with malignant gliomas, but not in healthy controls. Tumors of patients with detectable anti-survivin antibodies demonstrated survivin expression in at least 20% of the tumor cells as assessed by immunohistochemistry. This study concludes that patients with meningiomas and malignant gliomas can mount a high titer IgG immune response against the 'universal' tumor associated antigen survivin [114].

Anti-survivin antibody responses in patients with head and neck cancer were investigated by ELISA. High levels of anti-survivin were detected in patients with advanced stage. Interestingly, levels of anti-survivin fell after curative treatment. Moreover, 92.3% tested patients were positive for at least 1 of 3 markers. These findings demonstrated that anti-survivin antibody is present in the sera of a majority of patients with head and neck cancer [115].

Detection of autoantibodies to survivin in sera from patients with chronic hepatitis (CH) (57 patients) and hepatocellular carcinoma (HCC) (29 patients) by using ELISA, showed anti-survivin antibodies in 10 of 57 sera (17.5%) of (CH); and in 7 of 29 sera (24.1%) in HCC. These prevalence rates were consistent with results of previous studies [116, 117]. In addition, the levels of anti-survivin antibodies in HCC pa-

tients with hepatitis C virus (HCV) infection were significantly higher than those in the healthy controls and HCC patients with hepatitis B virus (HBV) infection. However, there were no significant differences in the levels of anti-survivin antibodies between HCV patients and HCC patients with HCV infection. Also, there were no significant differences in the levels of anti-survivin antibodies among the healthy controls, HBV patients and HCC patients with HBV infection. These results suggest that the levels of anti-survivin antibodies have no association with the progression of HCV or HBV to HCC [118].

The prevalence of anti-survivin and livin antibodies was examined in breast cancer patients with a specific enzyme linked immunosorbent assay (ELISA) using full length recombinant protein. Anti-livin antibodies were detected in excess of cutoff values in 15 of 46 breast cancer patients (32.6%), while anti-survivin were detected in 11 of 47 (23.9%). Sera from 46 breast cancer patients were assessed simultaneously with the anti-survivin and anti-livin ELISAs. Twenty-four sera (52.2%) were positive for 1 or both ELISAs using the respective proteins. These results suggest that combination ELISA for anti-livin antibodies and anti-survivin antibodies may be useful in detection of breast cancers [119].

To investigate the prevalence of mucosal autoantibodies to survivin in patients with human papillomavirus (HPV) associated cervical cancer and precursor lesions, cervical mucus from 117 HPV-associated cervical cancer and 80 normal controls were tested by ELISA using either full length recombinant survivin or survivin-derived peptides. This study reported that an increasing proportion of patients with cervical cancer (28.2%) have mucosal IgA antibodies reactive against recombinant survivin. Additionally, it showed that antibodies to survivin are detected in patients with oncogenic HPV-associated pre-malignant lesions and that the presence of such antibodies is related to the grade of lesion and to the level of expression of survivin in the cervical tissue [120].

To detect anti-survivin antibody in gastrointestinal cancer patients, blood samples were collected from 33 healthy blood donors and 63 gastrointestinal cancer pa-

tients. 25 of 63 gastrointestinal cancer patients (39.7%) were reactive with recombinant survivin protein by ELISA, whereas none of the control sera from healthy donors was reactive. In this study, antibody responses against survivin were not always apparent in all patients whose cancers expressed survivin; these results suggest that the site of tumor origin influences anti-survivin reactivity [116].

Another approach was performed to determine whether a mini-array of multiple TAAs would enhance antibody detection and be a useful approach to cancer detection and diagnosis. The mini-array of TAAs comprised full-length recombinant proteins expressed from cDNAs encoding c-myc, p53, cyclin B1, p62, Koc, IMP1, and survivin. Enzyme immunoassay was used to detect antibodies in 527 sera from six different types of cancer. Antibody frequency to any individual TAA was variable but rarely exceeded 15–20%. With the successive addition of TAAs to a final total of seven antigens, there was a stepwise increase of positive antibody reactions up to a range of 44–68%. Breast, lung, and prostate cancer patients showed separate and distinct profiles of reactivity, suggesting that uniquely constituted antigen mini-arrays might be developed to distinguish between some types of cancer. This study concluded that the detection of autoantibodies in cancer can be enhanced by using a mini-array of several TAAs as target antigens [117].

Detection of anti-survivin antibodies in the sera of lung (n=51) and colorectal (n=49) cancer patients was performed using ELISA. Eleven sera from lung cancer patients (21.6%) and four sera from colorectal cancer patients (8.2%) reacted with purified recombinant survivin. This is in agreement with [121], where a higher prevalence was seen for anti-survivin antibodies in patients with lung cancer.

Another study was conducted to detect autoantibodies to livin and survivin in sera from lung cancer patients. A cutoff value for positivity was determined as the mean absorbance +2SD for healthy control samples. As a result, 19 of 37 lung cancer patients (51.3%) were positive for anti-livin antibodies and 18 of 31 (58.1%) were positive for anti-survivin antibodies. Sera from 31 lung cancer patients were further as-

sessed simultaneously using anti-survivin and anti-livin ELISAs and 21 of them (71%) were positive for survivin, livin, or both. This suggests that antibodies against both survivin and livin were detected in lung cancer patients, which implies that sensitive assay for these antibodies can be used in combination for the detection of lung cancer. Nevertheless, further studies are necessary for the confirmation of this suggestion [122].

1.4.4. Antibody response affected by chemotherapy

Over the past decade, a number of new agents have become available for the treatment of metastatic non–small-cell lung cancer, including the taxanes, gemcitabine, and Vinorelbine. Most cytotoxic drugs have gross effects on the immune system, such as neutropenia and lymphopenia. However, their effects on tumor-specific immune responses are unknown.

Gemcitabine is a nucleoside analogue that is frequently used to treat non-small cell lung cancer. In a study conducted to investigate the effects of gemcitabine on antigen-specific antitumor immunity using a murine tumor cell line transfected to express influenza virus hemagglutinin (HA) , it was found that gemcitabine massively depletes lymphocyte numbers. Nevertheless it has a selective detrimental effect on the B-lymphocyte subset associated with a complete ablation of the antitumor antibody response [123].

Previous studies regarding chemotherapeutic agent immunotoxicity have not reported any selective B-lymphocyte toxicity. Most studies on patient populations have been performed in groups receiving combination chemotherapy, and thus it is difficult to separate the effects of any one agent. Children and young adults undergoing intensive chemotherapy for solid tumors and lymphomas have been studied for changes in peripheral blood lymphocyte populations following maximal hematological recovery after several chemotherapy cycles. It was reported that lymphocyte recovery did not occur between cycles in these patients, leading to severe B- and T-cell depletion [124]. B cells were nearly undetectable, and IgM was absent in 50% of the patients studied, whilst IgG levels were normal.

1.5. Aim of the project

As a consequence to the selective expression of survivin by malignant tissue, and the fact that it is ubiquitously expressed and required for maintenance of cancer cell growth, survivin has been considered as an attractive target in cancer immunotherapy protocols [125 , 126]. To this end, elegantly performed studies have identified spontaneous immune responses elicited by peptides of survivin [90, 92, 93, 127]. Humoral anti-survivin responses, on the other hand, mainly investigated in the context of cancer diagnosis and/or follow up, have been detected in patients with various forms of cancer, with a prevalence of antibody varying between 10% and 60% depending on the method utilised, the source of antigen and the type of cancer [116, 119, 121, 128]. In the same context, anti-survivin antibodies have been examined in patients with lung cancer. To the best of our knowledge, there are only 3 studies in small cohorts of patients, uncovering frequencies of anti-survivin antibodies in lung cancer varying between 10.7% and 58%, and also presenting inconsistent results regarding the levels of antibodies and their correlations with the type of cancer or the clinical parameters of the disease [117, 121, 122].

Our recent findings demonstrating a very low immunogenicity of survivin regarding the induction of CTL responses [129], coupled by the inconclusive results concerning the humoral anti-survivin response as described above, impose the need for a comprehensive and thorough investigation of anti-survivin antibodies in lung cancer. This study was scheduled in order to investigate in a large sample of patients with lung cancer and normal controls, the frequency and the levels of circulating anti-survivin antibodies. Their correlations with the parameters of the tumor and the disease were also explored. Finally, under the light of the reported immunomodulatory effects of radiotherapy and various chemotherapeutic agents used in lung cancer, like platinum, taxanes, gemcitabine, etc, their possible effect on the levels of anti-survivin antibodies was examined [130, 131].

2. MATERIALS AND METHODS

2.1. Serum samples

One hundred and fifty patients with lung cancer were examined. Thirty three of these were diagnosed with small cell lung cancer (SCLC) (31 males and 2 females, mean age 62.1±9.9years) and 117 with non-small cell lung cancer (NSCLC) (108 males and 9 females, mean age 64.2±9.3 years). In 22 patients with SCLC and 76 with NSCLC serial samples were collected post therapies (various combinations of surgery and chemotherapy including adjuvant chemotherapy) and during follow-up (those of interest are presented in results). The characteristics of the patients and their tumors are shown in (Table 2). Serum samples from 100 apparently healthy individuals served as controls (99 males and one female, mean age 68.2±5.8 years). The study conforms to the provisions of the Declaration of Helsinki, it was reviewed and approved by the University of Thessaly Ethics Committee, and all participants provided informed consent. Serum samples from patients and normal subjects were collected, aliquoted and stored at -80°C until the moment of testing.

2.2. Antigen

Survivin was purchased as full length recombinant protein with GST tag, (BIRC5) (1aa-142aa) from Abnova, USA (Cat. No H00000332-P01,) and was kept at -80^{0}C.

2.3. Buffers

Plates were coated with Carbonate/Bicarbonate buffer (pH 9.6) (Cat. No C3041, Sigma, USA). For each experiment, one capsule of 0.05M carbonate/bicarbonate buffer was dissolved in 100mL distilled water and stirred until completely dissolved. The pH was adjusted to 9.6 using 5M HCL and stored in a sterile container.

