EXPRESSIONS OF ANGIOGENIC MARKERS AND APOPTOTIC MARKERS IN SOFT TISSUE SARCOMA

By:

DR YUSRI BIN YUSUF

Dissertation Submitted In Partial Fulfillment Of The Requirement For The Degree Of Master Of Pathology (Anatomic Pathology)

UNIVERSITI SAINS MALAYSIA

UNIVERSITI SAINS MALAYSIA

2010

ACKNOWLEDGEMENTS

In the name of ALLAH, the Most Beneficent, and the Most Merciful

Praise ALLAH almighty, despite the obstacles, I finally able to complete this study.

First and foremost, I would like to express my heartful gratitude to my supervisor, Dr Thin Thin Win @ Safiya for her valuable time and tireless effort and professional guidance throughout the study. I am also thankful to my co-supervisor, Assoc. Professor Dr Hasnan Jaafar who helped me in the supervision.

I am indebted to Encik Rusli Jusoh, the most helpful technologist who is always energetic and ever willing to offer his skillful expertise in materializing this dissection.

For all my lecturers, Prof Dr Nor Hayati Othman, Dr Mukarramah, Assoc. Prof Dr Mutum Samarendra Singh, Dr Saleena Awang, Dr Nor Hidayah, Dr Venkatesh R Naik, Dr Md Salzihan, Dr Sharifah Emilia and Dr Shyamoli, I am forever thankful for their professional guidance.

I also wish to express my appreciation to my colleagues for their moral support and opinion.

Finally, I would like to dedicate this hard work to my beloved parents, for their endless love and supports throughout this study.

May our efforts be blessed by Allah and shall it benefit to mankind.

TABLE OF CONTENT

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Bax	Bcl-2 associated X protein (pro-apoptotic)
Bcl-2	B-cell lymphoma protein 2 (anti-apototic)
bFGF	Basic fibroblast growth factor
Caspase	Cysteine protease
EC	Endothelial cells
ECM	Extracellular matrix
FGF	Fibroblast growth factor
FNCLCC	French Federation of Cancer Centers Sarcoma Group Grading System
HCL	Hydrochloride
HIF-1 _	Hypoxia inducible factor - 1 alpha
HUSM	Hospital Universiti Sains Malaysia
IHC	Immunohistochemistry
MFH	Malignant Fibrous Histiocytoma
MMP	Matrix metalloproteinase
MPNST	Malignant Peripheral Nerves Sheath Tumor
MVD	Microvessel density
N	Normal
NCl	United States National Cancer Institute
PDGF	Platelet derived growth factor
PNET	Primitive neuroectodermal tumor
STS	Soft tissue sarcoma

TBS	Tris buffer saline
TC	Tumor cells
TGF-β	Tumor growth factor beta
TUNEL	Transferase mediated dUTP nick- end labelling
VEGF	Vascular endothelial growth factor
Vwf	von Willebrand Factor
WHO	World Health organization

Abstrak

Aktiviti angiogenesis dan apoptosis memainkan peranan yang penting untuk perkembangan sesuatu kanser. Bagaimanapun sehingga kini tiada kajian yang dibuat untuk mengkaji hubungan antara keduanya di dalam sel kanser termasuk kaitan antara kedua aktiviti ini di dalam sel kanser dan sel endotelium.

Kajian ini bertujuan untuk membandingkan ekspresi protein 'anti-apoptosis' Bcl-2 dan 'pro-apoptotic' Bax dalam sel kanser dan sel endotelium dalam STS. Selain itu kami juga menentukan kaitan antara aktiviti 'apoptosis' dan 'angiogenesis' dalam STS.

Kajian 'cross sectional' telah dijalankan dari April 2009 hingga Oktober 2010. Sebanyak 101 kes STS terlibat dalam kajian ini seperti liposarcoma (37), MFH (19), synovial sarcoma (6), leiomyosarcoma (14), MPNST (7), fibrosarcoma (9) dan rhabdomyosarcoma(9) . Potongan tisu daripada 'block' tisu arkib dikaji dengan kaedah immunohistokimia bagi menentukan ekspresi protein Bcl-2, Bax dan VEGF.

Ekspresi Bax dalam sel kanser (54.5%) adalah lebih tinggi daripada ekspresi Bcl-2 (44.6%), walaupun tidak signifikan secara analisa statistik. Kami juga mendapati kaitan yang signifikan bagi ekspresi Bcl-2 dan Bax dalam sel kanser dan sel endothelium ($p<0.001$). Bagi karektor kanser pula, satu-satunya penemuan yang signifikan adalah ekspresi Bcl-2 dengan 'histologic subtypes'. Dalam kajian ini kami dapat menunjukkan bahawa ekspresi VEGF adalah tinggi apabila ekspresi Bax rendah. Walaubagaimanapun, ekspresi VEGF didapati tiada kaitan dengan ekspresi Bcl-2, lokasi kanser, kedalaman kanser, saiz kanser, status margin dan status nodul limfa.

Kesimpulannya, Kajian ini menyokong peranan sel endotelium didalam proses 'tumoregenesis'. Oleh itu, rencatan pada faktor yang boleh menyebabkan survival pada sel endotelium selain pengaktifan pada faktor yang menyebabkan kematian sel endotelium boleh menjanjikan prospek yang berguna didalam kajian terapi kanser ini. Selain itu, ketiga-tiga protein yang digunakan didalam kajian ini terutamanya Bcl-2 sebenarnya berpotensi sebagai petunjuk untuk sesuatu entiti bagi membantu proses pendiagnosaan kerana reaksinya yang berbeza untuk setiap jenis 'histologic subtypes' didalam STS.

Aktiviti 'angiogenesis' yang tinggi berkemungkinan merencat apoptosis pada sel kanser yang menyebabkan perkembangan luar kawalan sel kenser . Walaubagaimanapun, aktiviti 'angiogenesis' tidak bergantung kepada aktiviti 'anti-apoptosis'. Justeru, kefahaman yang mendalam berkaitan

perhubungan antara aktiviti ‘angiogenesis’ dan ‘apoptosis’ ini boleh menjadi batu loncatan untuk lebih banyak lagi terapi kanser dapat ditemui pada masa depan.

Abstract

EXPRESSIONS OF ANGIOGENIC MARKERS AND APOPTOIC MARKERS IN SOFT TISSUE SARCOMA.

Dr Yusri Yusuf

MPath (Anatomic Pathology)

Department of Pathology, School of Medical Sciences, Universiti Sains, Malaysia, 16150, Kelantan, Malaysia.

Introduction: For tumor development and progression, angiogenesis and apoptosis play a crucial role. No study so far has tried to associate these two activities in tumor cells as well as correlation of both activities in the tumor and the endothelial cells.

Aim: This study was designed to compare the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax on the tumor cells and the endothelial cell of blood vessels supplying the tumor in soft tissue sarcoma. In relation to that, we also tried to associate the relationship between apoptotic and angiogenic activity in soft tissue sarcoma.

