TITLE

MOLECULAR MECHANISMS INVOLVED IN INTRAOCULAR TUMOR PROGRESSION

PART – 1 (RETINOBLASTOMA)

PART – 2 (UVEAL MELANOMA)

THESIS
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By

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CERTIFICATE

This is to certify that the thesis entitled

"**Molecular Mechanisms Involved in Intraocular Tumor Progression**"
Submitted by **Mallikarjuna Kandalam** ID. No **2005PHXF417** for award of Ph. D. Degree of the Institute embodies original work done by him/her under my supervision.

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“I dedicate this thesis to my ‘Wife’ who has been a great friend with love and support throughout my PhD course”

ABSTRACT OF THESIS – PART I

TO UNDERSTAND THE MOLECULAR MECHANISMS INVOLVED IN RETINOBLASTOMA PROGRESSION

Issues in Retinoblastoma and rationale of the study

Hurdles in killing the tumor cells:

For more than 100 years scientists have fanatically sought the fundamental origins of tumorigenesis, with the ultimate hope of discovering a cure. Indeed, these efforts have led to a significant understanding that multiple molecular and genetic aberrations, such as uncontrolled proliferation and the inhibition of apoptosis, contribute to the canonical characteristics of cancer. Despite these advances in our knowledge, a more thorough understanding, such as the precise cells, which are the targets of neoplastic transformation, especially in solid tumors, is currently lacking. An emerging hypothesis in the field is that cancer arises and is sustained from a rare subpopulation of tumor cells with characteristics that are highly similar to stem cells, such as the ability to self-renew and differentiate. In addition, more recent studies indicate that stem cell self-renewal pathways that are active primarily during embryonic development and adult tissue repair may be aberrantly activated in various cancers. It is well known that cancer stem cells have the characteristics of impaired apoptotic processes (Jagani and Khosravi, 2008). This phenomenon is most commonly associated with p53 mutations or loss of functional wild type p53 protein and the later has been demonstrated in retinoblastoma (RB) recently (Laurie et al., 2006). However, the recent discoveries of other p53 family proteins such as p63 and p73 added new insights and increases complexities to the analysis of p53 function. Reportedly, the truncated isoforms of p73 (ΔNp73) which is a putative antagonizer of p53 function (Wilhelm et al., 2010) is found to be overexpressed in several cancers (Nakagawa et al., 2002; Nekulova et al., 2010). Based on these evidences, the goal of maximizing tumor cell kill with conventional cancer therapy should also include specific cancer stem cell targets through pharmacological inhibition of self renewal pathways and provoke even a greater apoptotic response by activating the target protein (blocking ΔNp73 to restore p53 function). These could potentially restore the cell death response in the presence of DNA damage. However, the challenge of pursuing this approach will be to ensure an ample therapeutic manifestation, such that the nearly universal toxicities of chemotherapeutic agents on normal retinal cells are not similarly enhanced. Owing

to the complexities in RB biology, many more proteins and pathways need to be identified that would help in understanding the disease pathogenesis and to use novel proteins for target therapy. Differential protein profile of cancer tissues compared to its normal tissue of origin would help us in choosing them for targeted therapy and one can specifically target the tumor cells alone and spare the normal cells. Proteomics is a global approach that can identify the differentially expressed proteins in tumor cells relative to normal cells. These identified proteins can be functionally evaluated for its use in prognostic monitoring of the disease and for therapeutic interventions in RB.

Hence, as discussed above, my first part of thesis is focused on identifying the molecular determinants that cause impaired apoptosis during DNA damage response. Additionally, I have identified the differential proteins in RB tumors which are involved in antiapoptotic signals, increased proliferation and proteins involved in active transport and metabolic processes. Initially, we have characterized the RB tumors for the presence of cancer stem cell markers, ABCG2 (ATP binding cassette protein G2) and MCM2 (Minichromosome maintenance proteins). Immunohistochemical analysis demonstrated that tumors that had invaded local ocular structures such as the choroid, optic nerve and/or orbit expressed higher ABCG2 (ATP binding cassette G2) and MCM2 (Mini chromosome maintenance protein 2). Hence our study confirms that RB harbors cancer stem cells. Thus RB tumors can become more invasive with high proliferative potential coupled with impaired apoptotic mechanisms which are the hallmarks of cancer stem cell features. In continuation to this, we have further studied the proteins that are responsible for inhibition of apoptotic process in RB. As discussed above, beyond the inactivation of p53 function by MDM2, the additional proteins such as p63, p73 and their isoforms which may counteract normal p53 function have been studied in RB. Interestingly we observed that p63, p73 and their delta isoforms (truncated forms) were expressed in more than 50% of tumor samples. Delta p63 and delta p73 isoforms are known to have p53 pathway suppressive properties. Hence, our study for the first time revealed the possible additional factors that can counteract p53 normal function which may prevent tumor cell apoptosis during DNA damage response (chemotherapeutic exposure). In addition to the defective apoptotic processes, tumor cells use additional mechanisms for survival by upregulating the other pathways involved in tumor cell proliferation, angiogenesis, active transportation and cellular metabolic processes