To wash the plates two buffers were used: a). Phosphate Buffered Saline pH 7.4 (PBS) (Cat. No P5368, Sigma) and, b). PBS with 0.01% Tween 20 (Cat. No P9416, Sigma).

To block the non-specific binding sites on the plate, 5% Bovine Serum albumin BSA/ PBS (Cat. No A7888, Sigma) was freshly prepared for each experiment.

Table 2: Characteristics of patients and normal controls included in the study.

	NSCLC (*N*=117)	SCLC (*N*=33)	Controls (*N*=100)
Age			
range	40-80	42-81	53-72
mean±1SD	64.2±9.3	62.1±9.9	68.2±5.8
Sex (male/female)	108/9	31/2	99/1
Stage			
I	15	1	
II	11	3	
III	44	12	
IV	47	17	
Performance[&]			
0	71	20	
1	34	9	
2	7	3	
3	5	1	
Loss of weight[#]			
0	86	19	
1	31	14	
Death[**]			
0	35	8	
1	78	25	
Overall Survival (days)			
range	46-1165	66-916	
mean±1SD	495±265	360±194	
Surgery	31/117	0/33	
Chemotherapy	70/117	28/33	
Radiotherapy	21/117	7/33	

[&] ECOG Performance status; # 0= Less than 5% weight loss; 1 = Loss of weight >5%; **Alive = 0, Dead = 1

2.4. Reagents

Several reagents were used in this study as explained below:

Goat polyclonal to survivin with a concentration 0.50mg/mL was used for ELISA optimization (Cat. No 27468 Abcam, USA).

Rabbit polyclonal to goat IgG H&L (HRP) (Cat. No 6741, Abcam,) with a concentration 2mg/mL, was used as secondary antibody.

Anti-human HRP-conjugated secondary antibody, TMB chromogen developing agent, in addition to HRP stop solution were purchased from (Inova-San Diego, USA) and were used as recommended by the company.

2.5. Equipment

Plate Reader which supplied by (DAS Company, Italy) is an eight channel photometer for the sequential and simultaneous reading of micro-plate strips with 96 wells, with two optical filters (450 & 630 nm) and shaking capability.

Flat bottom 96 well cell cultures with lid, nonpyrogenic, polystyrene and sterile plates, supplied by (Costa USA) were used.

2.6. ELISA (Enzyme-Linked ImmunoSorbant Assay)

An ELISA was used to detect anti-survivin antibody in the blood of patients with lung cancer. ELISAs are performed in 96- well plates which permitting high throughput results. The bottom of each well is coated with a protein to which the antibody we want to be measured will bind.

2.6.1 Overview of methodology

The (ELISA) is the most commonly used antibody or antigen detection method. Preparation of ELISA microtiter plates involves three major steps: 1) binding antigen or antibody to the plate; 2) blocking non-specific binding sites on the plate; and 3) developing the plate to read each sample’s OD.

The first step in making a reliable ELISA is proper coating of the antigen onto the plate, also coating buffers should stabilize coated proteins, and this allow greater binding reactivity with the detection molecule, thereby enhancing the specific signal. Determination of optimal antigen concentration (or dilution) is one of the mandatory steps in developing an ELISA.

Plates were coated with survivin recombinant protein (BIRC5) in 0.05 M carbonate buffer, pH 9.6 overnight at 4^0C, and then plates were washed 4 times with 0.1% T20/PBS and 4 times with PBS, non-specific binding was prevented by adding blocking buffer and incubation overnight at 4°C. Primary antibody was added in duplicate in the microtiter plate wells, a positive control and a negative control are also included. Sera from different patients can be used concurrently (Fig. 8).

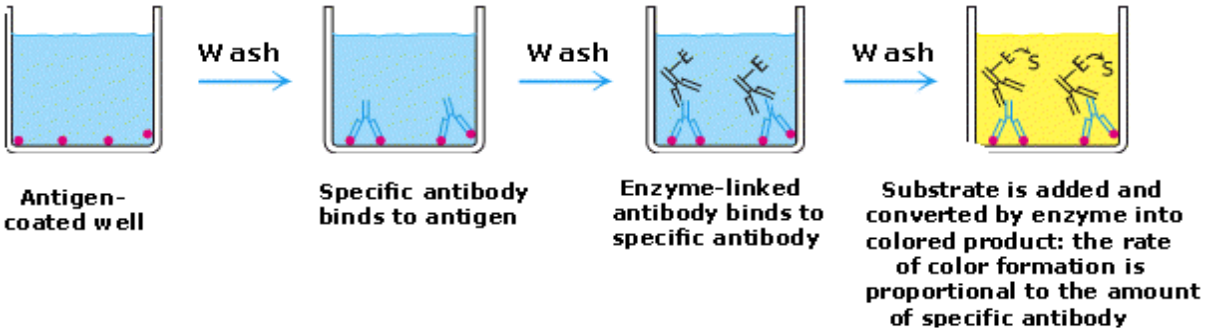

Figure 8: In indirect ELISA, the production of color indicates the amount of an antibody to a specific antigen.

After sufficient incubation at room temperature, the primary antibody is removed and weakly adherent antibodies are washed off as mentioned before. To detect the bound antibodies, a secondary antibody is added to each well and incubated for I hour at room temperature. The secondary antibody would bind to all human or goat antibodies. Attached to the secondary antibody is an enzyme. The enzyme can metabolize colorless substrates into colored products. After an incubation period the secondary antibody solution is removed and washed as before. Chromogen reagent was added for 1 hour at room temperature and plates were washed as before. When the enzyme reaction is complete, the entire plate is placed into a plate reader and the optical den-

sity (i.e. the amount of colored product) is determined for each well. The amount of color produced is proportional to the amount of primary antibody bound to the proteins on the bottom of the wells.

2.6.2. Optimization of blocking buffers

It is important to block the unoccupied sites on the surface of the well to reduce the amount of nonspecific binding of proteins during subsequent steps in the ELISA assay. The blocking buffer improves the sensitivity of the assay by reducing the background interference. A variety of blocking buffers in PBS, like skimmed milk (SM), human serum albumin (HAS) and (BSA) were tested to block un-reacted sites. To optimize blocking temperatures microtiter plates wells were blocked and incubated overnight at 4°C and 37°C with 5%: BSA/PBS, HAS/PBS, SM/ PBS. Blocking overnight with 5% BSA/PBS at 4°C was found to diminish significantly non-specific binding (Fig.9A and 9B).

2.6.3. Optimization of antigen concentration

The optimal concentration of survivin recombinant protein was determined as a coating antigen. 96-well microtiter plates were coated with 100 μL survivin full length recombinant protein concentrations between 10μg/mL and 0.3125 μg/mL in 0.05M carbonate buffer, pH 9.6, and plates were incubated overnight at 4°C. After removing the antigen solution, plates were washed. Non-specific binding was prevented by blocking overnight at 4°C with 5% BSA/PBS. After emptying the wells, plates were washed and, goat polyclonal anti-survivin antibody added at concentration 1 μg/mL as recommended by the company and the plates were incubated one hour at room temperature. Plates were washed again and rabbit polyclonal to goat IgG at dilution 1:16000 was added for 1 hour at room temperature and anti-survivin antibodies detected using chromogen reagent. 50% absorbance was calculated to be at 2 μg/mL survivin. (Fig.10). Similar titrated analyses were then performed for patients' sera at dilution 1:40 (Fig. 11).