Methodology: A cross sectional study was conducted from April 2009 to October 2010. 101 cases of soft tissue sarcoma consisted of liposarcom (37), MFH (19), synovial sarcoma (6), leiomyosarcoma (14), MPNST (7), fibrosarcoma (9) and rhabdomyosarcoma (9) were included in this study. Tissue sections that were retrieved from archieved tissue blocks were stained with immunohistochemical stain for Bcl-2, Bax and VEGF protein.

Results: Higher expression of Bax in tumor cells (54.5%) was seen compared to expression of Bcl-2 (44.6%) though statistically not significant. There were also significant association between the expression of Bcl-2 and Bax in tumor cells with endothelial cells ($p<0.001$). With tumor characteristics, the only significant association in our study was the expression of Bcl-2 in tumor cells with tumor histologic subtypes. In this study, we were able to demonstrate that the expression of VEGF is higher with weak expression of Bax in soft tissue sarcoma. However, the expression of VEGF was not associated

with expression of Bcl-2, tumor location, depth, size, margin, lymph nodes metastases and histologic subtypes.

Conclusion: This study supports the role of endothelial cells in survival and regression of tumor cells in tumoregenesis. Thus, the inhibition of endothelial cells survival factor or activation of endothelial cells death is a promising prospect as a candidate for tumor therapy. All the proteins used in this study might be potential as a useful marker for diagnosis of specific histologic subtypes especially Bcl-2 since there is diversity in expression amongst the various histologic subtypes in STS. An increase in angiogenic activity may inhibit apoptotic tumor cells death which leads to cell proliferation. However, the angiogenic activity was independent of anti-apoptotic activities. A better understanding of their relations probably provides the basis for more rational cancer therapies in the future.

Dr Thin Thin Win @ Safiya: Supervisor

Assoc. Professor Dr Hasnan Jaafar: Co-Supervisor

CHAPTER ONE

Introduction

1. INTRODUCTION

Soft tissue sarcomas are rare diseases that encompass a histologically and anatomically diverse group of neoplasm, which share a common embryologic origin from mesodermal tissue. Despite the relative rarity with annual incidence about 30/million, i.e. less than 1% of all malignant tumors but there is an increase in incidence and mortality trend, posing a significant diagnostic and therapeutic challenge due to their heterogeneity. In Malaysia, the tumor shows a slightly decreasing trend for newly diagnosed cases of cutaneous, subcutaneous and soft tissue sarcoma in both sex for Peninsular Malaysia, representing about 133 (1.4%) for male and 115 (1%) for female in year 2003 (National Cancer Registry, 2004) as compared to about 130 (0.6%) for male and 106 (0.5%) for female in 2006 (National Cancer Registry, 2006).

For tumor development and progression, many researchers have been looking into the apoptotic and angiogenic activities of cancer cells. Apoptosis activity depends partly on the balance between Bcl-2 which is anti-apoptotic and Bax which is pro-apoptotic gene expression. Most studies found that over-expression of Bcl-2 correlates with histological types, histological grades and prognosis. In previous studies, the Bcl-2 expression were 43% (Nakanishi. *et al*., 1997), 38.8% (Sabah. *et al*., 2007) and 32.2% (Tomoyoki. D. *et al.,* 2002 *)* whereas Bax expression were 92.8% (Sabah. *et al*., 2007) and 40.8% (Tomoyoki. D. *et al.,* 2002). Common immunoreactivity are seen in synovial sarcoma and MPNST (Kawauchi. S. *et al*. 1999 and Sabah. *et al*. 2007). Therefore, it may aid in differential diagnosis in synovial sarcoma (Suster. *et al*., 1997). In relation to the histologic grade and prognosis, the study by Sabah. *et al* 2007 revealed

the overexpression of Bcl-2 in high grade sarcoma may used as poor prognostic marker. In contrast, the study by Nakanishi*. et al*. 1997 showed the opposite view as the stronger Bcl-2 were associated with favourable prognosis whereas no correlation of both reported by Tomoyuki. D. *et al*. 2002 Only 2 studies have evaluated Bax expression in STS by immunohistochemistry. In this study, Bax expression was a common finding in STS (Kawauchi. S. *et al*., 1999 and Tomoyoki. D. *et al*., 2002) and no correlation with Bcl-2 and tumor grade was identified (Tomoyoki. D. *et al*., 2002and Sabah. *et al*., 2007). No study has been done on the expression of apoptotic marker in endothelial cells of blood vessel supplying the tumor. Hence one of our objectives is to look into this matter and correlate the findings with the expression of these markers in the tumor cells.

In relation to that, we will also study the angiogenic activities of the cancer cells as well as endothelial cells. The importance of angiogenesis in tumor progression and metastasis is well-recognized and its potential role in prognosis and therapeutic application has lead to many ongoing clinical researches. Angiogenesis can be studied by looking at the expression of angiogenetic markers such as vascular endothelial growth factor (VEGF) and hypoxia inducible factor 1α (HIF-1α). HIF-1 is heterodimer consisting of α and β subunits. HIF-1α expression is related to cellular hypoxia, whereas HIF-1β subunit is constitutively expressed independent of cellular hypoxia. HIF-1α is transcription factor that stimulate VEGF transcription and support the tumor growth and progression (Shintani. K. *et al.,* 2006). VEGF expression was diverse amongst the various histological subtypes. Leiomyosarcoma, synovial sarcoma and MFH were the more likely to over express VEGF than other histological types (Chao. C. *et al*. 2001, Potti A.

et al 2004. and Hong. T. *et al*., 2001). The expression of VEGF in STS was 75% by Chao. C. *et al*., 2001 but the expression was very low (25%) by Potti A. et al. 2004. The expression of HIF-1α was 71.4% by Shintani. K. *et al.,* 2006. However many studies showed no relationship between survival and VEGF status except for leiomyosarcoma as high VEGF was associated with shorter survival (Potti. A. *et a.,l* 2004). Microvessel density (MVD) is the best option for better quantitation of the angiogenetic activity. However after considering time limit, an evaluation of the VEGF to represent angiogenesis was considered more practical for this study.

No clinical study has correlated angiogenic and apoptotic activities in cancer cells. All of the studies on apoptosis and angiogenesis of STS so far were done separately. We attempt to do this and at the same time looking at the apoptotic and angiogenic activities in the tumor cell as well as endothelial cells of the blood vessels that supply the cancer cells.

CHAPTER TWO

Literature Review

2. LITERATURE REVIEW

2.1. General

Soft tissue tumors are defined as mesenchymal proliferations that occur in the extraskeletal, nonepithelial tissues of the body excluding the viscera, coverings of the brain and lymphoreticular system (Robbin and Cotran, 2004).

