Expression of Cell Adhesion, Invasion and Motility-associated Proteins in Retinoblastoma and the Study of Presence of Human Papillomavirus (HPV) in Retinoblastoma

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CERTIFICATE

This is to certify that the thesis entitled **Expression of Cell Adhesion, Invasion and Motility-associated proteins in Retinoblastoma and the study of presence of human papillomavirus (hpv) in retinoblastoma** submitted by **Ms.Adithi Mohan,** ID. No **2004PHXF017** for award of Ph. D. Degree of the Institute embodies original work done by her under my supervision.

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ABSTRACT

Retinoblastoma is a rare cancer of the eye that occurs in children. Most of the ~5000 cases of retinoblastoma diagnosed worldwide are children in developing countries, where advanced stage disease is common. There are limited therapeutic options for late-stage disease and eye salvage by enucleation is often the only option for these children. Predictably, metastatic disease is also more frequent in developing countries and the most important risk factors for metastatic disease are histopathological features such as invasion of the ocular coats and optic nerve of the eye. There is little information on the expression of genes that influence cell-cell adhesion, motility and/or invasiveness in retinoblastoma. The aim of my research has been to study the expression of some of these proteins in retinoblastoma and examine their correlation with invasiveness of the tumor.

Tumors that had invaded the local ocular structures such as the choroid, optic nerve and/or orbit expressed higher c-Src tyrosine kinase, phosphorylated (Active) Signal Transducer and Activator of Transcription 3 (pSTAT3), N-Cadherin, Epithelial Cell Adhesion Molecule (EpCAM), Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), Matrix Metalloproteinase (MMP)-2 and MMP-9, Tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2, T-cell Lymphoma Invasion and Metastasis 1 (Tiam1), inducible Nitric Oxide Synthase (iNOS) and nitrotyrosine (NT) than tumors that have not invaded these layers. The latter group of tumors (non-invasive) expressed higher E-Cadherin, α-Catenin and Motility-Related Protein-1 (MRP-1)/CD9 than invasive tumors. There were no differences in expression of β-catenin, endothelial NOS (eNOS) between the 2 groups of tumors. Rac1 and Cdc42 were expressed at infrequently in retinoblastoma. The changes in expression of tyrosine kinase and signal transduction molecules, cell-cell adhesion molecules, nitric oxide synthase molecule and extracellular matrix degrading molecules as tumors become more invasive suggest that these changes in expression may be associated with tumor invasiveness in retinoblastoma.

Correlative studies prove are useful to show an association between genes and a specific disease process, but to test whether tumors exhibit genetic changes in these pathways, we

carried out Fluorescent *In Situ* Hybridization (FISH) of the genes in the pathway that have shown genetic amplifications or deletions in other cancers. We anticipated that a genetic amplification or deletion would be selected for in invasive tumor cells compared to main mass of the tumor. Cadherin genes (E-cadherin, Cadherin-11, Cadherin-13 and Cadherin-15) on chromosome 16q showed genetic losses in >50% human tumors but of these, Cadherin-11 showed a genetic loss in invading human retinoblastoma cells. Western blot analysis of human and mouse retinoblastoma cell lines showed that cadherin expression was higher in Weri1 cells than Y79 cells and this correlates with their morphological characteristics. Our studies on cadherin expression in retinoblastoma cell lines also suggest that their expression may correlate with differences in adhesive properties (assessed by a cadherin-dependant cell-cell adhesion assay) of cells in culture. We are now testing the role of cadherin dependant cell-cell adhesion by using genetic approaches (cadherin-11 siRNA knockdown and cadherin-11 cDNA transfection) in the human tumor cell lines.

A second project was to determine whether Human Papillomaviruses (HPV) could be detected in Indian retinoblastoma. The rationale for this study is because a large proportion of patients in developing countries like Brazil and Mexico have sporadic retinoblastoma with an intact Rb1 gene, suggesting that alternative mechanisms of *Rb1* inactivation could play a role in the intiation of retinoblastoma. The HPV E7 protein is known to bind and inactivate pRb and the HPV subtypes 16 and 18 has been shown to be associated with retinoblastoma in Brazil and Mexico. We found that 47% (21/44) tumors were positive for HPV and that 57% (12/21) of these tumors were positive for HPV 16. HPV 18 was negative in all the tumors. Rb protein was absent in 71% (16/21) tumors that had HPV DNA. However, Rb was also absent in 86% (20/23) tumors that were HPV negative .There was no correlation of HPV positivty with Rb expression, clinical or histopathological features of the tumors. Further studies are required to determine whether the presence of HPV is required in the initiation of retinoblastoma.

Objectives of PhD

1. To study the expression of cell-cell adhesion, invasion and motility-related proteins in retinoblastoma and correlate with invasivness
2. To study the presence of Human Papillomavirus in Indian retinoblastoma

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ABBREVIATIONS

AMP	Acid Mucopolysaccharide
APC	Adenomatosis polyposis coli
ARFRP-1	Adp-Ribosylation Factor-Related Protein 1
BiAb	Bispecific Antibodies
BTB	*BR-C*, *ttk* and *bab*
CAS	Crk-associated substrate
cDNA	coding DNA
CNS	Central Nervous System
CSC	Cancer Stem Cell
Csk	C-Src tyrosine kinase
CT	Computed Tomography
CTGF	Connective Tissue Growth Factor
CXCR4	Chemokine Receptor 4
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
E-Cadherin	Epithelial Cadherin
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
EMT	Epithelial Mesenchymal Transition
eNOS	Endothelial Nitric Oxide Synthase
EpCAM	Epithelial Cell Adhesion Molecule
ERK	Extracellular signal-regulated kinase
FAK	Focal Adhesion Kinase
FISH	Fluorescent *In Situ* Hybridization
GAP	GTP Activating Protein
GDP	Guanosine Di Phosphate
GEF	GTP Exchange Factor
GSK-3β	Glycogen Synthase Kinase 3-Beta
GTP	Guanosine Tri Phosphate
HCl	Hydrogen Chloride
HPV	Human Papillomavirus
IL11	Interleukin 11
iNOS	Inducible Nitric Oxide Synthase
JNK	c-Jun N-terminal kinase
KCl	Potassium Chloride
MBII	Myc BoX II
MDM2	Mouse Double Minute 2
MDMX	Mouse Double Minute 4
MEK	Mitogen Activated Protein Kinase
$MgCl_2$	Magnesium Chloride
MLC	Myosin Light Chain
mM	milliMole
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
MRP-1/CD9	Motility-Related Protein-1/Cell Differentiation 9

N-Cadherin	Neuronal Cadherin
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NT	Nitrotyrosine
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol 3-Kinase
POZ	Pox virus and Zinc finger
pSTAT3	Phosphorylated Signal Transducer and Activator of Transcription 3
Rb	Retinoblastoma
siRNA	Short Interfering RNA
TAA	Tumor Associated Antigen
TCF	T-Cell Factor
Tiam1	T-cell Lymphoma Invasion and Metastasis 1
TIMP	Tissue Inhibitor of Metalloproteinase
uPAR	Urokinase Plasminogen Activator Receptor
UV	UltraViolet
VEGF	Vascular Endothelial Growth Factor
WASP	Wiskott-Aldrich Syndrome Protein
WISP	Wnt1-Inducible Signaling Pathway Protein

INTRODUCTION

Chapter 1

1.1. Tumor Initiation and Progression: Theories and Definitions

1.1.1 . Metastasis: Definition of terms

The formation of a tumor is a complex process and the transformation of a normal cell into increasingly neoplastic variants is called tumor progression. These changes involve both the genetic alterations i.e., the activation of oncogenes and the inactivation of tumor suppressor genes and epigenetic alterations, both of which affect multiple distinct regulatory circuits and function in a complementary fashion within a cell to create a neoplastic phenotype. Some of the earliest traits that enable tumor formation include replicative self-sufficiency, insensitivity to antiproliferative signals and resistance to apoptosis (Weinberg 2007b).

While these tumorogenic events are essential, they might not specifically mediate the steps of tumor dissemination and colonization of distant organs. These events collectively describe metastasis, or the spread of tumor cells from their original niche to distant tissue or organs. Metastasis accounts for 90% death in cancer patients (Kopfstein and Christofori 2006a). Metastasis includes several unique biological events such as loss of cellular adhesion, increased motility and invasiveness, intravasation (entry and survival in circulation), extravasation (exit into new tissue) and eventual colonization of a distant site to establish a metastatic focus (Chambers et al., 2002). In a recent review by Nguyen and Massague, a clear definition of different classes of metastasis genes is provided (Nguyen and Massague 2007). Genes that provide an advantage in a primary tumor such as cell motility, invasion and angiogenesis are *metastasis initiation* genes. Two good examples for the search of such 'necessary but not metastasis-sufficient' genes, would be those that mediate invasiveness of gliomas or vascularity of adenomas, as neither tumor metastasizes to other organs. *Metastasis progression genes* are defined as those that fulfil certain rate-limiting functions in primary tumor growth and other specific function in metastatic colonization. When expression or activity of metastasis progression genes is restricted to a particular target organ, they can be found within gene expression signatures that correlate primary tumors with the risk of organ-specific dissemination. The third

class of *metastasis virulence genes* provide selective advantages in the secondary site but not primary tumor and hence can be considered to complement the aggressiveness of metastatic tumor cells. As their altered expression or activity is at the secondary site, these genes would rarely be present in 'poor prognosis' gene expression signatures, i.e. the molecular signature defined to successfully predict poor prognosis for patients due to the metastatic potential of solid tumors (Nguyen and Massague 2007).

Work in the last several years has focused largely on the molecular basis of tumor initiation and the steps that cancer cells take toward metastasis has received less attention. The inherent difficulty to observe metastasis, a process that occurs within the body, it's ability to emerge after a long period of latency after primary tumor removal, such as in breast cancer and it's sudden regression, such as in paediatric neuroblastomas, are some of the clinical challenges in the field of metastasis research (Chambers et al., 2002; Nguyen and Massague 2007). I will provide a brief review of the theories and current concepts in the field of cancer metastasis.

1.1.2 . Metastasis Theories: Seeds, soils and signatures

One of the first theories in the field of metastasis came from clinical observations of tumor spread. For example, breast cancer metastasizes frequently to the bone and lungs while patients with colorectal cancer often develop initial metastases in the liver. In 1889, Stephen Paget proposed the 'Seed and Soil' hypothesis based the propensity of different types of cancers to metastasize to specific target organs. Hence, he proposed that tumor 'seeds' will arrest and proliferate exclusively in congenial 'soil' (the secondary organ) (Chambers et al., 2002). This theory was challenged by James Ewing in 1926 who postulated that metastatic propensities are dictated primarily by circulatory patterns – i.e. tumors cells are most likely to metastasize to organs that they have access through a vascular supply. Over the years, analyses of patient samples and experimental metastasis assays have shown that though circulatory patterns influence regional recurrences, distant metastases was more likely to be non-random, with no correlation to anatomically-defined haematogenous or lymphatic circulation patterns (Chambers et al., 2002; Gupta

et al., 2005).

The next big questions came from the identification that metastasis comprises several distinct steps (invasion, intravasation, survival in circulation, extravasation and growth in a secondary site) and that a tumors' success to metastasize depended on the fulfilment of all these events. Are there distinct molecular mediators of these various processes? What is the probability of these events occurring in a single cell or a clone of cells?

The observation that multiple genetic alterations were present in cancer cells strongly indicated that those alterations may accumulate in a stepwise manner during tumor progression. To prove this assumption, comparative analyses of genetic alteration in early and late stage tumors have been performed in a number of cancers, and it is seen that latter group typically harbor more alterations than the former group (Yokota 2000). One of the best genetic models of tumor progression is colon cancer and the identification of the sequential genetic changes associated with the transitions from normal colonic epithelium to colonic biopsies of different degrees of abnormalities, ranging from mildly deviant to aggressively malignant carcinoma. In landmark work, researchers discovered that about 90% early-stage adenomas showed loss of heterozygosity in the long arm of Chromosome 5 (i.e. 5q, and later the gene in this region was identified as adenomatous polyposis coli APC), and almost half of slightly larger adenomas had an additional K-ras mutation. About 60% of even larger adenomas tended to show Loss of Heterozygosity (LOH) on the long arm of chromosome 18 and about 50-70% of carcinomas showed LOH on the short arm of chromosome 17 (i.e. 17p, harbouring the Tp53 gene). Further, about 12% carcinomas have functional inactivation of the type-2 TGF-β receptor and that tumors bearing K-ras oncogenes rarely have mutant p53 alleles and vice versa. With the development of more sophisticated and sensitive tools to analysing tumor cell genomes, it is now known that that the series of genetic events in colon cancer progression does not represent an invariant sequence that defines the genetic paths followed by all colon carcinomas, but that they initiate with the loss of APC gene function and that the subsequent changes and precise order of the genes that are altered during progression are variable (Weinberg 2007b).

Applying Darwin's principles of natural selection, researchers explained that tumor progression could be viewed as a form of Darwinian evolution, with somatic evolution of a genetically diverse cancer-cell population under the selective pressures of an environment mediating the transition between progressive tumor stages (Gupta and Massague 2006; Nguyen and Massague 2007).

With the sequencing of the human genome and other technological advances in the 1990s, an important application to studying metastases was the classification of tumors based on genome-wide analyses. Gene expression profiling predicted 'poor' or 'good' prognosis in haematological malignancies (and later, solid tumors), hence further supporting that metastatic predisposition of tumors might already be fulfilled by traits of a primary malignancy. If pre-determined by selection at the primary tumor site, do additional changes by rare variants have only a complementary role? Or put slightly differently, how does metastasis depend on the acquisition of further genetic abnormalities beyond those that turn an incipient tumor to a locally aggressive malignancy?(Nguyen and Massague 2007)

A view that would solely depend on pre-determined traits would be inconsistent with the following observations. First, genes that are exclusively expressed by metastatic but not primary tumor cells and mediate events exclusive to metastasis and not primary tumorigenicity, have been identified in bone metastasis. Second, clinical literature has documented several instances where disseminated tumor cells lie dormant for years, before giving rise to overt metastases, suggesting that additional changes in the tumor cells and/or the niche of the secondary tumor site may have a role. This leads us to another question as to whether overt metastases arises from a progenitor pool present in early seeding at the secondary site, or from a later dissemination of cells acquiring their aggressiveness at the primary tumor. Which ever the answer may be, this would explain why cells derived from metastases have repeatedly been shown to have higher metastatic ability than the bulk population of a primary tumors (Gupta and Massague 2006; Nguyen and Massague 2007) which has lead to the proposal that metastases arise from rare variants within a primary tumor. However, as isolation of highly metastatic variants is by

in vivo selection or by culture, there is lack of definitive proof that this occurs naturally (Hynes 2003).

One study presents results that help to accommodate both views (Kang et al., 2003). Using a breast cancer cell line derived from a patient with metastatic breast cancer, MDA-MB-231, the authors obtained variants that metastasized exclusively to the bone and adrenal medulla by in vivo selection in immunodeficient mice. Array analyses of these variants also defined a signature (102 genes) that correlated well with potential for bone metastases. When the authors applied the pattern of 'poor prognosis' signature defined by an earlier study of human breast cancer samples (van't Veer et al., 2002) all the isolates, either of low or highly metastatic ability, fitted in with the poor prognosis signature. Hence the starting cell culture had already undergone a metastatic transition, but organ-specific metastases required an additional signature overlaid on the poor prognosis signature. They further showed that the 4 of the most highly over expressed genes of the bone metastases signature (IL11, CTGF, CXCR4 and MMP1), when co-expressed in different doublet combinations along with Osteopontin (a gene over-expressed in both bone and adrenal metastases) in parental MDA-MB-231 cells, endowed cells with a metastatic activity close to that of the highly aggressive cell populations endogenously expressing the entire bone metastatic gene set. This further showed that these genes were causally involved in metastases to the bone (Hynes 2003; Kang et al., 2003).

To test whether these highly metastatic variants were present in the bulk population of the original breast cancer cell line, the authors generated subclones in vitro with no selection and analyzed them for the levels of expression of the five genes from the earlier experiment. They found that variants expressing 4 or 5 genes were highly metastatic while those expressing 3 or fewer were metastatic to a lower degree and those expressing 1 or none were less metastatic than the bulk population. Therefore, the parental population did contain variants of low or high metastatic potential that globally expressed the poor prognosis signature and had specific variants which correlated with a tissue-specific (in this case, the bone) metastasis. Hence, this study combines features of both

models. However, the order in which these 2 events occur is unclear and may also differ in their relative importance among different tumors (Hynes 2003; Kang et al., 2003).

1.1.3 . Stem cells, Cancer Stem Cells (CSC) and the CSC Theory in Metastasis

In recent years, the discoveries of stem cells, and further demonstration of cancer stem cells, have also prompted theories of their contribution to cancer progression. A stem cell is defined by it's ability to proliferate, self-renew and most importantly, a feature that distinguishes it from progenitor cells in the same tissue, is it's ability to retain competence over time. Competence is defined as the ability of a single cell to produce all differentiated cell types of that tissue. Stem cells have been shown to be expressed in various tissues and their physiological functions to self-renew enables tissue homeostasis as well as to regenerate new cells after tissue injury. Several studies have also shown the expression and function of stem cells in cancer tissue, and these have been termed cancer stem cells (CSCs). Applying the same principles, CSC are defined by their enriched capacity to regenerate cancers using xenograft mouse models (and additionally, to re-capitulate features of the heterogenous tumor they were derived from) and to self-renew in serial transplantation assays. The questions regarding the origin of these cells – whether they arise from normal stem cells that have undergone neoplastic transformation, whether they arise from progenitor cells that have transformed and regained the ability to self-renew or thirdly, whether they arise from rare fusion events between stem and other cells – have been the subject of much debate. It is also possible that these theories may not be mutually exclusive (Corson and Gallie 2007).

The cancer stem cells theory seems to fit in well with some of the yet unexplained concepts of metastasis such as their ability to remain quiescent and be re-activated by secondary factors of the secondary 'niche', to generate a metastatic lesion after a period of dormancy. Secondly, since only few CSC are needed to initiate the metastasis, this could help reconcile that cancer cells can be detected in distant sites long before any detectable dissemination occurs at the primary tumors. Additionally, metastatic CSC maintain most of the genetic program acquired at the primary tumor site through self-

renewal but also evolve independently at the secondary site so that they can become resistant to treatments that are effective against primary tumors. Hence, there is not doubt that better understanding of the contribution of cancer stem cells to the metastatic cascade may help design better targeted therapies for tumor progression (Corson and Gallie 2007).

1.2. **Overview of Retinoblastoma**

1.2.1. Retinoblastoma – clinical information

Retinoblastoma is a malignant tumor of the immature retina and affects ~1:17,000 to 1:34,000 live births (Ellsworth 1969). Though a rare disease, it is the most frequent primary eye cancer in children under 15 years of age and accounts for 1% of all cancer-related deaths (Philip A.Pizzo and G.Poplack. 1993). Retinoblastoma occurs from loss or mutations of both alleles of the retinoblastoma gene (*Rb1*).

When left untreated, retinoblastoma is almost always fatal. Arising from a retinal progenitor cell of the inner nuclear layer of the retina, it grows and produces seeding in the eye, leading to retinal detachment, necrosis and invasion of ocular structures such as the choroid, optic nerve and orbit. These features are important risk factors for metastasis, or the spread of malignant cells to other parts of the body, which are most commonly the brain , bone marrow and lungs. Almost all untreated patients die of intracranial extension and disseminated disease within two years (Ellsworth 1969; Leal-Leal et al., 2006; Melamud et al., 2006).

Traditionally, patients with retinoblastoma underwent enucleation, or removal of the affected eye, as an approach to prevent metastasis and save life. Over the past century, significant advances in screening and treatment have lead to most children being cured of the primary cancer, particularly in developed countries. The most important recent advance in the management of retinoblastoma is the use of intravenous chemotherapy for tumor reduction, a technique of neoadjuvant chemotherapy termed "chemoreduction." This is followed by tumor consolidation with focal measures such as thermotherapy, cryotherapy, and plaque radiotherapy. This strategy provides reduced tumor volume and often permits consolidation with methods other than external beam radiotherapy. The shift in management is related to earlier detection of the disease, recognition of more effective chemotherapeutic agents, more focused local treatment modalities, and, most importantly, knowledge of the long-term risks of external beam radiotherapy (Shields and Shields 1999; Shields et al., 2004).

1.2.2. Retinoblastoma: Incidence, Heredity and Genetics

Early diagnosis is crucial in saving life and vision, and older children tend to have a worse prognosis, which is related to the fact that their tumors are diagnosed at a more advanced stage (Sanders et al., 1988; Erwenne and Franco 1989). Most children with retinoblastoma are diagnosed before the age of 3 years. Onset after five years of age is rare, but retinoblastoma has been reported in older children (Bovenmyer 1967) and adults (Mietz et al., 1997).

The retinoblastoma gene is a tumor suppressor gene, located on the long arm of chromosome 13 at region 14, that codes for the RB protein. Disease occurs from any mutation that inactivates both normal alleles. Approximately 70% of affected children have sporadic disease i.e, they are the only affected members of otherwise unaffected families. In these patients, both *Rb1* alleles are inactivated somatically in a single developing retinoblast, resulting mostly in unilateral disease, where one eye is affected. The remaining 30-40% of children have familial or heritable retinoblastoma and carry an *Rb1* inactivation in their germline. Subsequently, the loss or mutation of the second allele usually results in bilateral tumor development, where both eyes are affected. While a small proportion of children with familial disease develop unilateral tumors as a result of low penetrant mutations that partially disrupt *Rb1* function (Harbour 2001), some (~15%) children with sporadic disease can also develop bilateral tumors, which are most likely due to the early appearance of a somatic mutation in development resulting in mosaicism (Lohmann et al., 1997; Corson and Gallie 2007). Children with bilateral retinoblastoma tend to be younger (average age is 12 months) than children with unilateral retinoblastoma (about 24 months) (Sanders et al., 1988; Abramson and Servodidio 1992).

1.2.3. Retinoblastoma in Developing Countries:

1.2.3.1.Retinoblastoma in India - incidence, stage of presentation, and limited therapeutic options for late-stage disease

Most of the ~5000 cases of retinoblastoma diagnosed worldwide are children in

developing (low-income) countries, where the probability of their survival is lower than that of children in developed countries (Chantada et al., 1999; Chang et al., 2006; Ozdemir et al., 2007). This is mostly due to a low rate of early disease detection, limited resources of healthcare facilities and limited options for late-stage disease. With limited therapeutic options for late-stage disease, eye salvage by enucleation is often the only option for these children.

Diagnosis at a later age seems to be a common finding among developing countries (Senft et al., 1988; Gunalp et al., 1996; Leal-Leal et al., 2004). For example, the mean age of diagnosis of children with retinoblastoma in U.S.A is 14.6 months for bilateral cases and 23.5 months for unilateral cases (Augsburger et al., 1995). In India, the average age of presentation ranges from ~24 months to 41 months (Dhir et al., 1980; Schultz et al., 1993; Sahu et al., 1998; Shanmugam et al., 2005). This discrepancy may possibly be due to that the study by Sahu et al. has not been conducted in an ophthalmic hospital but a referral cancer hospital, where children are referred to a later stage of disease after primary treatment by an ophthalmologist. Later age was mostly associated with advanced stage disease at presentation - 74.5% patients had Reese Ellsworth Group IV and V disease in one series (Sahu et al., 1998), and 68% with Reese Ellsworth Stage V disease from another series (Shanmugam et al., 2005) and these include features such as multiple, large tumors and vitreous seeding. Predictably, the incidence of metastasis is also higher in developing countries, with frequencies ranging from 9% to 11%.

The management of advanced stage disease continues to be a challenge, with external beam radiotherapy and enculeation often employed in addition to chemoreduction, to save the child's life. In the study by Shanmugam et al., (Shanmugam et al., 2005), a total 21.6% (60 of 278 eyes) could be salvaged and the remaining 78.4% eyes were lost either due to primary enucleation and exeneration or enculeation due to conservative treatment failure. In another study at a tertiary eye care enter in India, enucleation due to tumors were 49% (74 of 150 patients) of the total cohort and retinoblastoma accounted for 74% (55 of 74) of these cases, further supporting the high incidence of late-stage disease in this population. Histopathologic analyses also reveal a higher incidence of choroidal and

optic nerve invasion of the enucleated eyes (Vemuganti et al., 2001; Biswas et al., 2003).

In India, the incidence of tumors with choroidal and optic nerve invasion, which are the high-risk factors for retinoblastoma, are higher than developed countries (Vemuganti and Honavar 2000; Biswas et al., 2003). Also, there is some indications that genetic background and modifier genes in the Asian population may also play a role in determining the disease phenotype and influence tumor aggressiveness (Pan et al., 2005). There is not much information on the expression of genes that influence cell-cell adhesion, motility and/or invasiveness in retinoblastoma. Hence, the first part of my research has been to study the expression of some of these proteins in retinoblastoma and examine their correlation with invasiveness of the tumor.

1.2.3.2. Human Papillomavirus in Retnoblastoma: An alternative mechanism for tumor development

Most of the cases of retinoblastoma from developing countries are in patients with no family history. There have been a few studies that show that 17 to 81% (Shimizu et al., 1994; Blanquet et al., 1995) of these non-familial cases have an intact *Rb1* gene and have suggested that an alternative mechanism of pRB inactivaton may exist. It is reasonably well known that there is a considerable overlap between epidemiological risk factors for retinoblastoma and HPV infection. The HPV E7 protein is known to bind and inactivate pRB (Dyson et al., 1989). Transgenic HPV16 mice overepressing E6 and E7 oncoproteins posses and intact retinoblastoma gene, but the ectopic expression of E6 and E7 in the retina leads to pRB inactivation and the development of retinoblastoma-like lesions (Albert et al., 1994). Recent studies from Mexico (Orjuela et al., 2000; Montoya-Fuentes et al., 2003) and Brazil (Palazzi et al., 2003) have shown an association between the presence of human papillomavirus and sporadic retinoblastoma suggesting that pRB may be inactivated by the HPV E6 and E7 oncoproteins in retinoblastoma that initiates in this country. There is no information available on the role of HPV in retinoblastoma in India and the second part of my research was to test for the presence of HPV DNA from retinoblastoma tumor tissue.

LITERATURE REVIEW

Chapter 2

2.1 Risk Factors for Retinoblastoma

By definition, a risk factor is a feature or characteristic of persons in the general population that is strongly associated with the disease of interest. Although risk factors are strongly associated with the disease, they are not necessarily causative of the disease (Augsburger 2003).

Over the years, several risk factors have been identified for retinoblastoma. These include: age at diagnosis and treatment, positive family history of retinoblastoma, deletion or inactivation of one allele of the retinoblastoma gene, laterality of disease and local invasion of the tumor (Finger et al., 2002a; Augsburger 2003). Looking at the factors that predict to metastasis, the most important predictors are invasion of the tumor into the uvea, orbit and optic nerve (Shields et al., 1994; Khelfaoui et al., 1996; Finger et al., 2002b). Bilaterality and delay in diagnosis are also important risk factors for metastatic disease (Finger et al., 2002a). Ethnicity and race have been proposed as risk factors by some investigators. In the US, higher incidence of retinoblastoma is somewhat higher among blacks compared to whites (Stiller and Parkin 1996). Higher incidences have also been reported in less affluent populations such as Africa (Stiller and Parkin 1996) and among the Asian versus non-Asian population in the U.K. (Muir et al., 1992). Older paternal age (DerKinderen et al., 1990; Moll et al., 1996; Sivakumaran et al., 2000), lower maternal intake of micronutrients during pregnancy (Orjuela et al., 2005) and paternal metal exposure (Bunin et al., 1990), radiation exposure (Hicks et al., 1984a) have been associated with sporadic heritable retinoblastoma, though these associations have been reported by a few studies and hence require further confirmation.

As the basis of this thesis is the expression of different molecules associated with histopathologic parameters, this chapter will summarize histopathological risk factors for retinoblastoma. Figure 2 shows the layers of the retina and choroid, the 2 layers that retinoblastoma grows into before invading extra-ocularly.

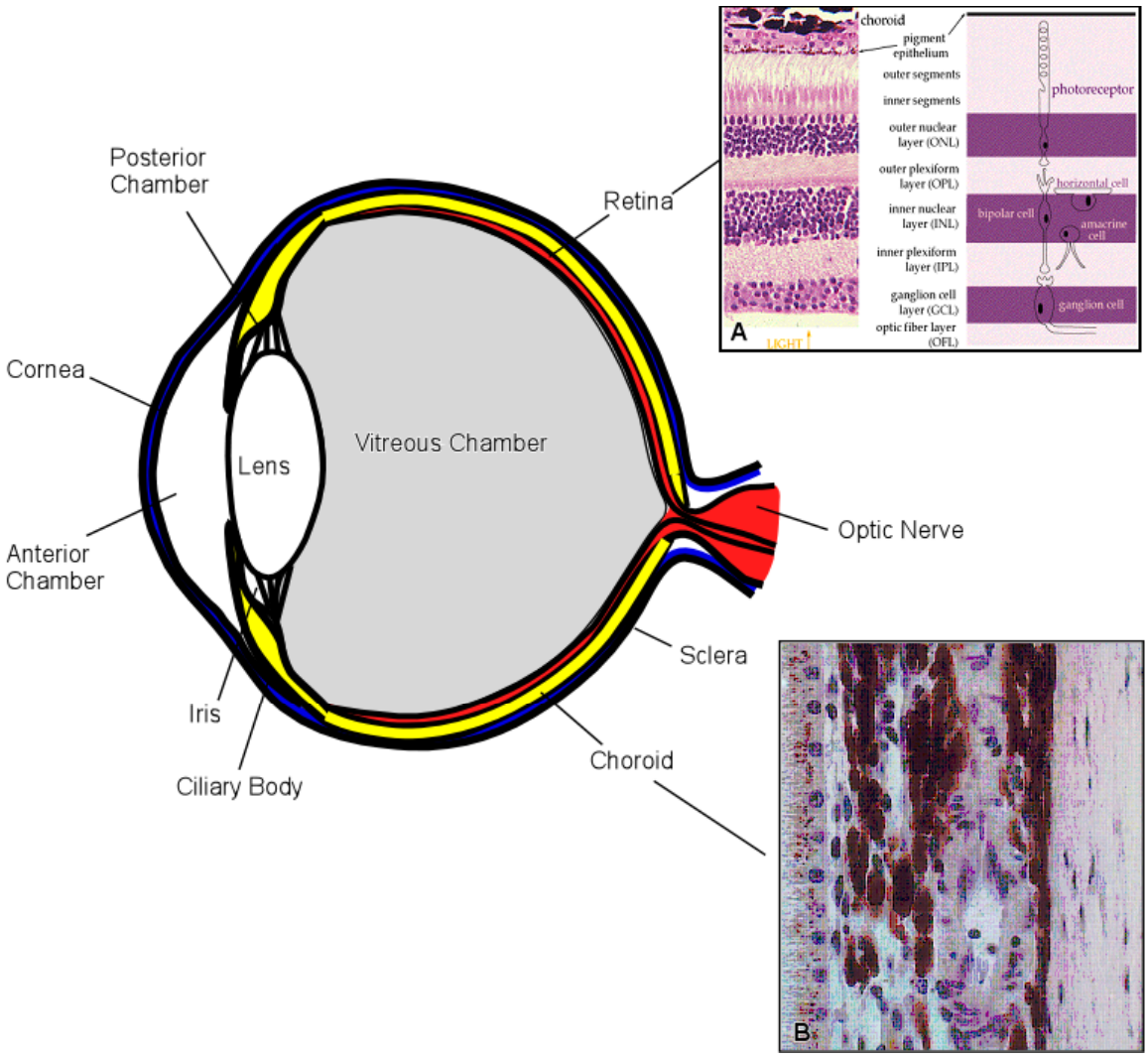


Figure 1: Structure of the Adult Eye (A) Structure of the Retina showing the nine components arranged in 'dark' nuclear layers containing cell bodies and 'light' plexiform layers containing axons and dendrites**; (B)** Structure of the Choroid showing darkly-colored spindle-shaped melanocytes interspersed between capillaries, called choriocapillaries,that lie beneath the Bruch's membrane**.**

Modified from the following web pages: ww.thalamus.wustl.edu/course/eyeret.html

2.2 Histopathology: Features and Risk Factors in Retinoblastoma

2.2.1 Histopathologic Features of Retinoblastoma

2.2.1.1 Macroscopic Appearance

Most retinoblastoma tissue is available after enucleation and depending upon the stage of the disease, the gross appearance differs. In general, the gross appearance of the tumor is white encephaloid, or brain-like with lighter foci of necrotic tissue or areas of calcification. The whitish tumor thickens, replaces and destroys part or the entire retina, and may totally fill the vitreous cavity. Necrotic tumors that have undergone acute infarction may have a blood-tinged, orange, or soupy, grayish appearance (Eagle 2000).

Four main tumor growth patterns have been recognized – exophytic, endophytic, mixed and diffuse infiltrative. The most common growth pattern, however, is a combination of exophytic and endophytic tumors. Exophytic tumors grow from outer retinal layers and extend underneath the subretinal space, causing secondary retinal detachment that may further lead to pupillary block and glaucoma. Invasion of tumor masses into the retinal pigment epithelium and erosion of the bruch's membrane leads to chorioidal invasion. With access to the rich vascular supply of the choroid, the risk of systemic dissemination is high in exophytic tumors (Eagle 2000; Finger et al., 2002a).

Endophytic tumors grow into the vitreous cavity, obscuring the retina, which remains attached. Retinoblastoma is a friable necrotic tumor and clusters of tumor cells detach from the main mass and tend to deposit on other areas of the retina, ciliary body, iris and lens. When tumor fragments invade the retina, it can be difficult to distinguish between secondary seeding and multifocal retinoblastoma and this is an important prognostic indication as multiple tumors are indicative of a germinal mutation. Seeds that follow the outflow of the aqueous humor may lead to floaters in the anterior chamber and spread to the trabecular meshwork, which drains into the main lymphatic vessels of the eye (Eagle 2000; Finger et al., 2002a).

Diffused infiltrative retinoblastoma occurs in less than 2% of cases and is the most difficult to distinguish in a clinical setting because they diffusely thicken the retina, grow slowly and do not form a discrete tumefaction (Eagle 2000; Finger et al., 2002a). Most of these tumors are anteriorly located, and when tumor cells detach into the vitreous and anterior chamber, they can masquerade as an inflamed eye (Bhatnagar and Vine 1991; Nemeth et al., 1992; Grossniklaus et al., 1998). When clinical diagnosis is in doubt, enucleation is usually not the first choice of treatment and may hence delay diagnosis in certain cases, which is a risk factor for metastatic retinoblastoma. In other cases however, the eye may be enucleated with a strong suspicion of retinoblastoma and histopathologic examination of the globe becomes extremely important for patient management. Some authors have proposed that this variant of retinoblastoma may have a relatively good prognosis due to it's slow growth, while others have noted that this type is more common in older children (Eagle 2000).

2.2.2 Microscopic Appearance

Microscopically, the predominant cell type is a poorly differentiated round cell with a large basophilic nucleus and scanty cytoplasm. Mitotic figures and fragments of apoptotic nuclear debris are usually present. As the tumor grows rapidly and outgrows it's blood supply, cuffs of viable-appearing tumor cells are present around blood vessels, beyond which (about 90 to 100 mm away) is a zone of necrotic cells that have lost their basophilic nuclear DNA and become pink or eosinophilic. Foci of dystrophic calcification develop in the necrotic parts of the tumor in many cases. Histopathologically, the calcific foci appear reddish-purple in hematoxylin-and-eosin sections, and the presence of calcium can be confirmed by the von Kossa or alizarin red stains. Electron microscopy suggests that calcification probably begins in the mitochondria of necrotic cells. Clinically, the demonstration of calcification by ultrasonography or computed tomography can help to differentiate retinoblastoma from other simulating lesions (Eagle 2000; Finger et al., 2002a).