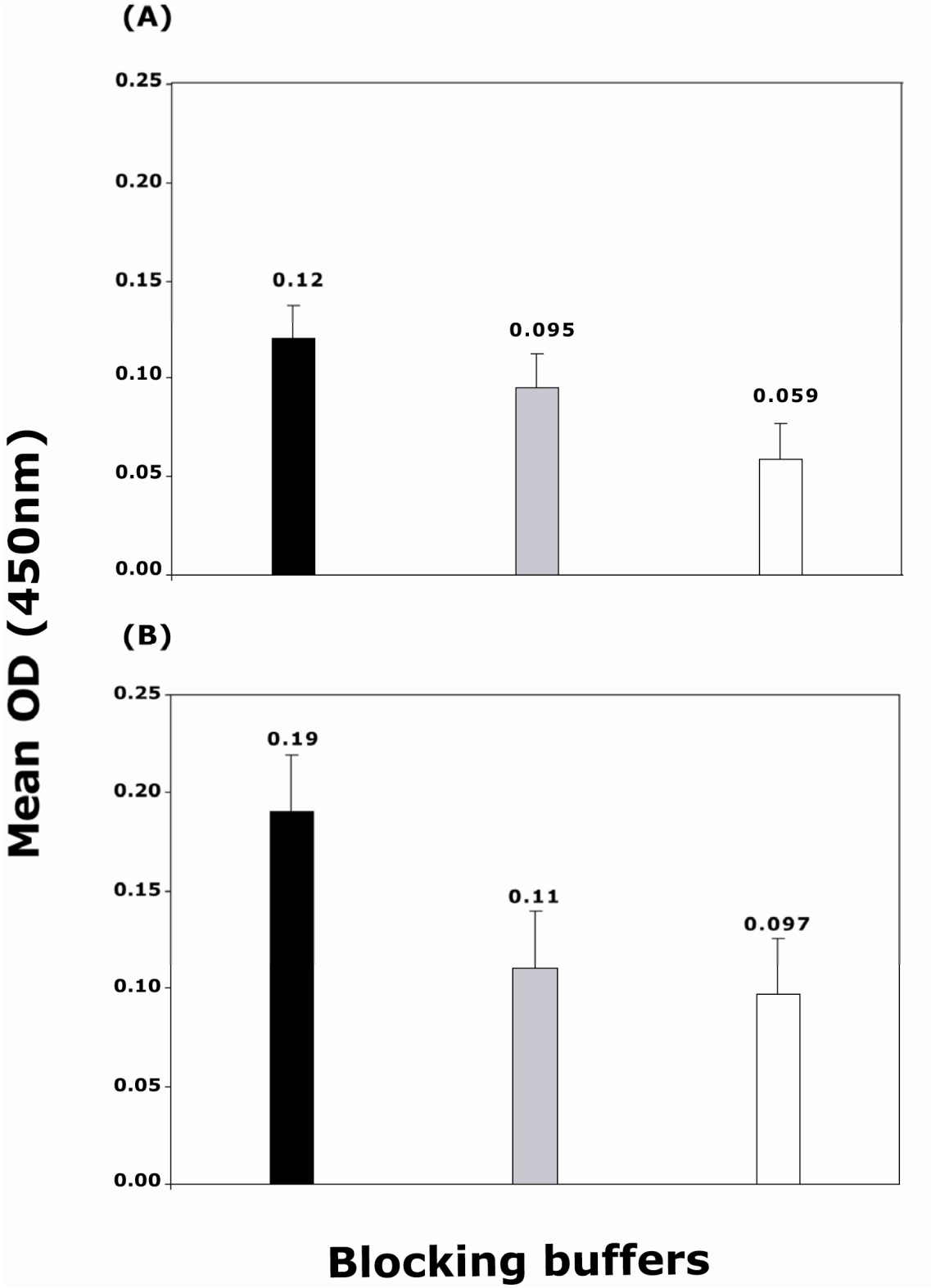

Figure 9: (A) Incubation overnight at 4°C or **(B)** Incubation overnight at 37°C. Blocking with 5% SM/PBS (black), with 5%HAS/ PBS (grey) and 5%BSA/PBS (white).

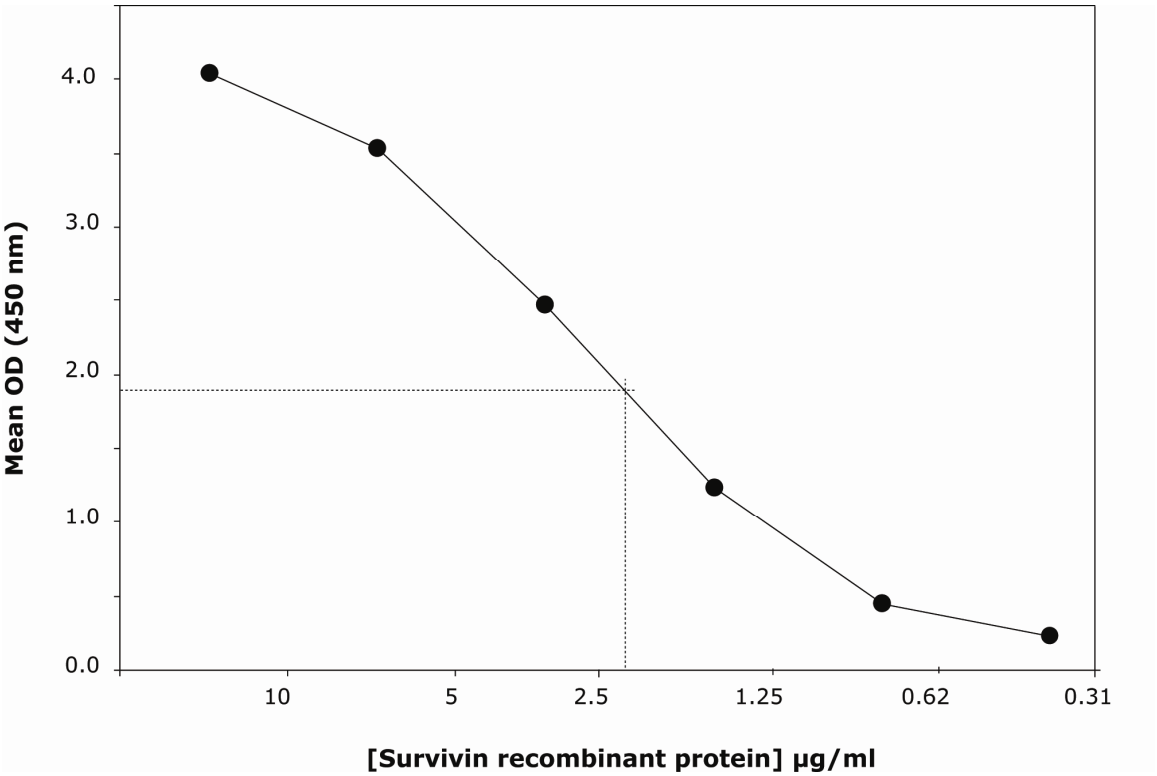

Figure 10: Antigen concentration optimization. Microtiter plates were coated and incubated with goat polyclonal Anti-survivin antibody. EC 50= 2 µg/ mL

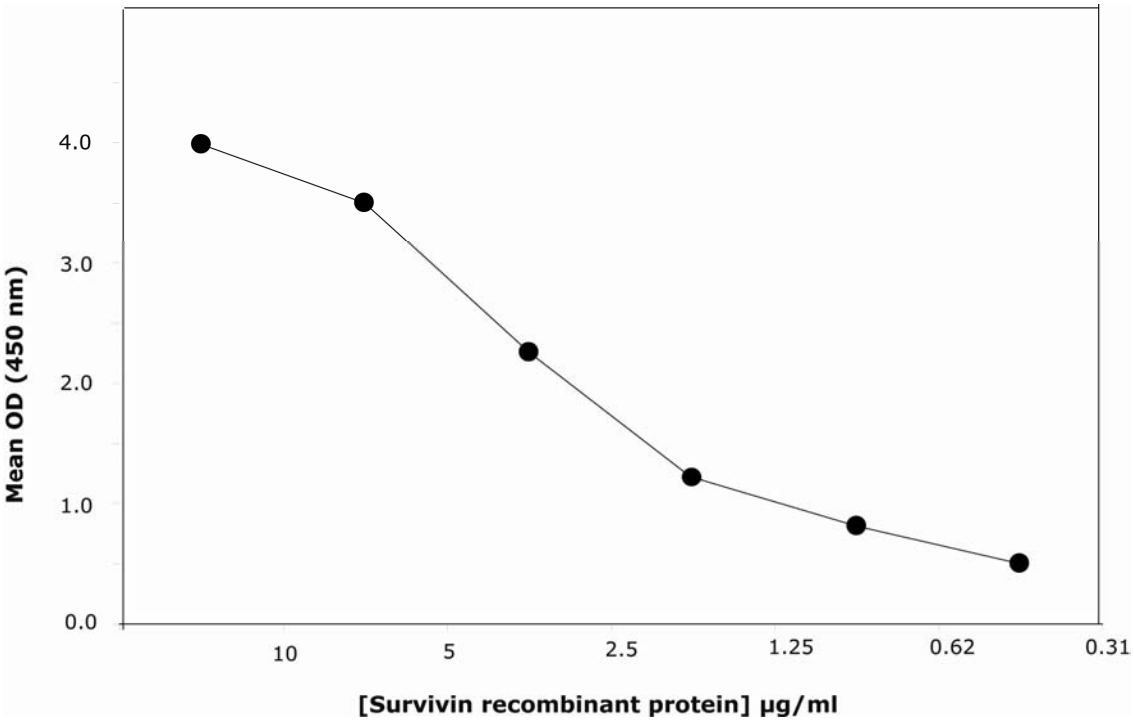

Figure 11: Antigen concentration optimization. Microtiter plates were coated and incubated with patient serum at dilution 1: 40. Absorbance was measured at 450 nm using an ELISA plate reader.

2.6.4. ELISA to measure anti-Survivin antibodies

Having setup the conditions of the ELISA, anti-survivin antibodies were measured. Plates were coated with 100 μL of a 2 μg/mL recombinant survivin protein (BIRC5) in 0.05 M carbonate buffer, pH 9.6 for 20 hours at 4°C. After removing the antigen solution, plates were washed 4 times with 0.1%T20/PBS and 4 times with PBS; non-specific binding was prevented by blocking for 20 hours at 4°C with 5% BSA/PBS. After emptying the wells, plates were washed as before and serum was added as duplicates for 1 hour at ambient temperature. A dilution of 1:40 in 1% BSA/PBS was used as it was proved appropriate for the discrimination between background noise and positivity [132]. Plates were washed as before and sheep anti-human IgG labeled with horse radish peroxidase (HRP) was added for 1 hour at ambient temperature and anti-survivin antibodies detected using chromogen reagent. Absorbance was measured at 450 nm using an ELISA plate reader.

Since sera could not all be tested at the same time and to eliminate inter-assay variability, each run of experiments included 1 positive serum sample (PS). All test sera were normalised against the ODs obtained each time against this serum and relative absorbance units were calculated as follows: Relative Absorbance Units (RAU) = $(OD_{\text{test sera}} - \text{blank})/(OD_{PS} - \text{blank}) \times 100$. Sera with an RAU>mean+2SD of the 100 normal sera were considered positive only after they were re-tested at serial dilutions.

To examine the performance of the ELISA, 3 different sera (1 with high, 1 with moderate and 1 with low anti-survivin antibody levels) were used. The inter-assay variability never exceeded 10% whereas intra-assay variability consistently ranged between 0% and 15%

2.6.5. Sera titration experiments

All positive sera from lung cancer patients were further diluted: 1/13, 1/40, 1/120/, 1/360, 1/1080 and 1/3241 in 1% BSA/PBS, and then subjected to ELISA as described before.

2.6.6. Preabsorption of sera with survivin.

To determine the specificity of the ELISA, all positive sera were pre-absorbed with the recombinant survivin. Serum samples (100 μL of a 1:100 dilution) were incubated with 30 μg/mL of recombinant survivin for 6 hours at 37 °C and then subjected to the anti-survivin ELISA as described above. After absorption, reactivity decreased significantly returning to values similar with those obtained by normal sera (Fig.13) ($p<0.001$), validating this way all readings obtained by our ELISA.