etc., In this milieu, the classical approach of studying one gene/protein at a time is now taken over by the recent technologies that can identify and quantify the complex and overall changes that occur during tumorigenesis. We have used 2DE (Two dimensional electrophoresis) and Mass Spectrometry (MS^2) proteomic approach to identify the differentially expressed proteins in RB using normal donor retinas as controls. Twenty seven distinct differentially expressed proteins were identified, including 16 upregulated 11 downregulated proteins. Interestingly, tumors with invasion showed significantly higher expression by 2DE-MSMS analysis of CRABP2 ($p<0.001$), Peroxiredoxin 6 (PRXD6; $p=0.025$), APOA1 ($p<0.001$), RCVRN ($p<0.001$) and significantly lower expression of CRABP1 ($p<0.01$) compared to tumors without invasion. Our study interestingly has revealed the highly expressed proteins such as Peroxiredoxin 6, alpha crystallin A that are involved in the inhibition of apoptosis and uncontrolled proliferation. These are coupled with downregulation of Peroxiredoxin 2 and Annexin V which represents reduced apoptosis. Expression of transferrin, T-complex zeta protein and recoverin was higher in RB tumors than in retina. These molecules contribute to increased proliferation of tumor cells.

To conclude, this study has revealed the molecular determinants involved in the prevention of tumor cell apoptotic process. Also the dynamic differential protein profile identified by proteomics revealed several altered proteins in RB which are involved in higher cellular proliferation, cellular metabolism, transport activity, resistant to apoptosis and other regulatory proteins. These alterations are the hallmarks of tumor progression. Thus further functional evaluation of these molecules in vivo, will give an insight to develop novel therapeutic interventions.

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PART 2

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Chapter 1

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2DE: Two Dimensional Electrophoresis
ABCG2: ATP Binding Cassette protein G2
APOA1: Apolipoprotein A1
CCTZ: T-complex protein Zeta
c-Met: HGF receptor
CRABP: Cellular retinoic acid binding protein
CRYAA: Alpha crystallin A
EGFR: Epidermal growth factor receptor
HGF: Hepatocyte growth factor
IGF-1R: Insulin-like growth factor receptor
LTD: Large tumor diameter
MALDI-TOF: Matrix Assisted Laser Desorption and Ionization-Time of Flight
MAPK : Mitogen-activated protein kinase
MCM2: Mini Chromosome Maintenance protein 2
MMP: Matrix metallo-proteinases
MS: Mass Spectrometry
NG: Nuclear grade
PRDX6: Peroxiredoxin 6
RB: Retinoblastoma
RCVRN:Recoverin
STMN1: Stathmin
TF: Transferrin
TIL: Tumor infiltrating lymphocytes
TUBB1: Beta tubulin

Symbols

μ: micro; α: alpha; β: Beta; Δ: delta; ζ: zeta; ↓: activation; ⊥: Inhibition

DECRIPTION CONTENTS PER CHAPTER

In chapter 1, we have studied the phenotypic characterization of retinoblastoma tumors for the presence of putative cancer stem cell markers by immunohistochemistry on RB paraffin sections. The markers studied were ABCG2 and MCM2 and their expression was correlated with clinicopathological features. **In chapter 2**, we studied the expression of p53 family proteins, p63 and p73 and their isoforms in retinoblastoma paraffin sections by immunohistochemistry and western blotting and associated with clinicopathological features. **In chapter 3**, we explored the differential proteomic profiling of retinoblastoma relative to normal retina using two dimensional electrophoresis (2DE) and Matrix assisted laser desorption and ionization-time of flight/time of flight (MALDI-TOF/TOF). Some of the differentially expressed proteins were validated by real time quantitative reverse transcriptase PCR (RT-Q-PCR) and immunohistochemistry.