Historically, the prognosis of retinoblastoma has been thought to be related to the amount

of differentiation of tumor cells, the signs of which are believed to be the formation of fibrils and rosettes. Flexner-Wintersteiner rosettes are believed to represent an early form of retinal differentiation and are composed of a ring of cuboidal cells surrounding a central lumen containing hyaluronidase-resistant acid mucopolysaccharide (AMP), similar to photoreceptor matrix AMP. The cells forming the rosettes are joined at their apices by intercellular connections (zonulae adherents), analogous to the external limiting membrane of the retina. The presence of abundant Flexner-Wintersteiner rosettes may have better prognosis. Flexner-Wintersteiner rosettes are more frequent in small, relatively early tumors that had not extended beyond the choroid or the surgical resection line. Whether this is directly associated with better prognosis or an incidenctal feature in younger patients who have a better prognosis is not known (Stannard et al., 1979). Flexner-Wintersteiner rosettes rarely are found in foci of metastatic retinoblastoma (Eagle 2000; Ellsworth and Boxrud 2000).

Homer-Wright rosettes are observed less frequently and are indicative of neuroblastic differentiation. They lack a central lumen, and their constituent cells encompass a central tangle of neural filaments. Wright rosettes are relatively nonspecific because they also occur in neuroblastoma and are a characteristic feature of cerebellar medulloblastoma (Eagle 2000; Ellsworth and Boxrud 2000).

Fleurettes are yet another type of arrangement and were described by Tso, Zimmerman and Fine in 1970. They as so called as they resemble a bouquet of flowers. They appear in the viable portions and believed to represent the greatest degree of differentiation. Retinal tumors composed entirely of fleurettes are thought to represent retinocytomas, a benign variant of retinoblastoma that is incapable of metastasis. Compared to retinoblastoma, mitotic activity is uncommon. In addition, calcification occurs in viable parts of retinocytomas. There are contradictary opinions on whether the presence of fleurettes signify better prognosis (Ts'o et al., 1969; Sevel et al., 1974; Mashiah and Barishak 1977) .

Brown reported that a retinoblastoma first forms true rosettes and with time, they decrease in number and change in pseudorosettes or anaplastic cells (Brown 1966). However, others have suggested that the length of time the tumor has been in the eye, rather than it's degree of differentiation, that counts in predicting prognosis (Herm and Heath 1956; Carbajal 1958; Mashiah and Barishak 1977). More recently, extensive analysis of early- and late-stage retinoblastoma has shown that rosette formation is not a hallmark of tumor differentiation, but represents extensive cell-cell contacts between cells that are present in both early- and late-stage tumors (Johnson et al., 2007). Hence, differentiation may not be a prognostic feature of retinoblastoma.

2.2.3 Histopathologic Risk Factors for Retinoblastoma

Although early detection has allowed for diagnosis of Rb before local extension, especially in developed countries, access to most tumor tissue is usually gained by enucleation or exenteration (removal of the eye, surrounding tissue and part of the bony socket, the orbit) of the eye. Histopathologic evaluation of the enucleated globe to identify high-risk factors for tumor spread play a crucial role in selecting patients for additional therapy (also called adjuvant therapy) to reduce the occurrence of metastasis. By increasing the risk of access of the tumor cells to the extraocular vasculature or along the optic nerve to the central nervous system, the following features are predictive of metastasis: massive choroidal invasion, infiltration of the optic nerve, scleral, extrascleral and extraocular extension (Shields et al., 1993; Shields et al., 1994; Khelfaoui et al., 1996; Finger et al., 2002b). One example of choroidal involvement is seen in Figure 2d.

2.2.4 Uveal Involvement

Involvement of the choroid is almost always seen in large tumors and there has been considerable debate in the field as to whether this is a risk factor and whether this alone necessitates the use of systemic chemotherapy for presumed sub-clinical metastasis. For instance, in a series of 289 patients, Shields et al. reported that eyes with choroidal invasion (with or without optic nerve involvement) were more likely to develop metastasis than those without choroidal invasion (Shields et al., 1993). In patients with

isolated choroidal invasion, there was a trend toward developing metastasis (Shields et al., 1993). In another retrospective study, Karcioglu et al. reported a significant correlation between the positivity of diagnosis for retinoblastoma metastasis (such as bone marrow aspirates and bone scans), with choroidal involvement with higher stage disease (stages III or IV of the Reese-Ellsworth classification) (Karcioglu et al., 1997).

The controversy of choroidal involvement as a risk factor for metastasis comes from the lack of sufficient histopathologic criteria to quantify choroidal invasion. Some of the earliest studies correlated 'significant' or 'considerable' choroidal extension with tumor dissemination and mortality, respectively, but did not quantify these definitions (Rootman et al., 1976; Stannard et al., 1979). Schilling et al. proposed a staging system for choroidal involvement from Stage I (infiltration of the retinal pigment epithelium without involvement of the Bruch's Membrane) to Stage IV (complete infiltration of the choroid with tumor extending laterally), where metastatic rate was recorded as 0.4% of patients of stages I- III to 12% of patients with stage IV disease. They concluded that this staging system was clinically significant and advised eyes enucleated for retinoblastoma with Stages II or III to be further examined by serial sections to rule out the possibility of Stage IV disease.

The mortality rate from scleral involvement has been well documented by many authors and ratio range from 57-77% (Carbajal 1958; Taktikos 1966). Most patients who have scleral involvement also have optic nerve involvement so is hard to discern whether scleral involvement by itself is a risk factor though it is likely to be so (Stannard et al., 1985). In patients with extension of retinoblastoma up to the level of optic nerve transection, scleral and extrascleral extension, adjuvant therapy of high-dose chemotherapy with the addition of orbital radiation has been recommended (Honavar and Singh 2005; Rodriguez-Galindo et al., 2007).

Larger tumors are also likely to extend into the iris and ciliary body which portends anterior chamber involvement. In these cases, poor response to therapy ultimately

requires enucleation (Haik et al., 1987). Poor prognosis has also been reported in tumors with invasion of the iris and is involved with hematogenous dissemination (Sevel et al., 1974).

2.2.5 Optic Nerve Involvement

Optic nerve involvement is the most well-established risk factor for metastasis in retinoblastoma. Clinical evaluation of optic nerve involvement by the physician has been aided by the usage of Magnetic Resonance Imaging (MRI), which aids in differentiating subretinal fluid from tumor and is particularly useful for preoperative detection of optic nerve invasion in patients with retinoblastoma (Schulman et al., 1986; Brisse et al., 2007). In addition, Ultrasonography and Computed Tomography (CT) are also useful for evaluating calcification of the tumor, extrascleral and optic nerve invasion (Finger et al., 2002c).

When tumor cells are detected in the optic neve, it's extent of involvement should be determined by histopathology and is crucial for selecting patients for adjuvant therapy (Figure 3 A-C). Serial sections of the enucleated eye and the surgical end of the optic nerve (which is collected separately during surgery) are used to detect the presence of tumor cells. It is generally accepted that tumor involvement anterior to the lamina cribrosa is not associated with greater mortality (Magramm et al., 1989; Shields et al., 1994; Chintagumpala et al., 2007). However, when tumor cells are present in the optic nerve posterior to the lamina cribrosa, the mortality rate is in the range of 13%–69% (Kopelman et al., 1987; Magramm et al., 1989; Shields et al., 1994; Chintagumpala et al., 2007). The different stages of optic nerve involvement are seen in Figure 2.

In a majority of cases, 8-10 mm of optic nerve is removed during enucleation. When there is evidence of active tumor >10mm, there is risk of tumor spread to the leptomeninges, orbital soft tissue, spinal fluid to base of the brain and to distant metastasis, most commonly to the bone (Ellsworth 1969; Finger et al., 2002a).

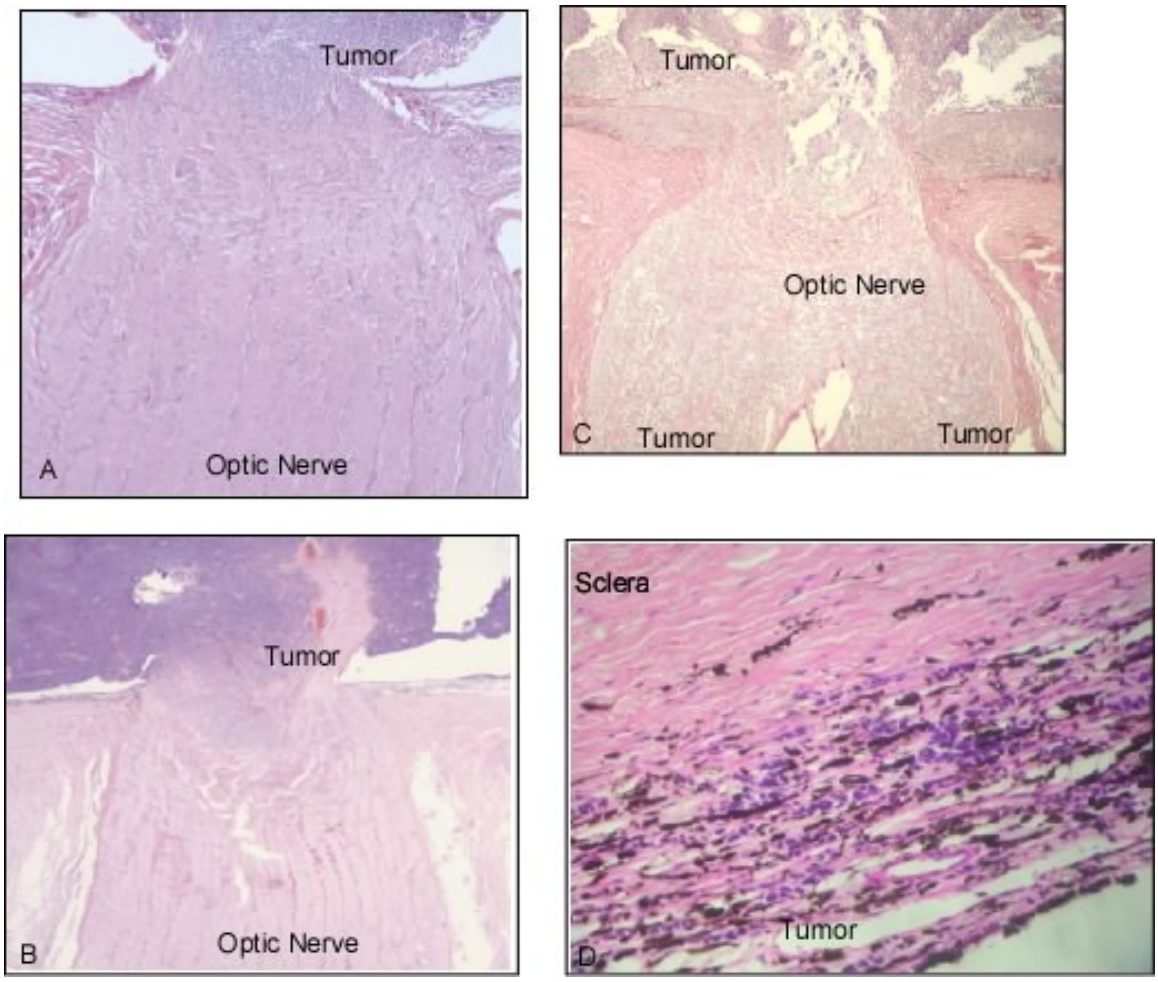


Figure 2: Histopatholgical high-risk features of retinoblastoma (A) Tumor invading the optic nerve anterior to lamina cribrosa; **(B)** Tumor invading past lamina cribrosa but away from surgical end of optic nerve; **(C)** Tumor invading entire optic nerve including surgical resection end. Also note invasion of adjoining choroid and sclera; **(D)** Tumor invading the choroidal layers.

Figures A, B and C are from Finger, P. T.et al., (2002). "Risk Factors for Metastasis in Retinoblastoma." Survey of Ophthalmology 47(1): 1-16.

2.2.6 Orbital Extension

Extension of the tumor outside the sclera into the orbit of the eye constitutes a very important risk factor for metastasis as it allows access of tumor cells to vascular and lymphatic channels outside the eye. Though microscopic orbital invasion is difficult to discover by clinical examination, larger extensions into the orbit is not as dependant on the histopathology of the globe as clinical examination and imaging may be diagnostic. In certain cases, fine needle aspiration biopsy (FNAB) or an open biopsy is possible (Finger et al., 2002a).

The most common chain of histologic events preceding orbital extension is massive involvement of the posterior choroid , post laminar optic nerve involvement (Khelfaoui et al., 1996) and extension along the scleral emissaria (Rootman et al., 1978). Orbital retinoblastoma is the worst prognostic factor for systemic metastasis and mortality rates vary from 25-95% (Rootman et al., 1978; Zygulska-Machowa et al., 1991; Honavar and Singh 2005).

There is no proven effective therapy for management of orbital retinoblastoma and combinations of external beam radiotherapy, chemotherapy, orbital exenteration, have met with variable results (Zygulska-Machowa et al., 1991; Kiratli et al., 1998; Honavar and Singh 2005). It has been suggested that a judicious and sequential combination of high-dose chemotherapy for orbital retinoblastoma followed by surgery, external beam radiotherapy, and extended chemotherapy will improve survival (Honavar and Singh 2005).

2.2.7 High-Risk Histopathologic Features and the Use of Adjuvant Therapy

Most of the statistical data that correlate the effectiveness of adjuvant chemotherapy with reduced incidence of metastasis in patients with high-risk histopathological features have been drawn from several retrospective studies which vary in their definition of high-risk features. Also, the chemotherapy regimens that have been used have varied between

different studies which further complicate the determination of efficacy of chemotherapy to minimize the risk of metastasis (Honavar and Singh 2005; Chintagumpala et al., 2007). A more recent study of the effectiveness of adjuvant therapy was performed in unilateral retinoblastoma patients with that underwent primary enucleation and categorized into a high risk group based on pre-determined histopathologic characteristics. The incidence of metastasis was 4% in patients who received adjuvant therapy compared with 24% in those who did not (Honavar et al., 2002).

More recently, a prospective multi-institutional clinical trial for unilateral patients has been initiated by the Children's Oncology Group (COG), where high-risk status will be assigned based on well-defined histopathologic features. These patients will be eligible to receive adjuvant chemotherapy consisting of six cycles of carboplatin, vincristine, and etoposide. Patients who do not have high-risk features as defined by the protocol do not require further therapy and will be observed. This will represent the first study in North America to prospectively evaluate histopathologic features and outcome in patients with unilateral disease (Chintagumpala et al., 2007).

2.2.8 Metastatic Retinoblastoma

Metastatic retinoblastoma is seen in fewer than 10% of patients in developed countries, but is a major cause of retinoblastoma-related mortality in developing countries (Kao et al., 2002; Honavar and Singh 2005; Gunduz et al., 2006; Ozdemir et al., 2007). Metastasis in retinoblastoma usually occurs within 1 year of diagnosis and when it is absent within 5 years of diagnosis the child is usually considered cured (Kopelman et al., 1987). Several studies have reported the risk of developing metastatic disease is higher in patients whose diagnosis has been delayed (Stannard et al., 1979).

In clinical practice, metastasis of retinoblastoma is unlikely in the absence of extra-ocular disease. Retinoblastoma metastasizes to the regional lymph nodes, central nervous system (CNS) and distant organs, such as the bone and liver. The most frequent site for

metastasis is the central nervous system and the prognosis of such patients is dismal (Ellsworth 1969; Gunduz et al., 2006; Leal-Leal et al., 2006). In the past, metastatic retinoblastoma was managed with conventional chemotherapy of vincristine, doxorubicin, cyclophosphamide, cisplatin, etoposide and radiotherapy yielded a few survivors. There are several reports now suggesting that high-dose chemotherapy with stem cell rescue, and in some studies, in combination with EBR for areas of bulky disease at diagnosis, was beneficial. Some of the long-term survivors were patients with metastatic disease not involving the central nervous system (CNS). It is rare for a patient with metastatic CNS involvement to survive using the therapies described above (Namouni et al., 1997; Dunkel et al., 2000; Rodriguez-Galindo et al., 2003; Chintagumpala et al., 2007).

The site of metastases and their frequency have been reported by several authors. Carbajal (Carbajal 1959) found a higher incidence of cranial and intracranial spread (75%) and conversely Taktikos (Taktikos 1966) reported a higher incidence of hematogenous involvement, only 29% having CNS involvement. Others have reported disease confined to intracranial contents, structures of the orbit, or spinal cord in 30-45% patients, whereas distant metastasis with or without cranial disease has been seen in 53-56% (Merriam 1950; Stannard et al., 1979; MacKay et al., 1984). While one report suggested that specific histopathological features were not predictive of metastatic patterns (MacKay et al., 1984), a couple have reported that the choroid was involved in all cases with hematogenous metastasis (Merriam 1950; Carbajal 1959).

McClean *et al.* propose 4 patterns of retinoblastoma metastasis. The first is a direct invasive spread of tumor along the optic nerve to the brain which can also seed the orbital tissue and adjacent bone, nasopharynx via the sinuses, or the cranium via the foramina. The second involves spread to the optic nerve and leptomeninges that disperse to the subarachnoid fluid from where they may spread to the spinal cord, distant sites of the brain and the contralateral optic nerve. Hematogenous dissemination, presumably via the orbit and/or choroid, which results in wide-spread metastasis to the lungs, bones and

brain characterizes the third pattern of metastasis. When the tumor is anteriorly located or where massive extra-ocular spread has occurred, tumor cells can enter the lymphatics of the conjunctiva and eyelids and travel to the regional lymph nodes and spread hematogenously (Chevez-Barrios et al., 2000).

Chapter 3

3.1 Tumor Initiation, Invasion and Metastasis in Retinoblastoma

As mentioned earlier, retinoblastoma is initiated by the loss or mutation of both alleles of the *Rb1* gene. In patients with a germline mutation in one allele, there is a 95% chance of loss of the other allele in a susceptible retinal cell in each eye, leading to early-onset, bilateral retinoblastoma. In patients with no heredity of a mutant *Rb1* allele, the chance of occurrence of 2 sporadic hits in the same retinal cell is much rarer and hence only one tumour forms, causing unilateral disease (Gallie et al., 1999).

The *Rb1* gene was the first tumour suppressor identified and over the last 15 years, there has been remarkable understanding of the functions of it's protein product, RB in regulating cell cycle, differentiation and apoptosis. Along with 2 other closely-related proteins, *p107* and *p130*, a core function of the pocket protein family is to negatively regulate the cell cycle by binding and inhibiting the E2F family of proteins. As cells progress from G1 to M phase, *Rb*, *p107* and *p130* are gradually inactivated by hyperphosphorylation by the CDK/Cyclin complexes, which relieve their inhibition of the E2Fs, resulting in induction of genes critical for cell cycle progression Hence *Rb1* loss by mutation leads mainly to uncontrolled cell proliferation, failed differentiation and apoptosis (Quill and O'Brien; Pacal and Bremner 2006).

Another important pathway that is inactivated in most cancers is the *Arf-MDM2/MDM4-p53* pathway. In normal cells, activation by various stress types such as DNA damage or oncogene activation leads to *p53* stabilization and induction of cell-cycle arrest through it's ability to induce expression of $p21^{WAF\text{-}1/CIP\text{-}1}$ and *14-3-3σ*, and/or apoptosis through it's ability to induce the expression of a number of pro-apoptotic genes, such as *Bax* (Weinberg 2007b). In retinoblastoma, several lines of evidence suggest that *p53* regulates apoptotic cell death. First, retinoblastoma is a chemosensitive tumour (Kingston et al., 1987). A majority of tumours have *p53*-immunoreactive cells in close association with dying regions or regions of complete cell death (Nork et al., 1997) and Divan et al. suggest that this is confined to poorly-differentiated tumours (Divan et al., 2001). Divan

and Burnier suggest that apoptosis could be triggered by hypoxia or nutrient starvation in these cells based on their localization to the outer zones of tumour foci (Burnier et al., 1990; Divan et al., 2001; Divan A 2001). Other studies have also shown that retinoblastoma express wild-type *p53* and that no *p53* mutation was detected in tumours (Nork et al., 1997; Schlamp et al., 1997; Gallie et al., 1999; Divan et al., 2001). Immunocytochemical analysis of 6 retinoblastoma cell lines showed abnormal cytoplasmic localization of wild-type *p53* in 4 lines including Y79 and Weri-Rb1, two of the most widely-used retinoblastoma cell lines. Both cytoplasmic and nuclear localization was observed in the other two, and the authors postulated that nuclear exclusion could be a mechanism of *p53* dysfunction in these cells. Three of thirteen invasive tumours also showed cytoplasmic localization in cells that were at the invasive edge while nuclear staining for *p53* was seen in cells in the main tumour mass, further suggesting a functional role for mislocalized *p53* (Schlamp et al., 1997).

Recently, Chen and colleagues proposed that retinoblastomas were intrinsically 'death-resistant' as studies on *Rb/p107* deficient mice showed that the cell of origin in these tumours survived *Rb/p107*-loss and stopped proliferating following terminal differentiation. Tumours arose from precursors that escaped this delayed growth arrest. Hence, the authors concluded that retinoblastoma arose from a precursor that had extended, not infinite, proliferative capacity, and that was intrinsically death-resistant, not death-prone(Chen et al., 2004)

In contrast to this death-resistant cell-of-origin theory, a recent study by Laurie et al. showed that retinoblastoma does inactivate the *p53* pathway after the loss of RB1. They showed that RB1-deficient retinoblasts undergo *p53*-mediated apoptosis and exit the cell cycle and that cells that amplify *Mdm4* (65% human tumours) or *Mdm2* (10% human tumours) were selected for during tumour progression. Mice lacking *p107*, *Rb* and *p53* develop 100% penetrant bilateral retinoblastoma that is aggressive and invasive, as compared to mice lacking *p107* and *Rb*, further suggesting the importance of *p53* inactivation in tumour development in mice, although they do not recapitulate the precise genetic change that occur in human retinoblastoma. In addition, *Mdm4* promoted the

progression from differentiated retinoblastoma with amacrine/horizontal cell features to a more immature cell with retinal progenitor features (Laurie et al., 2006).

There is considerable evidence that there are additional genetic changes that occur in retinoblastoma (Chen et al., 2002; Huang et al., 2003; Lillington et al., 2003; van der Wal et al., 2003; Amare Kadam et al., 2004; Marchong et al., 2004; Grasemann et al., 2005; Gratias et al., 2005). By karyotyping, cytogenetics and molecular genetics, these studies have shown specific chromosomal losses and gains and in some cases, the correlation of the altered expression and function of candidate oncogenes or tumour suppressors in these regions. Though these studies provide valuable information they have not yet been functionally proven as 'culprits' or even independently confirmed in most cases. This is due to various factors such as the limited number of laboratories world-wide that are active in this field, the limited number of commonly used cell lines (mainly Y79 and Weri-Rb1) and the difficulty in manipulating gene expression in these lines, the rarity of obtaining metastatic retinoblastoma in developed countries which are better equipped for such studies. Most importantly, no study has correlated specific chromosomal or molecular alterations with clinical or histopathologic features of retinoblastoma, which are important for understanding the how the tumour progresses (Corson and Gallie 2007).

Hence, apart from the conventional pathological parameters of tumour invasion such as choroidal and optic nerve involvement, there is a need to identify the molecular determinants of retinoblastoma invasion and metastasis. The underlying rationale in this strategy is that these proteins may play an important role in the maintenance of the transformed state and that targeting them (with antibodies, gene and transcript silencing or other strategies), might thereby improve current therapeutic strategies for late-stage disease. My research has focussed on examining the expression of genes that have been associated with cell adhesion, motility and invasion and correlating their expression with tumour invasion and differentiation in retinoblastoma.

3.2 Cell Adhesion, Migration and Invasion

The term 'invasion' indicates penetration into neighbouring territories and their

occupation. Invasion permits the entry into the circulation from where they can reach distant organs and eventually form secondary tumors, called metastasis (Mareel and Leroy 2003). One of the key early events in invasion of tumor cells is the remodeling of cell-cell and cell-matrix adhesion. It was known as early as the 1940s that the mutual adhesiveness of cancer cells is significantly weaker than corresponding normal cells (Corman 1944). Cell-cell adhesion is an important component of tissue homeostasis and regulation of processes such as cell-cell recognition, migration and cell sorting during embryonic development. Reduced cell-cell adhesiveness allows cancer cells to disrupt histolological structure, lose polarity and infiltrate stroma in a scattered manner. The functional units of cell adhesion consist of three classes of proteins – extracellular matrix proteins, cell adhesion receptors and cytoplasmic proteins that serve as linkers to the actin cytoskeleton. The cell adhesion receptors are usually transmembrane glycoproteins that bind on their extracellular surface to proteins in the cell's matrix or adhesion receptors of neighbouring cells and determine the specificity of cell-cell or cell-matrix interaction. On their intercellular surface, transmembrane proteins bind with cytoplasmic proteins that serve as linkers to the cell's cytoskeleton, regulate functions of adhesion molecules or transduce signals initiated by adhesion receptors. More than Cell adhesion molecules (CAMs) are now recognized. They include IgG-like CAMs, cadherins, selectins and integrins (Corman 1944; Okegawa et al., 2004; Weinberg 2007b).

The co-operation of different adhesive systems with generation of adhesive contacts at the cell's leading edge and breaking adhesive contacts and cytoskeletal retraction at the trailing edge enable cell migration. Focal adhesions are one of the most common type of adhesive contact that the cell makes with the extracellular matrix (ECM). Comprised on integrin receptors and associated cytoplasmic plaque proteins, focal adhesion are the major sites of actin filament attachment to the surface of contact and their formation is associated with cell spreading. Hence they represent sites of co-ordination between cell adhesion and cell motility.

3.3 c-Src Tyrosine Kinase and pSTAT3

Cellular Src was the first proto-oncogene described and acts as a central component of a number of signaling cascades from cell surface receptors. Src and it's family members (**S**rc **F**amily **K**inases) belong to a class of membrane-associated non-receptor tyrosine kinases that are enzymes that transfer phosphate groups from ATP to tyrosine residues on it's substrate proteins. Binding of ligands to their receptors result in phosphorylation of their cytoplasmic tails that act as docking sites to SH2-domain containing proteins such as Src. Once bound, they interact with other membrane-associated proteins and phospholipids to initiate a variety of different downstream signals. Src signaling has been implicated in cell fate decisions, proliferation, survival, differentiation and motility in both normal and transformed cells (Gallick 2003; Weinberg 2007a).

Src protein expression and activity is elevated in a number of cancers and is also often associated with later stages of disease, which may indicate that it facilitates malignant progression. The activation and functions of the Src family kinases has been most extensively studied in colon cancer (Cartwright et al., 1989; Cartwright et al., 1990). Studies on overexpression and downregulation of Src in colon carcinoma cell lines have shown that active Src may contribute to motility and invasiveness by upregulation of the matrix metalloproteinases (Nakagawa et al., 2000) and urokinase plasminogen activator receptor (uPAR)(Allgayer H and Jones T 1999) that facilitate degradation of the basement membrane, the disruption of cadherin-mediated cell/cell adhesion (Irby and T.J. 2002), resistance to detachment-induced cell death (anoikis) by regulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway(Windham et al., 2002) and in tumour angiogenesis by hypoxia-induced activation of Vascular Endothelial Growth Factor (VEGF)(Ellis et al., 1998).

Many of the oncogenic tyrosine kinases transmit signals through the STATs (Signal Transducers and Activators of Transcription). On activation by cytokine and growth factor receptor signaling, these cytoplasmic transcription factors dimerize and translocate

to the nucleus where they act to induce transcription of several genes. Although there are seven Stat proteins, STAT3 and STAT5 are the most commonly activated in a large variety of cancers. In particular, STAT3 and STAT5 signaling has been shown to prevent programmed cell death and/or enhance cell proliferation through regulating genes such as c-Myc, Cyclin D1, Bcl-X_L. Thus expression of these proteins by tumour cells is important for their survival and for the continuous growth of the tumour cells. Hence, we are interested in studying the expressions of pSTAT3 and c-Src Tyrosine Kinase in retinoblastoma.

3.4 E-Cadherin, N-Cadherin, α – catenin and β-catenin

Cadherins are integral membrane molecules that require calcium for their structure and function. Members of the classic type I cadherin family [E (epithelial), N (Neural) and P Placental)] have a conserved histidine-alanine-valine (HAV) cell recognition sequence in their first extracellular domain and calcium-binding motifs located between the 5 extracellular domains (EC1 to EC5) (Hirohashi and Kanai 1003; Van Aken et al., 2002). Cadherins mainly interact in a homophilic manner i.e. E-Cadherin binds selectively to E-Cadherin, when their extracellular domains form stable, parallel dimers. The catenins link the cytoplasmic domain of classical cadherins to the actin cytoskeleton of the cell. The catenins β- and γ- bind to the same conserved site on E-cadherin's cytoplasmic domain in a mutually exclusive manner and α-catenin links both bound β- and γ-catenin to the actin cytoskeleton (Hirohashi and Kanai 1003; Cavallaro et al., 2002). Hence, by anchoring actin cytoskeleton of adjacent cells, E-Cadherin is crucial for formation and maintenance of differentiated epithelial layers (Weinberg 2007a). A key step in the acquisition of motility and invasiveness in carcinomas is the loss of epithelial markers such as E-cadherin and replacement with mesenchymal markers that characterize cells of a motile phenotype, such as fibroblasts. This is called epithelial-mesenchymal transition (EMT) and epithelial cells that have undergone EMT often begin to make fibronectin, an extracellular matrix protein and express N-Cadherin, which enable their affinity with N-cadherin expressing stromal cells, such as fibroblasts and endothelial cells (Weinberg 2007b). Functional inactivation of the E-cadherin complex is a common feature in a

number of carcinomas and involves E-cadherin loss by inactivating mutations, epigenetic silencing, proteolytic cleavage and proteosomal degradation and/or mutations or tyrosine phosphorylation of α- and β-catenins (Hirohashi 1998; Cavallaro et al., 2002). Apart from it's role in the cadherin complex, β-catenin is also an important component of Wingless/Wnt signaling, a key pathway during development that enables cells to remain in a relatively undifferentiated state. When not associated with the cadherins, beta-catenin forms a complex with two other proteins, adenomatous polyposis (APC) and axin, that recruit GSK-3β which phosphorylates and marks it's for ubiquitylation and rapid degradation. When Wnt signaling is activated, GSK-3β is blocked which saves β-catenin from destruction. The accumulated β-catenin molecules translocate to the nucleus where they bind to Tcf/Lef DNA-binding proteins and activate transcription of genes such as Cyclin D1 and Myc (Weinberg 2007a). Like the cadherin complex, mutations of members of the Wnt pathway have been reported in a number of cancers, all of which promote stabilization of β-catenin and induce it's nuclear accumulation (Cavallaro et al., 2002). Until now, function of beta-catenin in both pathways does not suggest a simple relationship between cadherin and Wnt signaling. While in some systems, it has been demonstrated that sequestration of β-catenin by E-cadherin can compete with the β-catenin /TCF-mediated transcriptional activity of the canonical Wnt-signaling pathway, in other systems, loss of e-cadherin by mutation does not lead to an increased nuclear translocation and transcriptional activation of β-catenin (Kopfstein and Christofori 2006b; Weinberg 2007a). It has been suggested that beta-catenin may exist in two different molecular forms that act in adhesion and transcription, respectively and that a conformational change in the C-terminal end of β-catenin favors the nuclear function as opposed to a function in adhesion (Brembeck et al., 2006).

3.5 **Tetraspanins**

Tetraspanin proteins (also called the transmembrane 4 superfamily TM4SF) are surface membrane proteins that contain four transmembrane domains delimiting two extracellular regions of unequal size, as well as a particular fold in the large extracellular loop (Charrin et al., 2001). There are about 30 members of this family and these include the leukocyte

differentiation antigens CD9, CD37, CD53, CD63, CD81/TAPA-1, CD82/Kai1 and CD151/PETA-3.

All studied mammalian cells express several members of the family with the exception of erythrocytes that express none. A characteristic feature is the ability of these molecules to associate with one another and with other cell surface molecules forming molecular complexes that have been called the 'tetraspanin web'. These include association with integrins β1, lymphoid antigens CD4/CD8, HLA-DR MHC molecules and the membrane precursor of heparin-binding epidermal growth factor (HB-EGF) to name a few. With their diverse expression patterns and associations, members of this family have been implicated in cellular processes such as cell activation, proliferation, adhesion and motility and differentiation (Boucheix et al., 2001). Motility-Related Protein 1 MRP-1/CD9, KAI1/CD82, CD151 and ME491/CD63 have been shown to be involved in tumour cell metastasis. CD9 was first identified on the surface of B cell acute lymphoblastic cells and has subsequently been shown to be expressed on several cells of hematopoietic and non-hematopoietic origin. It's importance in cell migration was demonstrated when it was identified that the antibody against CD9 was the strongest inhibitor of cell motility in a lung adenocarcinoma cell line, MAC8. Reduced CD9 expression has been associated with poor prognosis in various cancers, including head and neck cancer (Mhawech et al., 2004), non-small-cell lung cancers (Higashiyama et al., 1995), breast cancer (Miyake et al., 1996), bladder cancer (Mhawech et al., 2003) and colon cancer (Cajot et al., 1997). In vitro studies of transfection of CD9 revealed that cell motility was suppressed in MRP-1/CD9 transfected cells (Ikeyama et al., 1993) and inhibited metastasis (Miyake et al., 2000; Takeda et al., 2007), further supporting it's functional role as a tumour suppressor.

3.6 **Epithelial Cell Adhesion Molecule (EpCAM)**

EpCAM or Epithelial Cell Adhesion Molecule is a 40 kDa transmembrane glycoprotein expressed on the basal and lateral surfaces of simple, pseudostratified and transitional epithelia (Spurr et al., 1986; Momburg et al., 1987). EpCAM mediates epithelium-

specific, calcium -independent homotypic cell– cell adhesions (Litvinov et al., 1994b) and are not likely to be involved in cell substrate adhesions (Litvinov et al., 1994a; Litvinov et al., 1994b).

Formation of EpCAM-mediated adhesion has a negative regulatory effect on adhesions mediated by classic cadherins, in direct relation to it's expression levels. EpCAM over-expression has been documented in a number of cancers such as those of the colon, rectum, prostrate, esophagus, breast, lung, head & neck, and kidney; and in some cases correlated with increased metastasis as well. However, normally non-adhesive cell lines have also been induced to aggregate with EpCAM transfection. Hence the dualistic role of EpCAM in different malignancies requires further analysis. EpCAM has also been considered a target for immunotherapy with bi specific antibodies- this helps in re-directing T cells towards tumour cells in a non MHC manner by cross linking tumour associated antigens such as EpCAM with the CD3 TCR receptor on Cytotoxic T cells (CTLs). There has been no study of EpCAM expression in retinoblastoma.