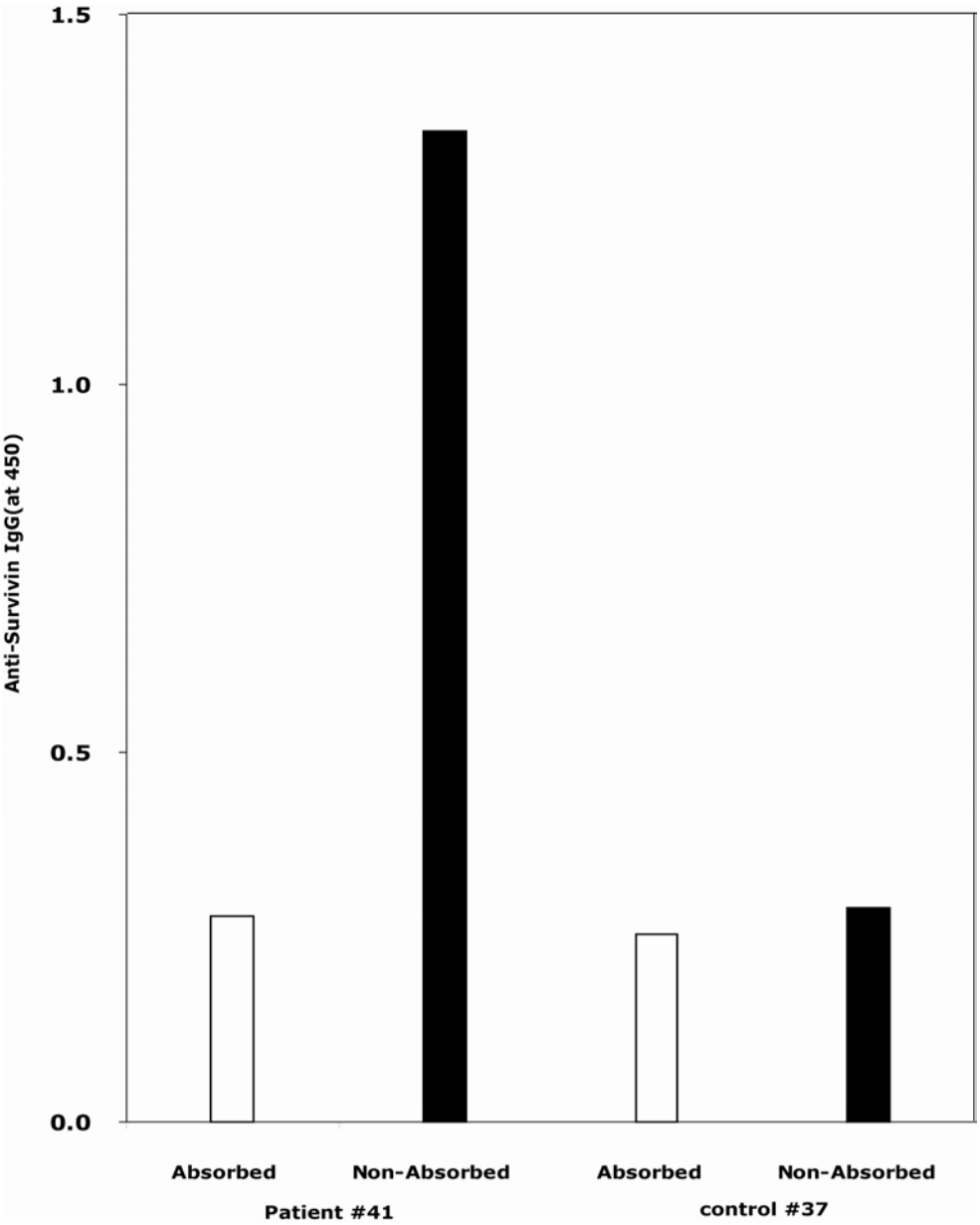

Figure 13: Specificity of survivin recognition was confirmed by lack of reactivity after Pre-absorption of sera with survivin protein.

2.7. Statistical analysis

Expression values are presented as mean±SD. Students' t-test, ANOVA, Fisher's exact test spearman's bivariate correlation and linear regression analysis were appropriately used. All analysis were performed with statistical software SPSS for Windows (version 11.5).

3. RESULTS

Upon diagnosis, patients with NSCLC had significantly higher levels of anti-survivin antibodies (11.5±15.2 RAU) both against patients with SCLC (3.1±2.1 RAU) and normal individuals (7.7±7.3 RAU) (Mann-Whitney U test, $p<0.001$, $p=0.009$, respectively) (Fig. 14). When a cutoff value for positivity was set to 20.9 RAU (mean+2SD of the normals), 9/117 (7.7%) patients with NSCLC appeared with an increased anti-survivin antibody level prior to therapy (46.3±33.5 RAU) versus none of the SCLC and 2% of the normal individuals. Differences in positivity observed amongst patients, did not differ significantly from that of controls neither in the case of NSCLC (odds ratio 3.6, 95% confidence interval 0.7-17.3) nor in that of SCLC patients (odds ratio 0.6, 95% confidence interval 0.03-12.6).

We next examined whether the levels of anti-survivin antibodies upon diagnosis correlated with any clinicopathological parameter (age, sex, performance status, loss of weight, stage of disease) and response to treatment (surgery, chemotherapy, and radiotherapy). No significant correlations were uncovered with either patient group (Table 3). Moreover, the overall survival time did not differ between patients with antibody levels greater or lesser than the cutoff value for positivity (20.9 RAU) (Fig 15A). The patients with an antibody value upon diagnosis >20.9 RAU had an overall median survival time of 452 days vs 415 days for patients with an antibody value <20.9 RAU ($p=0.395$).

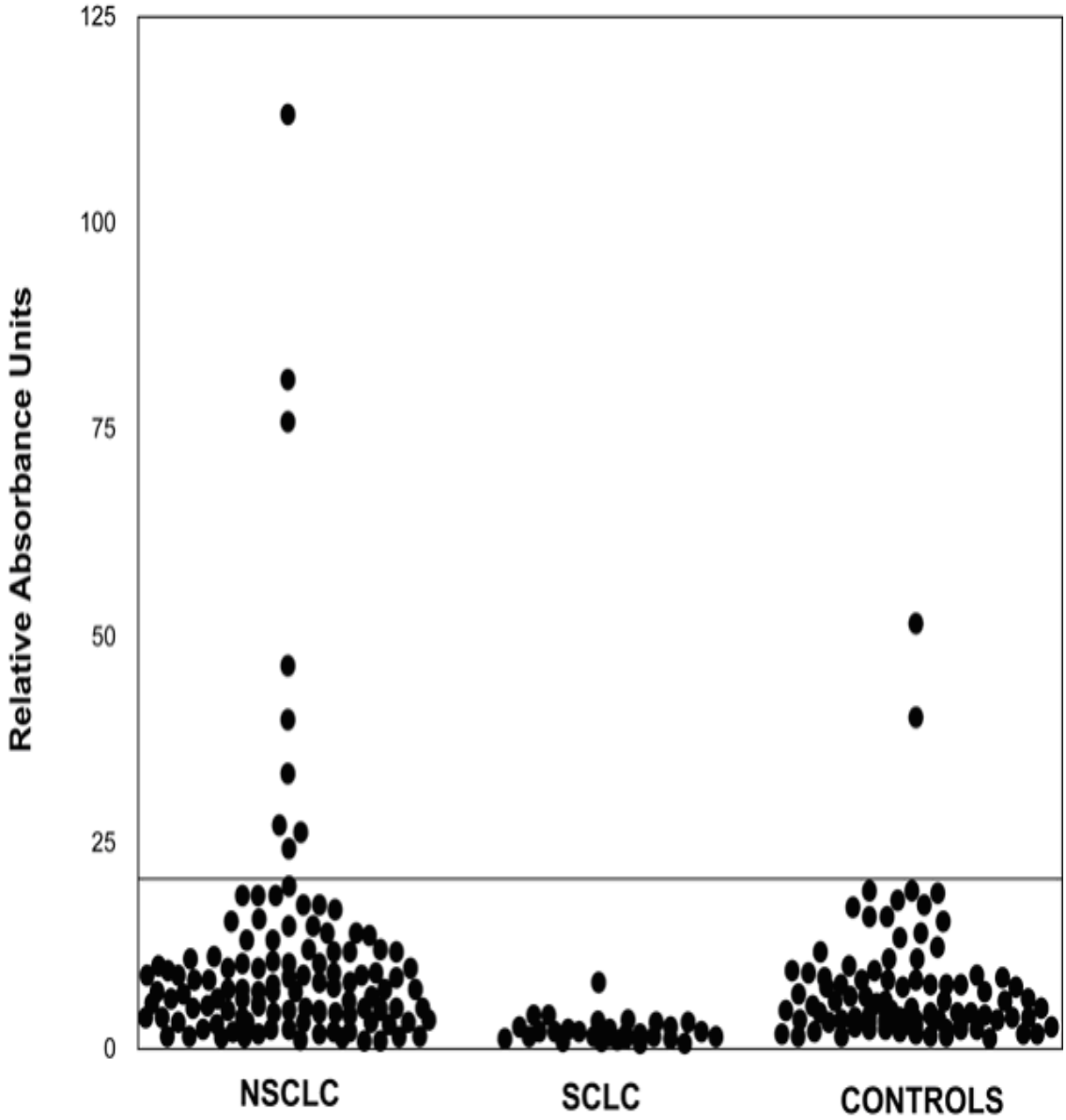

Figure 14: Anti-survivin antibodies detected by ELISA in sera from patients with NSCLC (n=117) and SCLC (n=33) in comparison to sera from age and gender matched healthy individuals (n=100). The results are mean values of two determinations and are represented as Relative Absorbance Units (RAU). The horizontal line indicates the cutoff value for positivity (mean+2SD of the 100 normal sera; 20.9 RAU).