Soft tissue sarcoma (STS) is a heterogenous group of malignant tumor that is traditionally classified based on a histogenetic basis according to the adult tissue they recapitulate. The new WHO classification of Soft tissue tumors 2002 incorporates detailed clinical, histological and genetic data. According to the WHO classification 2002, soft tissue sarcomas are classified as follows:

Table 2.1: WHO Classification of Soft Tissue Sarcoma 2002.

No.	Group	Tumors
1.	Adipocytic tumors	Liposarcoma
2.	Fibroblastic/myofibroblastic tumors	Fibrosarcoma
3.	Fibrohistiocytic tumors	Malignant Fibrous Histiocytoma (MFH)
4.	Skeletal muscle tumors	Rhabdomyosarcoma
5.	Smooth muscle tumors	Leiomyosarcoma
6.	Vascular tumors	Angiosarcoma and

		epithelioid hemangioendothelioma
7.	Chondro-osseous tumors	Mesenchymal chondrosarcoma and extraskeletal osteosarcoma.
8.	Tumors of uncertain differentiation	Synovial sarcoma, epitheliod sarcoma, alveolar soft part sarcoma, clear cell sarcoma, extraskeletal myxoid chondrosarcoma, PNET/extraskeletal Ewing tumor, desmoplastic small round cell tumors, extrarenal rhabdoid tumor, malignant mesenchymoma, neoplasms with perivascular epithelioid cell differentiation (PEComa) and intimal sarcoma.

However, there is an evolving classification of soft tissue sarcoma. In the most recent WHO Classification, so-called pleomorphic MFH is no longer regarded as a definable tumor type, but instead is synonymous with undifferentiated pleomorphic sarcoma, a diagnosis of exclusion for no more than 5% of adult sarcomas. It is now widely agreed that these tumors show no evidence of true histiocytic differentiation.

Following the creation of the prototypical form of MFH, the storiform-pleomorphic variant and other MFH variants were generated. So-called myxoid MFH is now termed myxofibrosarcoma to underline the fibroblastic line of differentiation of this neoplasm while so-called angiomatoid

MFH, undoubtedly a discrete entity, is now reallocated to the category of tumors of uncertain differentiation.

Similarly, the overwhelming majority of lesions formerly labelled inflammatory MFH are nowadays recognized to be dedifferentiated liposarcoma as a result of recent molecular analysis of the 12q13-14 chromosome region in a large series of retroperitoneal 'inflammatory MFH'. Some of the cases diagnosed as such in the past actually represented lymphomas of both Hodgkin and non-Hodgkin types, leiomyosarcomas and sarcomatoid carcinomas. Besides that, most example of so-called giant cell MFH are re-classified as either giant cell-rich osteosarcomas, giant cell tumor (osteoclastoma) of soft tissue, leiomyosarcoma with an osteoclastic giant cell reaction or as giant cell-rich anaplastic carcinoma.

Currently, accurate subclassification of pleomorphic sarcomas is mandatory as it enables recognition of non-sarcomatous lesions as well as pleomorphic neoplasm not associated with aggressive behavior. Furthermore, as myogenic differentiation predicts aggressive clinical behavior among pleomorphic sarcomas, precise histotyping allows prognostic stratification of patients. (Dei Tos A.P. *et al.* & Fletcher C.D.M., 2006).

By convention, the soft tissue sarcomas also include the peripheral nervous system because tumors arising from nerves present as soft tissue masses and pose similar problem in differential diagnosis and therapy. Embryologically, soft tissue is derived principally from mesoderm, with

some contribution from neuroderm (Enzinger and Weiss'e *et al.*, 2008). Therefore in most of the soft tissue sarcoma studies, the MPNST was also included.

In general, there is only very slight preponderance of STS in males compare to females and more common in older patient (Enzinger and Weiss's *et al*., 2008). The mean age is 65 years. However, the age-related incidences vary; embryonal rhabdomyosarcoma occurs almost exclusively in children and synovial sarcoma develop in late adolescence and young adulthood, whereas pleomorphic high grade sarcoma and liposarcoma dominate in the elderly (WHO, 2004)

STS can occur in any anatomical location but three fourths are located in the extremities (most common in thigh) and 10% each in the trunk wall and retroperitoneum. Of the extremity and trunk, one-thirds is superficial with median diameter of 5cm and two-thirds are deep seated with a median diameter of 9cm. Retroperitoneal tumor are often much larger before they become symptomatic and one tenth of the patients have detectable metastases (most common in the lung) at diagnosis of the primary tumors. (WHO, 2004)

The pathogenesis of most soft tissue tumors is still unknown. Most of these cancers had no clearly defined cause but several infrequent predisposing factors have been described such as genetic predisposition (mainly Recklinghausen diseases and bilateral retinoblastoma) and iatrogenic factors such as post-irradiation and postoperative chronic lymphoedema (Levi.F. *et al.*, 1999). MFH and angiosarcoma are the most common histological subtype of radiation-induced

STS. Radiation induced STS are usually high grade and developed at the peripheral borders of radiation fields (Brady M.S. *et al.,* 1992). The Stewart-Treves syndrome represents about 5% of all angiosarcomas (Roy P. *et al.* 2004, Kirova Y.M. *et al.* 2007 and Harvey E.B. *et al.* 1985). Recent study shows that 2.8% of STS are related to genetic syndrome (Penel N. *et al.,* 2008). Recklinghausen neurofibromarosis and bilateral retinoblastoma predominate. About 5% of patient affected by Recklinghausen neurofibromatosis develop MPNST. In contrast, recent study by Penel N. *et al*. 2008 revealed that most cases of adult STS are not related to well-established risk factors (radiation, genetic deseases and chronic lymphedema). However, in this study the diagnosis of genetic syndromes were based on clinical criteria. A systematic genetic testing can possibly modify those results. (Penel N. *et al*., 2008).

The two most widely used grading system for STS are the NCI system (United States National Cancer Institute) and the FNCLCC system (French Federation Nationale des Centre de Lutte Contre le Cancer). Guillou L. *et al.* 1997 performed a comparative study on both systems which showed both systems were of good prognostic value although grade discrepancies were observed in 34% of the cases and the use of the FNCLCC resulted in a better correlation with overall and metastases-free survival. (Guillou L. *et al*., 1997). Besides that, the FNCLCC is more universally used because it is more precisely defined and reproducible. (Golouh R. *et al*, Bracko M. *et al*. 2001). For Paediatric sarcoma, a separate grading system has been developed and appears to be of clinical use (Parham D.M. *et a.* 1995).

Table 2.2: Paediatric Oncology Group Soft Tissu Sarcoma Grading System.