3.7 Extracellular Matrix Degrading Enzymes – the MMPs, TIMPs and EMMPRIN

For cells to invade, cyclic attachment to and detachment from the extracellular matrix is necessary in a directed and controlled manner. This is achieved by the balance of localized proteolysis by active enzymes and their endogenous inhibitors. Based on their catalytic mechanisms and inhibitor sensitivities, 4 main groups of endoproteases are distinguished – cysteine, serine, aspartic and metalloproteinases (Stetler-Stevenson et al., 1993; Sternlicht and Werb 2001). Matrix metalloproteinases are distinguished by the presence of a zinc-binding motif in their catalytic site, their secretion as an inactive zymogen with in vitro activation by organomercurial agents, auto-removal of their N-terminal pre-domain and inhibition by the Tissue Inhibitor of Metalloproteinases (TIMPs). While most members of this family are secreted forms, the 3 Membrane type metalloproteinases (MT-MMPs) have transmembrane domains and are expressed at the cell surface. The secreted forms can also localize to the membrane when tethered by integrins, CD44, heparin sulphate proteoglycans or the extracellular matrix

metalloproteinase inducer (EMMPRIN). MMPs have distinct but overlapping substrate specificities, and together they can cleave almost all extracellular matrix components, some cell adhesion molecules and growth factors (Stetler-Stevenson et al., 1993; Sternlicht and Werb 2001; Egeblad and Werb 2002). Evidence for the role of MMPs in tumour invasion and metastasis comes from a variety of studies including in vitro studies of tumour cell lines, animal models and examination of human tumour tissues and sera for expression of mRNA and protein. In human tumours, some MMPs (such as MMP-2 and MMP-9) are also made by stromal cells. MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) have 3 cysteine-rich repeats within their catalytic domain that is required to bind and cleave collagen (Stetler-Stevenson et al., 1993; Sternlicht and Werb 2001; Egeblad and Werb 2002). MMP-2 expression is elevated and correlates with poor prognosis of breast, colon and gastric adenocarcinomas, esophageal carcinoma and head and neck squamous cell carcinoma, to name a few (Stetler-Stevenson et al., 1993; Sternlicht and Werb 2001; Egeblad and Werb 2002).

In both normal and pathologic conditions, the MMPs are regulated at the transcriptional, post transcriptional and protein levels via their activators, inhibitors and cell surface localization. The tissue inhibitors of metalloproteinases (TIMPs) are small (22–30 kDa), naturally occurring proteins that are capable of binding and inactivating MMPs by their ability to form non-covalent bonds with the latent and active forms of MMPs with a 1:1 stoichiometry. Four TIMPs have been identified (TIMP-1, TIMP-2, TIMP-3, and TIMP-4), and they differ in their MMP specificities, gene regulation and tissue-specific expression patterns (Birkedal-Hansen H 1993; Baker A.H 2000; Sternlicht and Werb 2001). Although the inhibitory effects of the TIMPs on tumour invasion and metastasis are seen when over expressed in tumour cells, they can also promote tumour growth, angiogenesis and regulate apoptosis, and some of these are independent of their ability to inhibit the MMPs (Sternlicht and Werb 2001; Jiang et al., 2002). Hence, the net effect of TIMP in tumourgenesis may depend on their levels and tumour microenvironment, as high levels may have a tumour-suppressing role due to it's dominant anti-MMP activity, while lower levels may facilitate tumour growth due to their anti-apoptotic effects (Jiang

et al., 2002).

3.8 Rac1, Tiam1 and Cdc42

With their paths cleared by the action of extracellular proteases, the advancement of invasive cancer cells depends on the continuous restructuring of the actin cytoskeleton in different parts of the cell, as well as the making or breaking of attachments between the migrating cells and the extracellular matrix (Weinberg 2007c).

The molecular control of actin assembly and disassembly is under control of the Rho family proteins, of which the three main subfamilies are Rho, Rac and Cdc42, each of which have distinct roles in organising the actin filaments, to enable changes in cell shape. Like the Ras proteins, Rho-like GTPases function as molecular switches by cycling between an active GTP-bound state and an inactive GDP-bound state. In response to stimulation by growth factor receptor tyrosine kinases such as EGFR and PDGFR, Rac regulates the formation of lamellipodia, that are broad, flat sheet-like structures at the cell's leading edge. In fibroblasts, Cdc42 is involved in the formation of filopodia that are spikelike structures with tightly-bundled actin filaments that help in sensing extracellular cues. Rho proteins stimulate the formation of actin filament bundles or 'stress fibres' at the trailing edge and focal adhesions, which are the establishment of new points of adhesion between the cell and the extracellular matrix during movement (Nobes and Hall 1995; Evers et al., 2000; Lozano et al., 2003; Malliri and Collard 2003; Weinberg 2007c).

Hence, the Rho family GTPases play important roles in the regulation of cell migration, having been found in cell-cell contacts and also downstream of integrin-extracellular matrix contacts. Their functions in cell-cell contacts are complex and dependent on the specific cell type, nature of the extra cellular matrix, relative levels of active Rac and Rho or possibly the specific exchange factors signalling to the Rho proteins (Evers et al., 2000; Lozano et al., 2003; Malliri and Collard 2003). For example, in epithelial MDCK cells, Rho and Rac1 are necessary for the formation and maintenance of E-cadherin-dependant cell-cell adhesions and their inactivation leads to dislocation of E-cadherin and disruption of cell-cell associations (Evers et al., 2000). Tiam1, the specific GEF for Rac

also localizes to adherens junction in non-motile cells and on a fibronectin or laminin matrix, Tiam1-mediated Rac activation reverts the oncogenic ras-induced fibroblastoid phenotype of MDCK cells into a non-invasive epithelioid phenotype by restoring E-cadherin-mediated adhesions in these cells. However, on a collagen matrix, Tiam1 localizes to the lamellae and stimulates the migration of these cells (Hordijk et al., 1997; Sander et al., 1998). In these Ras-transformed MDCK cells, Rac1 activity was downregulated and restoration of it's level by Tiam1 re-established cell-cell junctions. Activation of Rac1 was sufficient to destabilize cadherins junctions in normal human keratinocytes in a concenteration and time-dependant manner, and was independent of it's ability to induce lamellipodia formation (Braga et al., 2000).

With respect to invasion and metastasis, unlike their Ras cousins, activating mutations of the Rho proteins are rare. Elevated expression and/or activity of Rac (Rac1, Rac3) and Rho (RhoA, RhoB and RhoC) have shown to correlate with tumour stage or enhanced metastasis in various tumours, including breast cancer, melanomas, pancreatic ductal adenocarcinoma and testicular germ cell tumours (Lozano et al., 2003). Activation of Rho or Rac leads to increased MMP expression in different cell types- in HT1080 fibrosarcoma cells cultured in three-dimensional collagen gel, Rac1 is a mediator of MMP-2 activation and is required for Rac1-promoted cell invasion through collagen barrier (Zhuge and Xu 2001; Lozano et al., 2003). Tiam1-induced Rac1 inhibited invasion of the human renal carcinoma cell clearCa-28 by transcriptional up-regulation of tissue inhibitor of metalloproteinases-1 (TIMP-1) and post-transcriptional up-regulation of TIMP-2 (Engers et al., 2001). With the MMP-independent roles in tumour progression, it is conceivable that TIMP activation by Rac1 may be tumour-promoting.

Tiam1 has been implicated in both positive and negative regulation of tumour cell invasion. In colon carcinoma cells, increased Tiam1 correlates with increased migration and growth metastatic sites, reduced cellular adhesion and promoted anoikis-resistance (Minard et al., 2005; Minard et al., 2006). Tiam1 also contributes to the invasion and metastasis of 95D human giant-cell lung carcinoma cells (Hou et al., 2004). Tiam1 was also shown to be Wnt-responsive and up-regulated in mouse intestinal tumours and

human colon adenomas and Tiam1 deficiency significantly reduced the formation and growth of polyps *in vivo* (Malliri et al., 2006). In metastatic melanoma, overexpression of Tiam1 inhibits invasion and migration by increasing Rac activity and induction of stringent cell-cell contacts mediated by the Ig-like receptor ALCAM (activated leukocyte cell adhesion molecule) and actin redistribution to cell-cell junctions (Uhlenbrock et al., 2004).

3.9 Nitric Oxide Synthases - endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and nitrotyrosine (NT)

NO is a small messenger molecule that was first discovered as a potent vasodilator, known as the endothelium derived relaxing factor, produced and released by vascular endothelial cells (Palmer et al., 1987). It is now well established that NO has several diverse biological functions, and is produced by many cell types other than endothelium. 20,21 NO is synthesized from L- arginine by the action of NO synthases (NOS). There are three distinct isoforms of this enzyme, encoded by three different genes. Two of the NOS isoforms are constitutive and calcium/calmodulin dependent—the endothelial and neuronal types (eNOS and nNOS, respectively); the third is inducible (iNOS), and is not dependent upon calcium/calmodulin for its enzymatic action (Vallance and Leiper 2002). The produced NO can react with free radicals such as superoxide anions to form peroxynitrite, a potent nitrating agent. Peroxynitrite can cause oxidation of DNA and membrane phospholipids in addition to nitration of free or protein-associated tyrosines producing nitrotyrosine (NT). Thus, the occurrence of NT in tissues has been measured as a marker of peroxynitrite formation (Knowles and Moncada 1994; Griffith and Stuehr 1995)

Recent studies have investigated the expression and the activity of iNOS in human cancer. An increased level of iNOS expression and/or activity has been found in the tumour cells of gynecological malignancies (Thomsen et al., 1994), in the stroma of breast cancer (Thomsen et al., 1995), and in the tumour cells of head and neck cancer (Umar et al., 2003). However, as compared with the normal colon epithelium, colonic adenomas exhibit reduced iNOS but not eNOS or nNOS expression, and colorectal

adenocarcinomas also lost iNOS expression and exhibit a reduced level of eNOS and nNOS expression, suggesting that the loss of iNOS expression was associated with the progression of colon cancer (Xie and Fidler 1998). There is no information available about the expression of NOS in retinoblastoma.

METHODS

Chapter 4

4.1 Selection of Retinoblastoma Cases

Retinoblastoma cases from the records of the Ocular Pathology Department from the years 2001-2005 were selected based on the histopathalogical classification of invasion of the tumor into the ocular coats and differentiation. Clinical data on the age, sex, laterality and follow-up data were obtained from the medical records of each patient.

Histopathalogical classification was done in accordance to the recommendations for the reporting of tissues removed as part of the surgical treatment of common malignancies of the eye and its adnexa by the Association of Directors of Anatomic and Surgical Pathology (Folberg et al., 2003).

Hematoxylin and Eosin-stained tumour slides were reviewed by a trained pathologist (Dr. S. Krishnakumar) and invasion of the tumors into the choroid were classified as either focal invasion or diffuse invasion. For optic nerve invasion, prelaminar, post laminar and invasion of the surgical end of the optic nerve were analysed. Invasion of the tumour cells in orbital soft tissues were also looked for. Tumors were also described in terms of degree of differentiation and reported as well-, moderately- or poorly-differentiated (McLean et al., 1994).

1.1.1 poorly differentiated- cells with high nuclear to cytoplasmic ratios with a high mitotic index, pseudorosettes and Homer-Wright rosettes were found in such areas;

1.1.2 moderately differentiated-cells with moderate nuclear to cytoplasmic ratios, moderate mitotic index and possible pseudorosettes and Flexner –Winter Steiner Rosettes;

1.1.3 well-differentiated cells with a low nuclear to cytoplasmic ratio, a low mitotic index and the presence of Flexner- Wintersteiner rosettes and the presence of florets.

4.2 **Immunohistochemistry**

Formalin-fixed, paraffin-embedded blocks were retrieved and 5µm thick sections were taken on glass slides coated with 3-Aminoproplytriethoxy silane (Sigma Chemicals). They were then dewaxed and rehydrated in successive changes of Xylene and Methanol as follows: (a) Xylene 1 – 15 minutes; (b) Xylene 2 – 15 minutes; (c) 100% Alcohol – 4 minutes; (d) 80% Alcohol – 4 minutes; (e) 60% Alcohol – 4 minutes; (f) 40% Alcohol – 4 minutes; (g) distilled Water – 4 minutes. Antigen Retrieval was performed by a pressure-cooker method that has been standardized in our laboratory. Slides were placed in a slide holder and immersed in Citrate Buffer (0.1M Citric Acid, 0.1M Tri-Sodium Citrate, pH 6.0) and heated until two whistles. The cooker was then placed under running tap water for 20 minutes. Slides were rehydrated in a moist chamber with Tris-NaCl Buffer (TBS) (50 mM Tris, 150 mM NaCl, pH 7.6) for 5 minutes. Endogenous peroxidase activity was quenched with 3% Hydrogen Peroxide (Merck) for 10 minutes. Primary antibodies were diluted in TBS. Antibodies from Santa Cruz Biotechnology were 200µg/ml concentration. Dilutions were standardized in order to obtain maximum signal-noise ratio. The standardized conditions for each antibody are provided in the table below (Table 1). Biotinylated secondary antibodies and strepatividin- Horse Radish Peroxidase (HRP) were from a standard Dako LSAB + system- horseradish peroxidase kit (Dakocytomation, Glostrup, Denmark). The reaction was revealed by 3, 3'-diaminobenzidine tetrahydrochloride (DAB, DakoCytomation, Glostrup, Denmark) and the slides were counterstained with hematoxylin for 30 seconds. Slides were dried and mounted in DPX mountant.

Table 1: Details of Primary and Secondary Antibodies used for Immunohistochemistry

Antibody	**Catalog Number**	**Species (Clone)**	**Dilution in TBS**	**Duration of Incubation**	**Duration of Incubation of Link and Streptavidin-HRP and DAB**
c-Src	SC-5266	Anti-human Mouse Monoclonal (H-12)	1:25	Overnight at 4°C	1 hour each; DAB (1:50 dilution) for 5 minutes
pSTAT3	SC-8059	Anti-human Mouse Monoclonal (B7)	1:25	Overnight at 4°C	1.5 hour each; DAB (1:50 dilution) for 5 minutes
CD9	SC-13118	Anti-human Mouse Monoclonal (C4)	1:50*	Overnight at 4°C	1 hour each; DAB (1:50 dilution) for 5 minutes
E-Cadherin	SC-21791	Anti-human Mouse Monoclonal (67A4)	1:60	Overnight at 4°C	1.5 hour each; DAB (1:100 dilution) for 5 minutes
N-Cadherin	SC-8424	Anti-human Mouse Monoclonal (D4)	1:75	Overnight at 4°C	1.5 hour each; DAB (1:100 dilution) for 5 minutes
Alpha-Catenin	SC-7894	Anti-human Rabbit Polyclonal (H297)	1:100	Overnight at 4°C	1.5 hour each; DAB (1:100 dilution) for 5 minutes
Beta-Catenin	SC-7963	Anti-human Mouse Monoclonal (E5)	1:75	Overnight at 4°C	1.5 hour each; DAB (1:100 dilution) for 5 minutes
Epithelial Cell Adhesion Molecule (EpCAM)	GA 733.2 (from Dr. Lifen Ren-Heidenrich)	Anti-human Mouse Monoclonal	1:10	2 hours at Room Temperature	1 hour each; DAB (1:50 dilution) for 5 minutes
Matrix Metalloproteinase-2 (MMP-2)	SC-13594	Anti-human Mouse Monoclonal (2C1)	1:100	Overnight at 4°C	1.5 hour each; DAB (1:100 dilution) for 5 minutes
Matrix Metalloproteinase-9 (MMP-9)	SC-21733	Anti-human Mouse Monoclonal (2C3)	1:100	Overnight at 4°C	1.5 hour each; DAB (1:50 dilution) for 5 minutes
Tissue Inhibitor of Metalloproteinase-1 (TIMP-1)	SC-5538	Anti-human Rabbit Polyclonal (H150)	1:100	2 hours at Room Temperature	1 hour each; DAB (1:100 dilution) for 5 minutes

Tissue Inhibitor of Metalloproteinase-2 (TIMP-2)	SC-21735	Anti-human Mouse Monoclonal (3A4)	1:100	2 hours at Room Temperature	1 hour each; DAB (1:100 dilution) for 5 minutes
EMMPRIN	SC-21746	Anti-human Mouse Monoclonal (8D6)	1:50	2 hours at Room Temperature	1 hour each; DAB (1:100 dilution) for 5 minutes
Rac1	SC-	Anti-human Rabbit Polyclonal	1:50	Overnight at 4°C	1.5 hour each; DAB (1:100 dilution) for 5 minutes
T Lymphoma Invasion and Metastasis Suppressor (Tiam1)	SC-14023	Anti-human Rabbit Polyclonal (H300)	1:75	2 hours at Room Temperature	1 hour each; DAB (1:100 dilution) for 5 minutes
Cdc42	SC-8401	Anti-human Mouse Monoclonal (B8)	1:50	2 hours at Room Temperature	1 hour each; DAB (1:100 dilution) for 5 minutes
P63	SC-8431	Anti-human Mouse Monoclonal (4A4)	1:75	2 hours at Room Temperature	1 hour each; DAB (1:100 dilution) for 5 minutes
P73	SC-17823	Anti-human Mouse Monoclonal (E4)	1:75	Overnight at 4°C	1.5 hour each; DAB (1:100 dilution) for 5 minutes
iNOS	SC-651	Anti-human Rabbit Polyclonal	1:50	1.5 hours at Room Temperature	1 hour each; DAB (1:50 dilution) for 5 minutes
eNOS	SC-653	Anti-human Rabbit Polyclonal	1:50	1.5 hours at Room Temperature	1 hour each; DAB (1:50 dilution) for 5 minutes
Nitrotyrosine	05-233	Anti-human Mouse Monoclonal (1A6)	1:25		1 hour each; DAB (1:50 dilution) for 5 minutes

In addition to antigen retrieval by the pressure cooker method, these slides were trypsinised for 5 minutes at 37°C

4.3 **Immunoanalysis**

Tissue sections were read independently by two investigators without the knowledge of the results obtained by the other investigator. Furthermore, each investigator read all of the slides twice without the knowledge of the results obtained in the previous reading. Regional differences in staining patterns, i.e., whether the immunoexpression was different in the main tumor mass compared to the invading sites, were also looked for. Expression of each marker in the macroscopically normal-looking non-neoplastic retina of tumors was also reported. Average expression was calculated for the entire slide by scanning approximately 10 fields under 40 x magnifications. The scoring system used for each group of related proteins was reported as percentage positive cells with/out intensity of staining.

4.4 **Western Blotting and Gelatin Zymography**

4.4.1 Preparation of Human tumor lysates

All steps of this procedure were performed on ice. Retinoblastoma tumor tissue (~250 mg) was collected from a tumorous eyeball and stored in Phosphate-buffered saline (PBS, pH 7.0). The samples were homogenized in lysis buffer containing 50 mM Tris-HCl [pH 7.6], 5 mM EDTA, 150 mM Sodium Chloride, 0.1% phenylmethanesulphonylfluoride (PMSF, P7626, Sigma Aldrich, St. Louis, MD, USA) and 250 µl of 1mg/ml Proteinase Inhibitor Cocktail (P2714, Sigma). They were then passed through a 18-gauge needle, 3-5 times until all visible particles were dissolved. The samples were then centrifuged under cooling conditions at 5000 rpm until the supernatant was clear. For Rac and CD82, the samples were further spun at 10,000 rpm for 30 minutes and centrifuged at 26500 rpm for 4 hours on a Beckman XL-80 Preparative Ultracentrifuge at 4°C and the pellet fraction was collected. Protein estimation of the samples was done by the modified Hartree- Lowry method (Hartree 1972) as described below: The reagents prepared were as follows: (a) Reagent A: 2% Na_2CO_3 in 0.2 N

NaOH; (b)Reagent B: 0.5% CuSO4.5 H_2O in 1% Trisodium Citrate; (c) Reagent C: 49 ml of Reagent A + 1 ml Reagent B; (d) Standard Bovine Serum Albumin 0.1% and (e) diluted Folin- Ciocalteu reagent: freshly prepared 1:1 ratio of Folin-Ciocalteu reagent with Distilled Water. 5 ml of Reagent C were added to 50 µl of each sample, blank or standard BSA. After 10 minutes of incubation at room temperature, 500 µl diluted Folin's Reagent was added and the samples were vortexed. After 20 minutes of incubation, protein estimation was done in a UV Spectrophotometer (DU-640, Beckman) at 660 nm.

4.4.2 SDS-PAGE and Western blotting:

Fifty µg protein and equal volume sample loading buffer (50mm Tris/HCl, pH 6.5, 10% glycerol, 2% SDS, and 0.1% bromophenol blue) were boiled at 100°C for 5 minutes, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out. After SDS-PAGE, the gels were equilibrated in transfer buffer for 15 minutes and the protein bands were electrically transferred onto a nitrocellulose membrane (Hybond ECL, Amersham), at 100V for either 1 or 1.5 hour, depending on the estimated size of the protein of interest. The membranes were temporarily stained with Ponceau S and then incubated with 5% or 10% non-fat dry milk for an hour or overnight. The conditions for blocking of non-specific sites, primary and secondary antibody dilution and duration of incubation are given in the Table 2 below.

4.4.3 Western Blot Analyses

As tumor samples for westerns were obtained from the main tumor mass and not from an invading site, we did not attempt to correlate protein expression with histopathological features of the same tumor.

4.4.4 Gelatin Zymography for detection of MMPs

All steps of this procedure were performed on ice. Retinoblastoma tumor tissue (~250

mg) was collected and stored in 0.1 M Phosphate buffer (pH 7.4) containing 0.1% Triton X-100 and 0.02% Sodium Azide and stored at -20°C. At the time of protein lysate preparation, 500 µl of 10 mg/ml Phenylmethanesulphonyl fluoride (PMSF, P7626, Sigma Aldrich, St. Louis, MD, USA) and 10 µl of 1mg/ml Proteinase Inhibitor Cocktail (Sigma Aldrich, St. Louis, MD, USA) was added and the samples were homogenized in a glass homogenizer. They were then sonicated (VirSonic, Virtis, SP Industries Inc., Gardiner, NY) thrice for 10 seconds each, on ice. They were then incubated in 4°C for 15 minutes and centrifuged under cooling conditions (REMI C-24 Remi High Speed Cooling Centrifuge) at 5000 rpm for 5 minutes. The supernatant was collected and protein estimation was carried out by the Lowry method described previously. Ten- percent SDS-PAGE gels incorporated with 1 mg Gelatin (Merck Biochemicals) was prepared. 50 µg of each sample with equal volume of Native Loading Buffer (0.1 mg Bromophenol Blue, 2 ml Glycerol, 2.5 ml 0.5 M Tris-HCl at pH 6.8 and 5.5 ml MQ H_20) were loaded along with 12 µg Peripheral Blood Mononuclear cells and run at 150V for 90 minutes. The gels were renatured in 3 changes of 30 minutes each in 2.5% Triton X-100 and then washed with MQ H_20 3 times for 15 minutes each, and left overnight in LSCB Buffer (50 mM Tris, 0.2 M NaCl, 5mM $Cacl_2$, 0.02% Brij 35 and 0.02% Sodium Azide, pH 7.6) at 37°C. The gels were then stained with 0.5% Coomassie Blue for 90 minutes and destained in 10% acetic acid to reveal zones of digestion (Das et al., 1999).

4.4.5 Western Blots on Human and Mouse Retinoblastoma Cell Lines

Human retinoblastoma cell lines (Y79, Weri-1 and Rb355) and mouse retinoblastoma cell lines derived from a mouse model of retinoblastoma (*Chx-10 Cre Rb $^{Lox/+}$; p107$^{-/-}$; p53 $^{Lox/-}$;* manuscript in preparation) in the Dyer Lab (SJmRbl3, SJmRbl8, SJmRbl10 and SJmRbl12) were used for analyses of the cadherins and catenins. All steps of the procedure were done according to protocol using the glycine system described by Laemmli (Laemmli 1970) Briefly, the non-adherent cell lines (Y79, Weri-1, Rb355, SJmRbl3 and SJmRbl8) were grown to1-1.5 million cells/mL, and 10 mL of cells were

spun at 1000 rpm for 5 minutes and brought up in 2 mL 1X sample lysis buffer. The adherent cells were lysed in 2 mL 1X SLB and all samples flash frozen on dry ice and stored at -80°C. SDS-PAGE and western blotting was done as described previously.

4.5 *Fluorescent In Situ Hybridization (FISH) Analysis of Human Retinoblastoma*

Dual color fluorescence in situ hybridization was performed as previously described (Fuller et al., 2002) by the Dr. David Ellsion at the Pathology Department of St. Jude Children's Research Hospital, Memphis, TN. FISH was done on whole eye sections of 16 human retinoblastoma (Appendix I). Following deparaffinization, pre-treatment consisted of 30 minute steam cooking in citrate buffer with subsequent pepsin (4 mg/ml) digestion at 45°C for 30 minutes. Test and control probes were paired for dual-target hybridizations and were diluted 1:50 in DenHyb hybridization buffer (Insitus Laboratories, Albuquerque, NM). 10ul of the resultant hybridization mix was applied to the sections, with simultaneous denaturing of probe and target at 90°C for 13 minutes. Overnight hybridization at 37°C occurred in a humidified chamber. Post-hybridization washes included 50% formamide/1X SSC (5 minutes) and 2X SSC (5 minutes). DAPI (0.5 µl/ml) (Insitus Laboratories) was used as a nuclear counterstain, and the sections were viewed under a Nikon E800 fluorescent microscope with appropriate filters (Nikon Instruments, Melville, NY).

Table 2: Western Blotting Summary

Protein	**Blocking of non-specific sites**	**Primary Antibody (diluted in 1X Caesin or 1% NFDM)**	**HRP- tagged Secondary Antibody (diluted in 1XCaesin or 1% NFDM)**	**Detection Sysytem - Enhanced Chemiluminescent (ECL)**
c-Src	5% Non-fat dry milk for 60 minutes	1:250 for overnight at 4°C	1:2000 for 1 hour	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 5 minutes
pSTAT3	5% Non-fat dry milk for 60 minutes	1:250 for overnight at 4°C	1:2000 for 1 hour	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 2 minutes
CD9	5% Non-fat dry milk for 90 minutes	1:500 for overnight at 4°C	1:10,000 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 5 minutes
CD82	5% Non-fat dry milk for 1 hour	1:500 for 39 hours at 4°C	1:10,000 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 5 minutes
E-Cadherin	5% Non-fat dry milk for 90 minutes	1:500 for overnight at 4°C	1:10,000 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); Exposure at 5 minutes
N-Cadherin	10% non-fat dry milk for 90 minutes	1:2000 for overnight at 4°C	1:10,000 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); Exposure at 2 minutes
Alpha-Catenin	5% Non-fat dry milk for 1 hour	1:500 for overnight at 4°C	1:4000 for 2 hours	ECL System (Amersham); Exposure at 8 minutes
Beta-Catenin	5% Non-fat dry milk for 1 hour	1:300 for overnight at 4°C	1:2000 for 2 hours	ECL System (Amersham); Exposure at 1 minute
MMP-2	10% Non-fat dry milk for 1 hour	1:250 for overnight at 4°C	1:7500 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 5 minutes
MMP-9	10% Non-fat dry milk for 1 hour	1:100 for overnight at 4°C	1:7500 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 5 minutes
TIMP-1	5% Non-fat dry milk for 1 hour	1:300 for overnight at 4°C	1:4000 for 2 hours	ECL System (Amersham); Exposure at 2 minutes
TIMP-2	10% Non-fat dry milk for 1 hour	1:750 for overnight at 4°C	1: 15,000 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 5

				minutes
EMMPRIN	5% Non-fat dry milk overnight	1:200 for 2 hours at 4°C	1:7500 for 1 hour	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 5 minutes
Rac1	10% non-fat dry milk for 90 minutes	1:1500 for overnight at 4°C	1:10,000 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); 5 minutes
Tiam1	5% Non-fat dry milk for 1 hour	1:1000 for overnight at 4°C	1:25,000 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); Exposure at 10 seconds
Cdc42	10% Non-fat dry milk for 2 hours	1:500 for overnight at 4°C	1:10,000 for 2 hours	Supersignal West Femto Maximum Sensitivity Substrate (Pierce); exposure at 2 minutes
iNOS	5% Non-fat dry milk for 60 minutes	1:250 for overnight at 4°C	1:200 for 1 hour	
Beta Actin (Control)	5% Non-fat dry milk for 1 hour	1:1500 for overnight at 4°C	1:4000 for 2 hours	ECL System (Amersham); Exposure at 2 minutes

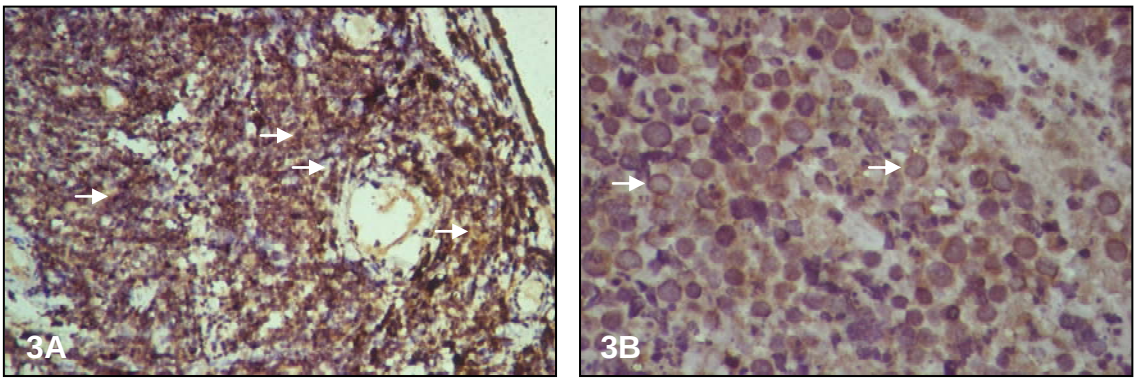
RESULTS AND DISCUSSION

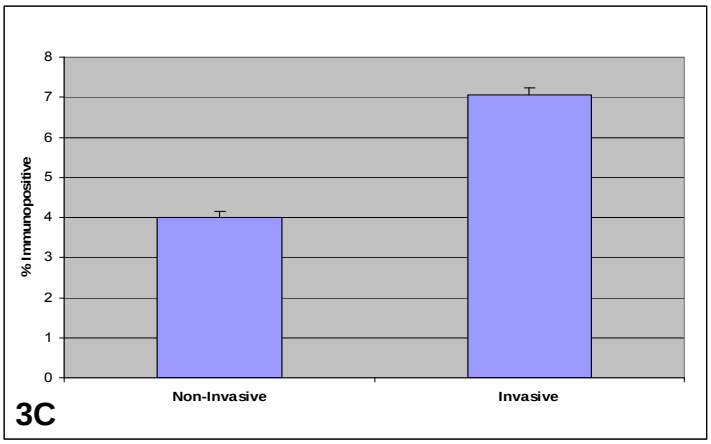
Chapter 5

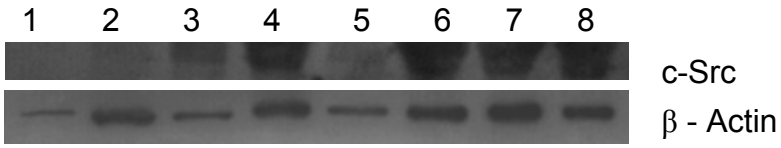
5.1 Higher expression of c-Src tyrosine kinase and pSTAT3 in invasive retinoblastoma compared to non-invasive retinoblastoma

The cohort of Src kinase and pSTAT3 included 18 tumors with no invasion and 22 tumors with invasion. The expression of c-Src tyrosine kinase and pstat3 were higher in the invasive tumors compared to non- invasive tumors ($P<0.01$) (Figures 3 and 4). Expression of c-Src kinase and pStat3 were also seen in normal retinal tissues. In the developing mouse retina, distinct Stat proteins, such as Stat1, Stat3, Stat5a and Stat6 were present in different cell populations in neuronal retina and in particular, Stat3 expression and activation gradually increased in the inner neuroblast layer and ciliary margin during development. In the adult mouse retina, Stat3 was detected in the inner nuclear layer and ganglion cells layers and activated Stat3 and Stat5a was seen in these layers and retinal pigment epithelium. This suggests that Stats may have signaling role in mammalian eye development (Zhang et al., 2003).

The increased expression of src kinase and stat3 proteins in the invasive RB suggests that these proteins could play an important role in the invasiveness of the retinoblastoma. Upregulated Src family kinases and pstat3 may directly contribute to multiple aspects of tumor progression, including blocking apoptosis, promoting proliferation and disruption of cell/cell contacts (Bromberg JF 1999; Irby and Yeatman 2000). Src and stat3 also play a critical role in signal transduction associated with cell-extracellular matrix interactions leading to activation of the MMP-2 and MMP-9 (Recchia et al., 2003; Xie et al., 2004; Kuo et al., 2006). Expression of stat3 in invasive RB could also contribute to the immune escape mechanisms in RB by suppressing tumor expression of proinflammatory mediators, (Wang et al., 2004) leading to immunosuppression in the tumor-infiltrating zone. Hence, Src kinase and Stat3 have many roles associated with tumor progression and further experiments are required to determine the functional relevance in retinoblastoma.







3D

Figure 3: c-Src expression in retina and retinoblastoma (A) c-Src immunoreactivity of a tumor invading the choroidal layers (Allred score 6) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** c-Src immunoreactivity in tumor cells invading the orbit (Allred score 6) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** c-Src immunoreactivity among invasive and non-invasive retinoblastoma**; (D)** Immunoblotting of c-Src in eight retinoblastoma tumors. Arrows indicate immunopositive cells.

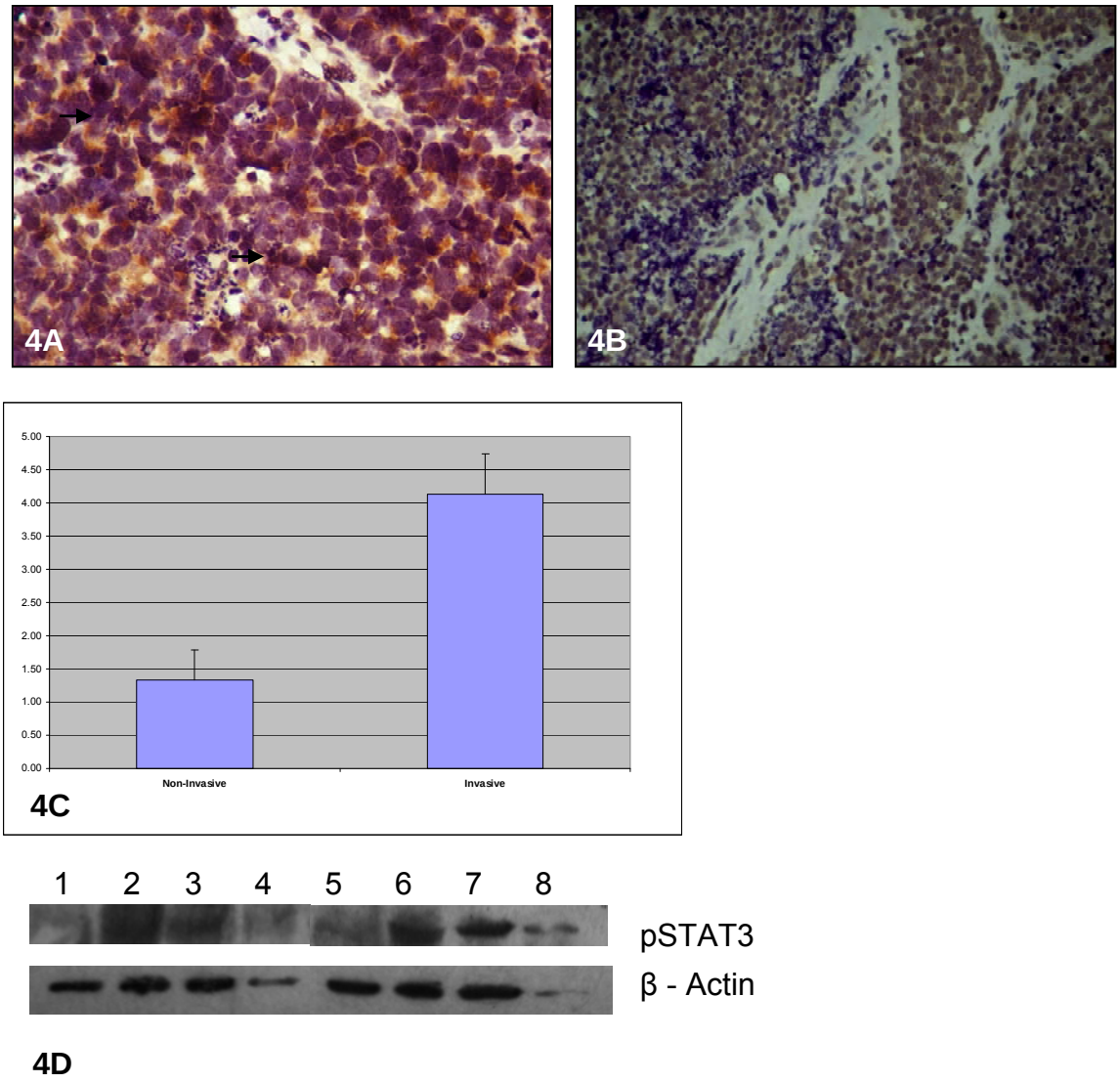


Figure 4: pSTAT3 expression in retinoblastoma (A) pSTAT3 immunoreactivity in a well-differentiated tumor (Allred score 6) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** pSTAT3 immunoreactivity in tumor cells invading the orbit (Allred score 6) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** pSTAT3 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of pSTAT3 in eight retinoblastoma tumors. Arrows indicate immunopositive cells.