Table 3: Correlations of anti-survivin antibody levels upon diagnosis with clinicopathological parameters and treatment for all patients (n=150).

		Anti-survivin antibodies		
		n	**Median (range)**	**p-value**
Age	≤65	82	5 (1-114)	0.087
	>65	68	7.5 (1-77)	
Sex	M	139	6 (1-114)	0.681
	F	11	6.8 (3-28)	
Performance Status[&]	0	91	6 (1-114)	0.949
	1	43	8 (1-82)	
	2	10	8 (1-15)	
	3	6	5.5 (3-20)	
Loss of weight	<5%	105	6 (1-114)	0.753
	≥5%	45	6 (1-82)	
Stage	I–II	30	8 (1-19)	0.270
	III-IV	120	6 (1-114)	
Response to Treatment[#]	PD	28	7 (1-82)	0.256
	DF	19	7 (4-34)	
	CR/PR	30	4 (1-114)	
	SD	21	5 (1-28)	

[*]p values were obtained after comparing for each group the antibody levels and every parameter; [&] ECOG Performance status; [#] Treatment refers to surgery and/or radiotherapy and/or chemotherapy and PD: progressive disease; PR: partial response; DF: disease free; SD: stable disease, CR: complete response.

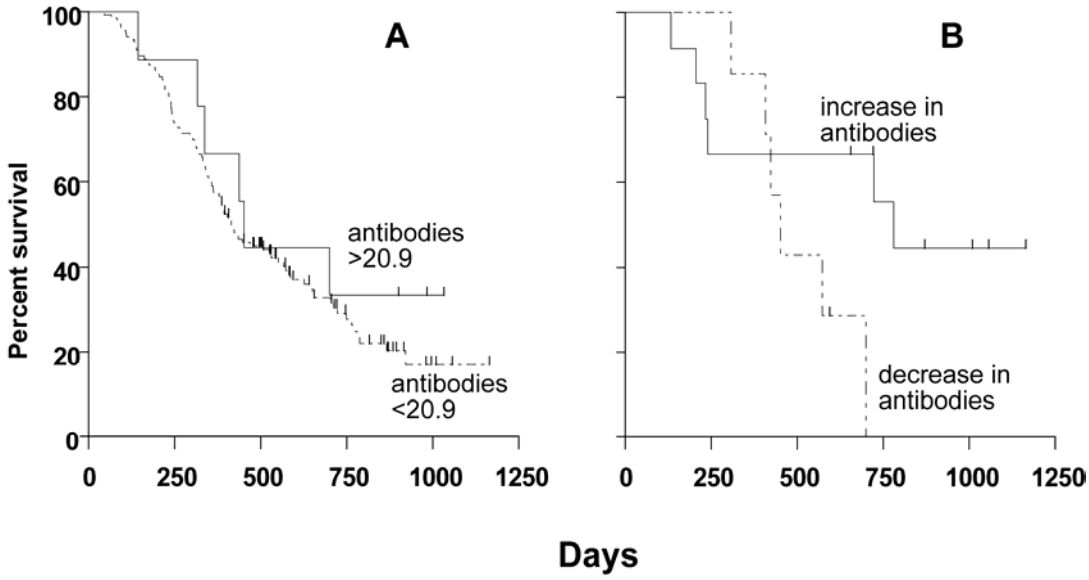

Figure 15: Kaplan-Meier survival curves. **A:** The overall survival time for all patients (n=150) is calculated on the basis of antibody levels upon diagnosis greater or lesser than the cutoff value for positivity (20.9 RAU) (median survival time: <20.9 =415 days, >20.9 =452 days, p=0.395). **B:** The overall survival time for patients with an increase or decrease in antibody levels post treatment is indicated (median survival time: increase in antibodies =781 days, decrease in antibodies =452 days, p=0.110).

Considering that for an antibody response to occur, an antigen needs to be seen by the immune system, resected tissues from 24 patients (NSCLC) were examined for the presence of mRNA transcript expression in a previous study of ours [46]. All the examined patient samples, representing a proportion of those tested for antibodies, expressed variable levels of survivin mRNA transcripts (Table 4). The expression levels appeared to be unrelated to the detected anti-survivin antibodies pre or post therapies, since even with a negligible antigen expression, antibodies could be detected. Interestingly however, as we had noted before [129], when we examined whether survivin specific CD8+ T cell precursors could be identified in the peripheral blood of some of these patients, none could be detected (Table 4). These findings suggest that the expression of survivin, does not necessarily relate to the anti-survivin B and T cell responses.

Table 4: Humoral and cellular responses of the NSCLC patients and the survivin mRNA expression levels in their respective tumor samples.

Patient No	Antibody levels*		T cells**	Survivin expression levels&
	pre	post		
9	12	NT&&	NT	23.5
15	16.3	22.5	<2.7	<1
22	4.5	NT	NT	<1
24	34	14	NT	<1
27	4.6	5.8	NT	<1
29	6.3	NT	<2.6	11.2
30	5.6	4.1	<1	75.3
32	6.8	4.1	<4.3	<1
39	18.2	16.3	<1	45.2
43	9	9.3	<3	5.1
50	12.6	NT	<4.3	8.7
52	113.9	84.9	<3.6	21.3
56	6.8	11.8	<2.4	12.5
57	3.6	54.2	<1.5	<1
59	10.6	64.7	NT	21.7
68	5.7	5.8	NT	<1
70	11.1	13.1	<3.8	18.2
76	4.7	5.6	<7.2	6.2
77	9.9	8.6	NT	<1
84	0.7	26.9	<5	25.5
89	3	4.0	NT	8.5
93	3.6	3.3	NT	<1
100	3.9	22.3	NT	<1
101	8.9	10.1	<6.3	37.6

*Antibody levels pre and post therapies and values represent RAU; **Peptide specific CD8 T cell responses measured at the time of diagnosis as described previously [129]. Values represent frequency per 10 million peripheral blood CD8 T cells; &Survivin mRNA expression levels in tumor samples adjusted to the expression in a testis reference sample (<1 expression level is considered as negative) as described previously [46]; && NT: Not tested.

Changes to the levels of anti-survivin antibodies in the NSCLC patients examined post therapies and during follow-up (n=76) were not statistically significant (repeated measures one-way analysis of variance with Bonferroni correction, $p>0.05$). However,

in 12 of these patients, with NSCLC, a significant increase by 2-38 (mean 8.2 ±10.4) times in the antibody levels was observed (p=0.02) that remained post therapies during follow-up. In 7 other NSCLC patients (one of them having not received any kind of therapy), a decrease was observed by 2-8 times (mean 0.4 ±0.14). Figure 16 presents representative patients with an increase or decrease in antibody levels post treatments and during follow-up whereas Table 5 presents the characteristics of all NSCLC patients examined post therapies and during follow-up.

We next examined whether in the NSCLC patients (n=19) alterations in the antibody levels correlated with the clinicopathological parameters (age, sex, performance status, loss of weight, stage of disease) and response to treatment (Table 6). No significant correlations were identified against any parameter examined, with the exception of a weak non-significant relation with an early stage of disease (p=0.054). Furthermore, the overall survival time between patients with an antibody increase or decrease post therapies and during follow-up did not present with any significant difference (Fig 15B). The patients with an increase in the antibody values post treatment had an overall median survival time of 781 days vs 452 days for patients with a decrease (p=0.110).

The strength of the antibody response was further evaluated. All positive sera were serially titrated. As presented in Fig 17, positive sera contained significant levels of anti-survivin antibodies with an average titre of 1/1270 which was clearly different from the two positive sera from normals that had an average titre of 1/360.

Finally, to ascertain whether treatment-induced lymphopenia was associated with alterations in anti-survivin antibody levels of NSCLC patients (n=76), correlations were determined between type of treatment and chemotherapy protocols (Table 7). No statistical significance was uncovered between any of the examined parameters and the antibody changes (p>0.05).

Table 5 Characteristics of NSCLC patients with or without variations in anti-survivin antibody levels.

(n=57)	**Antibody variations***		
	↓ (n=7)	↑ (n=12)	NC
Antibody change mean±1SD	0.4±0.14	8.2±10.4	1±0.3
Age (mean±1SD)	68.3±5.9 64.3±9.4	62.2±9.2	
Sex (male/female)	6/1	11/1	50/7
Stage			
I	1	1	9
II	0	3	5
III	4	3	21
IV	2	5	22
Performance[&]			
0	3	8	40
1	3	3	16
2	0	1	1
3	1	0	0
Loss of weight			
<5%	2	10	45
≥5%	5	2	12
Treatment	11	18	53
Surgery	2	4	16
Chemotherapy	4	8	39
Radiotherapy	1	3	11
Disease Status after treatment[#]			
PD	3	4	15
CR/PR	2	1	13
DF	1	4	14
SD	1	3	15

*↑ = Number of patients with antibody levels >2x pre-treatment value, ↓ = Number of patients with antibody levels <0.5x pre-treatment value, NC = Number of patients with no change in antibody levels; & ECOG Performance status; [#]PD: progressive disease; PR: partial response; DF: disease free; SD: stable disease, CR: complete response