Grade I

Myxoid and well-differentiated liposarcoma

Deep seated dermatofibrosarcoma protuberans

Well-differentiated or infantile ($\leq$4 yr old) fibrosarcoma or hemangiopericytoma

Well-differentiated malignant peripheral nerves sheath tumor

Extraskeletal myxoid chondrosarcoma

Angiomatoid (malignant) fibrous histiocytoma

Grade II

Sarcomas not specifically included in grades I and II and in which < 15% of the surface area shows necrotic and the mitotic count is $\leq$5 per 10hpf using a 40x objective; as secondary criteria, nuclear atypia is not marked and the tumor is not markedly cellular

Grade III

Pleomorphic or round cell liposarcoma

Mesenchymal chondrosarcoma

Extraskeeletal osteosarcoma

Malignant triton tumor

Alveolar soft part sarcoma

Sarcomas not included in grade I and in which >15% of the surface area shows necrosis or the mitotic count is $\geq$5 mitoses per 10hpf using a 40x objective

Marked atypia or cellularity is less predictive but may assist in placing tumors in this category

The major staging system for STS was developed by the International Union against Cancer (UICC) and American Joint Committee on Cancer (AJCC). This TNM system incorporates histological grade, tumor size and depth, regional lymph nodes involvement and distant metastases appear to be clinically useful and prognostic value.

The rapid advances in the molecular genetics understanding of soft tissue sarcomas show distinctive cytogenetic aberrations, most often reciprocal chromosomes translocations which are relatively tumor specific and thus diagnostically useful. (Hahn H.P. *et al*. 2005 and Antonescu C R. *et al.,* 2006). The well-characterized chromosomal abnormalities in soft tissue sarcomas are shown in table 2.3. Unfortunately, many of the more common sarcomas (the high grade spindle cell and pleomorphic sarcomas of later adulthood) have complex karyotypes without histotype-specific features. (Mertens F. *et al*. and Fletcher C.D.M. *et al*., 1998). Overall, abnormal cell populations can be detected by either cytogenetics or DNA ploidy studies in about 85% of all soft tissue sarcomas. (Mohamed A.N. *et al*., 1997).

Table 2.3: Cytogenetic aberrations in soft tissue sarcomas.

Tumor type	Cytogenetic changes	Gene rearrangement
Ewing's sarcoma/ peripheral primitive neuroectodermal tumor	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(17;22)(q33;q12) t(2;22)(q33;q12)	FLI-1-EWSR1 ERG-EWSR1 ETV1-EWSR1 EIAF-EWSR1 FEV-EWSR1
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1;13)(p36;q14)	PAX3-FOXO1A PAX7-FOXO1A

Myxoid /round cell liposarcoma	t(12;16)(q13;q11) t(12;22)(q13;q11-12)	DDIT3-FUS DDIT3-EWSR1
Synovial sarcoma	t(X;18)(p11.2;q11.2)	SSX1-SYT SSX2-SYT
Dermatifibrosarcoma protuberans/ giant cell fibroblastoma	t(17;22)(q22;q13)	PDGFB-COL1A1
Infantile fibrosarcoma	t(12;15)(p13;q25)	ETV6-NTRK3
Alveolar soft part sarcoma	t(X;17)(p11;q25)	ASPL3FE-T
Low-grade fibromyxoid sarcoma	t(7;16)(q33;p11)	FUS-CREB3L2
Angiomatoid MFH		ATF-1 rearrangement
Atypical lipomatous tumor/ well-differentiated liposarcoma	12q rings & giant markers	HMGA(2),CDK4 and MDM2 amplication.

Many studies have been designed to investigate the prognostic factors of soft tissue sarcomas. The overall grade and stage (including size) of soft tissue sarcomas, however determined, are the most significant prognostic factors (Costa J. *et a.l* 1984, Guillou L. *et al.* 1997 and Parham D.M. *et al.,* 1995). Age of patient and assessment of the surgical margins are also important (Li X.Q. *et al.,* 1996). Patients who presented with positive margins or a margin of 2mm or less had a worse survival than patients who had margins of greater than 2mm and wide margin, 5year survival, 47% versus 70% and 72% (Novais *et al.* 2010). Study by S. Vraa *et al.* 1998 shows the 5year local recurrence rate was 18% and the 5year survival rate was 75%.

Some investigators have focused their studies on immunohistochemical method. Among them, reductions in the expression of E-cadherin and catenins, proteins that are essential for forming intercellular junctions, were reported as prognostifications of a poor outcome in patients with soft tissue sarcoma, implying that decreases in cell to cell adhesion may cause tumor cell dissemination (Heslin M.J. *et al*., 1998). Ch'ng E.S. *et al.* 2007 revealed that CD100 expression and tumor sizes ($\leq$5cm and >5cm) were independent prognostificators for overall survival and CD100 expression was an independent prognostificator for disease free survival. We are interested in elaborating on angiogenetic and apoptotic marker expression in soft tissue sarcoma for further study to associate with prognostification.

2.2 Angiogenesis and apoptosis

2.2.1 General

Angiogenesis, the development of new blood vessels from the endothelium of the existing vasculature, is a multistage process whereby the angiogenic endothelial cells must proliferate, produce molecules able to degrade the extracellular matrix, change their adhesive properties, migrate, avoid apoptosis and finally, differentiate to new vascular tubes. All these processes are controlled by the signals received by endothelial cells from their environment, signals whose transduction pathways form cascades leading to gene transcription and a network of cross-talks determining the final behaviour of the cell. The pathways leading to entry into the cell cycle depend on the angiogenic signals received (fig. 2.1). Among them, binding of VEGF to the endothelial-specific receptor VEGFR2 is the main extracellular signal triggering an angiogenic response which leads to proliferation of the endothelium.