5.2 Higher CD9/MRP-1, E-Cadherin and Alpha Catenin in Non-invasive Retinoblastoma and higher N-Cadherin Expression in Invasive Retinoblastoma

The cohort for CD9/MRP-1, cadherins and catenins included 29 tumors with no invasion and 33 tumors with invasion. Of 62 tumors, we found higher expression of CD9, E-cadherin and α-catenin in the non-invasive tumors and higher expression of N-cadherin in the invasive tumors (all $P<0.01$) (Figures 5 to 9). By western blotting, we were able to see CD9 and E-cadherin expression in a minority of tumors while N-cadherin, α-catenin and β-catenin was expressed at different levels in a majority of tumors. In the non-neoplastic retina, N-Cadherin was expressed in outer and inner nuclear layers and the photoreceptors. CD9, E-Cadherin and catenins were negative.

As mentioned before, the E-cadherin/catenin complex plays an important role in establishing adherens-type junctions in epithelial cells. In most carcinomas, E-cadherin mediated cell-cell adhesion is lost as one of the first steps towards malignant progression. Inactivation of this system occurs by multiple mechanisms - inactivating mutations (Becker KF et al., 1994; Becker et al., 1994; Gayther SA et al., 1998; Gayther et al., 1998), promoter methylation (Hajra and Fearon 2002) transcriptional repression (Batlle E et al., 2000; Batlle et al., 2000) and tyrosine phosphorylation of it's components (Hirohashi 1998) – and these correlated with dedifferentiation and acquisition of an invasive phenotype. Additionally, in vitro models of loss of E-cadherin function increases invasive growth, and reintroduction of functional E-cadherin into cells with endogenous E-cadherin defects suppresses their invasive behavior, which further supports it's role as a tumor suppressor (Cavallaro et al., 2002).

With the loss of tight junctions, tumor cells acquire motile and invasive properties characteristic of mesenchymal cells and these include the expression of vimentin, an intermediate filament component and the fibroblastic marker, N-Cadherin. A type II adhesion molecule like E-Cadherin, N-Cadherin expression on tumor cells facilitates

homophilic interaction with other N-cadherin expressing cells such as fibroblasts in the stroma surrounding tumors. Unlike the tight cell-cell interactions, these are weak intermolecular interactions that favor motility. This change from E-Cadherin to N-Cadherin/Cadherin-11(another mesenchymal cadherin) expression is termed *cadherin switching* and has been seen in a number of primary tumors such as prostrate cancer (Tomita et al., 2000), pancreatic cancer(Nakajima et al., 2004) and breast cancer (Hazan et al., 1997) and tumor cell lines (Bussemakers et al., 2000; Hajra and Fearon 2002; Weinberg 2007d).

We saw a higher expression of N-Cadherin in invasive tumors compared to non-invasive tumors. In western blots, most tumors were positive for N-Cadherin. In an earlier study of the cadherin-catenin complex in retinoblastoma and normal retina, N-cadherin, but not E- or P-cadherin, was seen associated with α-catenin and β-catenin. In retinoblastoma, but not in normal retina, this complex showed irregular distribution and weak linkage to the cell cytoskeleton and acted as an invasion promoter (Van Aken et al., 2002). Marchong et al. (Marchong et al., 2004) showed that more than half of 71 RB showed the loss of cadherin-11 (CDH11) gene located in a 2.62 bp minimal region of chromosome 16q22 and that this loss corresponded to disease progression in a transgenic mouse model of RB. Consistent with these studies, our observation of an increase in expression of N-cadherin and loss of E-cadherin (also located on the 16q22 region) in invasive and poorly differentiated tumors suggest that retinoblastoma may undergo changes in cadherin expression that could aid tumor progression.

We saw reduced expression of α -catenin in invasive retinoblastoma compared to non-invasive tumors. As α -catenin connects both bound β- and γ-catenin to the actin cytoskeleton, loss of expression could result in weakening of cell-cell adhesion and tumor invasiveness. Abberent expression of α-catenin was seen in primary bladder cancer (Kashibuchi et al., 2007) and one report of invasive breast cancer (where abberant expression was defined as cytoplasmic immunoreactivity) (Nakopoulou et al., 2002) and significantly correlated with poor survival of patients. In patients with esophageal

squamous cell carcinoma (Setoyama, Natsugoe et al. 2007) and prostrate cancer (Nakanishi et al., 1997; Setoyama et al., 2007; van Oort et al., 2007), reduced expression was an independent prognostic factor to predict lymph node metastasis and clinical outcome. Homozygous deletions of the α -catenin gene and subsequent loss of expression have been reported in prostrate (Morton et al., 1993) and lung cancer cell lines (Shimoyama et al., 1992), and in the PC3 prostrate cell line, re-expression of full length α-catenin cDNA or microcell-mediated transfer of chromosome 5 (on which the gene for α -catenin, CTNNA1 is located) was sufficient to restore cell-cell contacts. Moreover, cells containing one or more copies of microcell-transferred chromosome 5 and stably expressing α -catenin produce fewer tumors that are slower growing when injected into nude mice (Ewing et al., 1995). Hence, these data suggest that α -catenin may function as a tumor suppressor.

We found that the expression of β -catenin was not significantly different between the invasive and non-invasive tumors. With the reduction of E-cadherin in invasive tumors, it could be that β – catenin stays at the membrane bound to N-Cadherin (as shown by Van Aken et al.(Van Aken et al., 2002)) or other cadherins. As mentioned before, β –catenin plays essential roles in 2 different cellular processes: calcium-dependant intracellular signaling via the cadherin complex (Rimm et al. 1995) and Wnt-mediated transcriptional activation of target genes such as c-Myc and cyclin D1 (Hecht et al. 1999; Tutter et al. 2001) which are relevant for tumor progression. How β –catenin binding to the cell surface cadherins or DNA binding proteins is regulated and the relationship between these two functions remains to be answered. Though some evidence does suggest that signaling and adhesion are tightly coordinated through competition for a common pool of β –catenin it is not yet clear whether this regulation occurs *in vivo* by changes in endogenous cadherin levels or function. A recent study found that there are distinct molecular forms of β –catenin in cells- an N-terminal phosphorylated form that is targeted for degeneration, an ICAT bound inactive form, a TCF-selective monomeric form and a form targeted to adhesion (β –catenin-α-catenin dimer) and a form that is competent for both signaling and adhesive functions. Hence, this model explains how

cells can control whether β– catenin is used independently in cell adhesion or nuclear signaling, or competitively so that the two processes are coordinated or integrated (Gottardi and Gumbiner 2004).

In another study of a small number of human retinoblastoma, β –catenin was predominantly membrane-bound and appeared in an irregular honeycomb pattern (Tell et al., 2006), similar to the immunoreactivities for N-Cadherin, α- and β –catenin seen by Van Aken et al (Van Aken et al., 2002). In one of four tumors, a small number of cells had nuclear-localized β– catenin and the authors suggested that this may represent a different cell type within the tumor or could be retinoblastoma cells that were phenotypically different from the rest of the tumor (Tell et al., 2006). In the LH $_{BETA}$ T$_{AG}$ mouse model of retinoblastoma, β –catenin was exclusively cytoplasmic or membrane bound in both early and late-stage tumors and no nuclear staining (a marker for constitutive Wnt/ β –catenin signaling) was seen (Tell et al., 2006). In cell culture experiments, activation of Wnt signaling lead to reduced tumor growth in vitro, induction of cell cycle arrest and elevation of β –catenin protein levels, further suggesting that the Wnt pathway may function as a tumor suppressor in retinoblastoma. Hence, further investigations are required to evaluate the role that β –catenin plays in these pathways in retinoblastoma.

The expression of MRP-1/ CD9 was found to be lower in the invasive tumors than the non-invasive tumors. As discussed earlier, reduced expression of CD9 and other tetraspanins such as CD83/KAI-1 and CD63 have been shown to be linked to tumor progression in many cancers. In carcinomas of the breast, lung, colon, pancreas and esophagus, the expression of CD9 in carcinomas of the have been reported to be prognostic indicators of better survival and an inverse correlation between it's expression in the primary tumor and the appearance of metastasis has been reported (Higashiyama et al., 1995; Miyake et al., 1995; Sho et al., 1998; Uchida et al., 1999; Hashida et al., 2003). How does MRP-1/ CD9 affect cell motility and invasion of cancer cells? Firstly, tetraspanins CD9, CD63, CD81, CD82, and CD151 are structural components of

lamellipodia and filopodia where they specifically complex with integrin α3β1 and modulate integrin-dependant actin reorganization through FAK phosphorylation (Berditchevski F. and E. 1999). Second, multiple lines of evidence suggest CD9 functions directly or indirectly to suppress tumor cell motility and/or invasiveness, and it is likely that it's influence in these processes is cell-type and microenvironment specific. For example, CD9 transduction into the epithelial cell line A549 and the non-epithelial cell line HT1080 resulted in downregulation of Wnt family genes such as Wnt1, Wnt 2b1 and Wnt 5a and their target genes WISP(Wnt1-Inducible Signaling Pathway Protein) -1, WISP-3, c-Myc, VEGF-A and MMP-26 (Huang et al., 2004). While Wnt1 stimulates the canconical Wnt/ β– catenin pathway that lead to changes in cell fate and/or cell transformation, Wnt5a signaling affects actin cytoskeleton organization and increases cell invasion (Weeraratna et al., 2002). In another study, CD9-transfected HT1080 cells also show decreased lamellipodia formation, changes in subcellular localization of actin modulating proteins Arp2 and Arp3 and downregulation of WAVE2 expression which suppressed cell motility through a Wnt-independent pathway (Huang et al., 2006). In contrast to these tumor suppressor functions, CD9 over expression has been reported to induce MMP-2 expression in melanoma cells (Hong et al., 2005) and endothelial-cell driven expression of CD9 is involved in transendothelial invasion of human multiple myleoma MM5.1 cells and murine 5T33MM *in vivo* (De Bruyne et al., 2006). Hence, further studies are required to elucidate it's role in retinoblastoma.

To summarize, the increase in N-cadherin and alpha catenin expression and loss of E-cadherin and CD9 expression in invasive RB may contribute to RB tumor invasiveness. However further functional studies are required to evaluate the role of β-catenin in RB.

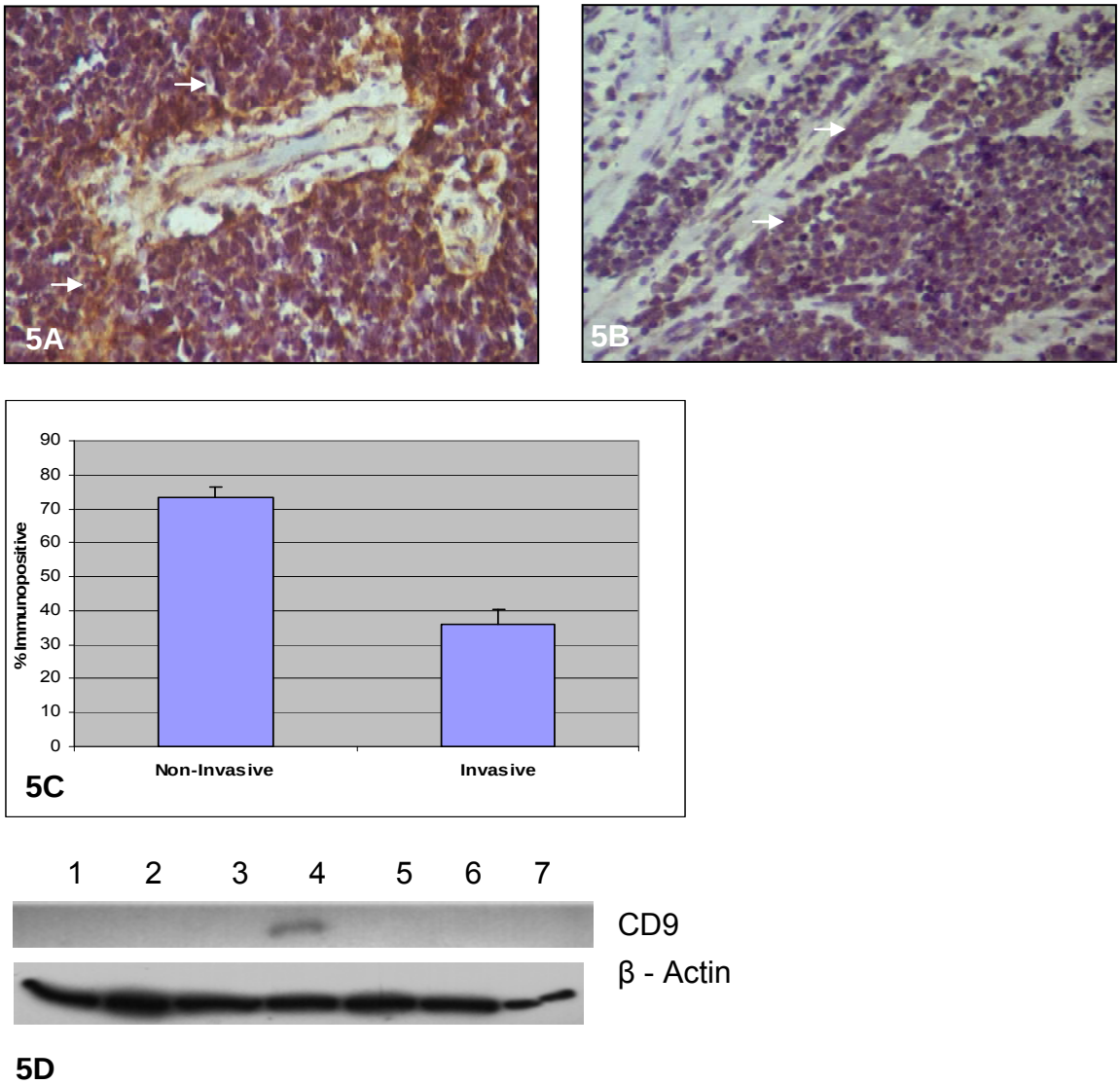
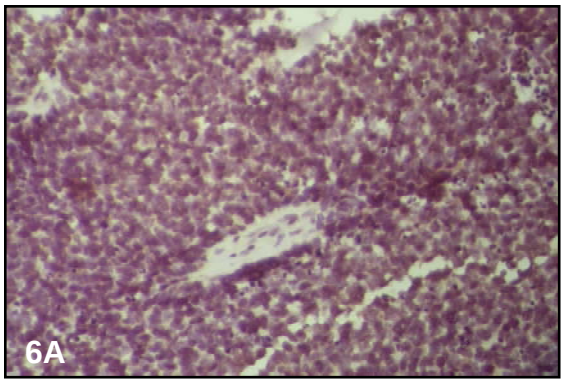
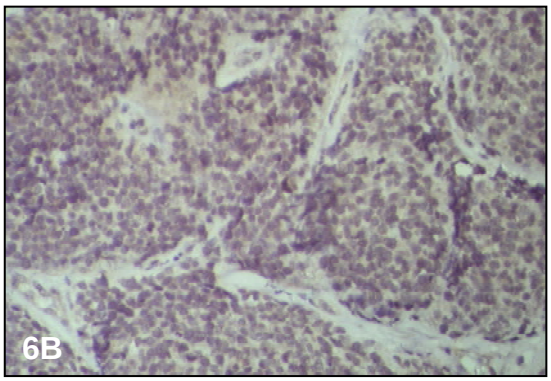
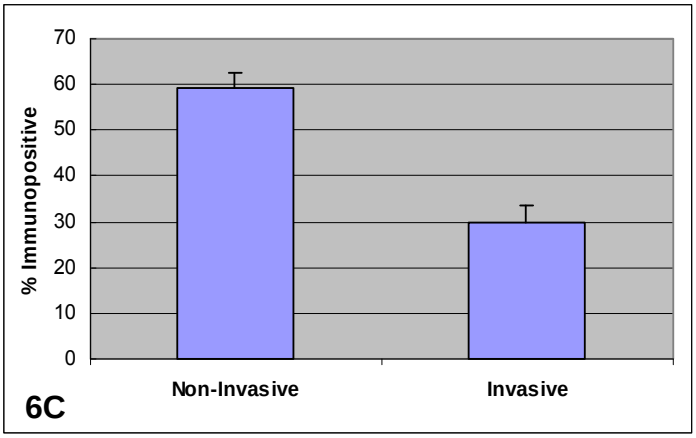


Figure 5: MRP-1/CD9 expression in retinoblastoma (A) CD9 immunoreactivity of a tumor without invasion (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** CD9 immunoreactivity in tumor cells invading the orbit (<50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** CD9 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of CD9 in seven retinoblastoma tumors. Arrows indicate immunopositive cells.







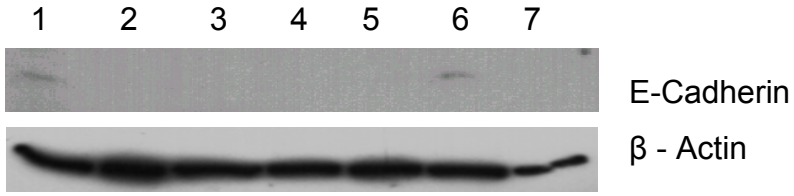
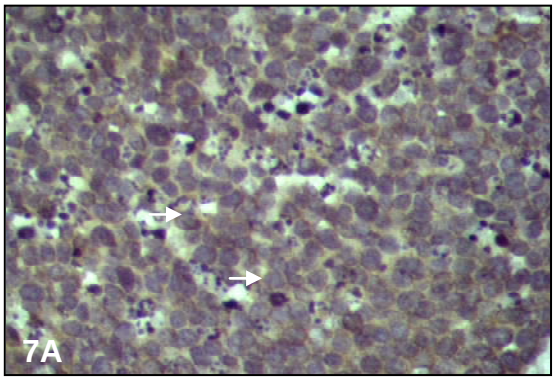
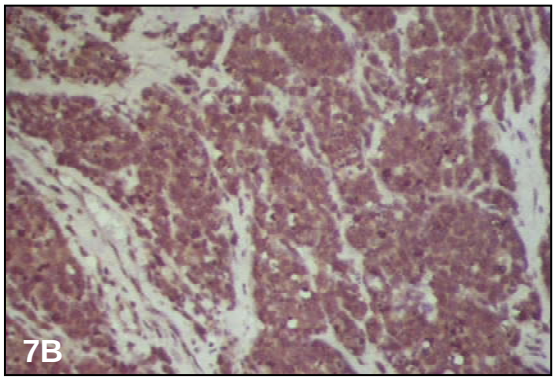
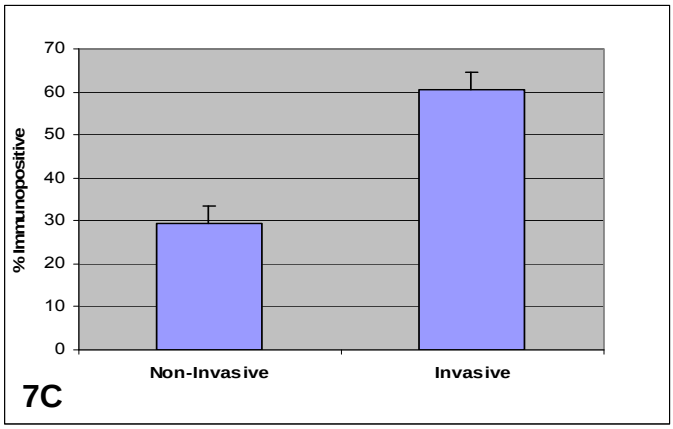


Figure 6: E-Cadherin expression in retinoblastoma (A) E-cadherin immunoreactivity of a tumor without invasion (50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** E-Cadherin immunoreactivity in tumor cells invading the orbit (<10% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** E-Cadherin immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of E-Cadherin in seven retinoblastoma tumors.







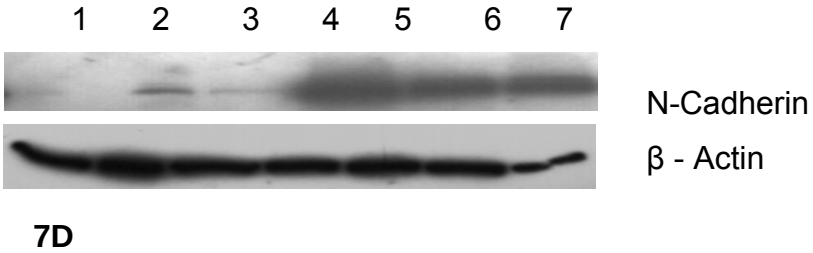


Figure 7: N-Cadherin expression in retinoblastoma (A) N-cadherin immunoreactivity of a tumor without invasion (<50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** N-Cadherin immunoreactivity in tumor cells invading the orbit (90% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** N-Cadherin immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of N-Cadherin in seven retinoblastoma tumors. Arrows indicate immunopositive cells.

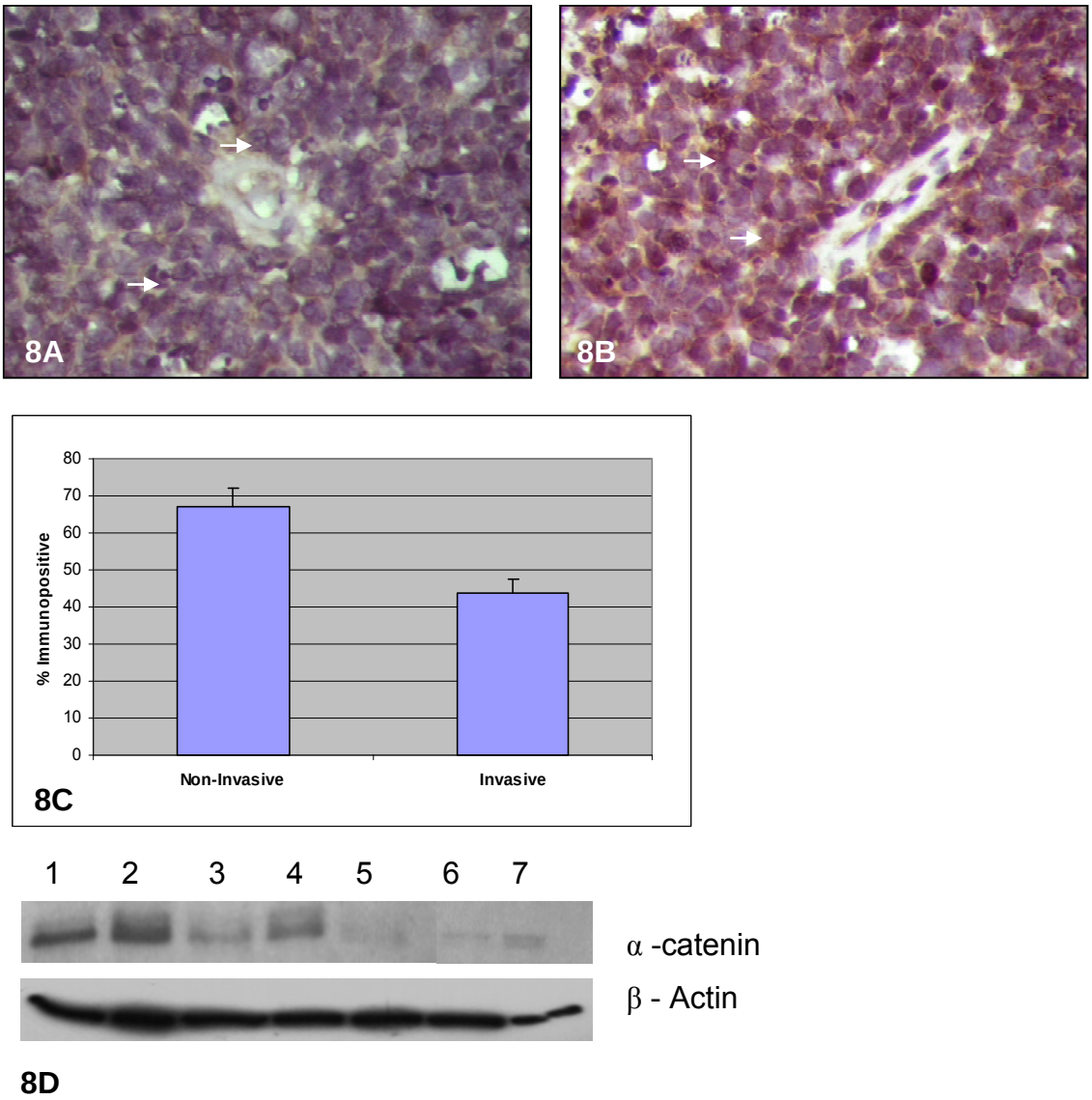


Figure 8: α -Catenin expression in retinoblastoma (A) α –catenin immunoreactivity of a tumor without invasion (<50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** α -catenin immunoreactivity in tumor cells (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** α-catenin immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of α -catenin in seven retinoblastoma tumors.

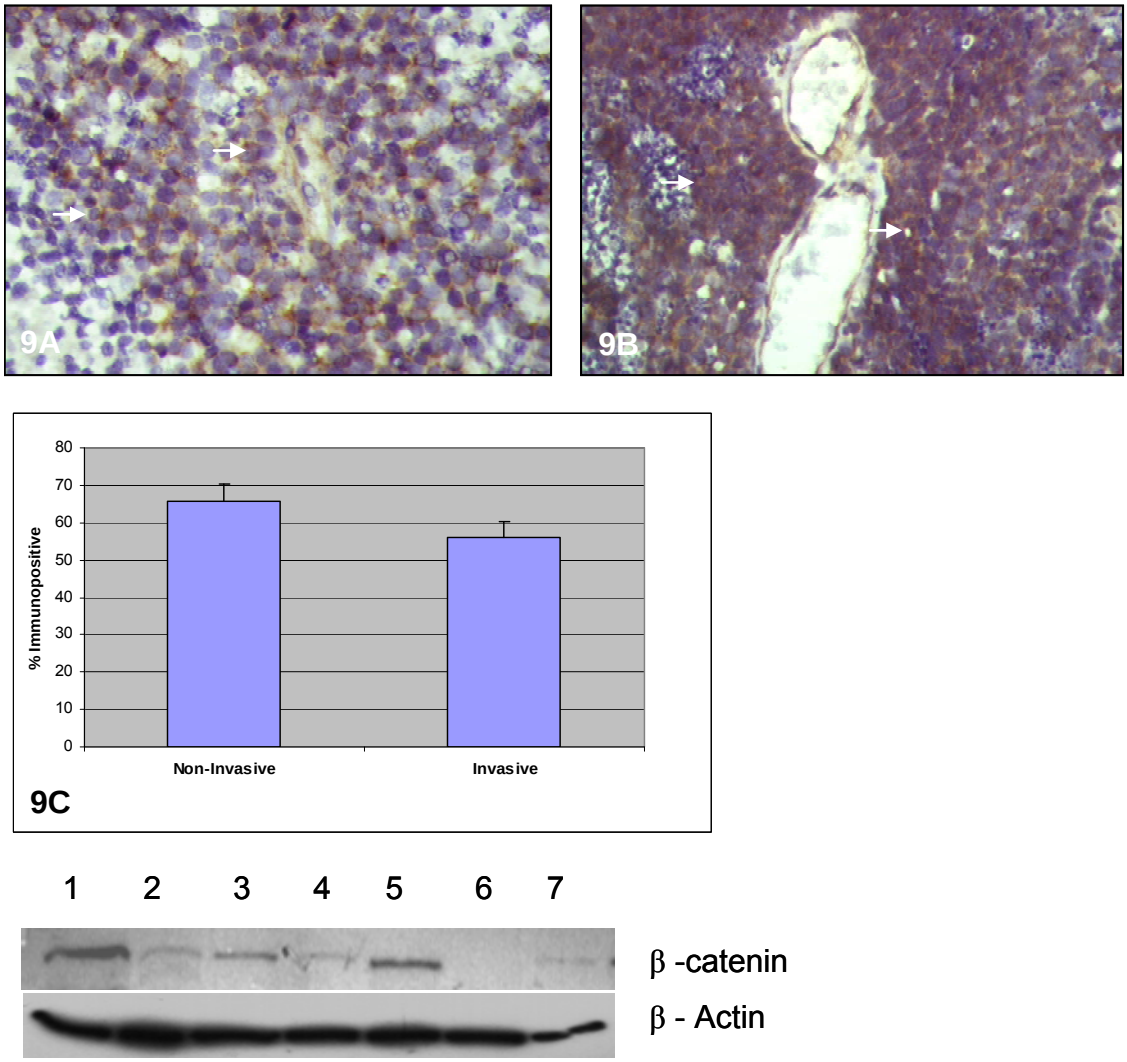


Figure 9: β-Catenin expression in retinoblastoma (A) β-catenin immunoreactivity of a tumor without invasion (50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** β-catenin immunoreactivity in tumor cells with invasion (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** β-catenin immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of β-catenin in seven retinoblastoma tumors.

5.3 Higher EpCAM Expression in Invasive retinoblastoma compared to non-invasive retinoblastoma

The cohort included 43 tumors of which 20 tumors without invasion and 23 tumors with invasion. Among 43 retinoblastoma, EpCAM reactivity was observed in 100% (43/43) tumors. Invasive tumors showed a higher expression of EpCAM than non-invasive tumors ($P<0.05$) (Figure 10).

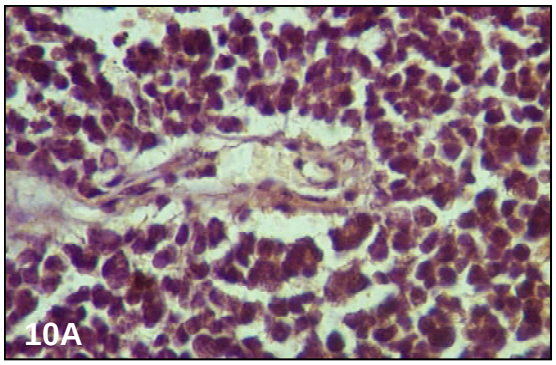
First identified as a tumor-specific antigen on several carcinomas of different origin, EpCAM is a calcium-independent cell-cell adhesion molecule not structurally-related to the four major families of cell adhesion molecule (CAM). It is a type I transmembrane glycoprotein with 2 Epidermal Growth Factor (EGF)-like extracellular domains and a short intercellular domain with binding sites for α-actinin. EpCAM is expressed in a wide spectrum of proliferating cells in normal adult epithelial tissues, such as lung, colon, pancreas, mammary gland and regenerating liver, but absent in normal liver, oral mucosa, gastric mucosa and uterine cervix. During transformation to neoplasia, de novo expression of EpCAM is seen in tissues which are normally negative and increased expression is seen in tissues with pre-existing EpCAM expression (Winter et al., 2003a; Winter et al., 2003b). In this context, EpCAM expression in the nuclear layers of the retinal tissue and it's overexpression in both early and late-stage tumors suggest that retinoblastoma may arise from cells which express EpCAM.

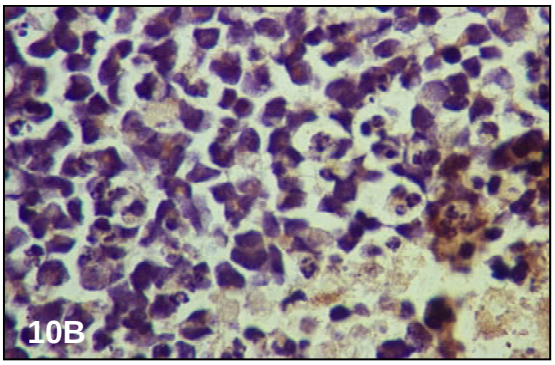
One of the ways by which EpCAM regulates cell adhesion is it's ability to influence cadherin-mediated cell-cell associations. Despite providing additional intercellular contacts between cells (Litvinov et al., 1994b), EpCAM overexpression affected the formation of cadherin-mediated junctional complexes by increasing the detergent soluble(and non-cytoskeletal associated) E-cadherin and β -catenin and a reduction in total levels of cellular α -catenin, which links E-Cadherin and β -Catenin to the actin cytoskeleton. As EpCAM interacts with α-actinin without the involvement of α- or β-catenin (Winter et al., 2003a), it's overexpression may competitively remove α-actinin

from cadherin junctions which in turn, could affect α-catenin expression or stability (Litvinov et al., 1997). The disruption of cadherin-mediated association and loss of differentiation may also be a reason for the association of EpCAM with epithelial cell proliferation and tumor progression (Litvinov et al., 1997). This is supported by the observation associations of EpCAM overexpression with poor prognosis in breast cancer(Gastl et al., 2000; Spizzo et al., 2004) , colon cancer (Salem et al., 1993; Packeisen et al., 1999) and the increase in EpCAM expression from low to high grade esophageal adenocarcinoma and prostrate adenocarcinomas (Winter et al., 2003b).

Our findings are of particular interest because of their clinical relevance. First, EpCAM may be a potential therapeutic target. Anti-EpCAM antibodies may be included in the treatment of retinoblastoma. The limitation for this would be as EpCAM is also expressed in normal retinal tissue and anti-EpCAM treatment may damage the normal retina. Second, EpCAM may be used as a tumor-associated antigen (TAA) and its application in immunotherapy using bispecific antibodies (BiAb). This is particularly relevant for tumors that downregulate Major Histocompatability Class molecules, like retinoblastoma, as this bypass their ability to be recognized and attacked by CD3 cytotoxic T cells. Bispecific antibodies are designed to recruit cytotoxic T cells by a common signaling molecule, such as CD3, against tumor cells bearing a frequently and differentially expressed tumor-associated cell surface antigen, such as EpCAM. EpCAM/CD3 single-chain bispecific antibodies significantly reduced tumor sizes of xenograft models of EpCAM-expressing SW480 colon carcinoma cell line, B16 melanoma cell line and ovarian carcinoma metastases, and this effect was dependent on activation of tumor-resident T cells (Schlereth et al., 2005; Schlereth et al., 2006).

Hence EpCAM expression in retinoblastoma opens new possibilities for antibody-based therapy. Since a majority of invasive retinoblastoma expressed higher EpCAM, EpCAM-targeted immunotherapy could be a possibility in retinoblastoma in the future.





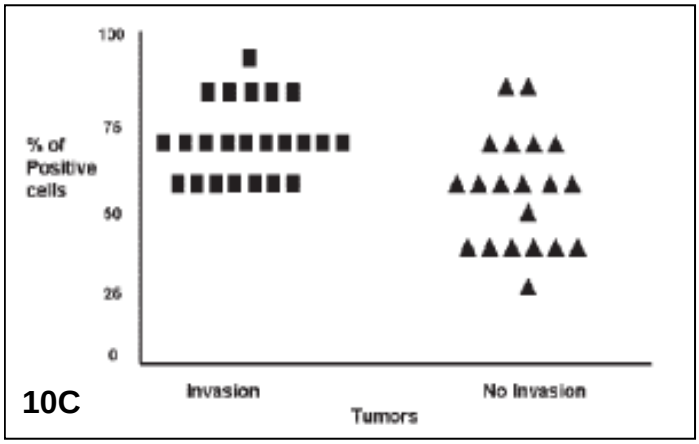


Figure 10: EpCAM expression in retinoblastoma (A) EpCAM immunoreactivity of a tumor without invasion (<50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** EpCAM immunoreactivity in tumor cells with invasion (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** EpCAM immunoreactivity among invasive and non-invasive retinoblastoma.