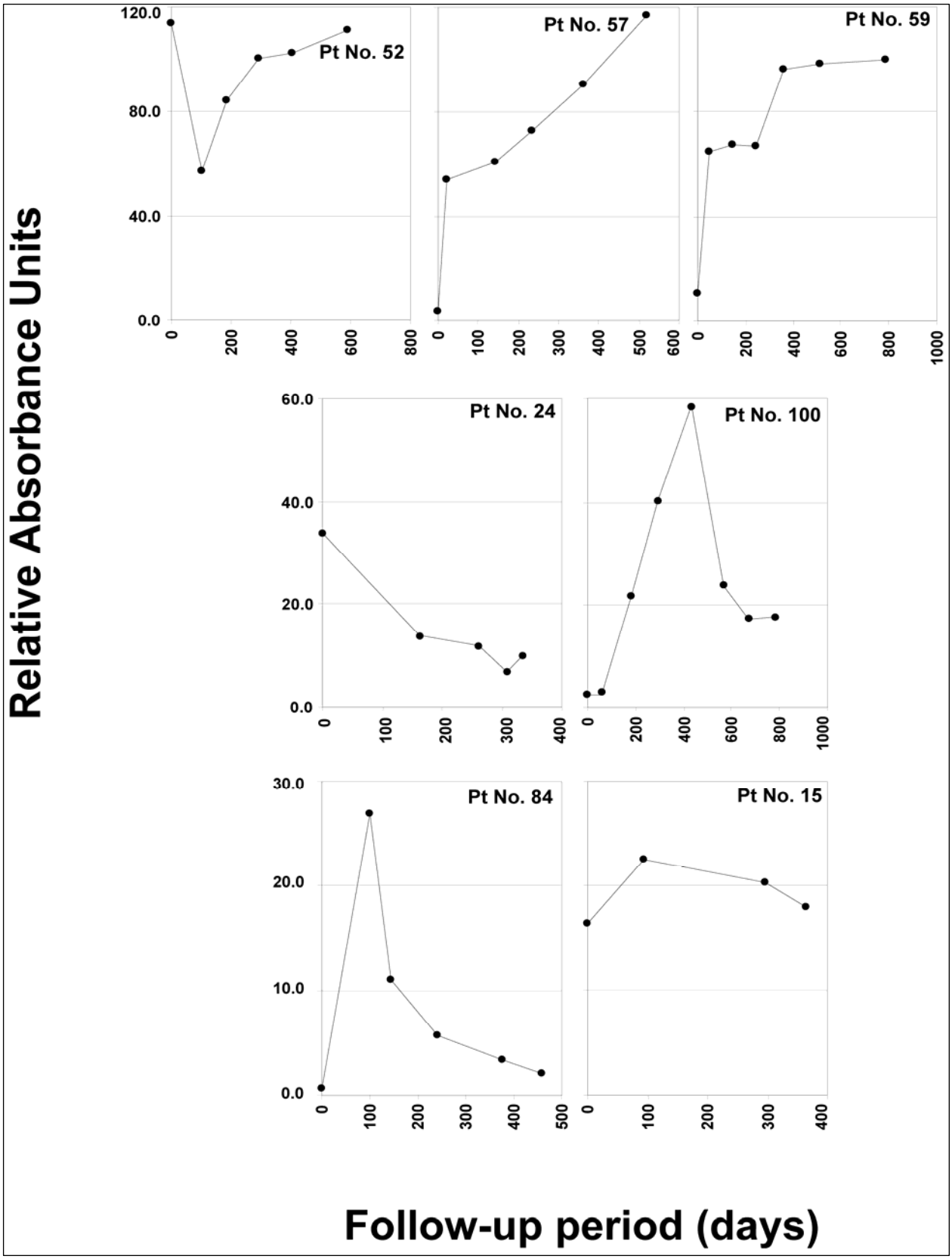

Figure16: Anti –survivin antibody variation in patients pre and post therapies. Plots represent Relative Absorbance Unit(y axis) observed during follow –up (x axis; days).

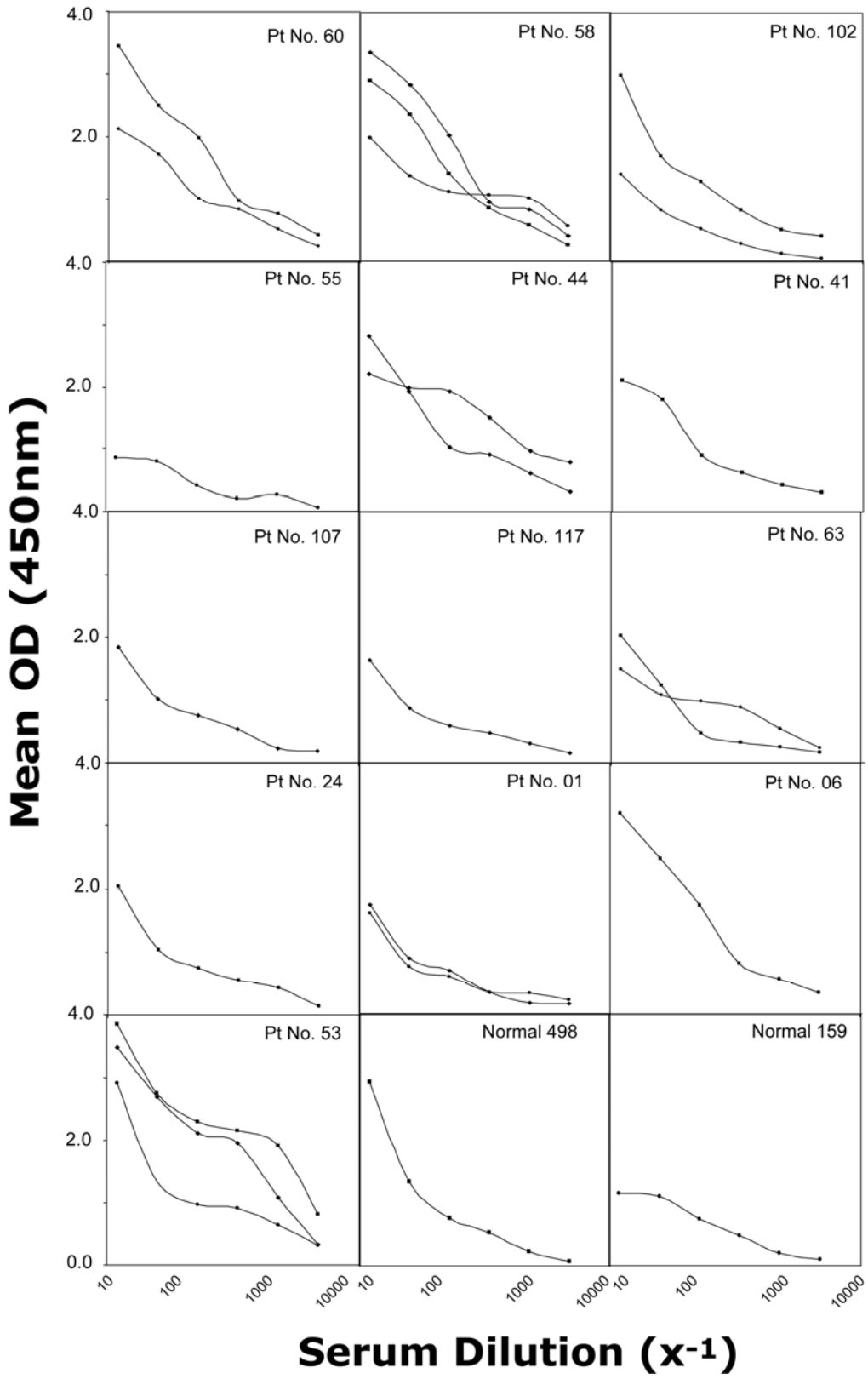

Figure 17: Positive sera titration. Y axis presents the OD at 450nM and x axis presents serum dilutions.

Table 6: Correlations of anti-survivin antibody level variations post treatment with clinicopathological parameters and treatment in patients with NSCLC (n=19)*.

		Anti-survivin antibodies		
		n	**Median (range)**	**p-value**
Age	≤65	12	13 (2-72)	0.165
	>65	7	8 (4-27)	
Sex	M	17	11 (4-72)	0.781
	F	2	28 (2-54)	
Performance Status&	0	11	18 (2-72)	0.876
	1	6	6 (4-27)	
	2	1	11	
	3	1	8	
Loss of weight	<5%	12	15 (2-72)	0.091
	≥5%	7	8 (4-27)	
Stage	I–II	5	54 (7-72)	0.054
	III-IV	14	10 (2-27)	
Response to Treatment#	PD	7	8 (2-13)	0.095
	DF	3	7 (4-18)	
	CR/PR	5	54 (14-72)	
	SD	4	14 (5-27)	

*p values obtained after comparing antibody level variations (before and after treatments) with every parameter; & ECOG Performance status; # Treatment refers to surgery and/or radiotherapy and/or chemotherapy and PD: progressive disease; PR: partial response; DF: disease free; SD: stable disease, CR: complete response.

Table 7: NSCLC patients with alterations in anti-survivin antibody levels in relation to chemotherapy treatment.

Chemotherapy protocols	Fold change*			Adj#	S**	R##	L&
	↑	↓	NC				
Paclitaxel (175 mg/m^2) + Vinorelbine (25 mg/m^2)	0	0	1	0	0	0	0
Paclitaxel (175 mg/m^2) + Gemcitabine (10^3 mg/m^2)	0	0	2	0	0	1	1
Paclitaxel (175 mg/m^2) + Carboplatin (6 AUC)	3	3	16	4	5	7	7
Docetaxel (75 mg/m^2) + Carboplatin (6 AUC)	1	1	5	0	0	2	2
Carboplatin (6 AUC) + Vinorelbine (25 mg/m^2)	0	0	1	0	0	0	1
Carboplatin (6 AUC) + Gemcitabine (10^3 mg/m^2)	1	0	7	0	0	3	2
Cisplatin (75 mg/m^2) + Gemcitabine (10^3 mg/m^2)	3	0	4	2	2	1	3
Cisplatin (75 mg/m^2) + Vinorelbine (25 mg/m^2)	4	2	9	9	10	7	5
Cisplatin (75 mg/m^2) + Docetaxel (75 mg/m^2)	0	0	3	0	0	1	1
No chemotherapy	0	1	9	0	5	1	2

*↑ = Number of patients with antibody levels >2x pre-treatment value, ↓ = Number of patients with antibody levels <0.5x pre-treatment value, NC = Number of patients with no change in antibody levels; #Number of patients who received adjuvant platinum-based chemotherapy; **Number of patients submitted to surgery; ##Number of patients who received radiotherapy; &Number of patients with post-treatment number of lymphocytes <1000/μL.