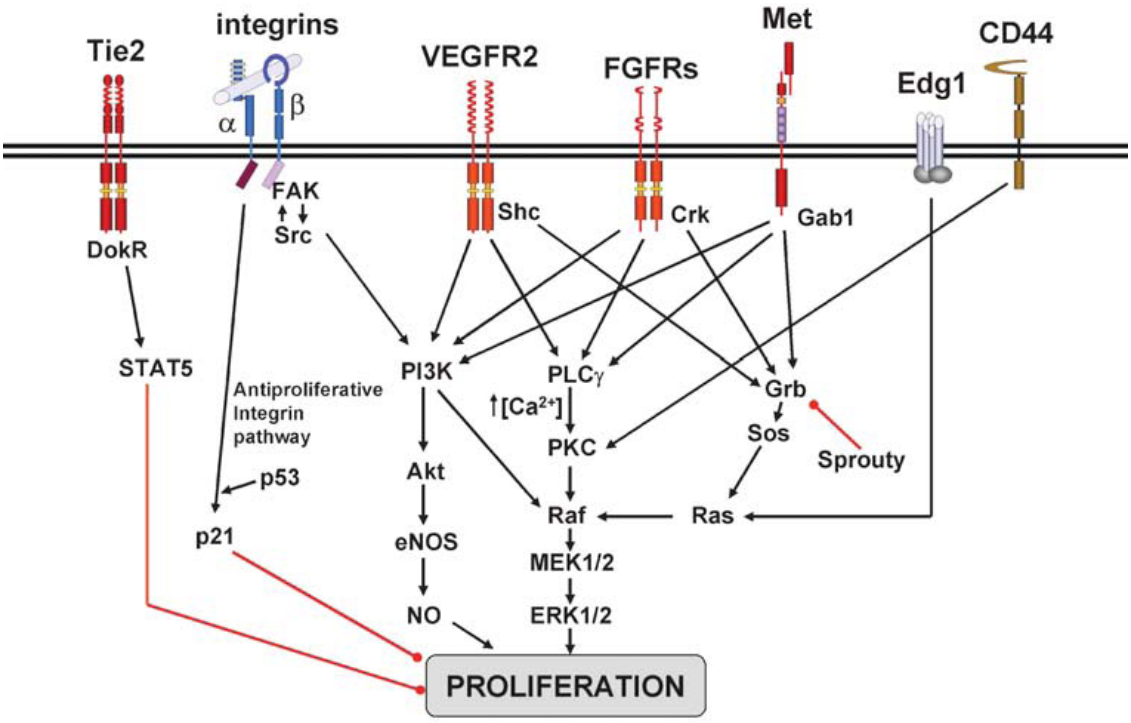

Figure 2.1: Main signaling pathways involved in proliferation of angiogenic endothelial cells.

Apoptosis is a normal process of programmed cell death involved in morphogenesis, vascular remodeling and elimination of neurons or cells from the immune system. Apoptosis seems to be essential in initiation of the angiogenic process (Nor J.E. *et al*., 1999). Caspases, a family of cysteine proteases, regulate apoptosis and it is controlled by apoptosis promoting and inhibitory factors (Adam J.M. *et al*., 2001). Angiogenic cells degrading the extracellular matrix should reinforce the mechanisms of apoptosis inhibition to avoid the risk of anoikis, i.e. apoptosis induced by lack of adhesion to the substrate (Frisch S.M. *et al*., 1997). For that reason, signals inducing endothelial cell migration must also promote cell survival. Endothelial cells repress their apoptogenic program through two main signaling pathways initiated from integrin-mediated attachment to the extracellular matrix and from survival factors such as VEGF and FGF2 (fig. 2.2).

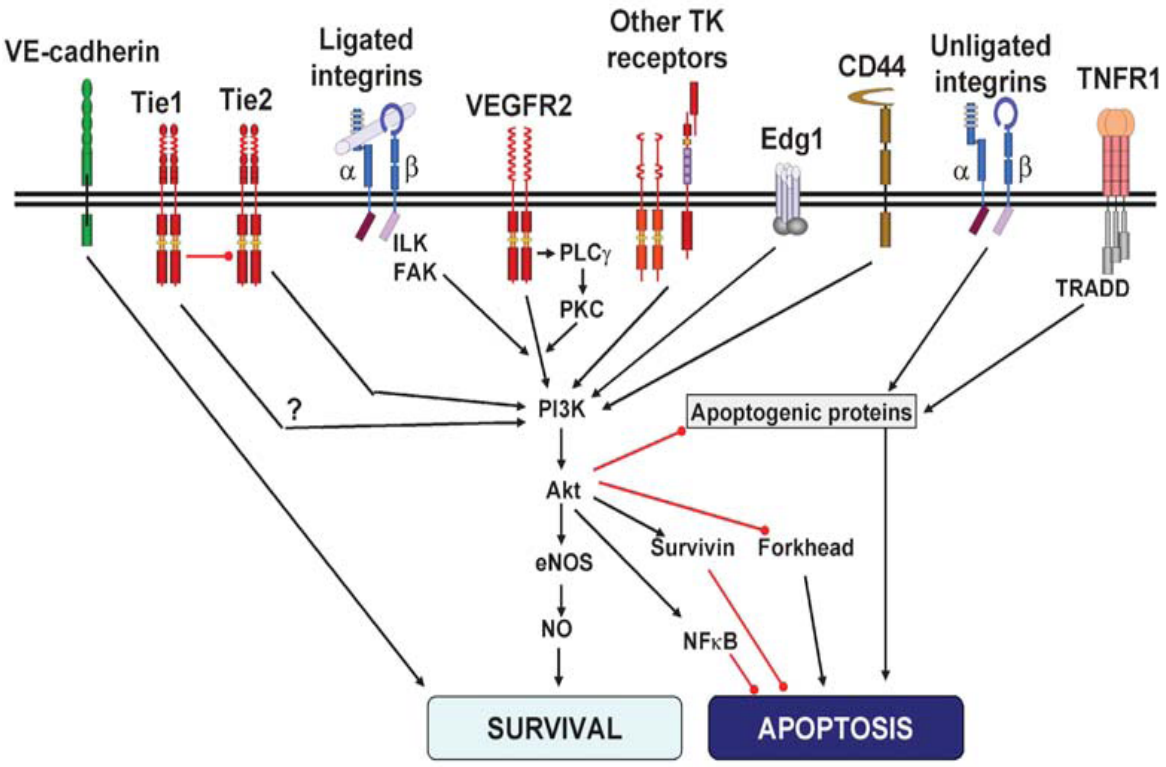

Figure 2.2: Main signaling pathways involved in survival of angiogenic endothelial cells.

Focal adhesions are specific areas of the cell surface where they are the origin of signals that activate or inhibit cellular processes such as proliferation, survival and migration of endothelial cells (fig. 2.3). The cytoskeletal changes associated to the formation of focal adhesions involve the development of stress fibers, lamellipodia and filopodia. These changes are mainly regulated by the members of the Ras superfamily of small GTPases RhoA, Rac and Cdc42. They control the cytoskeletal dynamics and also the cadherin function in endothelium (Van Wetering S. *et al.*, 2003). Rho and Rac are essential in the development of endothelial motility induced by VEGF (Soga Net *et al.*, 2001) and also control the mechanisms of polarization and migration in response to blood flow (Wojeiak-Stothard B. *et al.*, 2003).