5.4 Higher Expression of EMMPRIN, MMP-2, MMP-9, TIMP-1 and TIMP-2 in Invasive Retinoblastoma

There were 29 tumors with no invasion and 33 tumors with invasion. Invasive tumors displayed significantly higher expressions for EMMPRIN, MMP-2 and MMP-9, TIMP-1 and TIMP-2 ($P<0.001$ for all) (Figures 11 to 15). On western blotting, almost all tumors were positive for the MMPs and TIMPs though the levels of expression were variable. Normal retina did not express EMMPRIN, MMP-2, MMP-9 or TIMP-1. Retinal Pigment Epithelium was positive for EMMPRIN. TIMP-2 was positive in inner and outer nuclear layers. The retinal blood vessels showed faint expression of MMP-2.

What is the role of MMPs in retinoblastoma invasion? First, MMPs could degrade the extracellular matrix and contribute to invasiveness in these tumors. Our observations of MMP-2 and MMP-9 expression in retinoblastoma are in agreement with other studies on MMP-2 and MMP-9 expression in retinoblastoma. Surti et al. showed that 2/4 invasive tumors were positive for MMP-9 while all 4 non-invasive tumors were negative. They also showed that all 4 invasive tumors were positive for MMP-1 and MMP-2. Among the non-invasive tumors, all were negative for MMP-1 and 1/4 were positive for MMP-2 expression (Surti et al., 2003). Examination of the LHßTag murine transgenic retinoblastoma model showed that MMP-9 was strongly upregulated and MMP-2 was weakly upregulated by gelatin zymography, an assay that tests for active MMP-2 and/or MMP-9 by their ability to cleave gelatin, a product of collagen which is an important component of basement membranes (Cebulla et al., 2005). In situ zymographic analysis of human retinoblastoma also showed that gelatinase activity was upregulated and was highest along vascular-like structures within the tumor and also along the edges of the tumor (Cebulla et al., 2006), which further suggests that the matrix degradation could be associated with tumor invasion.

Secondly, MMPs could contribute to tumor angiogenesis in retinoblastoma. Both MMP-2

and MMP-9 have been implicated as positive regulators of tumor angiogenesis (Itoh et al., 1998; Bergers et al., 2000) and in a transgenic model of pancreatic islet cell carcinogenesis, MMP-9 contributed to the induction of angiogenesis by releasing sequestered Vascular Endothelial Growth Factor (VEGF) (Bergers et al., 2000). Retinoblastomas that have invaded the choroid and/or optic nerve have significantly higher vessel densities than tumors without local invasion (Rossler et al., 2004) and quantification of the tumor's relative vascular area, and not extent of choroid or optic nerve invasion, could help identify patients with retinoblastoma at high risk for disease dissemination after enucleation (Marback et al., 2003). VEGF is expressed at both mRNA and protein level in retinoblastoma and is hypoxia-inducible in Y79 retinoblastoma cells (Kvanta et al., 1996; Pe'er et al., 1997). The VEGF Receptors, Flt-1 and KDR, are also expressed in retinoblastoma (Stitt et al., 1998). Hence, it will be interesting to test whether VEGF is regulated by MMP-9 in retinoblastoma.

The mechanisms involved in MMP upregulation in retinoblastoma are unknown. One of the ways by which MMP-2 is activated is by CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN) or basigin. Tumor cell associated EMMPRIN have been shown to induce the production and/or activation of various MMPs, such as MMP-1, MMP-2 MMP-3 and MT1-MMP and MT2-MMP (Sameshima et al., 2000; Caudroy et al., 2002; Suzuki et al., 2004). EMMPRIN was expressed at higher levels in invasive tumors ($P<0.001$, Mann Whitney U Test) suggesting that EMMPRIN may play a role in tumor cell invasion by activation of MMP-2 at the cell surface.

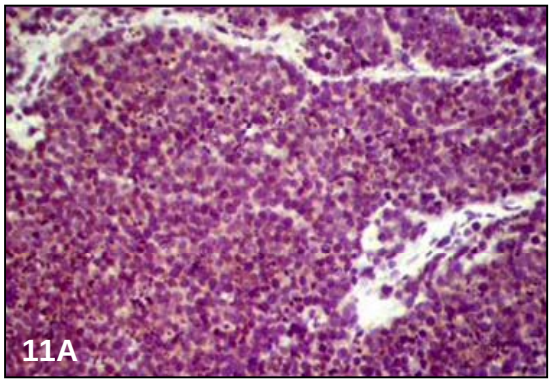
The expressions of TIMP-1 and TIMP-2 was more in invasive tumors With respect to differentiation, there were higher expressions of TIMP1 ($P<0.01$) and TIMP2 ($P<0.05$) in the poorly differentiated tumors, as compared to moderately- and well-differentiated tumors (that were grouped together). The exact of role of TIMPs in cancer progression remains poorly understood. Initial studies on the MMP/TIMP balance showed that high levels of TIMPs were associated with inhibition of cell invasion and that inhibiting their

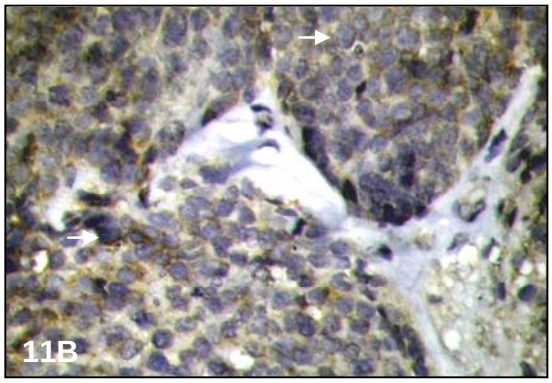
activity led to increased growth, invasion and/or metastasis. For example, overexpression of TIMP-1 inhibited tumor growth and metastasis of melanoma and suppressed metastatic ability of gastic cancer cells. Antisense-mediated reduction of TIMP1 was associated with increased tumor invasivness and metastatic potential of mouse 3T3 cell line (Khokha et al., 1989). involved in the inhibition of MMPs thereby imparting an antitumoral effect, (Hicks et al., 1984b). Interestingly, TIMP-2 binds to pro-MMP-2, via it's C-terminal domain, and to MT1-MMP by it's N-terminal domain, following which a neighboring TIMP-2 free MT1-MMP cleaves and activates the tethered pro-MMP-2. The N-terminal of TIMP-2 is MMP inhibitory and while low-to-moderate levels facilitate MMP-2 activation, at higher levels TIMP-2 inhibits the activation of MMP-2 by saturating free MT-MMPs that are needed to remove the MMP-2 prodomain (Sternlicht and Werb 2001; Jiang et al., 2002). If high levels of TIMPs are not MMP inhibitory in retinoblastoma, what role do they play?

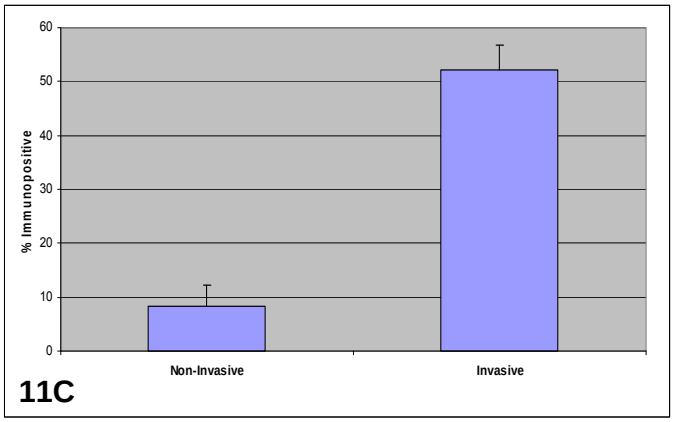
In contrast to their anti-MMP activity, TIMP-1 and TIMP-2 have also been shown to potentiate cell growth (Hayakawa et al., 1992; Hayakawa et al., 1994) and have anti-apoptotic properties and some of these are independent of their MMP-inhibition. The effects of TIMPs on angiogenesis are multifunctional and paradoxical. By inhibiting MMP's pro-angiogenic activity or by a direct effect on endothelial cell proliferation, such as the ability of TIMP-2 to inhibit the outgrowth of basic FGF-stimulated endothelial cells (Murphy et al., 1993), TIMPs show anti-angiogenic effects. As some MMPs such as MMP-2, MMP-7 and MMP-9 generate potent anti-angiogenic inhibitors such as angiostatin from plasminogen (Sang 1998), the TIMPs that inhibit these MMPs may have pro-angiogenic effects. With these multiple and paradoxical roles, further investigation is required to determine the functional significance of TIMP expression in retinoblastoma.

In conclusion, it is clear that in addition to MMP-2, MMP-9, TIMP-2 and TIMP-1, EMMPRIN, also has important roles in retinoblastoma invasion. Their co- localization

could further aggravate invasiveness and contribute to local immunosuppression in the tumor environment. Thus, targeting any one mechanism may be insufficient to reduce tumor invasiveness. Further, studies are clearly needed to understand these complex pathways, which contribute to tumor aggressiveness before considering potential therapeutic targets for the treatment or prevention of invasive retinoblastoma.







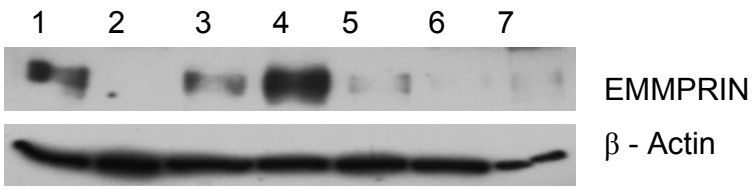


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Figure 11: EMMPRIN expression in retinoblastoma (A) EMMPRIN immunoreactivity of a tumor without invasion (50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** EMMPRIN immunoreactivity in tumor cells with invasion (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C) (D)** EMMPRIN immunoreactivity among invasive and non-invasive retinoblastoma; **(E)** Immunoblotting of EMMPRIN in seven retinoblastoma tumors

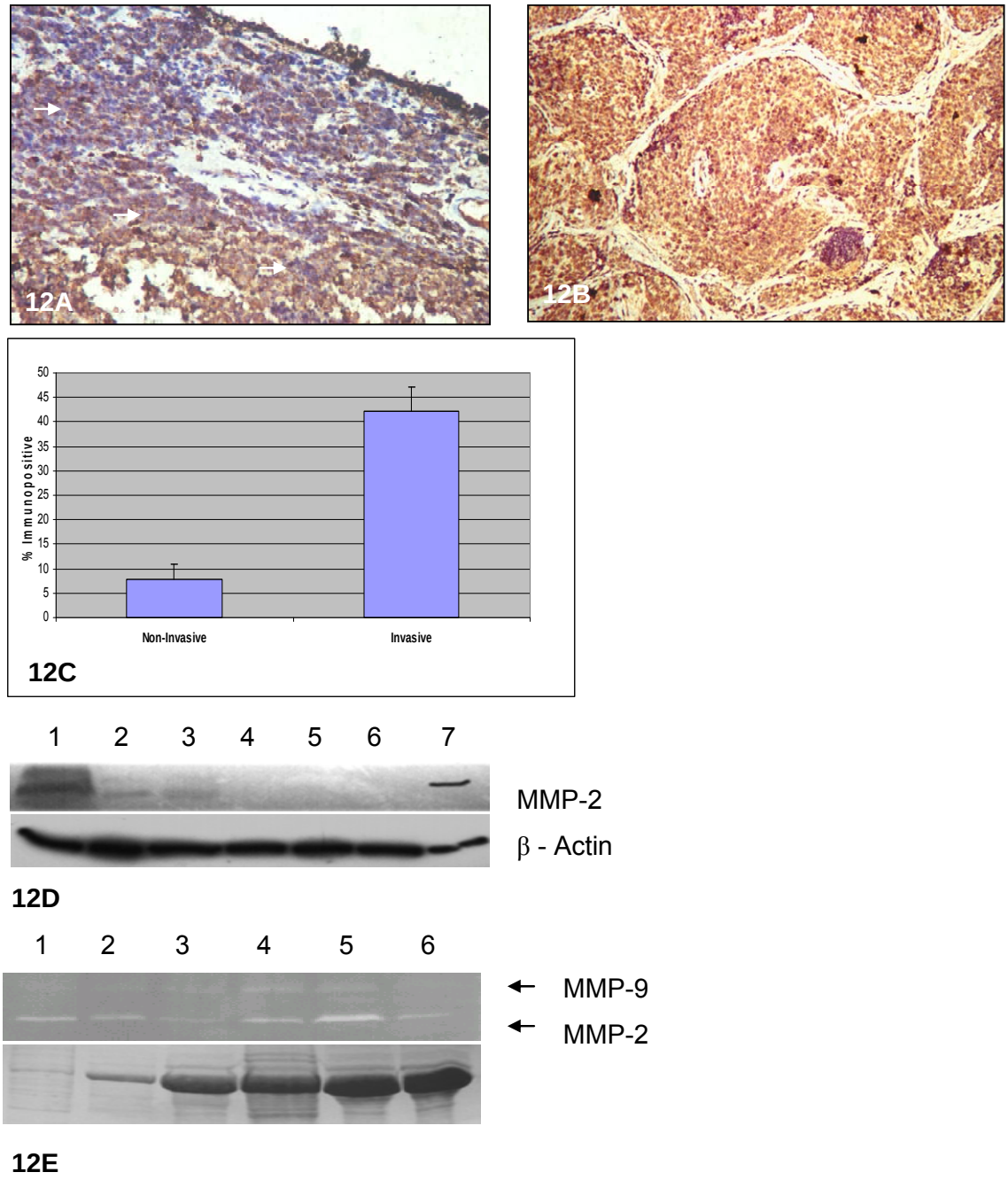


Figure 12: MMP-2 expression in retinoblastoma (A) MMP-2 immunoreactivity of a tumor with choroidal invasion (50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** MMP-2 immunoreactivity in tumor cells with optic nerve invasion (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** MMP-2 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of MMP-2 in seven retinoblastoma tumors; **(E)** Upper panel shows gelatin zymography of retinoblastoma tumors showing bands at ~92

and 72 kDa, probably corresponding to MMP-9 and MMP-2, respectively ; lower panel shows ponceau staining of tumors.

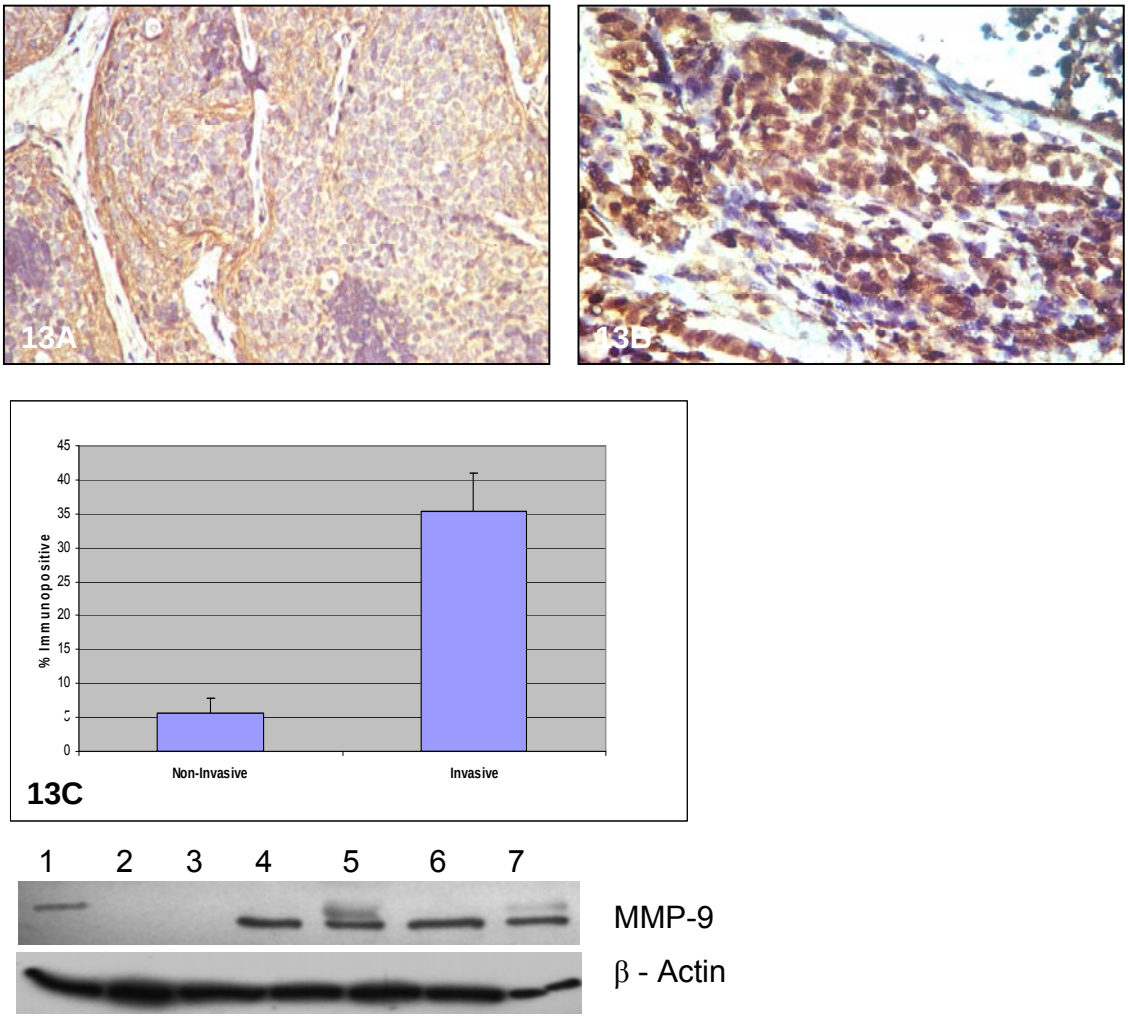


Figure 13: MMP-9 expression in retinoblastoma (A) MMP-9 immunoreactivity of a tumor with optic nerve invasion (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** MMP-9 immunoreactivity in tumor cells with choroidal invasion (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** MMP-9 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of MMP-9 in seven retinoblastoma tumors

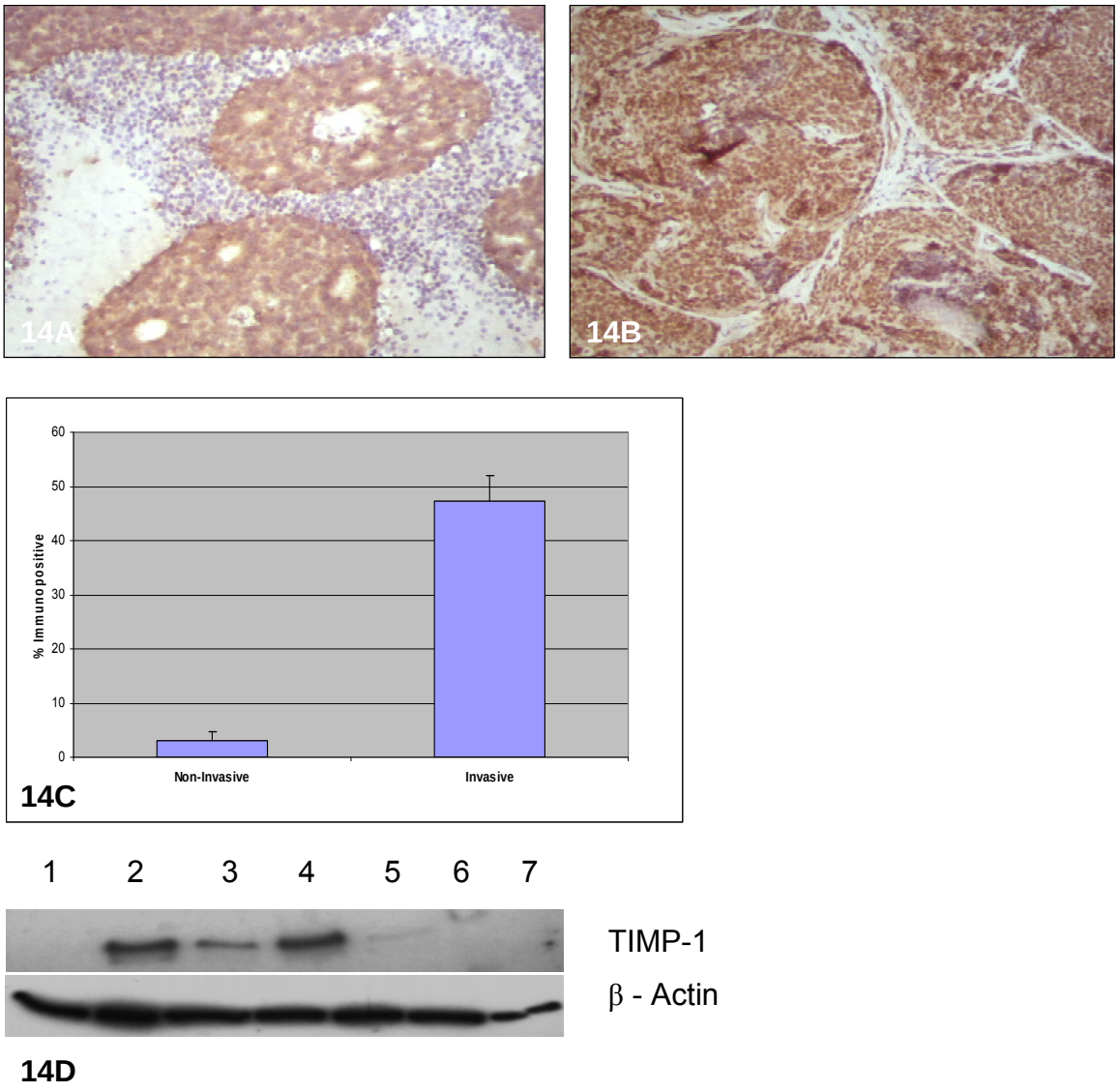


Figure 14: TIMP-1 expression in retinoblastoma (A) TIMP-1 immunoreactivity of a tumor surrounding blood vessels (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** TIMP-1 immunoreactivity in tumor cells with optic nerve invasion (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** TIMP-1 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of TIMP-1 in seven retinoblastoma tumors

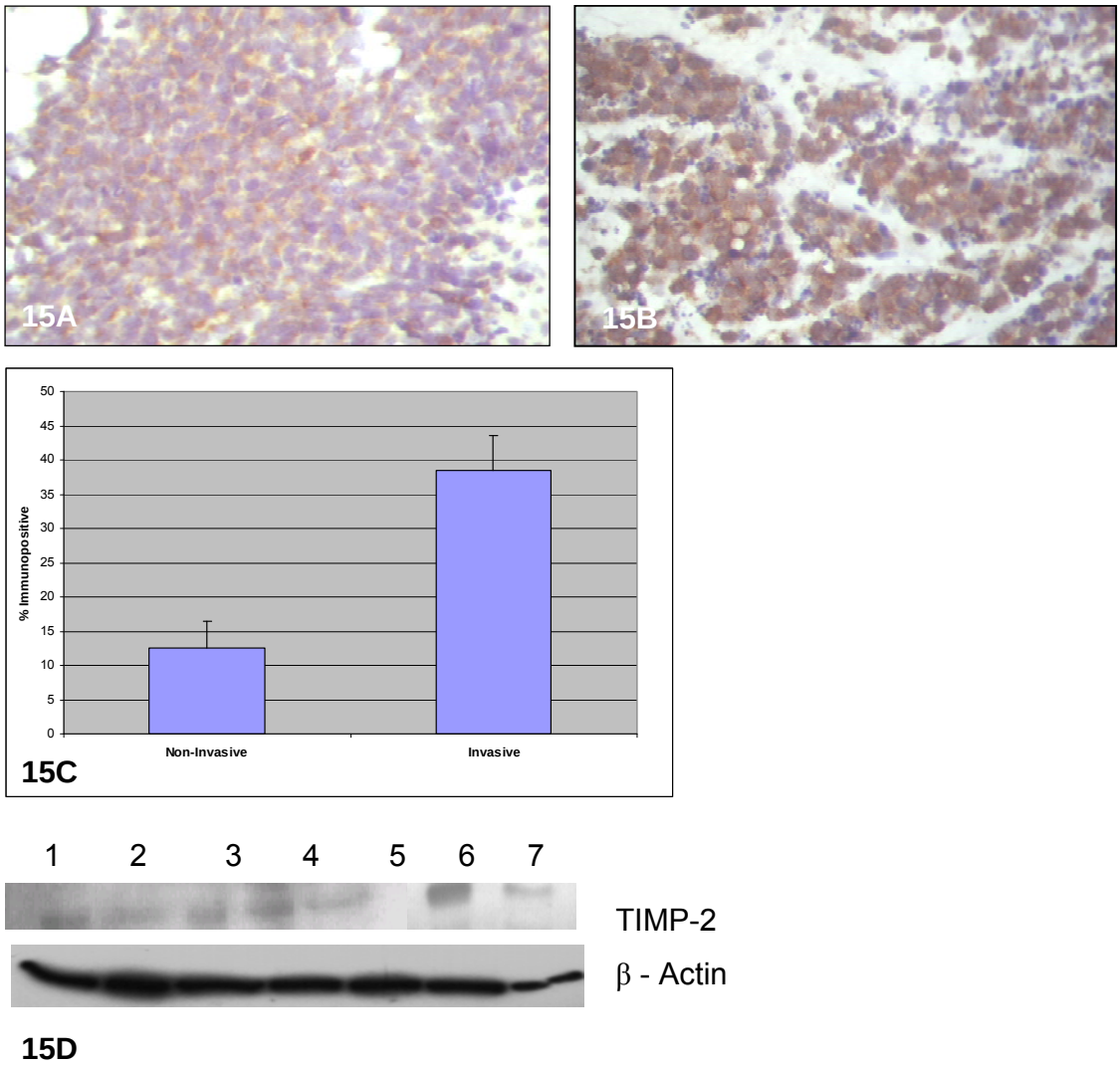


Figure 15: TIMP-2 expression in retinoblastoma (A) TIMP-2 immunoreactivity of a tumor surrounding blood vessels (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** TIMP-2 immunoreactivity in tumor cells invading the orbit (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain)**; (C)** TIMP-2 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of TIMP-2 in seven retinoblastoma tumors

5.5 Higher Tiam1 Expression in Invasive Retinoblastoma and Low Rac1 and Cdc42 Expression in all Retinoblastoma

The cohort included 32 tumors with no invasion and 35 tumors with invasion. There was no significant difference in Rac1 or Cdc42 expression between invasive tumors and non-invasive tumors ($P>0.05$) (Figures 16 to 19). By Western blotting, Rac1 was expressed in 7/20 tumors and Cdc42 in 8/20 tumors. Normal retina was negative for Rac1 and Cdc42.

Tiam1 was observed in a majority of the tumors 90% (60/67) and tumors with invasion showed significantly higher expression of Tiam1 compared to tumors with no invasion ($P <0.01$). In western blots, Tiam1 was expressed in all tumors but the immunoreactive bands were at ~110 kDa and ~70 kDa, unlike the full length band at 200 kDa from the 3T3 cell lysate (positive control). A study by Qi et al. reported that apoptosis induction by Fas stimulation of human Jurkat T lymphocytes, serum withdrawal of rat PC12 cells, and ceramide treatment of murine HMN1 neuroblastoma motor neurons resulted in caspase-3 dependent Tiam1 cleavage and generated a product with 75kDa. Using an N-terminal mutant of Tiam1, the authors mapped the caspase cleavage site at amino acid position 993 and demonstrated that this produced a stable COOH fragment which produced the 75 kDa band seen in vivo. This product failed to localize to the membrane and was defective in activation of Rac and it's downstream effector Jun Kianse (JNK) and Serum Response factor (SRF). The authors also observed a 120 kDa band in PC12 and HMN1 cells in anti-Tiam1 immunoprecipitations but this band was not seen consistently, nor was it competed away by preincubation of the Tiam1 antibody with the antigenic peptide. (Qi et al., 2001). We were also able to demonstrate the presence of caspase-3 in retinoblastoma.

Tiam1 was localized to the nucleus and cytoplasm in retinoblastoma. This may explain why Rac1, it's specific effector is expressed infrequently in retinoblastoma. Nuclear localization of Tiam1 has been reported when it interacted with c-Myc, and this interaction inhibited the transactivation and apoptosis activities of the latter. This

interaction required the N-terminal domain (1-392 aa) and the Myc Box II (MBII) domain in the N-terminal domain of c-Myc. In Rat-1 and Rat-1/c-Myc cells, Tiam1 was localized to the nucleus when plated on uncoated slides. When plated on fibronectin, membrane ruffle localization of Tiam1 was detected as early as 30 minutes in Rat-1 cells, whereas it took 50 minutes in Rat-1/c-Myc plated under the same conditions, showing that overexpression slowed down Tiam1 mobilization to membrane ruffles and cytoplasm from the nucleus. While the authors showed that Tiam1 negatively regulated the apoptotic activities of c-Myc, the regulators of apoptosis were not identified (Otsuki et al., 2003).

Interestingly, the N-terminus (1-392 aa) of Tiam1 also interacts with the tumor metastasis suppressor nm23H1 and this interaction inhibited the amount of GTP-bound Rac1, activation of c-Jun kinase in vitro and peripheral accumulation of Tiam1 (Otsuki et al., 2001). In a previous study of nm23H1 expression in retinoblastoma (Krishnakumar et al., 2004), higher expression was seen in non-invasive compared to invasive tumors.

To summarize, the low expression of Rac1 and Cdc42 suggest that their expression may be inhibited or that the other family members may be expressed in retinoblastoma. The mechanism for the nuclear localization of and the functional role Tiam1 in the nucleus is not known, and it is probable that it is cleaved to generate a C-terminal fragment that lacks Rac activation and that does not localize to the membrane. The lack of membrane accumulation may be relevant to decreased cell motility or decreased intracellular adhesion in tumor progression.

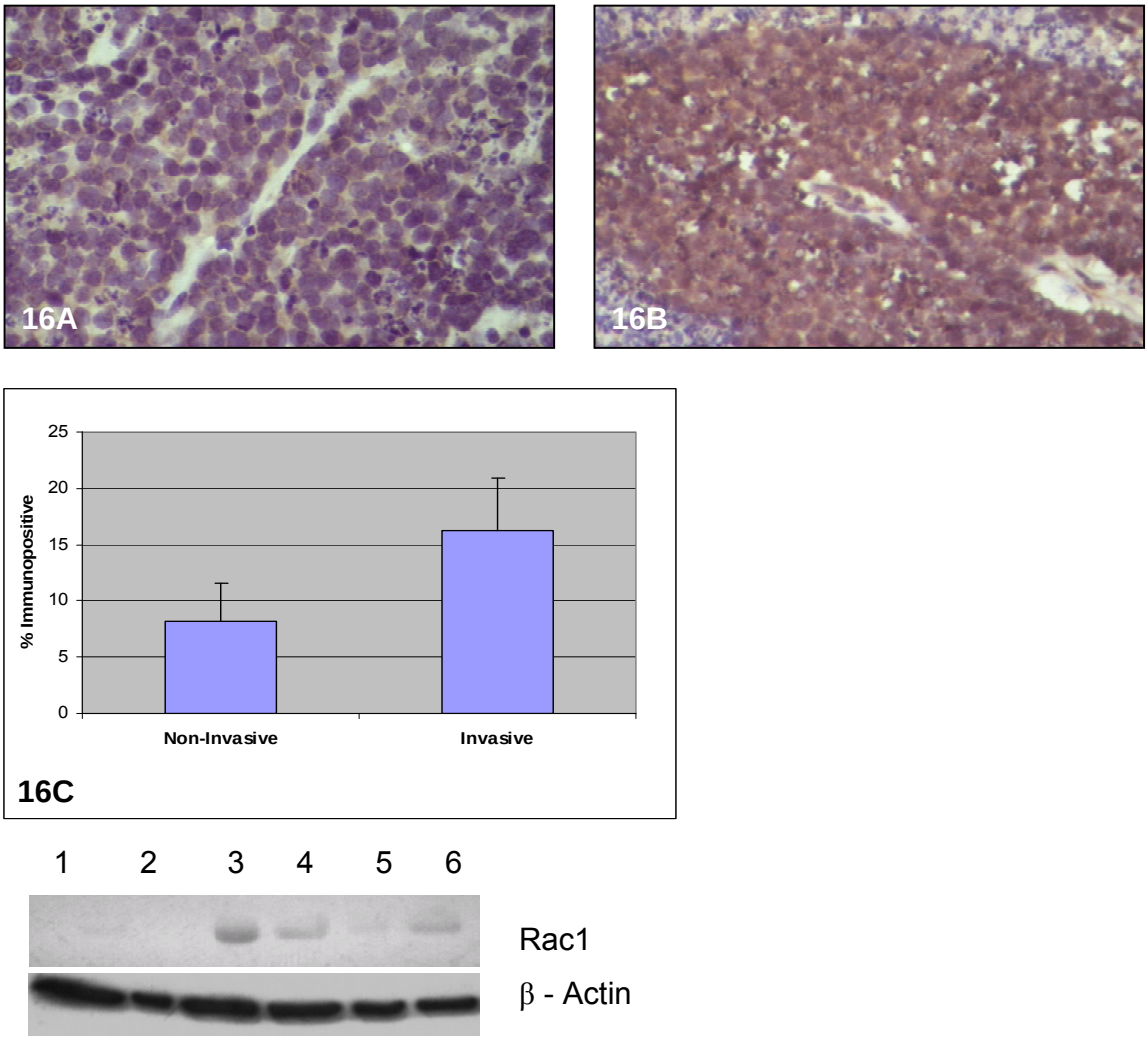
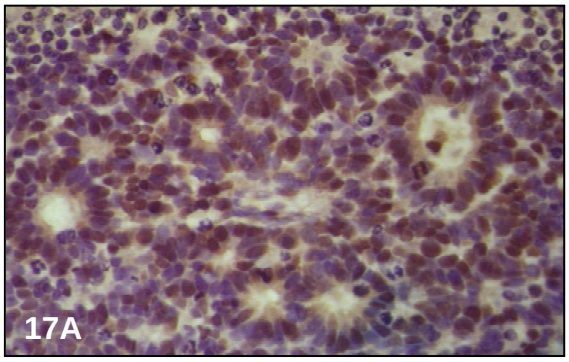
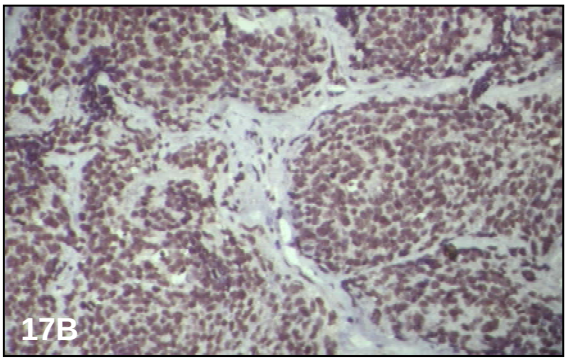
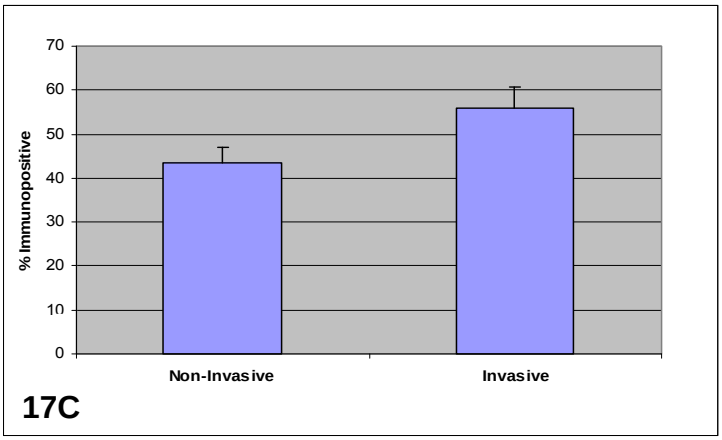
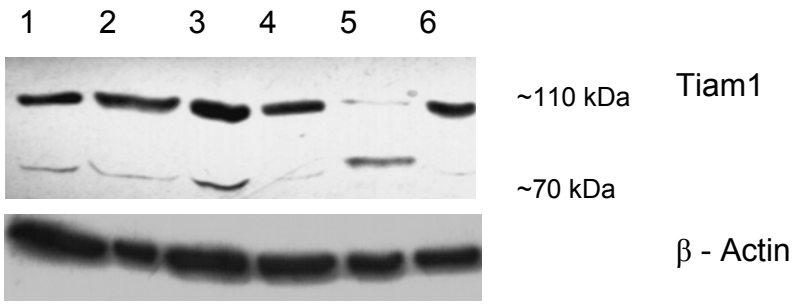