4. DISCUSSION

Since the mid-1970's, there has been an increasing demand for sensitive, relatively simple assays for use in basic research and clinical diagnosis. Over the same period, many practical considerations have led to the need to adapt existing assays and to develop novel ones without the use of radioisotope tracers, which have been commonly used in assay development for decades. The assay format that most closely meets all of these criteria in many situations is the Enzyme-Linked Immunosorbent Assay (ELISA). Today, most ELISAs follow one of three strategies: Indirect ELISA, typically used to screen for antibodies; Sandwich (or antigen capture) ELISA, to assay the amount of target antigen which is present; or Competitive ELISA, to define antigenic specificity or to increase the specificity of an assay when samples contain cross-reacting species. Choosing the best format depends on the intended application of the assay; the type of samples to be analyzed; the availability of reagents.

There are many parameters which influence the results obtained in an ELISA. These include: antibody quality and concentrations, incubation times, incubation temperatures, blocking & detection reagents and its concentration, and substrate type and quality. The ELISA screening system established in this study has been standardized using goat mAbs with specificity for survivin recombinant antigen.

The blocking buffer should improve the sensitivity of the assay by reducing the background interference. An individual blocking buffer will not be compatible with every system; for this reason, a variety of blockers in both Tris buffered saline (TBS) and phosphate buffered saline (PBS) are available. The most important parameter when selecting a blocker is the signal/noise ratio, measured as the signal obtained with a sample containing the target analyte, as compared to that obtained with a sample without the target analyte. Using inadequate amounts of blocker will result in excessive background and a reduced signal: noise ratio. Using excessive concentrations of blocker may mask antibody-antigen interactions or inhibit the enzyme, again causing a reduction of the signal/noise ratio. When developing any ELISA, it is important to test

several different blockers for the highest signal/noise ratio in the assay. No single blocking agent is ideal for every occasion since each antibody-antigen pair has unique characteristics. In this study three different blocking buffers were tested (5%: SM/PBS, HAS/PBS and BSA/PBS) at different incubation temperatures (4°C, 37°C), and 5% BSA/PBS at 4°C overnight incubation was chosen as a proper blocker.

The ELISA sensitivity and specificity depend also on the quality of used sera which contain the antibodies specific to the coated antigen. Ideally, the positive control serum should have a high concentration of antibodies which have to react specifically only with the coated antigen. These antibodies should not interact with other reagents or even with the solid phase (plastic material).

This study examined the presence of antibodies against survivin, a widely occurring tumor-associated protein, in sera from lung cancer patients. Using an ELISA technique, anti-survivin antibody response was detected in lung cancer patients. Specificity of survivin recognition was confirmed by preincubation of the sera with soluble survivin.

The immune response against cancer cells is complex, involving the interaction of many different cell types and cell products, and it is not possible to consider cell-mediated and antibody-mediated responses in isolation. Functional antitumor immunity requires a response initiated by potent antigen-presenting cells, such as dendritic cells, and the engagement of a variety of effector cells not only cytotoxic T cells, but also T-helper cells and B cells, which initiate antibody immunity. However, it is well recognised that cytotoxic T lymphocytes (CTL) constitute one of the most important effector mechanisms of anti-tumour immunity [133]. CTL are able to perform tumour-specific recognition via their clonal T cell receptors (TCR) generated via a somatic recombination mechanism. Activated CTL can mediate specific destruction of tumour cells by the release of lytic components and direct cell–cell interaction. In general, CTL are CD8+ and are therefore class I major histocompatibility complex (MHC)-restricted,

even though in some instances CD4+ class II MHC restricted T cells have also been shown to perform cytolytic activities [134].

Over the past decade, it has been demonstrated that cancer is immunogenic, and multiple tumor antigens have been identified in cancer patients. It is now possible to potentially harness the immune response elicited by cancer growth as a potential diagnostic tool. Currently, only a limited number of tumor markers for non-small cell lung cancer (NSCLC) are available, and NSCLC heterogeneity makes the identification of a single sensitive and specific marker unlikely. Antibodies to tumor-associated proteins may expand the number of available tumor markers for lung cancer, and may be used together in a serum profile to enhance sensitivity and specificity.

Humoral immunity to tumor-associated proteins is receiving renewed attention as the appropriate measure of cancer development. This is because antibody responses can be generated against non-circulating proteins expressed on the cell surface, irrespective of their amount. Antibody measurement is a rather stringent process aiming to: a). Increase assay sensitivity, b). Evaluate responses to a panel of proteins rather than an individual tumor antigen, c). Evaluate the level of antibody response (titer or absolute amount of immunoglobulin), d). Identifying the classes of the reactive antibodies, and finally, e). Determining the most appropriate form of the antigen to be used in the assay, for example, "captured" protein, recombinant protein, or even peptides.

The process of carcinogenesis involves genetic instability, with one estimate of over 11000 genomic alterations occurring in a cancer cell [135]. Genetic and epigenetic changes mean that cancer cells can produce novel proteins and over-express proteins normally present at low levels [136]. Multiple peptides are processed from each protein, allowing multiple different epitopes or TAAs from tumour-related proteins to be presented on the cell surface [19], even at early stage of the disease, autoantibodies to tumor-associated antigens (TAAs) such as HER-2/neu, c-myc, ras, cyclin B1, p53, p62, human telomerase reverse transcriptase (hTERT), and survivin have been

identified in patients with cancer [106-113]. The detection of autoantibodies may be used as an adjunct to more standard serologic tests being evaluated to aid in cancer diagnosis, clinical reports of autoantibodies against TAA detected in cancer patients with much higher frequency than control donors demonstrate that autoantibodies can be raised against both intra- and extracellular proteins, that circulating autoantibodies can be found in both early stage as well as preinvasive tumors, and that TAA autoantibodies can be identified in high risk patients who are not yet tumor bearing. All these characteristics would be a benefit for a diagnostic assay. However, autoantibodies can also be associated with benign disease or even be detected at lower levels in tumor bearing individuals than controls. Moreover, measurement of a single autoantibody will not provide the adequate sensitivity needed for a diagnostic test. These observations underscore the need to fully characterize an autoantibody response across multiple populations prior to clinical development.

The survivin protein regulates both cell division and cell survival and is overexpressed in the vast majority of human cancers [68]. Current evidence suggests that survivin's dual function as an anti-apoptotic protein and a chromosomal passenger protein makes it a factor favoring cancer progression. In fact, the gene encoding survivin is notable for its high degree of tumor-specific expression, ranking among the top five most tumor-specific genes in the human genome based on comparisons of the number of times survivin transcripts appear in tumors compared with normal cells and tissues [137]. In normal cells, survivin is produced only in small amounts and only briefly during mitosis. However, in tumors, the survivin protein is continuously present at excess levels, suppressing apoptosis and aiding in cell division [68].

Consequently, Survivin has emerged as a hot target for cancer therapy, with a variety of strategies already articulated for nullifying survivin in tumors, including (a) small molecule antagonists that block interactions of survivin with critical partner proteins ; (b) antisense oligonucleotides that reduce survivin expression; (c) ribozyme-mediated inhibition of survivin expression [138]; and (d) gene therapy using dominant negative mutants of survivin that induce cell cycle arrest and apoptosis [139]. Target-

ing of survivin by RNA interference in a multitude of cancer cell lines leads to an increase in susceptibility to DNA-damage induced programmed cell death, such as by chemicals or radiation.

This indicates that high levels of survivin mediate resistance to chemotherapy and radiation [140]. This is clinically significant in that patients harboring tumors expressing high survivin have poorer clinical outcomes [134]. Current evidence suggests that survivin plays an essential role in tumor maintenance by facilitating proliferation, angiogenesis, multi-drug resistance and by preventing apoptosis [141]. However, whether survivin plays a role in the initiation or establishment of tumors still remains unclear.

This study was undertaken to further explore the immunogenicity of survivin, an overexpressed TAA that, due to its nodal role in the survival of cancer cells, is considered as an ideal target for immunotherapy. It has been demonstrated by several groups that survivin is an immunogenic protein eliciting both humoral and cellular immune responses.

As the success of cancer immunotherapy is dependent on using suitable tumor-associated antigens (TAAs), an ideal TAA should have several important features. First, TAA should induce T cells that recognize tumors but not normal cells. Second, TAA should be expressed in tumors from a significant proportion of the patients and in a significant proportion of tumor cells. Third, it is important that TAA belongs to molecules, which are obligatory for the survival of tumor cells. Immunization against such TAA(s) would overcome tumor escape attributable to antigenic variation because losing the survival-related molecule may result in tumor cell death. At this time, there are very few molecules that fit these criteria. Survivin may be one such rare candidate.

Efforts were made in our laboratory to examine the anti-survivin humoral response under the light of our recent finding that HLA-A restricted peptides of survivin do not induce detectable CTL responses [129] as well as bearing in mind that the results of all three existing studies examining the anti-survivin antibody response in lung cancer [117, 121, 122] are inconclusive. In general, the autoantibody responses asso-

ciated with cancer have attracted limited attention despite the fact that they may provide useful information about the helper T cell response against TAA. Instead they have been extensively studied in the context of been utilised as biomarkers for diagnosis [142].

In the present study we used the largest in the literature cohort of lung cancer patients and controls, and we have shown that autoimmunity against survivin in these patients did not differ significantly than that observed in cancer-free individuals. The statistically significant difference in the concentration of antibodies, observed in the group of NSCLC, obviously represents the effect of extreme values (11.5±15.2 RAU), with only three of these values from patients, being above the upper limit of those detected in normal individuals. This finding becomes even more intriguing considering that survivin was found to be overexpressed in nearly all NSCLC [46] yet, at the same time; this was not accompanied by a detectable anti-survivin CTL response [129]. Taken into account all the above, we could consider that either survivin truly appears with a low immunogenicity or tolerance against it is easily induced in cancer patients. Regarding the latter, it is not amazing for the immune system to recruit tolerizing mechanisms in order to protect important molecules, for the survival of the cells, from autoreactivity.