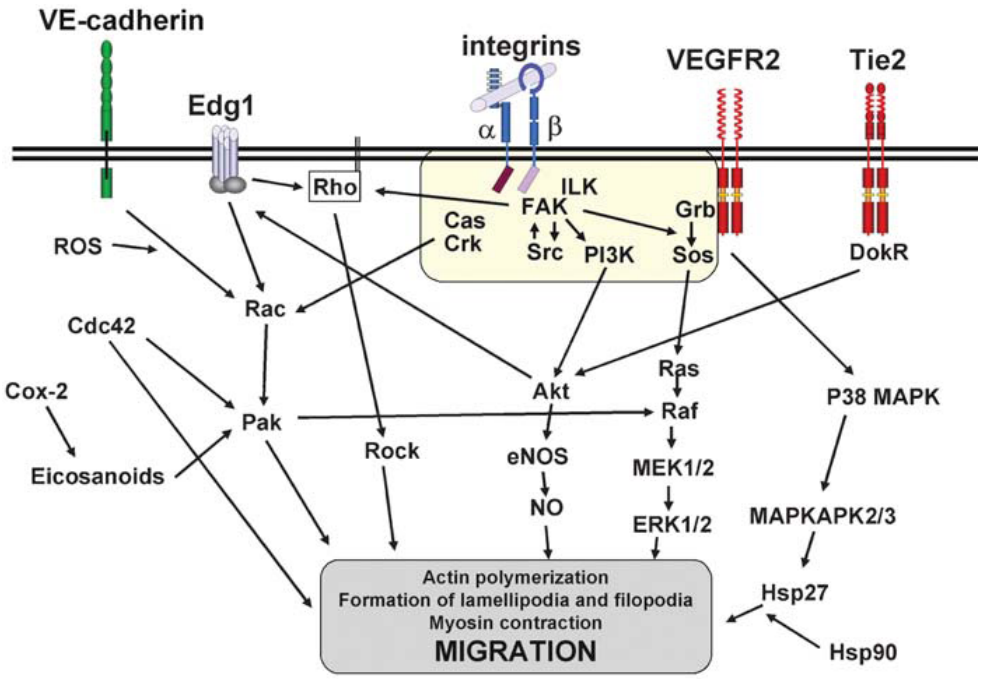

Figure 2.3: Main signaling pathways involved in migration of angiogenic endothelial cells. The yellow box represents the signaling complex recruited by integrins.

Migration of endothelial cells is finally achieved by controlled cell adhesion, cytoskeletal reorganization and localized degradation of the extracellular matrix. In fact, the degradation of the basal lamina of the endothelium is one of the earliest events in angiogenesis. Degradation of the extracellular matrix is performed by a proteolytic arsenal exquisitely regulated (figure 2.4).

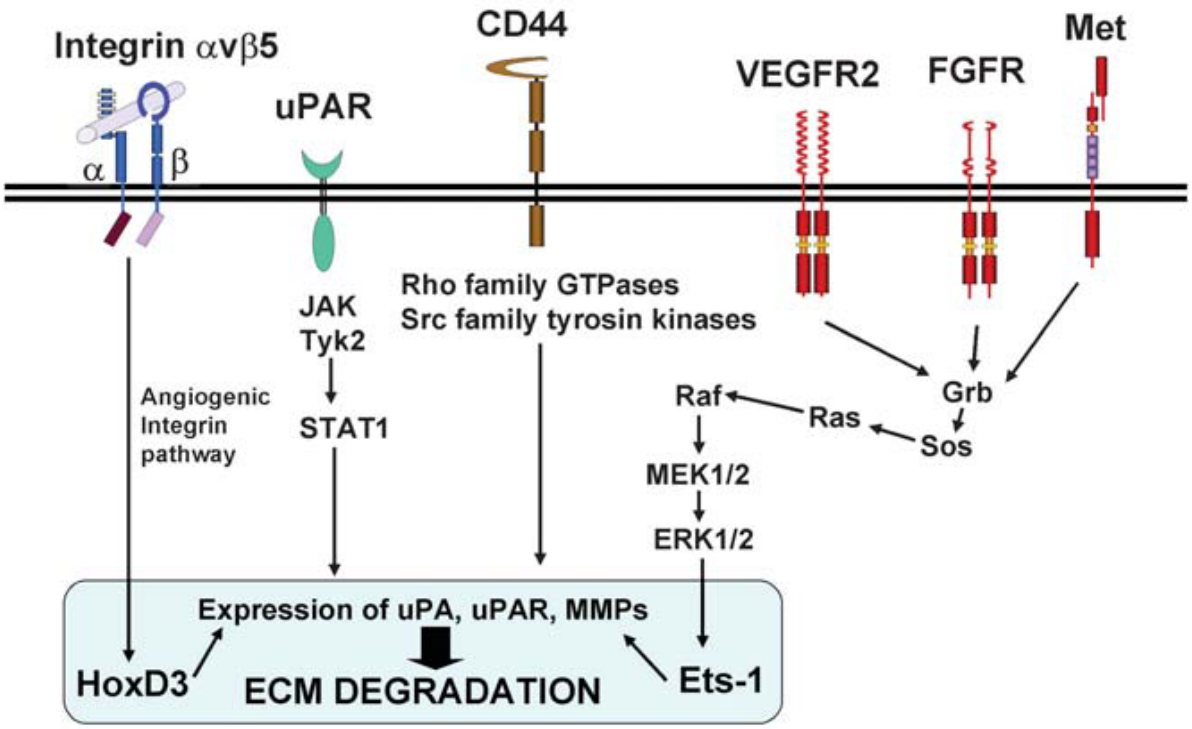

Figure 2.4: Main signaling pathways involved in extracellular matrix degradation induced by angiogenic endothelial cells.

After proliferation and migration, endothelial cells should be able to recover quiescence, produce a new basal lamina, recruit perivascular cells and form lumenized tubules (figure 2.5).

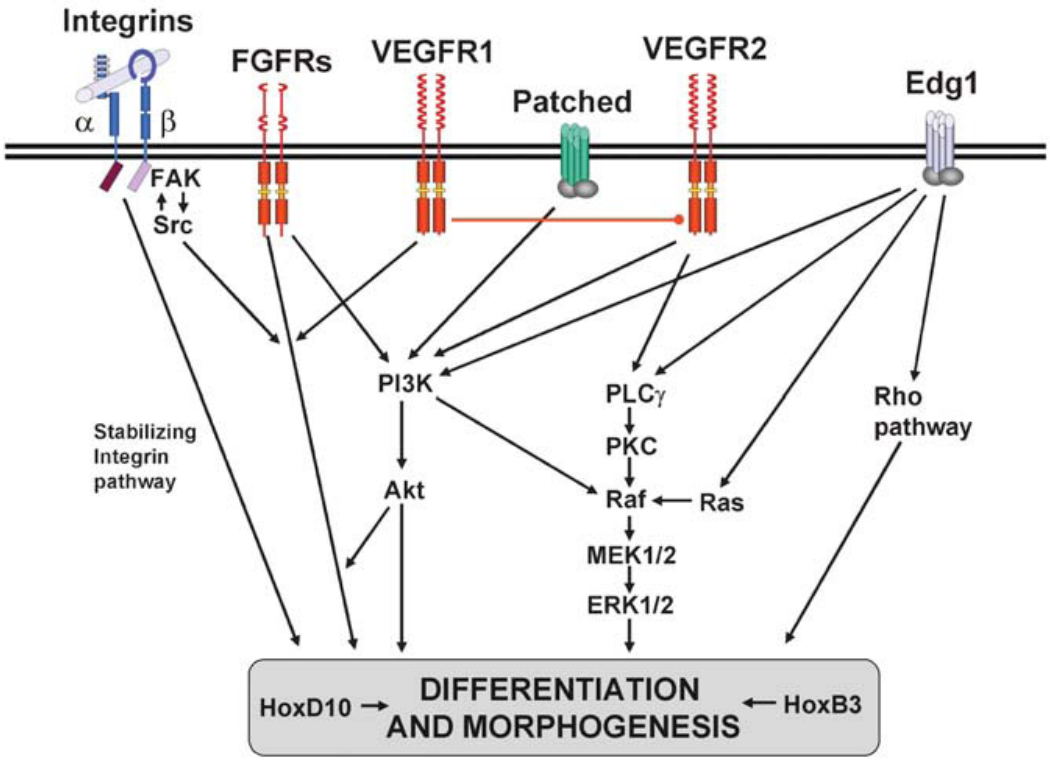

Figure 2.5: Main signaling pathways involved in differentiation and morphogenesis of angiogenic endothelial cells.