Figure 16: Rac1 expression in retinoblastoma (A) Rac1 immunoreactivity of a tumor surrounding blood vessels (<10% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** Rac1 immunoreactivity in tumor cells (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** Rac1 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of Rac1 in six retinoblastoma tumors









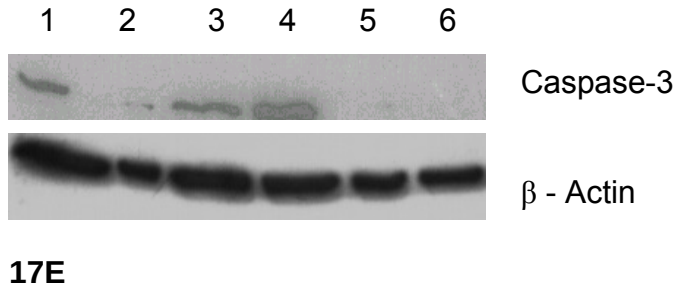


Figure 17: Tiam1 expression in retina and retinoblastoma (A) Tiam1 immunoreactivity of tumor arranged in rossettes (50% cells positive) (diaminobenzidine

chromogen with hematoxylin counterstain); **(B)** Tiam1 immunoreactivity in tumor cells invading the optic nerve (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** Tiam1 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of Tiam1 in six retinoblastoma tumors; **(E)** Immunoblotting of Caspase-3 in six retinoblastoma tumors

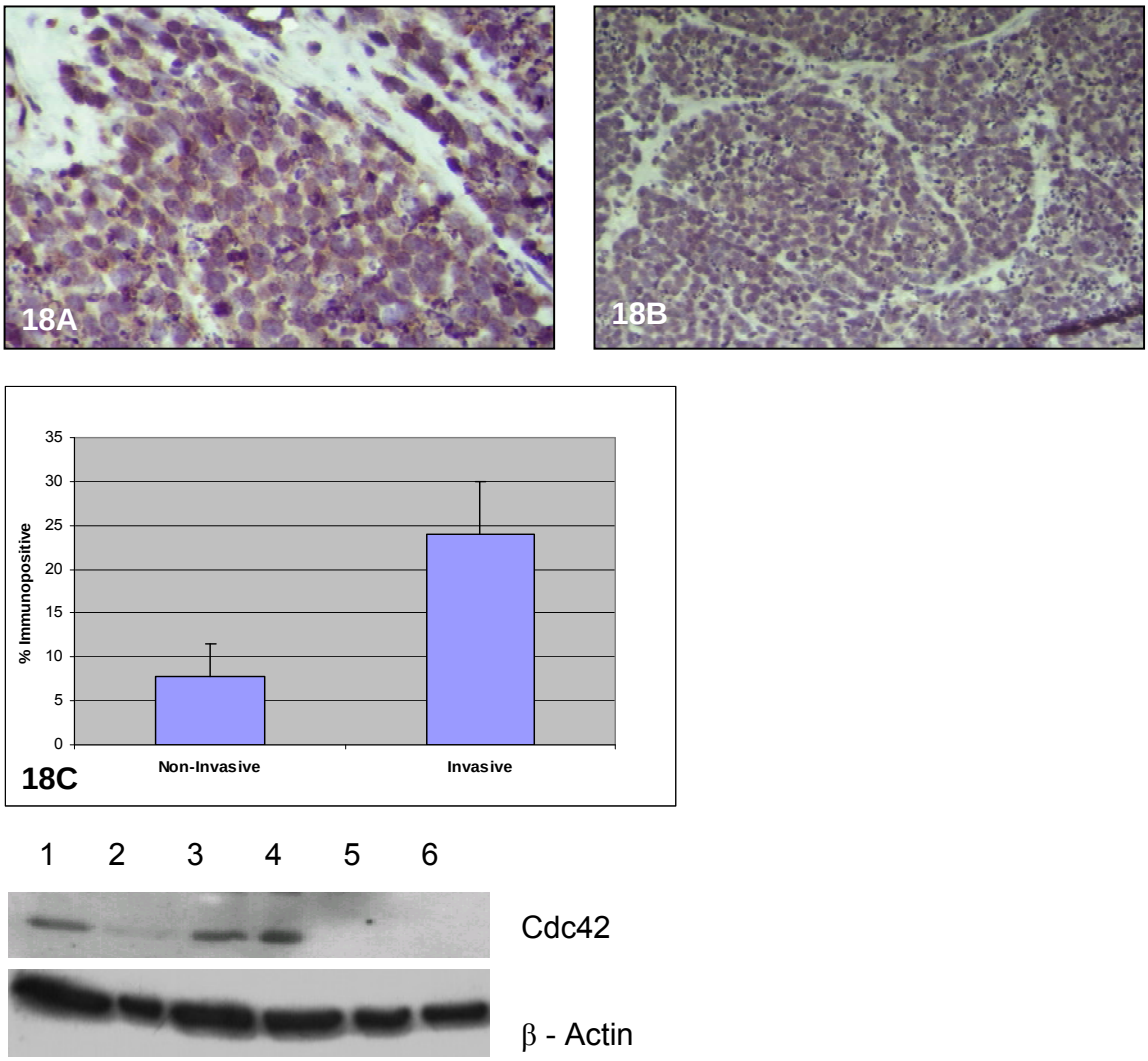


Figure 18: Cdc42 expression in retina and retinoblastoma (A) Cdc42 immunoreactivity of tumor invading the optic nerve (<10% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** Cdc42 immunoreactivity in tumor cells invading the optic nerve (<10% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** Cdc42 immunoreactivity among invasive and non-invasive retinoblastoma; **(D)** Immunoblotting of Cdc42 in six retinoblastoma tumors

5.6 Higher inducible Nitric Oxide Synthase (iNOS) and Nitrotyrosine (NT) in Invasive Retinoblastoma

NO has been implicated in diverse processes in the retina such as normal phagocytosis of the retinal outer segment, control of ocular blood flow, retina and choroidal neovascularization, regulation of aqueous humor dynamics and retinal neurotransmission and phototransduction (Goldstein et al., 1996; Becquet et al., 1997; Ando et al., 2002). Earlier reports have demonstrated iNOS expression in Mueller cells and the RPE, nNOS (neuronal NOS) expression in photoreceptors and bipolar cells (Goldstein et al., 1996) and both eNOS and nNOS expression in endothelia of choroidal and retinal vasculature (Chakravarthy et al., 1995). In our study which included 17 non-invasive and 17 invasive tumors, eNOS, iNOS and NT were expressed in the ganglion cell layer, inner and outer nuclear layers and in the retinal pigment epithelial tissue. They were negative in the optic nerve. In addition, eNOS expression was also seen endothelial cells of retinal blood vessels.

Secondly, eNOS, iNOS and NT are expressed in retinoblastoma. When their expression was compared with invasiveness, eNOS was seen expressed in both the groups, but the expressions of iNOS and NT were significantly higher in tumors with invasion ($P<0.01$) (Figures 19 to 21).

In general, low levels of NO have homeostatic functions in regulation of vascular tone, neurotransmission and memory formation and prevention of blood clotting, Upregulation of it's levels have been implicated in inflammation and immunological disorders, artherosclerosis and cancer. It is likely that it's role in disease processes depends on the levels of it's expression, cell type and the biological targets it modulates via it's effects on transcription factors, elements of signal transduction pathways, mRNA stability and translation and DNA methyltransferases (Crowell et al., 2003; Xie and Huang 2003).

In the past few years, data regarding the promoting effects of iNOS on tumor

development in vivo have been mounting. A consistent association between up-regulation of iNOS and Nitrotyrosine in tumor cells and/or tumor-associated cells of the thyroid, (Patel et al., 2002), prostrate (Baltaci et al., 2001), bladder (Swana et al., 1999), lung (Marrogi et al., 2000), skin (melanoma) (Ekmekcioglu et al., 2000), pancreas (Vickers et al., 1999) and in some cases, correlated with poor survival. On the other hand, role of iNOS in tumors of the breast and colon are contradictory as some studies show that iNOS expression increases with tumor progression while other suggest it's expression is downregulated or lost during tumor progression (Crowell et al., 2003).

A number of activities may contribute to the tumor-enhancing effects of NO. This highly potent toxic molecule can cause DNA damage (Clemons et al., 2007), increased vascular permeability and tumor blood flow by vasodilation (Fukumura and Jain 1998), prevention of apoptotic cell death by upregulating heat-shock proteins, cyclooxygenase-2, or heme oxygenase or as a consequence of NO/Superoxide interaction (Brune et al., 1998), and suppression of the immune system (Lejeune et al., 1994). On the other hand, many reports also indicate that NO can inhibit neoplasia. NO can induce apoptosis by upregulating p53 and caspases (Brune et al., 1998), can decrease tumor growth and metastasis (Dong et al., 1994; Thomsen and Miles 1998) by cytotoxicity of NO or it's products such as peroxynitrate, decreased cell cell adhesion (Xie and Huang 2003) and inhibit angiogenesis by inhibiting proliferating of endothelial and smooth muscle cells (Thomsen and Miles 1998; Xie and Huang 2003)or by downregulating MMP-2 expression (Shariftabrizi et al., 2006).

Though it has the capacity to be tumor-promoting or tumor-inhibitory, low levels of NO appear to increase the tumor-promoting effects of NO, whereas high levels are cytostatic/cytotoxic. For example, iNOS activity in genetically engineered human colon cells and with increased growth and angiogenic potential was at least 1–2 orders of magnitude lower than that associated with antitumor actions such as cytotoxicity and apoptosis (Jenkins et al., 1995). Whereas biological systems where inhibitory effects of NO was seen, used continous, high levels of NO or exogenous NO donors in

macrophages or endothelial cells and high levels produced by iNOS gene transfection in tumor cells (Crowell et al., 2003). It has also recently been pointed out that although it is clear that NOS-expressing cells produce NO, it remains unknown whether NOS directly synthesizes NO. Either NO or other reactive nitrogen species such as NO or peroxynitrite may be the immediate products of NOS. Moreover, exogenous NO donors enhance differentiation and apoptosis and inhibit angiogenesis and could account for the discepancies between in vitro and in vivo observations. Although much is known about how NO functions, is regulated and in turn influences gene expression, not much is known about NO-mediated signaling pathways *in vivo*.

In conclusion, eNOS, iNOS and NT are expressed in retinoblastoma. At present, the role of NO in tumor biology is still poorly understood. The complex biological actions of this ubiquitous signaling molecule necessitate careful experimentation to adequately assess it's contribution in retinoblastoma.

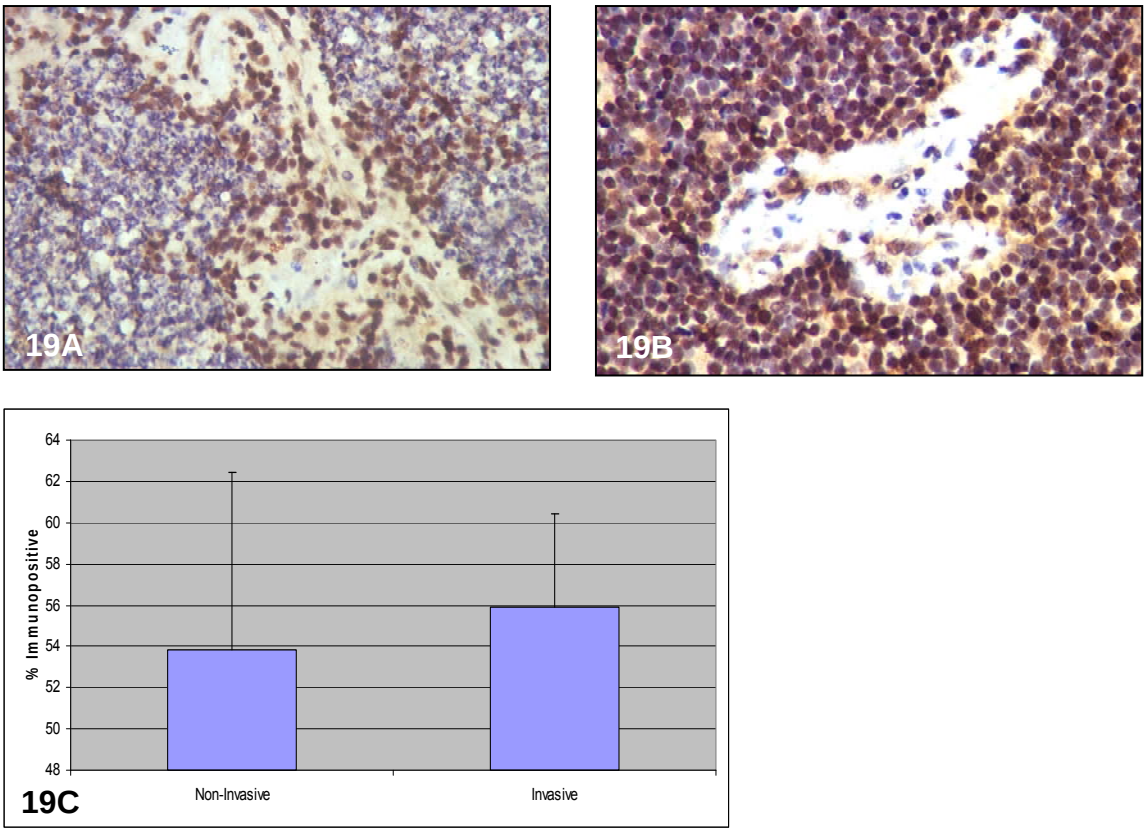


Figure 19: eNOS expression in retinoblastoma (A) eNOS immunoreactivity of tumor cells (<50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** eNOS immunoreactivity in tumor cells (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** eNOS immunoreactivity among invasive and non-invasive retinoblastoma

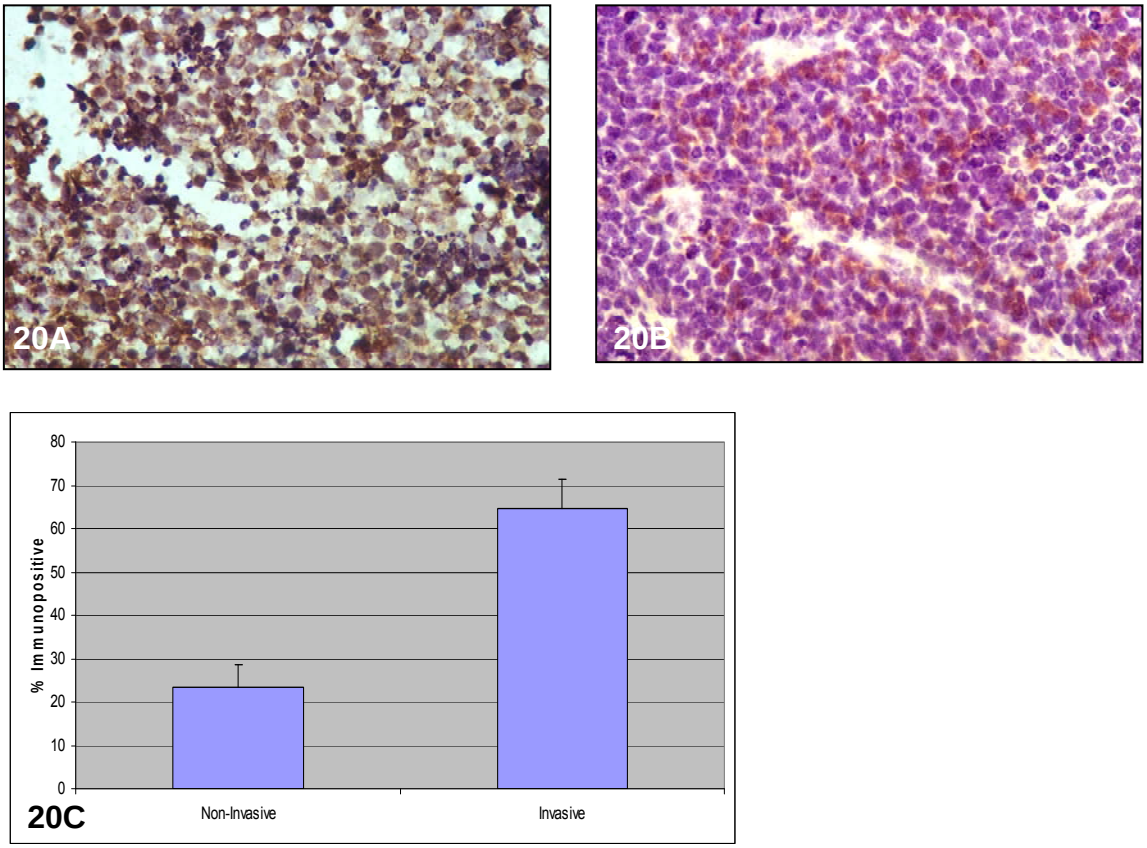


Figure 20: iNOS expression in retinoblastoma (A) iNOS immunoreactivity of tumor cells (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** iNOS immunoreactivity in tumor cells invading the optic nerve (50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** iNOS immunoreactivity among invasive and non-invasive retinoblastoma

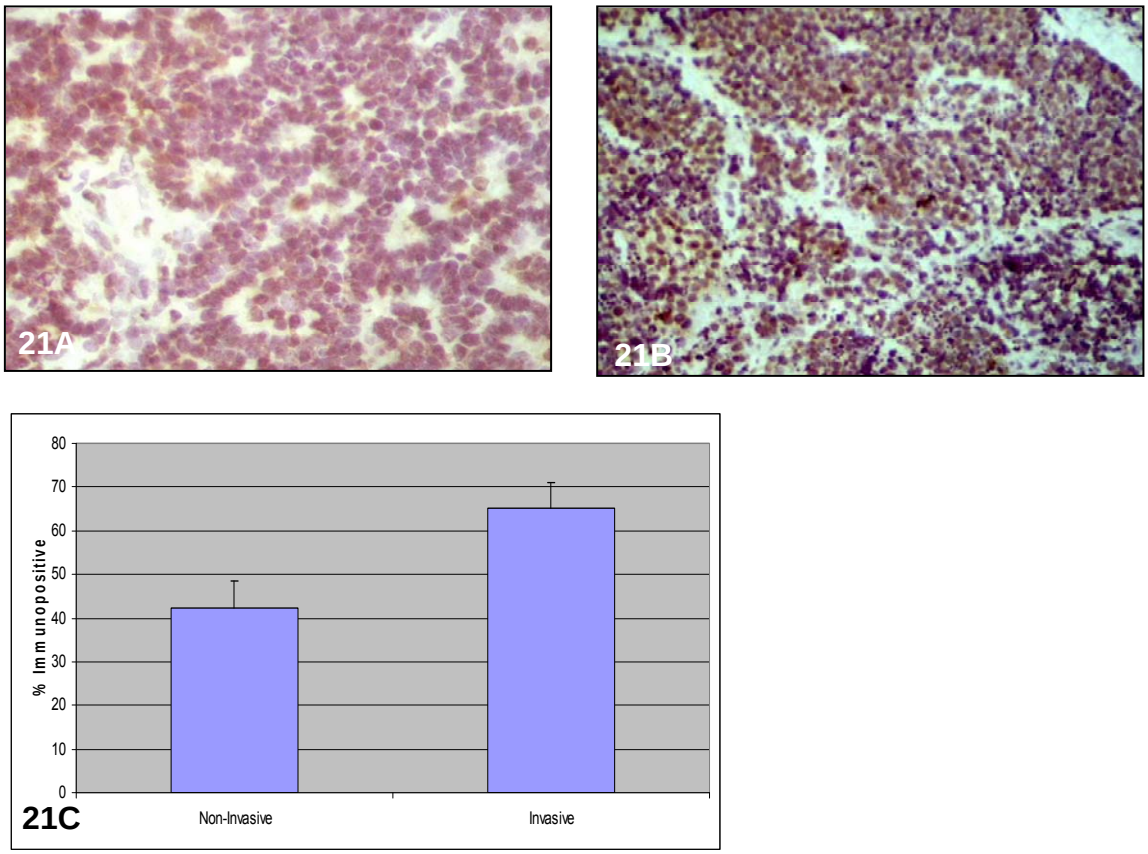


Figure 21: NT expression in retinoblastoma (A) NT immunoreactivity of tumor cells (>50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(B)** NT immunoreactivity in tumor cells invading the choroid (50% cells positive) (diaminobenzidine chromogen with hematoxylin counterstain); **(C)** iNOS immunoreactivity among invasive and non-invasive retinoblastoma

5.7 Src tyrosine kinase can regulate cadherins and catenins to modulate cell-cell adhesion

To suppment our studies of expression of cadherin, catenins and Src in human tumor samples, I was interested in studying the expression of these molecules in human and mouse tumor cell lines. As a starting point, we investigated whether these pathways we connected and below is a summary of their interactions. These pathways are represented schematically in Figure 22.

As mentioned earlier, elevation in Src protein expression and activity in a number of cancers is also often associated with stage of disease, which strongly suggests that it facilitates malignant progression. The elevation or activation of Src has often been associated with the induction of adhesion changes and the promotion of the mesenchymal phenotype (Martin 2001; Frame 2002). Src's role in adherans junctions was suggested by the fact that tyrosine phosphorylation in general, and v-Src activity in particular, is associated with the disruption of cadherin mediated cell-cell adhesions(Volberg et al., 1992; Avizienyte et al., 2002; Irby and Yeatman 2002). Some of the adhesion substrates that are tyrosine phosphorylated by v-Src include focal adhesion kinase (FAK), p190Rho-GAP, cortactin, Beta-catenin and $p120^{CTN}$ and it has been suggested that these changes could contribute to the concomitant loss of cell-cell adhesion observed in different v-Src transformed cells (Figure 24).

5.7.1 Src signaling to E-Cadherin involves integrin:

Studies from KM12C colon cancer cells has shown that Src kinase activity is required to facilitate the dynamic formation and retraction of membrane protrusions where it induces the assembly of alpha-v/beta-1 integrin adhesion complexes, expression of the mesenchymal marker vimentin and the suppression of E-cadherin localization and function (Avizienyte et al., 2002). This change from the predominant cell-cell contact to the cell-matrix/substratum is likely through Src-induced peripheral accumulation of phosphorylated MLC (myosin light chain) through signals from the mitogen activated

protein kinase cascade involving Mitogen Activated Protein Kianse (MEK), Extracellular signal-related kinase (ERK) and it's substrate MLC Kinase (MLCK) (Avizienyte et al., 2004). Another study on the human hepatocellular carcinoma cells line KYN-2 have shown the possible involvement of beta-1 and beta-5 integrin in the inhibition of cadherin-mediated cell-cell adhesion through the tyrosine dephosphorylation and activation of c-Src, and this plays an important role in intrahepatic metastasis of human HCC.

5.7.2 Src signaling through p120 may reduce E-cadherin mediated cell adhesion:

p120 was originally singled out in a lineup of several prominently phosphorylated Src substrates because it was phosphorylated by v-Src and not by a transformation defective v-Src variant (v-Src/G2A) rendered cytoplasmic by a mutation of it's amino-terminal glycine residue (Mariner et al., 2001). In mouse L fibroblasts , tyrosine phosphorylation of p120 depended on it's association with E-cadherin and was involved in the v-Src-mediated reduction of E-cadherin mediated adhesion, although the mechanism by which tyrosine phosphorylated p120 inactivates E-cadherin is not known. In these cells, the interactions between B-catenin with cadherins and alpha-catenin was stable under all conditions tested, and only p120 interactions with cadherins seemed to be weak (Ozawa and Ohkubo 2001). Alternatively, other reports suggest that p120 could have positive effects on cadherin clustering in certain circumstances. These multiple effects are probably tightly regulated by it's subcellular localization, isoform expression and post-translational modification by phosphorylation (Yap et al., 1998; Thoreson et al., 2000).

Recent studies have shown that one of the main roles of p120 is to regulate cadherin turnover i.e. determine the amount of cadherin at the cell surface by increasing cadherin half-life after it's arrival at the cell surface. This is further supported by extrapolation of siRNA data on normal cells, where p120 is present at relatively high levels, and suggest a model by which cadherin endocytosis could be modulated by signaling to p120, which could be affect phosphorylation. Hence, the p120 on/off rate could be dynamically

modulated such that it's binding retains cadherin at the surface and it's dissociation promotes cadherin internalization (Davis et al., 2003). This is further supported by data from the pathology literature also show that frequent loss or downregulation of p120 seen in a number of cancers, suggesting that it could be an early event in tumor progression, maybe even before E-cadherin is lost (Thoreson and Reynolds 2002; Reynolds and Roczniak-Ferguson 2004). Interestingly, there might exist a competition of other proteins for the JMD binding domain of E-cadherin such as Hakai, the E3 ubiquitin ligase that targets E-cadherin for ubiquitination and internalization (Fujita et al., 2002).

On the other hand, if E-cadherin was lost either before or by mechanisms independent of p120, p120 mislocalization to the cytoplasm may promote cell motility and/or invasion by the induction of the dendritic/branching phenotype by the regulation of the family of GTPases – either by activation of Rac1 and Cdc42 by binding the GEF Vav-2 (Noren et al., 2000) and/or by it's inactivation of RhoA (Anastasiadis et al., 2000). Nuclear localization of p120 is also increased in E-cadherin deficient cells and this could have implications on it's interaction with Kaiso, a BTB/POZ transcription factor that have important roles in development and cancer, and kinesin, a microtubule-associated motor protein though the significance of this interaction is unknown (Thoreson and Reynolds 2002).

5.7.3 Src interactions with FAK and GTPases:

One puzzling aspect of Src's functional interactions with cytoskeletal regulators is the Src-induced antagonism of Rho via activation of p190-Rho GAP. Activation of a temperature sensitive v-Src induced association of tyrosine phosphorylated p190 with p120 (RasGAP) and stimulation of p120(RasGAP)-associated RhoGAP activity, although p120 (RasGAP) itself was not a target for phosphorylation by v-Src in chicken embryo cells. These events required the catalytic activity of v-Src and were linked to loss of actin stress fibres during transformation. Though Rho is required for the generation of necessary contractile forces, Src-induced antagonism of Rho-induced contractility may be

required at specific times and places within migrating cells (Fincham et al., 1999).

Another important regulator of Src activity is Focal Adhesion Kinase, a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of proliferation and migration of normal and tumor cells. FAK integrates signals from growth factor receptors, such as EGFR, and integrins and regulate cell migration by involvement of the Crk-associated substrate (CAS) family. Cellular Src bound to FAK phosphorylates CAS proteins leading to the recruitment of a Crk family adaptor molecule and activation of a small GTPase and c-Jun N-terminal kinase (JNK) promoting membrane protrusion and cell migration (Cox et al., 2006).

5.8 Human and mouse retinoblastoma cell lines express components of the Src pathway

We looked at the expression of some of the Src pathway components in human (Y79, Weri1 and RB355) and mouse (SJmRbL3, SJmRBL8, SJmRBL10 and SJmRbL12) retinoblastoma cell lines (Figure 23). All cell lines expressed Csk (C-Src tyrosine kinase), a known negative regulator of Src tyrosine kinase (Okada et al., 1991) at different levels. They all also expressed total Src but not phospho-Src, which is an indicator of active Src. The absence of active Src may be because of the presence of Csk.

E-Cadherin was seen expressed in all the cell lines but the expression levels varied. N-cadherin was expressed at high levels in the mouse adherent cell lines (SJmRBL10 and SJmRbL12) and the control Cos7 cells, a kidney green monkey cell line that has been immortalized by an origin defective Simian Virus (SV40). Among the non-adherent cell lines (Y79, Weri1, RB355, SJmRBL3 and SJmRBL8), Weri1 cells expressed N-Cadherin and cadherin-11 at a stronger intensity than the Y79 cell line. The catenins α-, β- and p120 catenin were also expressed at higher levels in the adherent cells than the non-adherent cells. Hence, the cell-cell adhesion components are expressed by all human and mouse retinoblastoma cells, with high expression levels in the adherent cells than the

non-adherent cells.

It is interesting to note that this type of heterogeneity of tumor cells in culture (adherent and non-adherent cells) have been reported in almost all efforts of primary human retinoblastoma cells in culture (Reid et al., 1974; McFall et al., 1977; Griegel et al., 1990). For example, the Weri1 cells were derived from a 1-year-old patient with no family history of retinoblastoma (McFall et al., 1977) and the initial passages of cells in culture yielded 2 distinct population of cells – the adherent and the non-adherent cells- but only the non-adherent cells were propagated as the Weri1 cells in culture. The adherent cells were not maintained as they underwent senescence in culture. The authors proposed that these were tumor-associated fibroblasts not immortalized by *Rb1* gene inactivation. As it is interesting to examine differences in cell-cell adhesion between the 2 populations and the changes that occur during establishment of cells.in culture, our lab developed the mouse retinoblastoma cell lines and we are in the process of perfoming an extensive genetic and expression analysis of the cell lines compared to each other and to the tumor from which they were originally derived from.

5.9 Genetic loss of Cadherin-11 in Human Retinoblastoma

To test whether tumors exhibit genetic changes in the pathway, we carried out Fluorescent *In Situ* Hybridization (FISH) of the genes in the pathway that have shown genetic amplifications or deletions in other cancers. We anticipated that a genetic amplification or deletion would be selected for in invasive tumor cells compared to main mass of the tumor. These results are summarized in Table 3. We observed that E-cadherin, Cadherin-11, Cadherin-13 and Cadherin-15 were lost in atleast 50% tumors, but that Cadherin-11 showed a genetic loss in 44% (5/9) invading tumor cells compared to the main tumor mass. As mentioned earlier, cadherin-11 loss has been reported previously (Marchong et al., 2004) but this is the first time a regional loss of cadherin-11 has been reported. Hence, the loss of cadherin-11 may have a role in tumor invasion. In order to test cadherin function, we are now taking genetic (cadherin knockdown and

overexpression) and functional (cadherin dependant cell-cell adhesion) approaches in retinoblastoma cell lines.

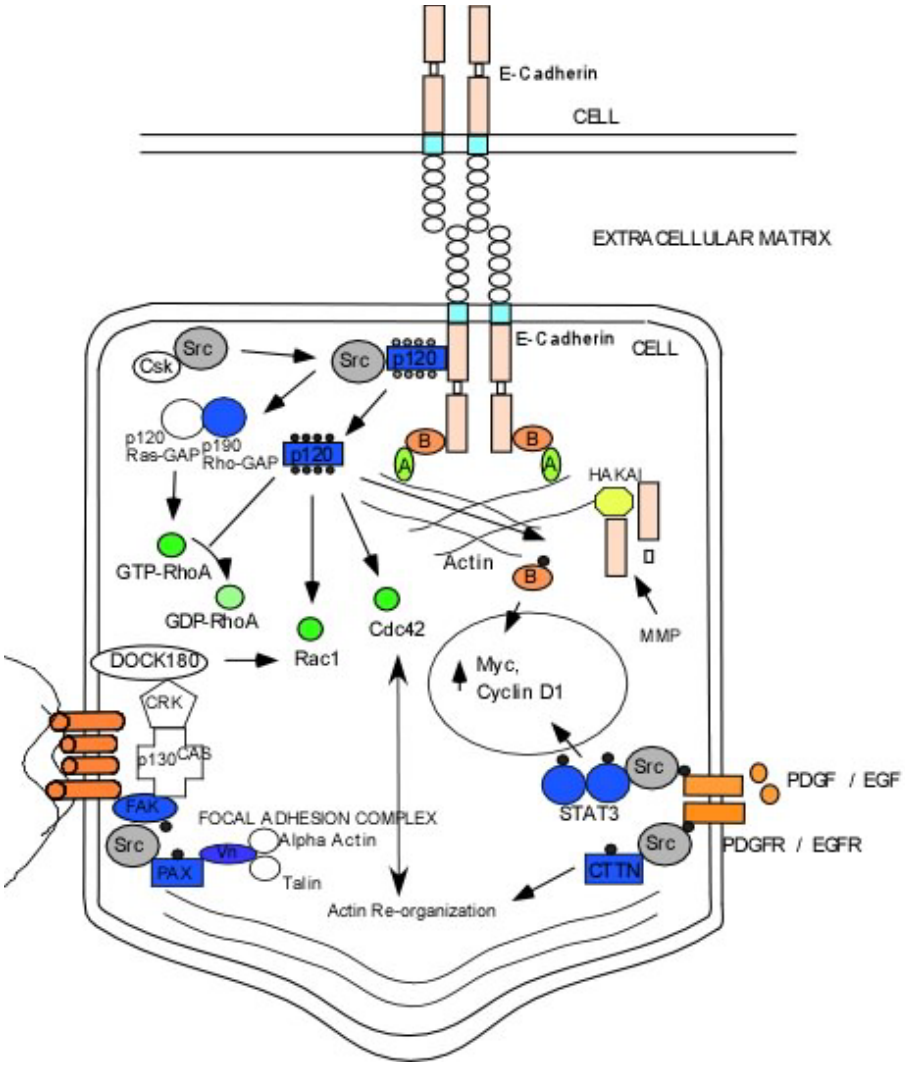


Figure 22: Cell adhesion, invasion and motility proteins that are associated with invasion in Retinoblastoma. The scheme represents some of the cell-cell adhesion molecules such as the cadherins and the cell-matrix adhesion molecules, the integrins, and some of the components that connect the 2 pathways, such as Src tyrosine kinase. Some of the effectors of Src signaling are shown in blue shading

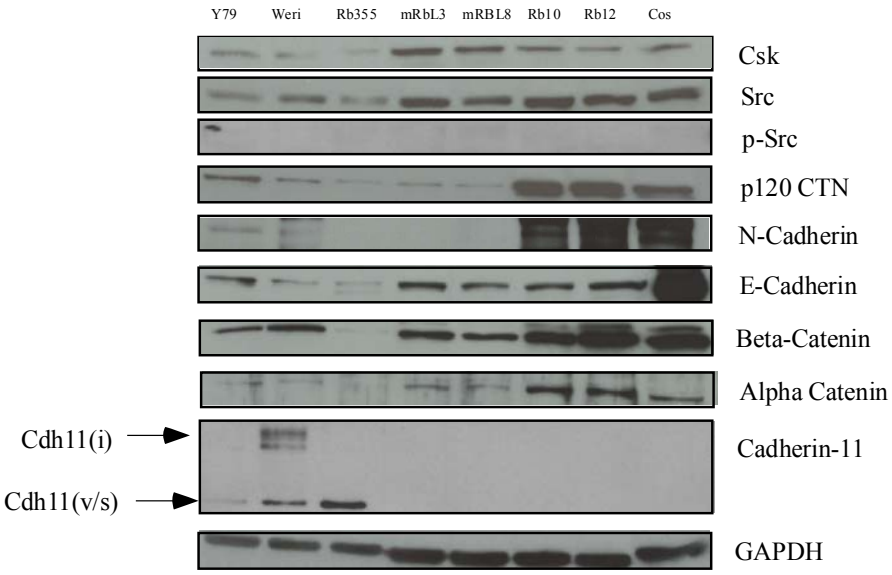
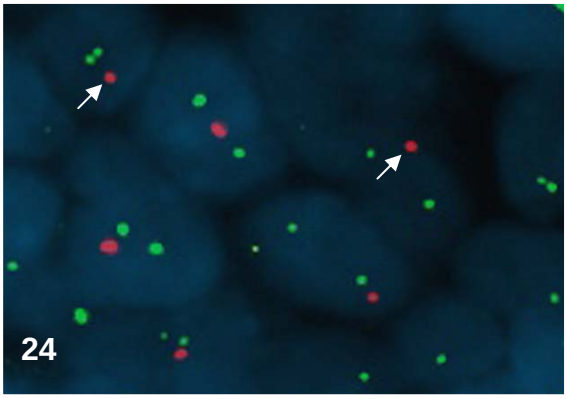


Figure 23: Human and mouse retinoblastoma cell lines express different components of the Src pathway. All retinoblastoma cells express Csk and total Src but not active Src (phosphorylated Src). The cadherins, N- and E-, are seen more in the Weri1 cell lines (among the non-adherent cells) and in the adherent cells lines Rbl10 and Rbl12 (abbreviated for SJ mRBl10 and SJmRbl12). The catenins are expressed more strongly in the adherent cell lines as well.