Efforts to correlate the antibody concentration with clinical parameters of the patients or to identify the characteristics of those appearing with elevated levels at diagnosis did not reveal any significance. Taking into account that this study utilized the largest cohort of lung cancer patients, this finding is in accordance with those pertaining that, at least when detected alone, anti-survivin antibodies do not present with any diagnostic utility [116, 117].

Cytotoxic chemotherapy, surgery, and radiotherapy are the major modalities used in cancer treatment. Because cytotoxic drugs target dividing cells, the metabolic similarities between normal and neoplastic cells lead to low therapeutic indices and high toxicity. Bone marrow is particularly susceptible to cytotoxic damage, and the

majority of cytotoxic drugs cause neutropenia and, to some degree, lymphopenia, thrombocytopenia, and anemia.

Radiation therapy and chemotherapy can be applied as an initial treatment (neoadjuvant therapy) with the purpose to shrink the tumor for complete resection of borderline respectable tumors (stage III). Alternatively, radiation and chemotherapy can be used after surgery for eradication of micrometastases (adjuvant chemotherapy) or prevention of local relapse (radiation therapy).

Different chemotherapeutic agents have been shown to induce different forms of cell death, and may have different immunological consequences [143]. Chemotherapy may interact to augment immunotherapy by several different mechanisms, including increased T cell infiltration enhanced antigen cross presentation to dendritic cells (DCs) and tumour cell sensitization to killing by cytotoxic lymphocytes [144, 145, 146].

Some chemotherapeutic agents are probably more immunogenic than others. For example, cyclophosphamide has long been investigated for an ability to deplete Treg at doses that relatively spare the rest of the lymphocyte population [147]. Targeted therapies also have the potential to interact with the immune system. For example, the c-kit tyrosine kinase inhibitor, imatinib, has been reported to activate DCs and overcome tumour-induced T cell tolerance [148].

Agents used to treat hematological malignancies in particular can cause severe lymphopenia and profound defects in cell-mediated immunity leading to opportunistic infections [149].

What has been less clear is the effect of chemotherapy on antitumor immunity. Clearly, the destruction of tumor cells by cytotoxic drugs increases the load of tumor antigen reaching the draining lymph node. The fact that this increased load fails to generate an immune response that controls minimal residual disease is assumed to be due to immune suppression. However, this has never been systematically evaluated. Most tumors express an array of antigens that could act as targets for their immune-

mediated destruction, and a number of potential therapies have emerged to exploit this [150].

Chemo- and radiotherapy kill cancer cells mainly by triggering apoptosis or programmed cell death [151]. In human cancer cells and tissues, the expression of four members of the anti-apoptotic inhibitor of apoptosis (IAP) protein family (XIAP, cIAP1, cIAP2 and survivin) has been investigated as a potential factor for chemoresistance, based on their ability to inhibit the key molecules of the apoptotic machinery, the caspases [152]. As mentioned before survivin is involved in both proliferation and apoptosis and its localization is important for its prognostic properties [125, 153]. Highly proliferative tumors are more sensitive to chemotherapy suggesting that survivin expressing tumors might be more sensitive to chemotherapy [154]. Conversely, the anti-apoptotic function of survivin would suggest that these tumors would be refractory to cytotoxic treatments, e.g. endocrine therapy.

Accumulating evidence over the last decade indicates that the host immune system contributes to the anti-cancer therapeutic outcome, since cancer cell death elicited by radiotherapy and some chemotherapeutic agents seems to be immunogenic [155 , 156]. Moreover, they induce reduction of the tumor mass directly affecting in this way its immunosuppressive properties that can lead to reversal of tumor-induced immune tolerance [157]. Platinum-based regimes, like those used in 86% of NSCLC patients in this study, have been proved in vitro able to increase the sensitivity of prostate carcinoma tumor cells to the lethal and lytic effects of tumor-infiltrating lymphocytes as well as the sensitivity of colon carcinoma tumor cells to antigen-specific CTLs [158, 159]. Our study, however, did not reveal any in vivo correlation between the treatment with cisplatin or carboplatin and the antibody response of the patients.

Chemotherapy-induced lymphopenia has been regarded as a factor that compounds on the anti-tumor immune response. Treatment with gemcitabine which is a nucleoside analogue that is frequently used to treat non-small cell lung cancer, it is also active in other malignancies, either alone or in combination with cisplatin, a constituent of the regimens used in 22% NSCLC patients in this study, is known to have a

detrimental effect on humoral immune responses [160], without compromising specific anti-tumor cellular immunity. Taxanes also given in 46% of our NSCLC patients reduce CD19+ cell numbers in animal models [161]. Again, lymphopenia did not prove in our study to relate to the antibody response of the patients.

Notwithstanding the absence of correlations, as discussed above, 19 out of the 150 examined patients, all with NSCLC appeared with tremendous and constant increases or decreases in their anti-survivin antibody levels, a fact that cannot be overlooked. Even amongst these patients, no relation was observed either with any treatment regimen or with clinicopathological parameters. To this end, not even the removal of the antigenic stimulus can explain the extreme decreases observed in antibody concentration, since 5 out of 7 patients with decrease had not been subjected to surgery. The great increases, however, argue against a low immunogenicity of survivin but rather are in support of its strict tolerogenicity that could account for the limited anti-survivin antibody response generally. In favor of this are recent data indicating that the number and the function of regulatory T cells are negatively affected by chemotherapeutic agents, such as taxanes [162, 163]. In such a case, anti-survivin autoimmunity might reflect an effectiveness of chemotherapy. In any case, changes of anti-survivin antibody levels during cancer treatment seem to be an interesting phenomenon that requires attention and warrants further investigation. It is an intriguing question, whether anti-survivin antibodies could also serve as an early predictive marker in patients with lung cancer.

5. ΠΕΡΙΛΗΨΗ

Προσδιορισμός της χυμικής ανοσιακής απάντησης έναντι της σαρβιβίνης σε ασθενείς με καρκίνο του πνεύμονα

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ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ – ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ
ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

Εισαγωγή: Τα μέχρι στιγμής δεδομένα που αφορούν στην αυτόματη έναντι της σαρβιβίνης χυμική ανοσιακή απάντηση είναι αμφιλεγόμενα. Επιπρόσθετα, παρά το γεγονός ότι ο θάνατος των νεοπλασματικών κυττάρων μετά από ακτινοθεραπεία ή χορήγηση χημειοθεραπευτικών φαρμάκων φαίνεται να είναι ανοσογονικός, λίγα είναι γνωστά για την πιθανή επίδραση της αντικαρκινικής θεραπείας στις χυμικές απαντήσεις στον καρκίνο. Σκοπός της παρούσας μελέτης ήταν, ο χαρακτηρισμός της αυτόματης χυμικής ανοσιακής απάντησης έναντι του ευρέως εκφραζόμενου αντιγόνου των όγκων σαρβιβίνη, η οποία μπορεί να εκλυθεί από τον όγκο, σε ασθενείς με καρκίνο του πνεύμονα.

Υλικό και Μέθοδοι: Δείγματα ορού από 33 ασθενείς με πρωτοδιαγνωσθέν μικροκυτταρικό καρκίνο πνεύμονα (SCLC) καθώς και από 117 ασθενείς με πρωτοδιαγνωσθέν μη μικροκυτταρικό καρκίνο πνεύμονα (NSCLC) και δείγματα ορού από 100 φυσιολογικούς μάρτυρες αναλύθηκαν με ELISA για την ανεύρεση αντισωμάτων έναντι της σαρβιβίνης.

Αποτελέσματα: Ως κατώφλι θετικότητας για την ELISA τέθηκε η μέση τιμή ± 2 SD (mean±2SD) των τιμών των φυσιολογικών μαρτύρων. Σε ποσοστό 7.7% των ασθενών με NSCLC (OR 3.6, 95% CI: 0.7-17.3), σε κανέναν από τους ασθενείς με SCLC (OR 0.6, 95% CI: 0.03-12.6) και στο 2% των φυσιολογικών μαρτύρων βρέθηκαν αυξημένοι τίτλοι αντισωμάτων έναντι της σαρβιβίνης. Από τον προσδιορισμό του τίτλου των αντισωμάτων σε 76 ασθενείς με NSCLC μετά τη θεραπεία και κατά τη διάρκεια της παρακο-

λούθησής τους προέκυψαν 12 ασθενείς στους οποίους ο τίτλος αυξήθηκε κατά 2-38 φορές ενώ σε 7 ο τίτλος μειώθηκε κατά 2-8 φορές. Καμία στατιστικά σημαντική συσχέτιση δεν αποκαλύφθηκε μεταξύ του τίτλου των αντισωμάτων κατά τη διάγνωση ή των μεταβολών του μετά τη θεραπεία, με οποιαδήποτε κλινική και ιστολογική παράμετρο, την ανταπόκριση των ασθενών στη θεραπεία και την επιβίωση τους.

Συμπέρασμα: Στον καρκίνο του πνεύμονα η σαρβιβίνη φαίνεται να μην προκαλεί ισχυρές χυμικές ανοσιακές απαντήσεις. Παρόλα αυτά, είναι πιθανό, μετά τη θεραπεία και κατά την παρακολούθηση των ασθενών να εκλύεται ισχυρή χυμική απάντηση με αντισώματα έναντι της σαρβιβίνης, η κλινική σημασία των οποίων παραμένει άγνωστη. Τα ευρήματα αυτά σε συνδυασμό με δεδομένα που αφορούν στην έκφραση της σαρβιβίνης και τις σχετιζόμενες με αυτή Τ κυτταρολυτικές απαντήσεις στον καρκίνο του πνεύμονα υποδεικνύουν την υψηλή πιθανότητα ανάπτυξης ανοχής από το συγκεκριμένο αντιγόνο.

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