Despite the high specificity of the signaling system triggering an angiogenic response in endothelial cells, there are no specific signaling pathways or transcription factors regulating the entire angiogenic process or any of its stages (Sonein F. *et al.*, 1999). That means that regulation of endothelial behavior during angiogenesis is the result of a very complex network of intracellular signaling systems that trigger, control and terminate the process. This is expected from a process leading to a radical transformation in a cell type that is extraordinarily stable in the absence of the angiogenic signal. Knowledge of this network may be at least as necessary and useful as the knowledge of the extracellular signals which initiate angiogenesis in order to find angiogenic inhibitors or activators with therapeutic utility. It is important to emphasize that several angiogenic signaling pathways, such as the MAP kinases or the PI3K/Akt cascades, are shared with those leading to tumor cell proliferation and invasion. It should not be surprising that

future angiogenic inhibitors able to interfere with some intracellular signaling pathway also exhibit antitumoral activity.

2.3 Role in tumor development

Neovascularisation is a requirement for solid tumour growth beyond 1–2 mm in diameter (Folkman J. *et al*., 1991). Blood vessels play an important role in tumorigenesis supplying nutrients and oxygen, disposing of metabolic waste products and allowing metastatic spread. Tumour growth is dependent on the balance between increasing cell numbers through proliferation and decreasing numbers through apoptosis and necrosis (Holmgren L. *et al.*, 1995). Potentially a small genetically unstable hypoxic tumour exposed to a hostile environment will develop mutations eventually producing a phenotype able to resist apoptosis and therefore have a survival advantage (Harris A.L. *et al*., 1997).

The angiogenic process is a balance between stimulatory and inhibitory factors. A change that favours stimulation may trip an ‘angiogenic switch’ allowing the tumour to induce the formation of microvessels from the surrounding host vasculature (Hanahan *et al*., 1996). These regulatory factors may be endocrine, coming from the circulation, paracrine, from the adjacent tumour, stromal and inflammatory cells or extracellular matrix (ECM) and:or autocrine, from the endothelial cells themselves. Tumours promote angiogenesis by secreting or inducing the release of growth factors that stimulate endothelial cell migration and proliferation, proteolytic activity and capillary morphogenesis. There may be inhibition or loss of the synthesis of

antiangiogenic factors, such as thrombospondin-1, that suppress normal angiogenesis (Weinstat Saslow D.R. *et al*., 1994).

Apart from being spatially and temporally restricted, microvascular endothelial cells behave in a similar manner to invasive tumour cells. Hypoxia and hypoglycaemia induce local proangiogenic cytokines and growth factors leading to changes within endothelial cells. There is an increase in adhesion molecule expression and release of proteolytic enzymes. Plasminogen activators and collagenases create a gap in the basement membrane through which endothelial cells from post-capillary parent venules migrate into the perivascular stroma and form a capillary bud. Further proteolysis of the ECM produces chemotactic degradation products and releases bound growth factors. The capillary bud undergoes cell proliferation and sprout extension before maturation and lumen formation. The endothelial cells in these new vascular loops are abnormal in shape and size with wide cell junctions and an irregular, leaky basement membrane. These new vessels leak plasminogen, fibrinogen and platelets leading to extravascular fibrin deposition and hypercoaggulation. Inflammation plays an important part in tumorigenesis (Polverini P.J. *et al.*, 1984) and macrophage infiltration also correlates with angiogenesis (Leek R.D. *et al*., 1996).

Overall, growth factors play a crucial role in angiogenesis and malignancy (Fig. 2.6). Growth factors secreted by tumour, inflammatory and stromal cells bind to soluble growth factor receptors in the stroma (Hannekan A. *et al.*, 1995) where they can be released by proteolytic enzymes secreted by an invading tumour. Growth factors may be chemotactic and mitogenic, targeting kinase receptors expressed by endothelial and tumour cells. Tumour cell growth factor production will lead to self-proliferation and indirectly mediate growth by stimulating tumour

vessel proliferation. Several growth factors have been investigated in soft tissue sarcoma. This review will concentrate on the growth factors that have been studied most closely in relation to angiogenesis.

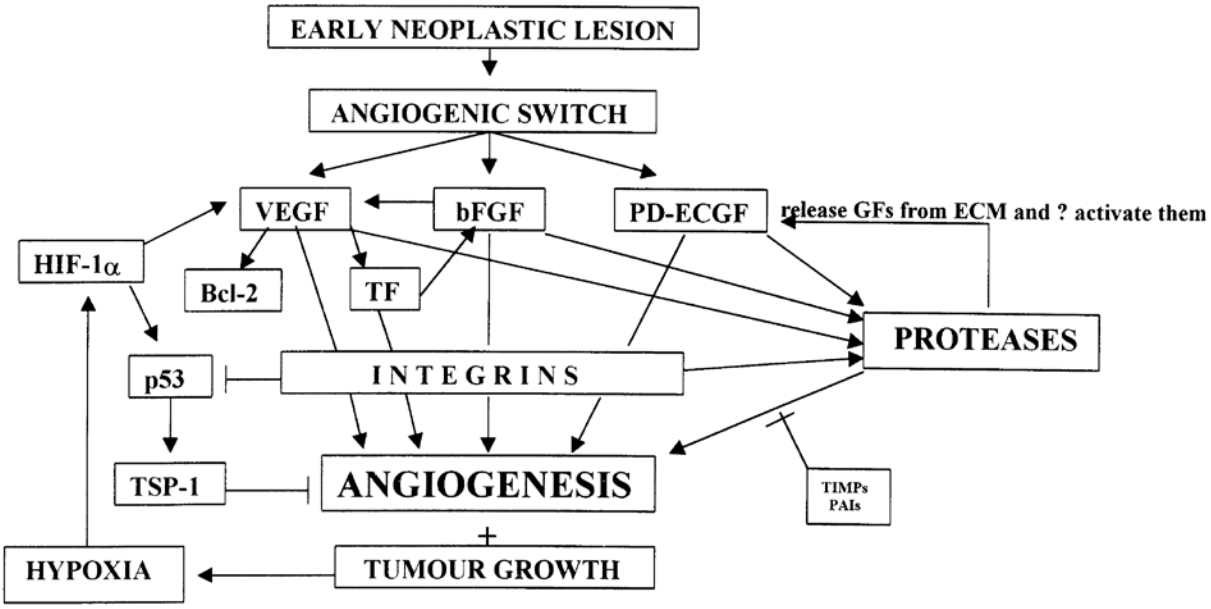

Figure 2.6: Growth factors and the induction of angiogenesis. GFs, growth factors; PAIs, plasminogen activator inhibitors; TSP-1, thrombospondin-1.