Gene	GENETIC LOSS	
	Main Tumor	Invading Cells
E-Cadherin	50% (8/16)	12.5% (1/8)
N-Cadherin	12.5% (2/16)	50% (1/2)
Cdh11	56% (9/16)	44% (4/9)
Cdh13	56% (9/16)	11% (1/9)
Cdh15	56% (9/16)	11% (1/9)
PDGFR β	Normal	Normal

Figure 24: Fluorescent *In Situ* Hybridization (FISH) of Cadherin genes in human retinoblastoma. FISH on human retinoblastoma for E-Cadherin (pink) versus the control (green) probe. Arrows indicate cells with a single copy of E-Cadherin gene; **Table 3: Summary of FISH analysis of human retinoblastoma (N=16) for the genes in the Src pathway.** Genetic loss was scored in the main tumor mass and the invading tumor cells.

CONCLUSIONS

Invasive retinoblastoma express higher c-Src tyrosine kinase, active Signal Transducer and Activator of Transcription 3 (pSTAT3), N-Cadherin, Epithelial Cell Adhesion Molecule (EpCAM), Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), Matrix Metalloproteinase (MMP) MMP-2 and MMP-9, Tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2, T-cell Lymphoma Invasion and Metastasis 1 (Tiam1), inducible Nitric Oxide Synthase (iNOS) and nitrotyrosine (NT) than tumors that have not invaded the choroid/optic nerve and/or orbit. Non-Invasive tumors express higher E-Cadherin α-Catenin and Motility-Related Protein-1 (MRP-1)/CD9 than invasive tumors. There were no differences in expression of β-catenin, endothelial NOS (eNOS) between the 2 groups of tumors. Rac1 and Cdc42 were expressed at infrequently in retinoblastoma.

In western blots, Tiam1 protein in retinoblastoma was of lower molecular weight than their full length forms. Though further investigations are required to determine the mechanism, this might serve as a mechanism for regulating their activities. Though it is possible that the cleaved Tiam1 is defective is signaling to Rac1, this is yet to be tested in retinoblastoma.

We have also demonstrated cadherin-11 genetic loss in invading human retinoblastoma cells. Our studies on cadherin expression in retinoblastoma cell lines suggest that their expression may correlate with differences in adhesive properties of cells in culture. We are now carrying out functional analyses to test the role of cadherin dependant cell-cell adhesion by using genetic approaches (siRNA, cDNA transfection) and calcium-dependancy of these cells in culture (which is a readout of cadherin function)

The changes in expression of tyrosine kinase and signal transduction molecules, cell-cell adhesion molecules, nitric oxide synthase molecule and extracellular matrix degrading molecules as tumors become more invasive suggest that the increase or decrease in expression may be associated with tumor invasiveness in retinoblastoma.

THE STUDY OF PRESENCE OF HUMAN PAPILLOMAVIRUS (HPV) IN RETINOBLASTOMA

Chapter 6

6.1 Human Papillomavirus in Retinoblastoma: An alternative mechanism for tumor development

Most of the cases of retinoblastoma from developing countries are in patients with no family history. There have been a few studies that show that 17 to 81% (Shimizu et al., 1994; Blanquet et al., 1995) of these non-familial cases have an intact *Rb1* gene and have suggested that an alternative mechanism of pRB inactivaton may exist. It is reasonably well known that there is a considerable overlap between epidemiological risk factors for retinoblastoma and HPV infection. The HPV E7 protein is known to bind and inactivate pRB (Dyson et al., 1989). Transgenic HPV16 mice overepressing E6 and E7 oncoproteins posses and intact retinoblastoma gene, but the ectopic expression of E6 and E7 in the retina leads to pRB inactivation and the development of retinoblastoma-like lesions (Albert et al., 1994). Recent studies from Mexico (Orjuela et al., 2000; Montoya-Fuentes et al., 2003) and Brazil (Palazzi et al., 2003) have shown an association between the presence of human papillomavirus and sporadic retinoblastoma suggesting that pRB may be inactivated by the HPV E6 and E7 oncoproteins in retinoblastoma that initiates in this country. There is no information available on the role of HPV in retinoblastoma in India and the second part of my research was to test for the presence of HPV DNA from retinoblastoma tumor tissue.

6.2 DNA Extraction and PCR for Human Papillomavirus Testing in Retinoblastoma

Forty four fresh tumor samples and ten non-neoplastic ocular tissues (donor retinal tissues) were used for HPV screening in the study and DNA extraction was done by QIamp kit method. The extracted DNA was quantified at 260nm wavelength of UV spectrophotometer. Adequacy and qualities of DNA was evaluated by PCR amplification of human beta globin gene.

6.2.1 PCR: β –Globin

Extracted DNA was subjected to PCR for β globin targeting of the 248 base pair fragment of the β- globin gene. PCO4 (5'-GAAGAGCCAAGGACAGGTAC-3') and GH20 (5'-CAACTTCATCCACGTTCACC-3') primer were used as forward and reverse primer respectively. The reaction mixture contained 10mM of Taq buffer, 1.5mM MgCl2, 1.5U of Taq polymerase, 200µm each dNTP and 0.125□m of each primer. The conditions for the PCR were initial denaturation of 94°C for 4 min, followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 45 seconds and a final extension at 72°C for 5 min. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

6.2.2 Nested PCR for HPV using General Primers

The degenerate MY09/MY11 primer set was used for the amplification of HPV DNA. The primer set FP 5' CGTCCMARRGGAWACTGATC 3' and RP 5' GCMCAGGGWCATAAYAATGG 3' (R=A+G, Y=C+T, M=A+C, W=A+T) was capable of amplifying a wide spectrum of HPV types to produce a PCR product of 450 bp. The amplification mixture consisted of 1x PCR buffer, (10mM Tris/HCl, pH 8.3, and 1.5mM MgCl2), 200µM of each dNTP, 100 pmol of each primer, 2.5 units of Taq DNA polymerase (Bangalore Genei) and 500 ng of DNA in a final volume of 25 µl. Thirty five amplification cycles were completed as follows: 45 seconds at 94°C, 45 seconds at 45°C, and 45 seconds at 72°C. Each batch of samples included negative controls containing

water and positive control DNA from an HPV positive SiHa cell lines.

The L1 consensus GP05/GP06 primer set, a non-degenerate primer (GPO5: 5'-TTTGTTACTGTGGTAGATAC-3' and GP06: 5'-GAAAAATAAACTGTAAATCA-3') was used for 2nd round, which detects a PCR product of approximately 150 bp. Thirty five amplification cycles were completed as follows: initial denaturation was for 5 minutes at 94°C, followed by denaturation for 1 minute at 94°C, annealing for 2 minutes at 45°C, extension for 90 seconds at 72°C, and a final extension step of five minutes at 72°C. Each batch of samples included negative controls containing 1st round negative products and positive control DNA from an HPV positive SiHa cell lines from 1st round product.

6.2.3 Nested PCR for HPV 16 (E6, E7 region)

The HPV 16 primer set (FW: 5'CCCAGCTGTAATCATGCATGGAGA 3' and RW: 5'GTGTGCCCATTAAACAGGTCTTCCA 3') was capable of amplifying a wide spectrum of HPV types to produce a PCR product of 272 bp. The amplification mixture consisted of 1x PCR buffer, (10mM Tris/HCl, pH 8.3, 50mM KCl, and 1.5mM MgCl2), 200µM of each dNTP, 100 pmol of each primer, 2.5 units of Taq DNA polymerase and 500 ng of DNA in a final volume of 25 µl. Forty amplification cycles were completed in a perkin Elmer 2600 model. Each batch of samples included negative controls containing water and positive control DNA from HPV positive SiHa cell lines.

Two microlitres of the first round product were used for second round PCR, which amplified the PCR product of 162 base pairs. The amplification mixture consisted of 1x PCR buffer, (10mM Tris/HCl, pH 8.3, 50mM KCl, and 1.5mM MgCl2), 200µM of each dNTP, 100 pmol of each primer, 2.5 units of Taq DNA in a final volume of 25 µl. Fifteen amplification cycles were completed in a perkin Elmer 2600 model using (FW: 5' TTGCAACCAGAGACAACTGA 3' and RW: 5'GCACAACCGAAGCGTAGAGT 3') specific primer for HPV16.

6.2.4 Semi-Nested PCR for HPV 18 (E6, E7 region)

The primer set (Forward primer: 5' CGACAGGAACGACTCCAACGA 3' and Reverse primer : 5'GCTGGTAAATGTTGATGATAAACT 3') was capable of amplifying a wide spectrum of HPV types to produce a PCR product of 202 bp. The amplification mixture consisted of 1x PCR buffer, (10mM Tris/HCl, pH 8.3, and 1.5mM MgCl2), 200µM of each dNTP, 100 pmol of each primer, 2.5 units of Taq DNA polymerase and 500 ng of DNA in a final volume of 25 µl. Each batch of samples included negative controls containing water and positive control DNA from HPV positive HeLa cell lines.
Two microlitres of the first round product was used for second round Semi Nested PCR, [where forward primer (FW; 5'CGACAGGAACGACTCCAACGA 3') of first round and reverse primer (RW: 5'TTTCATCGTTTTCTTCCTCTGA 3') of second round were used for amplification], which amplifies a PCR product of 172 base pairs. The amplification mixture consisted of 1x PCR buffer, (10mM Tris/HCl, pH 8.3, 50mM KCl, and 1.5mM MgCl2), 200µM of each dNTP, 100 pmol of each primer, 2.5 units of Taq DNA in a final volume of 25 µl. Fifteen amplification cycles were completed in a perkin Elmer 2600 model. All PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

6.2.4.1 Positive control

HPV positive fresh tissue of cervical condyloma was used as a control while cervical carcinoma cell lines (including HeLa cell lines that contain 10-50 copies of HPV 18 per cell and SiHa cell lines which contain 10-50 copies HPV 16) were also used as additional positive controls.

6.2.4.2 PCR sensitivity and specificity

To determine the sensitivity of the nested PCR, we performed PCR on serial dilutions of the SiHa cell DNA and HeLa cell DNA for HPV 16 and 18 respectively. The sensitivity of nested HPV 16 and HPV 18 semi-nested PCR was found to detect 12.5ng of HPV

DNA.

6.3 **Immunohistochemistry**

Immunohistochemical detection of Rb (Phosphorylated and unphosphorylated Rb) on paraffin wax embedded tumor sections was performed using a mouse monoclonal antibody (IF-8; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:50. Staining for Rb was visualized with the streptavidin-biotin immunoperoxidase technique using the Dako LSAB + system with horseradish peroxidase (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. For negative control, the same procedure was performed in the absence of the primary antibody, whereas endothelial cells and other normal cells were stained (internal positive control) on the paraffin sections. Detection of Rb protein was defined as tumors having >10% detectable nuclear staining Slides were examined independently by two pathologists who were blinded to the HPV status of the tumor tissues and to the clinical stage of the patient.

6.4 Detection of Human Papillomavirus (HPV) in Indian Retinoblastoma

Our study confirms the earlier studies where there was an association of HPV DNA with retinoblastoma (Orjuela et al., 2000; Montoya-Fuentes et al., 2003; Palazzi et al., 2003). In contrary, recently Gillison et al (Gillison et al., 2007) have shown that pRb-inactivating human DNA tumor viruses do not play a role in the development of RB. However, the study was carried out in USA and hence there could be a possible variation in epidemiological risk factors association with development and progression in Asian Indian retinoblastoma.

Among the 44 tumors in the study, HPV DNA was detected in 21/44 (47%) of tumors. In addition, HPV 16 was detected in 12/21(57%) tumors containing HPV. HPV 18 was no detected in any of the tumors. Ten non-neoplastic donor retina samples were for HPV genome and it was seen that all the controls were negative for HPV. Eight of 44 sporadic tumors were immunoreactive for Rb suggesting that Rb is present in a subset of these tumors. Rb protein was absent in 71% (16/21) tumors that had HPV DNA. However, Rb was also absent in 86% (20/23) tumors that were HPV negative. Representative PCRs and immunostaining pictures are shown in Figure 25.

There was no correlation between the HPV positivity and Rb expression, invasion or differentiation of tumors. There was also no correlation between the HPV expression and age at diagnosis of children with RB. Further studies are required to determine whether the presence of HPV is required in the initiation of retinoblastoma.

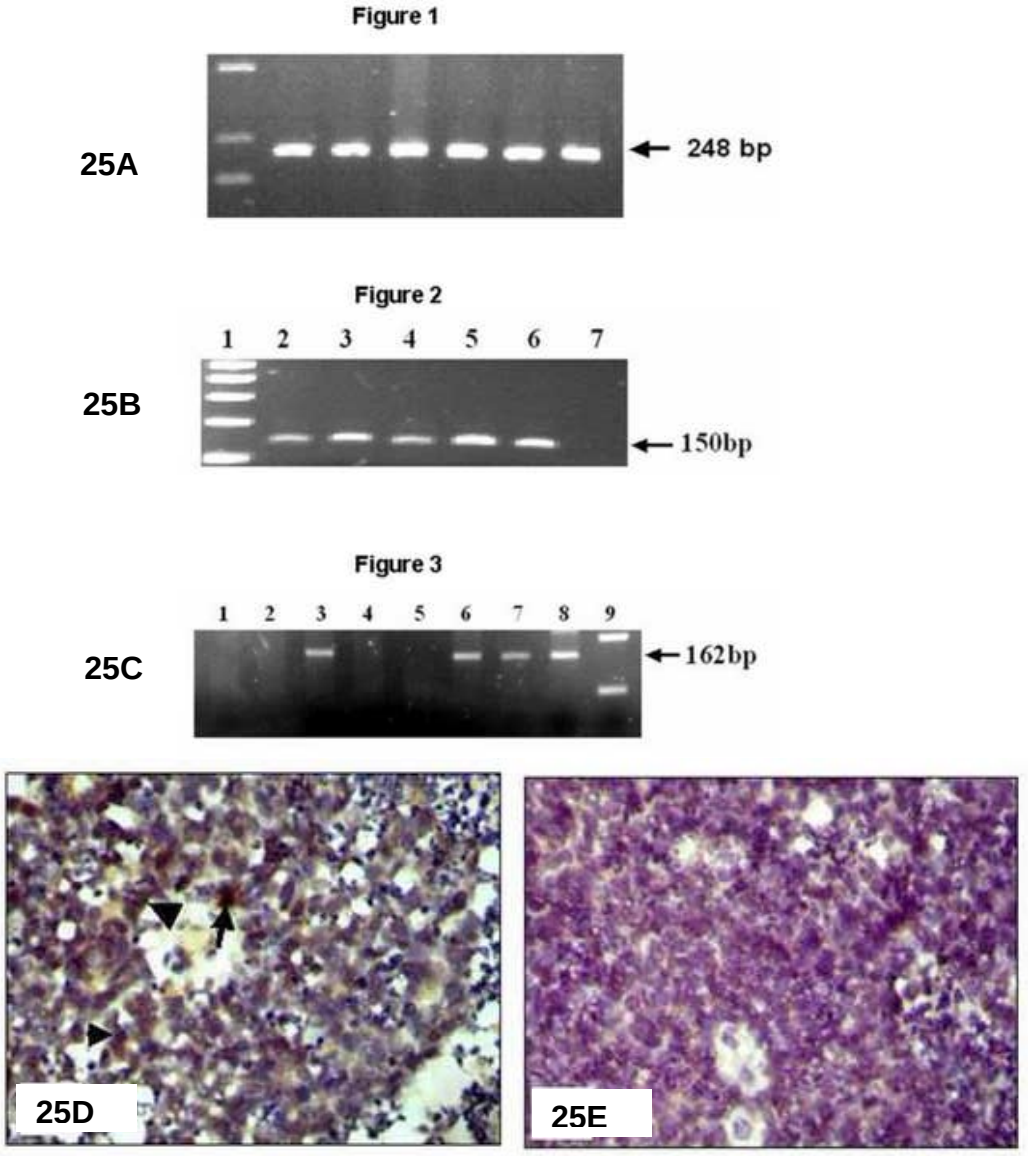


Figure 25 HPV Expression in Retinoblastoma (A) Molecular weight marker (100 bp), Lanes 2 to 7: β-actin (248 bp) expression in retinoblastoma; (**B)** Lane 1: molecular weight marker (100bp), Lanes 2-6: positive for HPV DNA (2nd round product) in retinoblastoma, lane 7: negative control; **(C)** Lanes 1&2: negative controls (1st and 2nd round), Lanes 3, 6&7: positive HPV 16 DNA in retinoblastoma (2nd round product), Lane 8: HPV DNA 16 positive in HeLa cell line (positive control); **(D)** Pictomicrograph shows the nuclear expression of Rb in the tumor with no invasion and with positive HPV DNA. Magnification – 40X (DAB staining with hematoxylin counter stain); **(E)** Pictomicrograph shows the absent nuclear expression of Rb in the tumor with no invasion and with negative HPV DNA. Magnification – 40X (DAB staining with hematoxylin counter stain)

Table 4: Summary of HPV positivity and immunostaining for pRB in human retinoblastoma

Tumors	**HPV Positive**	**HPV Negative**
pRb status		
pRB positive	5	3
pRb negative	16	18
HPV 16	12	-
HPV18	-	-
Total	**21**	**23**

SPECIFIC CONTRIBUTIONS AND SCOPE OF FUTURE WORK

Understanding the expression of proteins associated with cell adhesion, motility and invasion serves as a starting point to test whether these molecules can work synergistically with current therapies to improve treatment for late-stage retinoblastoma. Typically, tests for targeting the drugs in animal models of retinoblastoma follow studies in cell culture. Some of the molecules in the pathways studied in retinoblastoma have already been tested in other cancers. For example, recently the N-Cadherin small molecule inhibitor ADH-1 has entered Phase II clinical trials in combination with Melphalan in the treatment of melanoma in the United States.

However, further studies on the expressions of these molecules in retinoblastoma cell lines and experiments in cell culture to test their roles in cell adhesion and invasion are required before we can determine their use in retinoblastoma treatment.

With respect to the presence of HPV DNA in retinoblastoma, we find that ~50% tumors express HPV of which 54% express HPV-16. HPV-18 was absent in retinoblastoma from our cohort. The presence of HPV did not correlate with retinoblastoma protein expression. Further investigation is required to determine the role of HPV in retinoblastoma.

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Appendix I

Summary of FISH Analysis on Primary Human retinoblastoma

Genes chosen for FISH Analysis

	Gene	GenBank Accession	Human Chromosome
1.	N-Cadherin	NM_001792	18q11.2
2.	E-Cadherin	NM_004360	16q22.1
3.	EGFR (ERBB1)	NM_005228	7p12.3-p12.1
4.	ErBB2 (HER2)	NM_004448	17q21.1
5.	PDGFRA	NM_006206	4q12
6.	PDGFRB	NM_0062609	4q12
7.	CTTN (cortactin)	NM_005231	11q13
8.	Cadherin 11	NM_001797	16q21-q22.1
9.	Cadherin 15	NM_004933	16q24.3
10.	Cadherin 13	NM_001257	16q24.2-q24.3

Details of Primary Tumors used in FISH Analysis

MR #	Case #	Seeds	Choroid	Optic Nerve	Subretinal
	S-89-00706	yes	no	no	no
	S-90-00310	no	no	no	yes
	S-03-00111	yes	no	no	no
	S-04-00426	no	yes	no	yes
	S-04-00118	yes	no	no	np
	S-04-00665	yes	no	yes	yes
	S-98-05980	yes	yes	no	no
20279	S-03-01450	no	no	yes	yes
20817	S-04-00502	yes	no	no	no
22027	S-05-00512	no	yes	no	yes
22223	S-05-00793	yes	no	no	no
23811	S-06-00958	yes	yes	yes	no
17813	S-00-01481				
18403	S-01-00880				

Appendix II

Table 1A: Clinical and Immunohistochemical Data of c-Src and pSTAT3 in Retinoblastoma with Invasion

S NO.	Laterality	Age/ Sex	Clinicopathological features	c-Src expression (Allred Score)	PStat3 expression (Allred Score)
1.	UL	3 Y/F	OS: PD, Diff Ch Inv	7	7
2.	UL	3 Y 11 mon/M	OS: PD, Focal Ch inv	6	7
3.	BL	3 Y/M	OS: PD, Diffuse Ch, Post Lam ON Inv	7	7
4.	UL	2 Y/M	OS:PD, Focal Ch, Pre Lam ON Inv	6	6
5.	UL	21 Y/F	OS: PD; diffuse Ch and post lam ON invasion	8	8
6.	UL	1/F	OD: PD, Diff Ch Inv	8	8
7.	UL	2 Y/F	OD: PD, Surgical End Inv	7	7
8.	BL	2 Y 10 mon /M	OS: PD; Diffuse Ch, Post Lam ON Inv	8	8
9.	UL	3 Y/M	OS: PD; diffuse Ch and post lam ON invasion	7	7
10.	UL	13 mon/F	OD: PD, Focal Ch, Post Lam ON Inv	6	6
11.	UL	7 Y/M	OS:MD, Post Lam ON Inv	8	7
12.	UL	2 Y/F	OD:PD, RPE, Post Lam Inv	6	8
13.	BL	3 Y 6 mon/M	OS: PD, Diffuse Ch inv	8	8
14.	UL	3 Y/M	OD:PD, Focal Ch, Pre Lam ON Inv	8	5
15.	UL	9 mon/M	OS:MD, Focal Ch, Pre Lam ON Inv	6	4
16.	UL	4 Y/F	OS:PD, Laminar ON Inv	7	6
17.	UL	3 Y 6 mon/F	OD: MD; Focal Ch, Pre Lam ON Inv	6	5
18.	BL	2 Y/F	OS: PD, Focal Ch Inv	6	8
19.	BL	10 mon/M	OD:WD, Diff Ch, Pre Lam ON Inv	6	6
20.	UL	3 Y 2 mon/M	OS: PD, Diffuse Ch, Pre Lam ON Inv	8	8
21.	UL	2 Y/F	OS: MD, focal Ch, pre lam ON invasion	8	6
22.	UL	7 Y/M	OD: PD, Rectus Orbital Inv	8	8

Y; Years; mon: months; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; Focal Ch Inv: Focal Choroidal Invasion; Diff Ch Inv: Diffuse Choroidal Invasion; Pre Lam ON Inv: Pre-laminar Optic Nerve Invasion; Post Lam ON Inv: Post-laminar Optic Nerve Invasion; bld vss inv: invasion of tumor into blood vessels; UL: Unilateral Disease; BL:Bilateral Disease; M: Male; F: Female.

Table 1B: Clinical and Immunohistochemical Data of c-Src and pSTAT3 in Retinoblastoma without Invasion

S NO.	Laterality	Age/ sex	Clinicopathological features	c-Src expression	PStat3 expression
1.	UL	11 mon/F	OD:WD	4	4
2.	UL	5 Y/M	OS: PD	4	0
3.	UL	7mon/M	OD: WD	5	4
4.	UL	2 Y 2 mon /M	OD: WD	4	4
5.	UL	1 Y/ F	OD: WD	3	0
6.	BL	1 Y/F	OS: MD	4	0
7.	BL	1 Y 11 mon/M	OD: MD	3	0
8.	UL	6 Y/F	OD: PD	5	0
9.	BL	1 mon/M	OS: WD	3	0
10.	UL	4 Y/F	OD: MD	4	0
11.	BL	1 Y 2 mon/F	OD: PD	5	4
12.	BL	5 mon/M	OS: WD	4	4
13.	BL	3 Y 4 mon /M	OS: PD	5	0
14.	UL	3 Y/F	OS: WD	4	4
15.	BL	3/M	OS: PD	4	0
16.	UL	8 Y/M	OS:PD	3	0
17.	BL	5 mon/M	OD: WD	4	0
18.	UL	4 Y/M	OD: PD	4	0

Y; Years; mon: months; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral Disease; BL: Bilateral Disease; M: Male; F: Female; UK: Unknown.

Table 2A: Clinical and Immunohistochemical Data of CD9, Cadherins and Catenins in Retinoblastoma with Invasion

S. No.	Laterality	Age/ Sex	Clinicopathalogical features	CD9 % Positivity	E-Cadherin % Positivity	N-Cadherin % Positivity	Alpha Catenin % Positivity	Beta Catenin % Positivity
1.	UL	3 Y/M	OD:PD, focal Ch, Pre Lam ON Inv	60	<20	40	0	70
2.	UL	7 Y/M	OD:PD, Diff Ch Inv	40	30	80	10	80
3.	UL	7 Y/M	OS:MD, Post Lam ON Inv	50	20	80	40	40
4.	UL	1 Y 6 mon /M	OD: PD, Pre Lam ON Inv	70	30	60	50	60
5.	UL	4 Y/F	OD: MD, focal Ch Inv	70	20	50	30	80
6.	UL	4 Y/F	OS: PD; Laminar ON Inv	70	70	70	40	60
7.	BL	3 Y 6 mon/M	OS: PD, Diff Ch Inv	40	20	80	40	80
8.	BL	2 Y 10 mon /M	OS: PD; Diffuse Ch, Post Lam ON Inv	70	40	80	60	70
9.	UL	3 Y 6 mon /F	OD: MD, Focal Ch, Pre Lam ON Inv	80	70	80	60	70
10.	UL	2 Y/F	OS:MD, Focal Ch, Pre Lam ON Inv	20	<10	20	40	70
11.	UL	13 mon/F	OD: PD, Focal Ch, Post Lam ON Inv	10	<20	60	20	70
12.	UL	3 Y/M	OS:PD, Post Lam ON Inv	20	10	60	40	50
13.	BL	2 Y 3 Mon /F	OD: PD, Post Lam ON Inv	40	30	70	40	60
14.	UL	3 Y/F	OS: PD, Diff Ch Inv	40	10	60	30	20
15.	UL	2 Y/F	OD: PD, surgical end Inv	5	60	80	80	20
16.	UL	4 Y/F	OD: WD, Lam ON Inv	30	30	40	20	60
17.	UL	1 Y/F	OD: PD, Diff Ch Inv	40	30	60	40	60
18.	BL	2 Y/F	OS:PD, Focal Ch Inv	90	40	5	60	80
19.	BL	8 Y/F	OS:PD, Pre Lam ON Inv	40	40	60	40	30
20.	UL	2 Y/M	OS:PD, Focal Ch, Pre Lam ON Inv	30	<10	40	20	40
21.	BL	2 Y/M	OS: MD, Focal Ch Inv	60	60	40	70	0
22.	UL	3 Y 2 mon/M	OS: PD, Diff Ch, Pre Lam ON Inv	0	<10	30	80	0
23.	UL	2 Y/M	OS:PD, Post Lam ON Inv	0	30	80	70	40

24.	UL	7 mon/M	OD:WD, Diff Ch Inv	30	5	80	40	60
25.	UL	3 Y/M	OD: PD, Focal Ch, Post Lam ON, meningeal sheath and bld vess. Inv	0	40	70	60	80
26.	UL	5 mon/M	OS: MD, Focal Ch Inv	30	40	70	50	80
27.	UL	4 Y/F	OD: PD, Focal Ch, Pre Lam ON Inv	20	20	80	60	60
28.	UL	3 Y 6 mon/M	OD: PD, Focal Ch, Post Lam ON Inv	10	40	90	60	80
29.	UL	6 Y/M	OS: PD, Focal Ch, Post Lam ON Inv	10	50	80	0	50
30.	UL	7 Y/M	OD: PD, Rectus Orbital Inv	30	10	60	0	40
31.	BL	1 mon/M	OD:MD, Diff Ch Inv	40	40	90	80	80
32.	UL	3 mon/M	OS: PD, Pre Lam ON Inv	0	30	50	70	80
33.	UL	2 Y/F	OS :PD, Diff Ch, Post Lam, meningeal sheath Inv	40	40	5	40	30
Y; Years; mon: months; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; Focal Ch Inv: Focal Choroidal Invasion; Diff Ch Inv: Diffuse Choroidal Invasion; Pre Lam ON Inv: Pre-laminar Optic Nerve Invasion; Post Lam ON Inv: Post-laminar Optic Nerve Invasion; bld vss inv: invasion of tumor into blood vessels; UL: Unilateral Disease; BL:Bilateral Disease; M: Male; F: Female.								

Table 2B: Clinical and Immunohistochemical Data of CD9, Cadherins and Catenins in Retinoblastoma without Invasion

S No.	Laterality	Age/ Sex	Clinicopathalogical features	CD9 % Positivity	E-Cadherin % Positivity	N-Cadherin % Positivity	Alpha Catenin % Positivity	Beta Catenin % Positivity
1.	BL	5 mon/M	OS: WD	85	40	30	80	90
2.	UL	4 Y 6 mon/ M	OS:MD	80	60	5	60	80
3.	BL	3/M	OS: PD	80	70	70	80	60
4.	UL	4/F	OD:PD	80	20	10	60	60
5.	UL	2 Y 6 mon/F	OD:PD	80	70	50	<10	80
6.	UL	4 Y/M	OD: PD	90	70	40	90	80
7.	UL	11 mon/F	OS: PD	90	70	70	80	0
8.	BL	1 Y 11 mon/M	OD: MD	80	20	<10	0	0

9.	UL	7 mon/M	OD: PD	90	40	5	30	0
10.	UL	1Y 4mon/M	OD: WD	90	70	20	60	80
11.	UL	18 mon/M	OD: WD	80	40	5	70	70
12.	UL	3 Y/F	OS: WD	80	70	40	80	80
13.	UL	5 Y/M	OS: PD	80	50	40	60	70
14.	UL	1 Y/F	OD: WD	70	70	30	90	70
15.	UL	2 Y 2 mon/ M	OD: WD	80	70	60	80	70
16.	UL	2 Y 6 mon/F	OD:WD	95	80	20	70	80
17.	UL	5 Y/M	OS:MD	80	80	40	90	50
18.	UL	3 Y/M	OS: PD	70	60	80	80	80
19.	BL	1 Y/M	OS:MD	60	80	5	70	90
20.	BL	2Y 6 mon/M	OS:PD	40	70	10	80	60
21.	BL	3 Y /F	OD:PD	90	40	20	90	70
22.	BL	1 month 10 days /M	OD:PD	70	70	30	80	90
23.	UL	5 mon/M	OD:WD	70	80	10	90	80
24.	UL	10 mon/F	OS:WD	80	70	10	80	80
25.	BL	5 Y/F	OD: PD	40	70	60	90	40
26.	UL	6 mon/M	OS:WD	60	60	10	60	60
27.	UL	1 Y/M	OS:WD	40	30	40	0	60
28.	BL	1 mon/M	OS: WD	40	40	30	80	90
29.	UL	6 Y 6 mon/F	OD:PD	50	60	5	60	80

Y; Years; mon: months; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral Disease; BL: Bilateral Disease; M: Male; F: Female; UK: Unknown.

Table 3A: Clinical and Immunohistochemical Data of EpCAM in Retinoblastoma with Invasion

S No.	Laterality	Age/ Sex	Clinicopathalogical features	EpCAM % Positivity
1.	UL	2 Y/F	OD: PD, surgical end Inv	70
2.	UL	1 Y/F	OD: PD, Diff Ch Inv	90
3.	UL	2 Y/F	OS: MD, Focal Ch, Pre Lam ON Inv	60
4.	UL	3 Y/F	OS: PD, Diff Ch Inv	70
5.	BL	2 Y/F	OS:PD, Focal Ch Inv	70
6.	UL	7 Y/M	OD: PD, Rectus Orbital Inv	80
7.	UL	13 mon/F	OD: PD, Focal Ch, Post Lam ON and central retinal vessel Inv	80
8.	UL	4 Y/F	OS: PD; Laminar ON Inv	70
9.	BL	2 Y 10 mon/M	OS: PD; Diffuse Ch, Post Lam ON Inv	80
10.	UL	1Y 6 mon/M	OD: PD, Pre lam ON invasion	70
11.	UL	21/F	OS: PD; diffuse Ch, Post lam ON inv	80
12.	BL	3 Y/M	OS: PD, Diffuse Ch, Post Lam ON Inv	70
13.	UL	3 Y 11 mon/M	OS: PD, Focal Ch inv	60
14.	UL	3 Y 2 mon/M	OS: PD, Diffuse Ch, Pre Lam ON Inv	70
15.	UL	3 Y 6 mon/F	OD: MD; Focal Ch, Pre Lam ON Inv	70
16.	BL	3 Y 6 mon/M	OS: PD, Diffuse Ch inv	80
17.	UL	3 Y/M	OD:PD, Focal Ch, Pre Lam ON Inv	60
18.	UL	2 Y/M	OS:PD, Focal Ch, Pre Lam ON Inv	70
19.	UL	7 Y/M	OD:PD, Diff Ch Inv	60
20.		2/F	OS: PD; Focal Ch, Post Lam ON Inv	60
21.	UL	4/F	OD: PD, focal Ch, Pre Lam ON invasion	70

22.	BL	2 Y 3 mon/F	OD: PD, Focal Ch, Pre Lam ON Inv	60
23.	BL	4 Y/F	OS: PD, Pre Lam On Invasion	40

Y; Years; mon: months; M: Male; F: Female; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral disease; BL: Bilateral Disease; Diff Ch Inv: diffused choroidal invasion of tumor; Focal Ch Inv: Focal Invasion of tumor cells into choroids; Pre Lam ON Inv: Pre-laminar invasion of Optic Nerve; Post Lam ON Inv: Post-laminar invasion of the Optic Nerve.

Table 4B: Clinical and Immunohistochemical Data of EpCAM in Retinoblastoma without Invasion

S NO	Laterality	Age/sex	Clinical features	EpCAM % Positivity
1.	BL	1 Y 6 mon/M	OS: WD	80
2.	UL	7 mon/M	OD: PD	70
3.	UL	1Y 4mon/M	OD: WD	40
4.	BL	5 mon/M	OS: WD	60
5.	UL	6 Y 6 mon/F	OD:PD	60
6.	UL	7/F	OS: PD	40
7.	BL	5 mon/M	OD: WD	40
8.	BL	1 Y 11 mon/M	OD: MD	60
9.	UL	3 Y/F	OS: WD	70
10.	UL	8 Y/M	OS:PD	30
11.	UL	7 mon/M	OD: WD	80
12.	UL	4 Y/M	OD: PD	60
13.	UL	1 Y/F	OD: WD	40
14.	UL	2 Y 2 mon /M	OD: WD	70
15.	UL	5 Y/M	OS: PD	60

16.	UL	18 mon/M	OD: WD	40
17.		3y/M	OS: MD	60
18.	UL	2 Y/F	OD:MD	50
19.	UL	4 Y/F	OD: MD	70
20.		1/F	OS:MD	40
Years; mon: months; M: Male; F: Female; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral disease; BL: Bilateral Disease.				

Table 6A: Clinical and Immunohistochemical Data of EMMPRIN, MMP-2, MMP-9, TIMP-1 and TIMP-2 in Retinoblastoma with Invasion

S. No	Laterality	Age/Sex	Clinicopathological Features	EMMPRIN % positivity	MMP-2 % positivity	MMP-9% positivity	TIMP-1% positivity	TIMP-2% positivity
1.	UL	1/F	OD: PD, Diff Ch Inv	80	80	80	60	60
2.	UL	9 mon/M	OS:MD, Focal Ch, Pre Lam ON Inv	0	0	20	0	0
3.	UL	4 Y/F	OS:PD, Laminar ON Inv	40	30	0	0	60
4.	BL	3 Y 6 mon/M	OS: PD, Diffuse Ch inv	80	80	70	70	80
5.	UL	2 Y/F	OD: PD, Surgical End Inv	70	90	80	80	0
6.	UL	3 Y/M	OD: PD, Focal Ch, Post Lam ON	70	40	60	70	80
7.	UL	6 Y/M	OS: PD, Focal Ch, Post Lam ON Inv	50	50	60	40	0
8.	BL	3 Y/M	OS: PD, Diffuse Ch, Post Lam ON Inv	70	80	70	80	60
9.	UL	7 Y/M	OD:PD, Diff Ch Inv	50	30	30	40	20
10.	BL	2 Y 3 mon/F	OD: PD, Post Lam ON Inv	0	20	30	50	60
11.	UL	13 mon/F	OD: PD, Focal Ch, Post Lam ON Inv	30	30	20	50	30
12.	UL	3 Y/M	OD:PD, Focal Ch, Pre Lam ON Inv	60	50	30	0	30
13.	UL	4 Y/M	OS:PD Focal Ch, Orbital inv	50	60	40	50	0
14.	UL	3 Y 6 mon/F	OD: MD; Focal Ch, Pre Lam ON Inv	80	20	20	60	0
15.	UL	1Y 6 mon/M	OD: PD, Pre Lam ON Inv	40	40	30	70	0
16.	UL	3 Y 2 mon/M	OS: PD, Diffuse Ch, Pre Lam ON Inv	80	20	0	50	70
17.	UL	5 mon/M	OS: MD, Focal Ch Inv	70	60	0	40	30
18.	BL	2 Y 10 mon /M	OS: PD; Diffuse Ch, Post Lam ON Inv	70	50	70	70	60

19.	BL	14 Y/M	Lymph Node Biopsy [OU: (OD) PD, Diffuse Ch Inv , Post Lam ON Inv]	0	0	0	80	0
20.	UL	2 Y/M	OS:PD, Focal Ch, Pre Lam ON Inv	80	0	0	50	30
21.	BL	1 mon/M	OD: MD, Diff Ch Inv	80	60	0	70	60
22.	UL	7 Y/M	OD:PD , Rectus orbital Inv	90	80	70	80	90
23.	BL	2 Y/M	OD: WD, Focal Ch Inv	10	0	0	0	0
24.	UL	7 Y/M	OS:MD, Post Lam ON Inv	40	50	0	0	40
25.	UL	3 Y/F	OS:PD, Diff Ch inv	30	0	0	50	50
26.	BL	2 Y/M	OS: MD, Focal Ch Inv	10	0	60	20	0
27.	UL	4 Y/F	OD: MD, Focal Ch Inv	40	30	0	0	40
28.	UL	2 Y/ M	OS:PD, Post Lam ON Inv	60	60	0	80	60
29.	BL	2 Y/F	OS: PD, Focal Ch Inv	60	70	80	60	80
30.	UL	2 Y/F	OS: MD, Focal Ch, Pre Lam ON Inv	60	30	60	60	40
31.	UL	7mon/M	OD: WD, Diff Ch Inv	60	60	70	30	40
32.	UL	3 Y/F	OS:PD, Diff Ch Inv	60	70	80	50	60

Y; Years; mon: months; M: Male; F: Female; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral disease; BL: Bilateral Disease; Diff Ch Inv: diffused choroidal invasion of tumor; Focal Ch Inv: Focal Invasion of tumor cells into choroids; Pre Lam ON Inv: Pre-laminar invasion of Optic Nerve; Post Lam ON Inv: Post-laminar invasion of the Optic Nerve.

Table 6B: Clinical and Immunohistochemical Data of EMMPRIN, MMP-2, MMP-9, TIMP-1 and TIMP-2 in Retinoblastoma without Invasion

S No.	SNH No.	Laterality	Age/ Sex	Clinicopathalogical features	EMMPRIN % positivity	MMP-2 % positivity	MMP-9% positivity	TIMP-1% positivity	TIMP-2 % positivity
1.	489-03	UL	1 Y 6 mon /M	OD: WD	20	0	0	0	30
2.	140-04	UL	4 Y/M	OD: PD	0	0	0	0	30
3.	214-03	UL	5 Y/M	OS: PD	0	0	0	0	0
4.	407-03	BL	4 mon/M	OS: MD	0	0	0	0	0
5.	423-03	UL	2 Y 2 mon /M	OD: WD	60	0	0	0	0
6.	1002-03	BL	1 Y 11 mon/M	OD: PD	0	0	0	0	0
7.	334-03	UL	3 Y/F	OS: WD	0	0	0	0	40
8.	227-03	UL	1 Y/ F	OD: WD	0	30	0	20	0
9.	102-04	UL	2 Y 6 mon/F	OD: PD	0	0	0	0	0

10.	536-03	UL	1 Y 4 mon/M	OD: MD	0	0	0	0	50
11.	320-01	UL	14 Y/M	OS: PD	0	20	20	0	50
12.	153-04	BL	3 Y/M	OS: PD	0	0	0	0	0
13.	58-03	UL	8 Y/M	OS:MD	0	0	0	0	0
14.	640-04	UL	2 Y 6 mon/F	OD:WD	10	0	20	0	0
15.	660-04	UL	6 mon/M	OS:WD	10	0	30	0	0
16.	669-04	UL	3 Y/M	OS: PD	0	50	0	0	0
17.	580-03	UL	7 mon/M	OD: PD	0	0	20	30	20
18.	582-04	UL	1 Y/M	OS:WD	0	0	30	0	0
19.		BL	3 mon/F	OD: MD	0	0	0	0	0
20.	716-04	UL	8 Y/F	OD:PD	20	30	0	0	0
21.	1278-03	BL	3 Y 4 mon /M	OS: PD	70	40	0	0	40
22.	771-03	UL	4 Y/F	OD: PD	0	0	0	0	30
23.		UL	11mon/F	OD: PD	0	10	10	20	0

Years; mon: months; M: Male; F: Female; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral disease; BL: Bilateral Disease.

Table 7A: Clinical and Immunohistochemical Data of Rac1, Tiam1 and Cdc42 in Retinoblastoma with Invasion

S No.	Laterality	Age/Sex	Clinicopathalogical Features	Rac1 % positivity	Tiam1 % positivity	Cdc42 % positivity
1.	UL	5 mon/M	OS: MD, Focal Ch Inv	0	60	0
2.	UL	3 Y 2 mon/M	OS: PD, Diff Ch, Pre Lam ON Inv	0	70	0
3.	UL	4 Y/F	OS: PD, Laminar Inv	0	70	40
4.	UL	3 Y 6 mon /F	OD: MD, Focal Ch, Pre Lam ON Inv	60	70	90
5.	UL	2 Y/ M	OS:PD, Focal Ch, Pre Lam On Inv	0	60	0
6.	UL	3 Y/F	OS: PD, Diff Ch Inv	0	0	0
7.	UL	2 Y/F	OD: WD, Focal Ch, Pre Lam ON Inv	0	80	0
8.	BL	3 Y 6 mon/M	OS: PD, Diff Ch Inv	60	90	80
9.	UL	1 Y/F	OD: PD, Diff Ch Inv	0	80	60
10.	UL	7 Y/M	OS:MD, Post Lam ON Inv	0	40	0
11.	UL	4 Y/F	OD: WD, Lam ON Inv	0	30	0
12.	UL	7 mon/M	OD: WD, Diff Ch Inv	0	80	80
13.	BL	2 Y 10 mon /M	OS: PD, Diff Ch, Post Lam ON Inv	40	70	80
14.	BL	8 Y/F	OS:PD, Pre Lam ON Inv	60	0	0
15.	UL	4 Y/F	OD: MD, Focal Ch Inv	0	40	0
16.	UL	2 Y/M	OS:PD, Post Lam ON Inv	60	80	70
17.	UL	7 Y/M	OD: PD, Rectus Orbital Inv	90	90	90
18.	UL	2 Y/F	OD: PD, Surgical End Inv	0	90	0
19.	BL	7 mon/M	OS: PD, Focal Ch Inv	0	50	0
20.	UL	3 Y 6 mon/M	OD: PD, Focal Ch, Post Lam ON Inv	0	40	0
21.	BL	2 Y/F	OD:PD, Ch, Post Lam ON In	0	70	40
22.	UK	8 Y/F	OS:PD, Ch, Post Lam, Surgical End Inv	70	70	60
23.	BL	1 Y 7 mon/F	OD: PD, Focal Ch, Post Lam ON Inv	0	80	0
24.	BL	7 mon/M	OD:PD, Focal Ch, Post Lam ON Inv	30	60	0
25.	UL	2 Y/M	OS: PD, Ch, Post Lam Inv	0	70	0

26.	UL	5 Y/M	OD:MD, Diff Ch, Post Lam ON Inv	0	80	0
27.	UL	5 Y/M	OD:PD, Ch, Surgical End Inv	0	40	0
28.	UL	3 Y/F	OD: PD, Ch, Surgical End Inv	0	80	70
29.	UL	4 Y/M	OS: PD, Diff Ch, Surgical End Inv	0	0	0
30.	BL	5 Y/F	OD: WD, Focal Ch, Lam ON Inv	60	70	80
31.	UL	2 Y/F	OS: MD, Focal Ch, Pre Lam ON Inv	0	40	0
32.	UL	2 Y/F	OD: PD, Diff Ch, Surgical End Inv	0	0	0
33.	BL	3 Y/M	OS: PD, Pre Lam ON Inv	0	70	0
34.	UL	6 mon/F	OS:PD, Focal Ch, Surgical End Inv	0	40	0
35.	BL	2 Y/M	OS: MD, Focal Ch Inv	40	0	0

Y; Years; mon: months; M: Male; F: Female; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral disease; BL: Bilateral Disease; Diff Ch Inv: diffused choroidal invasion of tumor; ON: Focal Ch Inv: Focal Invasion of tumor cells into choroids; Pre Lam ON Inv: Pre-laminar invasion of Optic Nerve; Post Lam ON Inv: Post-laminar invasion of the Optic Nerve; UK: Unknown.

Table 7B: Clinical and Immunohistochemical Data of Rac1, Tiam1 and Cdc42 in Retinoblastoma without Invasion

S No.	Laterality	Age/Sex	Clinicopathological Features	Rac1 % positivity	Tiam1 % positivity	Cdc42 % positivity
1.	UL	4 Y/F	OD:PD	0	30	0
2.	UL	4 Y 6mon/M	OS:MD	0	60	0
3.	UL	2 Y 2 mon/M	OD: WD	0	20	0
4.	BL	3 Y/F	OD: PD	0	40	0
5.	UL	1 Y/F	OD: WD	0	20	0
6.	UL	11 mon/F	OD:WD	0	60	50
7.	UL	4 Y/M	OD: PD	40	80	0
8.	UL	2 Y 6 mon/ F	OD:WD	0	40	0
9.	UL	14 Y/M	OS: WD	0	0	0
10.	UL	5 Y/M	OS: PD	0	40	0
11.	BL	2 Y 6 mon /M	OD:PD	0	70	70
12.	BL	1 mon 10 days /M	OD:PD	0	20	0
13.	BL	5 mon/M	OS: WD	0	40	0

14.	BL	2 Y 6 mon/M	OS:PD	0	20	0
15.	UL	6 mon/M	OS:WD	0	40	0
16.	UL	5 mon/M	OD:WD	0	70	0
17.	BL	3 Y 4 mon /M	OS: PD	0	60	0
18.	UL	1 Y/M	OS:WD	0	30	0
19.	UL	5 Y/M	OS: MD	0	40	0
20.	UL	2 mon/M	OD:WD	0	70	0
21.	UL	10 mon/F	OD:WD	0	30	0
22.	UL	1 Y 2 mon/M	OD:WD	0	0	0
23.	BL	4 mon/M	OD:WD	0	70	0
24.	UL	1 Y 4 mon/M	OS:PD	60	50	0
25.	UL	2 Y/F	OD:MD	60	30	0
26.	UL	17 mon/M	OD:PD	60	50	60
27.	BL	11 mon/M	OD:WD	0	50	0
28.	UK	3Y/M	OD:PD	0	30	0
29.	UL	11 mon/F	OS:PD	0	50	70
30.	UL	10 mon/F	OS:WD	40	30	0
31.	UL	6 Y 6 mon/F	OD:PD	0	60	0
32.	UL	3 Y/F	OS: WD	0	50	0
Y; Years; mon: months; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral Disease; BL: Bilateral Disease; M: Male; F: Female; UK: Unknown.						

Table 8A: Clinical and Immunohistochemical Data of Endothelial Nitric Oxide Synthase (eNOS), Inducible Nitric Oxide Synthase (iNOS) and Nitrotyrosine (NT) in Retinoblastoma with Invasion

S. No	Age/Sex	Clinicopathological Features	eNOS	iNOS	NT
1.	3 Y/M	OS:PD, Diff Ch, Post Lam ON Inv	+++	+++	+++
2.	4 Y/F	OS:PD, Post Lam ON Inv	+++	++	+++
3.	2 Y 10 mon/M	OS:PD, Diff Ch,, Pre Lam ON Inv	+++	+++	++
4.	21/F	OD: PD, Pre and Post Lam ON, Surgical end Inv	++	+++	++
5.	1 Y/F	OD:PD, Diffuse Ch Inv	+++	+++	+++
6.	4Y/F	OD: WD, Pre lam ON Inv	++	++	++
7.	7 Y/M	OD:PD, Diffuse Ch Inv	++	++	+
8.	4 Y/F	OD:MD, Focal Ch Inv	+	Neg	++
9.	4 Y/F	OD:MD, Focal Ch and Pre Lam ON Inv	++	+++	+++
10.	1 Y 6 mon/M	OD: PD, Pre lam ON Inv	++	+	++
11.	5 mon/M	OS: MD, Focal Ch Inv	+++	+++	+++
12.	3 Y/M	OS: PD, Diff Ch, Post Lam ON Inv	+++	+++	++
13.	2 Y/F	OS: PD, Surgical End Inv	++	+++	+++
14.	3Y 6mon/M	OS: PD, Diff Ch Inv	+++	+++	+++
15.	4 Y/M	OS: PD , Diff Ch Inv	+	++	++
16.	7 Y /M	OS:MD, Post Lam ON Inv	++	++	+
17.	2 Y 6 mon /F	OS:PD, Diffuse Ch Inv	++	++	+++

Y; Years; mon: months; M: Male; F: Female; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral disease; BL: Bilateral Disease; Diff Ch Inv: diffused choroidal invasion of tumor; ON: Focal Ch Inv: Focal Invasion of tumor cells into choroids; Pre Lam ON Inv: Pre-laminar invasion of Optic Nerve; Post Lam ON Inv: Post-laminar invasion of the Optic Nerve; UK: Unknown.

Table 8B: Clinical and Immunohistochemical Data of Endothelial Nitric Oxide Synthase (eNOS), Inducible Nitric Oxide Synthase (iNOS) and Nitrotyrosine (NT) in Retinoblastoma without Invasion

S. No	Age/Sex	Clinicopathological Features	eNOS	iNOS	NT
1	1 Y 6 mon/M	OD: WD	++	++	++
2	4/M	OD: PD	+++	++	++
3	5/M	OS:PD	+++	+++	++
4	4 mon/M	OS:WD	+++	++	+
5	2 Y 2 mon/M	OD: WD	+++	+	+
6	3 Y/ F	OS:WD	+	+	++
7	3 Y 6 mon/F	OD:PD	+	+	+
8	8 Y/M	OS:PD	+	Neg	+
9	1/F	OD:WD	++	+	++
10	3/F	OD:PD	+++	+	++
11	1 Y/F	OD:PD	+++	+	+
12	4Y/F	OS:PD	+++	Neg	+
13	1 Y 6 mon/M	OS:WD	+	Neg	++
14	1 Y 4 mon/M	OD:WD	+++	+	++
15	1 Y 6 mon/M	OD:MD	+	+	+
16	7 mon/M	OD:WD	+	+	+++
17	4 Y 6 mon/M	OS:MD	+++	++	+++

Y; Years; mon: months; OD: Right Eye; OS: Left Eye; PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated; UL: Unilateral Disease; BL: Bilateral Disease; M: Male; F: Female; UK: Unknown.

List of Publications and Presentations Relevant to Thesis

Publications

1. **Adithi M***, Nalini V, Kandalam M, Krishnakumar S. Expression of Matrix Metalloproteinases and Their Inhibitors in Retinoblastoma. J Pediatr Hematol Oncol. 2007 Jun; 29(6):399-405.

2. **Mohan A**, Nalini V, Mallikarjuna K, Jyotirmay B, Krishnakumar S. Expression of motility-related protein MRP1/CD9, N-cadherin, E-cadherin, alpha-catenin and beta-catenin in retinoblastoma. Exp. Eye Res. 2007 Apr; 84 (4):781-9.

3. **Adithi M***, Venkatesan N, Kandalam M, Biswas J, Krishnakumar S. Expressions of Rac1, Tiam1 and Cdc42 in retinoblastoma. Exp. Eye Res. 2006 Dec; 83 (6):1446-52.

4. Laurie NA, Donovan SL, Shih CS, Zhang J, Mills N, Fuller C, Teunisse A, Lam S, Ramos Y, **Mohan A**, Johnson D, Wilson M, Rodriguez-Galindo C, Quarto M, Francoz S, Mendrysa SM, Guy RK, Marine JC, Jochemsen AG, Dyer MA. Inactivation of the p53 pathway in retinoblastoma. Nature. 2006 Nov 2; 444(7115):61-6.

6. **Mohan A**, Mallikarjuna K, Venkatesan N, Abhyankar D, Parikh PM, Krishnakumar S. The study of c-Src kinase and pStat3 protein expression in retinoblastoma. Exp. Eye Res. 2006 Oct; 83(4):736-40.

7. **Adithi M***, Nalini V, Krishnakumar S. The role of nitric oxide synthases and nitrotyrosine in retinoblastoma. Cancer. 2005 Apr 15; 103(8):1701-11.

10. Krishnakumar S, **Mohan A**, Mallikarjuna K, Venkatesan N, Biswas J, Shanmugam MP, Ren-Heidenreich L. EpCAM expression in retinoblastoma: a novel molecular target for therapy. Invest Ophthalmol Vis Sci. 2004 Dec; 45 (12): 4247-50.

Presentations:

1. "Expression of MMP-2 and MMP-9 in Retinoblastoma" at the "Exclusive Meet on Ophthalmic Research" Conference, August 20-22, 2004 at Fishermans' Cove, Chennai, India.
2. "Role of Nitric Oxide Synthases and Nitrotyrosine in Retinoblastoma" at the 28th All India Cell Biology Conference, December 1-3, 2004 at Punjab University, Chandigarh, India.
3. "Role of Matrix Metalloproteinases and their inhibitors in Retinoblastoma'" at the 24th Annual Convention of Indian Association for Cancer Research, February 9-12, 2005 at the Institute of Cytology and Preventive Oncology (ICPO), Noida, India.
4. "Role of Matrix Metalloproteinases and Their Inhibitors in Retinoblastoma" at the 2005 Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), May 1- 5, 2005 at Ft. Lauderdale, Florida, U.S.A. Was awarded the **Susruta Travel Grant** by ARVO.
5. 'Molecular Pathways in Retinoblastoma: Part II' at the Indian Eye Research Group (IERG) 2005 Meet, July 30-31, 2005 at the L.V. Prasad Eye Institute, Hyderabad, India. Was awarded the **CCLRU Travel Grant** by the IERG.
6. "Expression of Serine/Arginine-Rich Protein Specific Kinase 1(SRPK1) in Retinoblastoma" at the 2006 Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), April 30 – May 4, 2006 at Ft. Lauderdale, Florida, U.S.A.

Biography of Adithi Mohan

Education Ph.D., Cancer Biology, 2008 (Expected)
Birla Institute of Technology and Science, Pilani, India

M. Sc. Human Genetics, 2002
University of Leeds, Leeds, U.K

B. Sc. Zoology, 2001
Stella Maris College (Autonomous), Chennai, India

Academic and Professional Honors

2005 Susrata Travel Award, 2005 Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), Ft. Lauderdale, Florida, U.S.A.
2005 CCLRU Travel Award, Indian Eye Research Group (IERG) 2005 Meet, L.V. Prasad Eye Institute, Hyderabad, India
2001 Proficiency Prize, Department of Zoology, Stella Maris College, India
2000 Second Rank Holder-Recipient of the Dr. (Mrs.) Hannah John Cash Award & Silver Medal, Department of Zoology, Stella Maris College, India
1999 Proficiency Prize Winner, Department of Zoology, Stella Maris College, India

Experience:

2006–present International Research Scholar, St Jude Children's Research Hospital, Department of Developmental Neurobiology, PI: Dr. Michael Dyer
Research: Understanding Tumor Invasion and Metastasis in Retinoblastoma

2004-present Graduate Student, Birla Institute of Technology and Science, Pilani, India PI: Dr. S. Krishnakumar (Department of Ocular Pathology, Vision Research Foundation, Chennai, India)
Research: Understanding Tumor Invasion and Metastasis in Retinoblastoma

2004-2005 Project Assistant, L. V. Prasad Eye Institute, Hyderabad, India, Molecular Genetics Laboratory, PI: Dr. Chitra Kannabiran
Research: Identifying mutations in genes coding for ATP-Binding Cassette Transporter ABCR (ABCA4) and Peripherin/ Retinal Degeneration Slow (RDS) in patients with Retinitis Pigmentosa

2002 Research Assistant, University of Leeds, Leeds, U.K, School of Biology, PI: Dr Marie-Ann Shaw
Research: Identifying sequence variation in an intergenic region between IL-4 and IL-13 in relation to parasitic and allergic responses

Publications relevant to thesis

1. **Adithi M***, Nalini V, Kandalam M, Krishnakumar S. Expression of Matrix Metalloproteinases and Their Inhibitors in Retinoblastoma. J Pediatr Hematol Oncol. 2007 Jun; 29(6):399-405.
2. **Mohan A**, Nalini V, Mallikarjuna K, Jyotirmay B, Krishnakumar S. Expression of motility-related protein MRP1/CD9, N-cadherin, E-cadherin, alpha-catenin and beta-catenin in retinoblastoma. Exp. Eye Res. 2007 Apr; 84 (4):781-9.
3. **Adithi M***, Venkatesan N, Kandalam M, Biswas J, Krishnakumar S. Expressions of Rac1, Tiam1 and Cdc42 in retinoblastoma. Exp. Eye Res. 2006 Dec; 83 (6):1446-52.
4. Laurie NA, Donovan SL, Shih CS, Zhang J, Mills N, Fuller C, Teunisse A, Lam S, Ramos Y, **Mohan A**, Johnson D, Wilson M, Rodriguez-Galindo C, Quarto M, Francoz S, Mendrysa SM, Guy RK, Marine JC, Jochemsen AG, Dyer MA. Inactivation of the p53 pathway in retinoblastoma. Nature. 2006 Nov 2; 444(7115):61-6.
6. **Mohan A**, Mallikarjuna K, Venkatesan N, Abhyankar D, Parikh PM, Krishnakumar S. The study of c-Src kinase and pStat3 protein expression in retinoblastoma. Exp. Eye Res. 2006 Oct; 83(4):736-40.
7. **Adithi M***, Nalini V, Krishnakumar S. The role of nitric oxide synthases and nitrotyrosine in retinoblastoma. Cancer. 2005 Apr 15; 103(8):1701-11.
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7. "Expression of MMP-2 and MMP-9 in Retinoblastoma" at the "Exclusive Meet on Ophthalmic Research" Conference, August 20-22, 2004 at Fishermans' Cove, Chennai, India.
8. "Role of Nitric Oxide Synthases and Nitrotyrosine in Retinoblastoma" at the 28th All India Cell Biology Conference, December 1-3, 2004 at Punjab University, Chandigarh, India.
9. "Role of Matrix Metalloproteinases and their inhibitors in Retinoblastoma'" at the 24th Annual Convention of Indian Association for Cancer Research, February 9-12, 2005 at the Institute of Cytology and Preventive Oncology (ICPO), Noida, India.
10. "Role of Matrix Metalloproteinases and Their Inhibitors in Retinoblastoma" at the 2005 Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), May 1- 5, 2005 at Ft. Lauderdale, Florida, U.S.A. Was awarded the **Susruta Travel Grant** by ARVO.
11. 'Molecular Pathways in Retinoblastoma: Part II' at the Indian Eye Research Group (IERG) 2005 Meet, July 30-31, 2005 at the L.V. Prasad Eye Institute, Hyderabad, India. Was awarded the **CCLRU Travel Grant** by the IERG.
12. "Expression of Serine/Arginine-Rich Protein Specific Kinase 1(SRPK1) in Retinoblastoma" at the 2006 Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), April 30 – May 4, 2006 at Ft. Lauderdale, Florida, U.S.A.

Professional Organizations and Committees:

2004-2006 Association for Research in Vision and Ophthalmology (ARVO)
2007-2008 American Association of Cancer Research (AACR)

Biography of Dr. S. Krishnakumar

Designation: Professor of Pathology and Pathologist

Department/Institute/University: Department Of Ocular Pathology and Stem Cell Laboratory Vision Research Foundation, Sankara Nethralaya, 18, college Road, Chennai, 600 006, Tamil Nadu, India, Phone: 91-44-28271616, Fax: 91-44-28254180,

Email: **drkrishnakumar_2000@yahoo.com**

Date of Birth: 26.01.1967, **Sex** (M/F), Male **SC/ST** : Nil

Education (Post-Graduation onwards & Professional Career)

Sl No.	Institution Place	Degree Awarded	Year	Field of Study
1	Kilapauk Medical College, Madras university	MBBS	1994	Medicine, Surgery
2	Institute of Child Health and Hospital for children, Egmore, Madras Medical College.	Special Trainee	1995-96	Pediatric Hematology
3	Madras University, Chennai	MD	1996-98	Pathology, cytology
4	Doheny Eye Institute at Keck School of Medicine, University of Southern California (USA)	Research fellowship	2000	Ophthalmic Pathology
5	Ocular Surface Center Miami	Visiting Scholar	2002	Corneal stem Cells
6	Retinoblastoma Center Texas Children Hospital Baylor college of Medicine, Houston, USA	Visiting Scholar	2003	Retinoblastoma
7	Doheny Eye Institute at Keck School of Medicine, University of Southern California (USA)	Visiting Scholar	2003	Ocular Tumors
8	Vinod Labhasetwar, Ph.D. Associate Professor of Pharmaceutics, Biochemistry and Molecular Biology Department of Pharmaceutical Sciences 986025 Nebraska Medical Center Omaha, NE 68198-6025	Visiting Scholar	2005	Nanoparticles and PLGA Scaffold for 3D growth of tumor cells

9.	Dr. Uday Kompella, Ph.D., Associate Professor, Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, 985840 Nebraska Medical Center, Omaha	ICMR Fellowship	1-1-2007 to 31-1-2007	Nanotechnology and Ocular drug delivery
10.	Dr. Raghuraman Kannan Assistant Professor Department of Radiology Director, Nanoparticle Production Core Facility, University of Missouri-Columbia Columbia, MO 65212	DBT Associateship	March – August 2007	Nanotechnology fellowhsip

Position and Honors

Position and Employment (Starting with the most recent employment)

Sl No.	Institution Place	Position	From (Date)	To (date)
1	Sankara Nethralaya	Post Doc Fellow	April 1998	March 1999
2	Sankara Nethralaya	Assistant Pathologist and Lecturer	April 1999	March 2005
3	Sankara Nethralaya	Reader and Pathologist	April 2005	Till date

Honors

Academic distinctions attained

1998 Best Outgoing student in MD Pathology 1998 (Madras University)

2004 XVI International Congress of Eye Research Travel Fellowship for Young Investigator to present the work on Retinoblastoma and uveal melanoma at Sydney

2005 Selected by National Eye Institute, USA as a participant for collaborative research between US and India in Eye Research

2006: Selected as DBT Associate and awarded short-term Overseas Fellowship Award for 6 months in nanotechnology (2005-2006)

Professional Experience and Training relevant to the Project

Trained in cell culture and handling the corneal stem cell project from ICMR

B. Publications (Numbers only) 60

Books : chapters 2. Research Papers, Reports : .....General articles :.Nil

Patents : . Nil .Others (Please specify)

Selected peer-reviewed publications (Ten best publications in chronological order)

1. Mohan A, Nalini V, Mallikarjuna K, Jyotirmay B, Krishnakumar S. Expression of motility-related protein MRP1/CD9, N-cadherin, E-cadherin, alpha-catenin and beta-catenin in retinoblastoma. Exp Eye Res. 2007 Jan 9; [Epub ahead of print]
2. Mallikarjuna K, Vaijayanthi P, Biswas J, Krishnakumar S. Expession of Epidermal Growth Factor Receptor, Ezrin, Hepatocyte Growth Factor, and c-Met in Uveal Melanoma: An immunohistochemical study. Curr Eye Res. 32:1-10, 2007.
3. Adithi M, Nalini V, Krishnakumar S. The role of nitric oxide synthases and nitrotyrosine in retinoblastoma. Cancer. 2005 Apr 15;103 (8):1701-11
4. Krishnakumar S, Mohan A, Mallikarjuna K, Venkatesan N, Biswas J, Shanmugam MP, Ren-Heidenreich L. EpCAM expression in retinoblastoma: a novel molecular target for therapy. Invest Ophthalmol Vis Sci. 2004 Dec;45(12):4247-50.
5. Krishnakumar S, Sundaram A, Abhyankar D, Krishnamurthy V, Shanmugam MP, Gopal L, Sharma T, Biswas J. Major histocompatibility antigens and antigen-processing molecules in retinoblastoma. Cancer. 2004 Mar 1;100 (5):1059-69.
6. Krishnakumar S, Kandalam M, Mohan A, Iyer A, Venkatesan N, Biswas J, Shanmugam MP. Expression of Fas ligand in retinoblastoma. Cancer. 2004 Oct 1;101 (7):1672-6
7. Krishnakumar S, Mallikarjuna K, Desai N, Muthialu A, Venkatesan N, Sundaram A, Khetan V, Shanmugam MP. Multidrug resistant proteins: P-glycoprotein and lung resistance protein expression in retinoblastoma. Br J Ophthalmol. 2004 Dec;88 (12):1521-6
8. Krishnakumar S, Lakshmi A, Shanmugam MP, Vanitha K, Biswas J. Nm23 expression in retinoblastoma. Ocul Immunol Inflamm. 2004 Jun;12(2):127-35.

9. Agarwal M, Biswas J, Krishnakumar S,, Shanmugam MP. Retinoblastoma presenting as orbital cellulitis: report of four cases with a review of the literature. Orbit. 2004 Jun;23(2):93-8.
10. AmirthaLakshmi S, Pushparaj V, Krishnamurthy V, Biswas J, Krishnakumar S, Shanmugam MP. Tetraspanin protein KAI1 expression in retinoblastoma. Br J Ophthalmol. 2004 Apr;88(4):593-5.
11. Biswas J, Das D, Krishnakumar S, Shanmugam MP.Histopathologic analysis of 232 eyes with retinoblastoma conducted in an Indian tertiary-care ophthalmic center. J Pediatr Ophthalmol Strabismus. 2003 Sep-Oct;40(5):265-7.
12. Shanmugam MP, Lakshmi A, Biswas J, Krishnakumar S. Prognostic significance of Fas expression in retinoblastoma. Ocul Immunol Inflamm. 2003 Jun;11(2):107-13

List maximum of five recent publications relevant to the proposed area of work

1) Mohan A, Kandalam M, Ramkumar HL, Gopal L, Krishnakumar S. Stem cell markers: ABCG2 and MCM2 expression in retinoblastoma. **Br J Ophthalmol**. 2006 Jul;90(7):889-93. Epub 2006 Mar 23.
2) Sudha B. Arumugam SK, Sastry TP, Sitalakshmi G, Krishnakumar S . Phenotypic Characterization of Human Limbal Epithelial Cells Expanded on Chitosan. **(CORNEA-D-06-00167) (accepted in Cornea)**
3) Sudha B. Geetha KI, Sitalakshmi G, Madhavan HN, Krishnakumar S. Expression of Pluripotent Stem cell Marker ABCG2 in Cultured Limbal Epithelial Cells on Intact Human Amniotic Membrane**. (CORNEA-D-06-00024) (accepted in Cornea)**

Research Support: Ongoing Research Projects

Sl No.	Title of Project	Funding Agency	Amount (in Rs.)	Date of sanction and Duration
	Article I. Principal Investigator of the projects			
1	To study the presence of Human Papilloma Virus 16 and 18 Genome in Retinoblastoma from Indian Children with Sporadic RB	Vision Research Foundation, Chennai	5,00,000	April 2004 – October 2006 Duration: 2 years
2	To understand the Biology of Corneal Stem cells	ICMR (Commenced on August 2005)	19,00,000 For 3 years	August 2005– August 2008 Duration: 3 years
3	"Proteomic profiling for Retinoblastoma progression: Differential display analysis for the _expression of intracellular proteins between non-invasive and invasive tumors and tumor cell lines"	Department of Biotechnology (Commenced on March 2007)	29,10,000	March 2007 – February 2010 Duration: 3 years
4	To study the modulation of multidrug resistant proteins expression, function and in vitro antitumor effect on Y79 retinoblastoma cells by curcumin longa derivative	ICMR (Commenced on November 2006)	19,00,300	1st November 2006 - 30th October 2009 Duration: 3 years
5	To study the effect of FAS (Fatty acid Synthase) inhibitors on Y79 retinoblastoma cell line	ICMR (Commenced on March 2007)	17,00,000	March 2007 – February 2010 Duration: 3 year
6	To study the effect of small interfering RNA (siRNA) on the EpCAM expression of Y 79 retinoblastoma cell line and its correlation with invasion and migration properties of cell line	Department of Science and Technology (Commenced on February 2007)	25,00,000	5th February 2007 - 4th January 2010 Duration: 3 years
7	To identify and characterize the retinal stem/progenitor properties of Human Iris Pigment Epithelial (IPE) cells and Ciliary Epithelial (CE) cells in vitro	Department of Biotechnology (Project sanctioned)	13,24,400	Duration: 1 year
8	Efficacy of EpCAM-conjugated drug loaded biodegradable nanoparticles for drug therapy in Retinoblastoma (Co-PI)	Department of Biotechnology	96,00,000	Duration: 3 years 1st August 2007 – July 2010

Completed Research Projects (State only major projects of last 3 years)

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
Article II.	**Principal Investigator of the projects**			
1	Role of Tetraspanin, p53 family proteins, Rac 1-Tiam 1 signal transduction, Cadherins, Catenins, Proteases and its inhibitors in Retinoblastoma and correlation with aggression.	ICMR Ref No: 5/4/6/12/2003-NCD II & Iris code: 2002-03970)	17,940.00 for 2 years	January 2004 – December 2005 (2 years)
2	Study of Ezrin, EGFR, IGF-IR, HGF,C-Met,C-Fos, C-Jun in uveal Melanoma correlating with known clinicopathological parameters and with proliferation marker MiB-1	ICMR (5/4/6/3/03-NCD-II)	7,64,280.00 for 1 year	January 2005 – December 2005 (1 year)