The VEGF family consists of VEGF, placental growth factor and VEGF-B, -C, -D and –E. VEGF binds with high affinity to two tyrosine kinase receptors, VEGFR-1 (Flt-1) (Hannekan A *et al*., 1995) and VEGFR-2 (Flk-1:KDR) (Terman B I *et al.*, 1992). VEGF is the most potent and specific growth factor for endothelial cells. Hypoxia is a common finding in tumours. Hypoxia increases VEGF expression (Shweiki D. *et al*., 1992) by inducing VEGF transcription via hypoxia-inducible factor-1a (Brigstock D.R. 2002) and by stabilizing VEGF mRNA (Levy A.P. *et al* 1996). Hypoxia also increases the synthesis of VEGF receptors (Walten Berger J. *et al.*, 1996). Wild-type p53 down-regulates VEGF promoter activity (Mukhopadhyay D *et al.*, 1995) whilst mutant p53 has the opposite effect (Keiser A *et al*., 1994). Vascular permeability is increased by VEGF allowing tumour dissemination into the circulation and the leakage of

plasma proteins and fibrin deposition into the ECM (Dvorak H.F. *et al*., 1995) that lead to activation of proteases and allows ECM degradation. Transfection of VEGF cDNA leads to an increase in tumour growth, vascularity and metastases (Senger D.R. *et al*., 1994-95) and this effect can be inhibited using VEGF antibodies (Kondo S et al 1993 and Zhang H.T. *et al*., 1995). The ability of tumour cells to produce VEGF may lead to resistance to apoptosis and therefore tip the balance in favour of proliferation and tumour growth.

VEGF tumour immunostaining is usually cytoplasmic especially in the perinuclear region. High levels of expression of VEGF and their receptors are found in many solid tumours including breast (Brown L.F. *et al*., 1993), colon (Brown L.F. *et al*., 1993), ovary (Olson T.A. *et al*., 1994), cervix (Guidi A.J. *et al.*, 1995), kidney (Brown L.F. *et al.*, 1993) and non-small cell lung carcinoma (Mattern J. *et al.*, 1995, Holm C. *et al.*, 1996, Onta Y. *et al*. 1996 and Takanami I. *et al.* 1987). There is diversity seen in VEGF expression amongst the various histologic subtype of STS whereby leiomyosarcoma, carcinosarcoma and MFH are more likely to reveal overexpression of VEGF than the other histologic subtype but no relationship between survival and VEGF status in any subtypes of STS, except leiomyosarcoma (Potti A. *et al.*, 2005). High intensity VEGF expression in soft tissue sarcomas are correlates with advanced tumor grade (Chao C. *et al*. 2001 and Pakos E.E. *et al.* 2005). Mutation of p53 is very frequently found in soft tissue sarcoma and this may also play a role in up-reulating VEGF (Zhang I. *et al*., 2000).

Neoplastic transformation is brought about through the activation of oncogenes or the inactivation of tumour suppressor genes. Lung cancer is associated with the expression of several

of these genes including p53, bcl-2, *myc*, *ras* and Rb (Sekido Y. *et al*., 1998). The rate of tumour growth is defined by the balance between cell proliferation and cell loss by necrosis and apoptosis (programmed cell death). Successful tumour therapies largely rely on an induction of endogenous apoptotic pathways (Hickman J.A. *et al*., 1995).

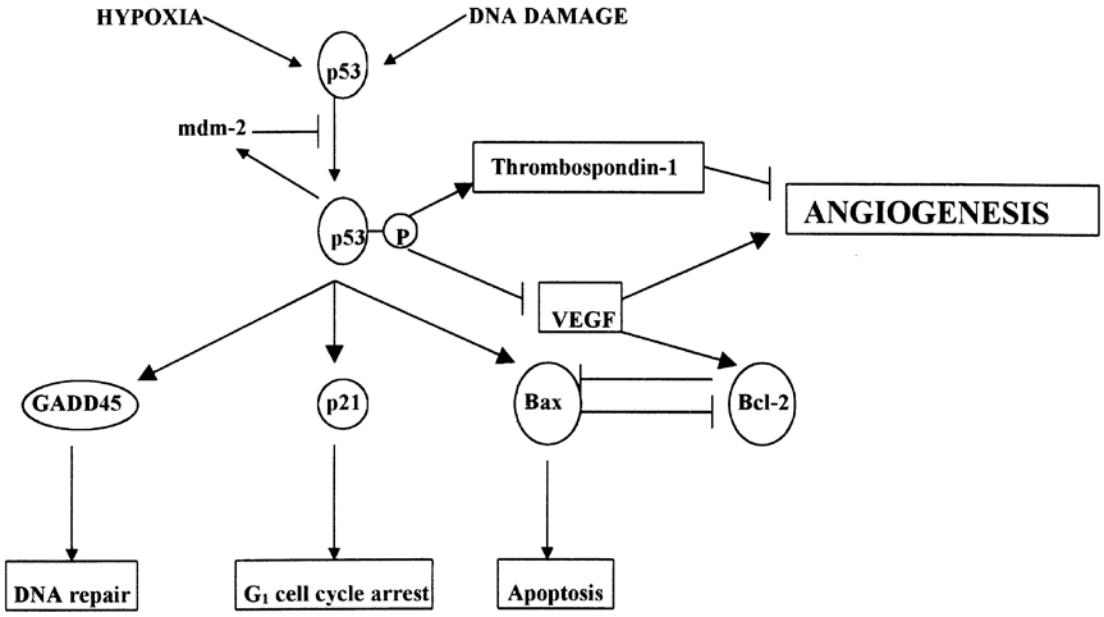

Figure 2.7: p53, bcl-2 and apoptosis. P53-P_phosphorylated p53.

Bcl-2 is a member of a family of genes involved in the regulation of apoptosis which includes proapoptotic and antiapoptotic proteins such as Bax proteins and Bcl-2 proteins respectively. The ratios of different family members seem to determine the survival or death of cells after apoptotic stimuli (Oltvai ZN *et al.*, 1993). The bcl-2 gene differs from conventional oncogenes as it can neither promote growth nor directly lead to cellular transformation. Raised bcl-2 levels protect from the induction of apoptosis by wild-type p53 (Chiou S.K. *et al*., 1994) whilst p53 down-regulates bcl-2 gene-expression (Miyashita T. *et al.*, 1994). The Bcl-2 expression was