PART 1

GENERAL INTRODUCTION

INTRODUCTION AND LITERATURE REVIEW

1. Literature Review

1.1. Retinoblastoma – clinical information

Retinoblastoma is the most common primary intraocular cancer of the immature retina in the pediatric population 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). When left untreated, retinoblastoma is almost always fatal. When retinoblastoma is treated in the early stages by enucleation, the cure rates approach 95% (Advani et al., 1994; Byrne et al., 1995; Eng et al., 1993). 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).

Leukocoria is the most common presenting sign in patients with retinoblastoma. In a retrospective review by Abramson et al, (Abramson et al., 1998) the observation of leukocoria correlated with the presence of advanced disease. Because a retinoblastoma in the posterior pole has to be sufficiently large to generate a leukocoria reflex, leukocoria is a late presenting sign with poorer prognosis for globe salvage, but nonetheless has a good survival rate (approximately 88% at 5 y)(Balmer et al., 2006; Shields et al., 2004). Strabismus is the second major presenting sign of retinoblastoma, detected in approximately 1 in 5 retinoblastoma patients in the United States (Abramson et al., 1998). A small tumor in the fovea or foveola or tumor-associated subretinal fluid in the macula can significantly reduce visual acuity and result in strabismus. In contrast to leukocoria, tumors presenting with strabismus as the initial sign are associated with a higher survival rate and a higher chance of globe salvage (Balmer et al., 2006; Shields et al., 2004).

Enucleation or removal of the affected eye is a choice of treatment for later-stage disease, an approach to prevent metastasis and save life due to limited therapeutic options. 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. Retinoblastoma – Genetics and Epidemiology.

Disease occurs from any mutation that inactivates both normal alleles of retinoblastoma gene, a tumor suppressor gene located on the long arm of chromosome 13 at region 14, that codes for the RB protein. Approximately 30-40% of children has familial or heritable retinoblastoma and carries 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. The remaining 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. 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).

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 (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 incidence of heritable retinoblastoma is remarkably constant among the various populations of the world, with no sex or race predilection and no significant environmental or socio-economic predisposing factors (Buckley 1992). In contrast to heritable retinoblastoma, there are marked geographic differences in the incidence of nonheritable, unilateral retinoblastoma. Sporadic, unilateral retinoblastoma seems to occur more commonly in poorer, tropical, and subtropical regions of the world. Preliminary evidence suggests a possible viral etiology (human Papilloma virus) or perhaps a diet deficient in fruits and vegetables (Orjuela et al., 2000; Orjeula et al., 2005).

1.3. Retinoblastoma in Developing Countries:

The cumulative lifetime incidence of retinoblastoma is 1 in 18,000 to 30,000 live births worldwide (Abramson 1990). Diagnosis at a later age seems to be a common finding among developing countries (Senft et al., 1988; Leal-Leal et al., 2004). Due to a low rate of early disease detection, limited resources of healthcare facilities and limited options for late-stage disease in developing countries, 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). Most of the ~5000 cases of retinoblastoma diagnosed worldwide are children in developing (low-income) countries.

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 enucleation 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 enucleation 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 are 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).

2. Structure of the Eye

For the purpose of this thesis, I will provide a brief description of the human eye with specific details on layers and structures that are relevant for retinoblastoma tumor invasion.

The mature retina consists of 3 nuclear layers - the rods and cone cell bodies in the outer nuclear layer (ONL), horizontal, bipolar, muller and amacrine cell bodies in the inner nuclear layer (INL), and ganglion and amacrine cells in the ganglion cell layer (GCL) (Figure 1). The axons of the retinal ganglion cells converge to a point where they leave the posterior surface of the eye and travel down the optic stalk. Gradually, the inner layer of the optic stalk encroaches on the cavity of the stalk, until the outer and inner layers fuse. This stalk, along with the optic axons, is called the optic nerve. The axons of the two optic nerves meet at the optic chiasma and grow backward down the optic tract to transmit visual stimuli to the tectum region of the midbrain (Snell and Lemp 1998; Gilbert 2006).

3. Histopathologic features of retinoblastoma tumor:

3.1 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.

Figure 1

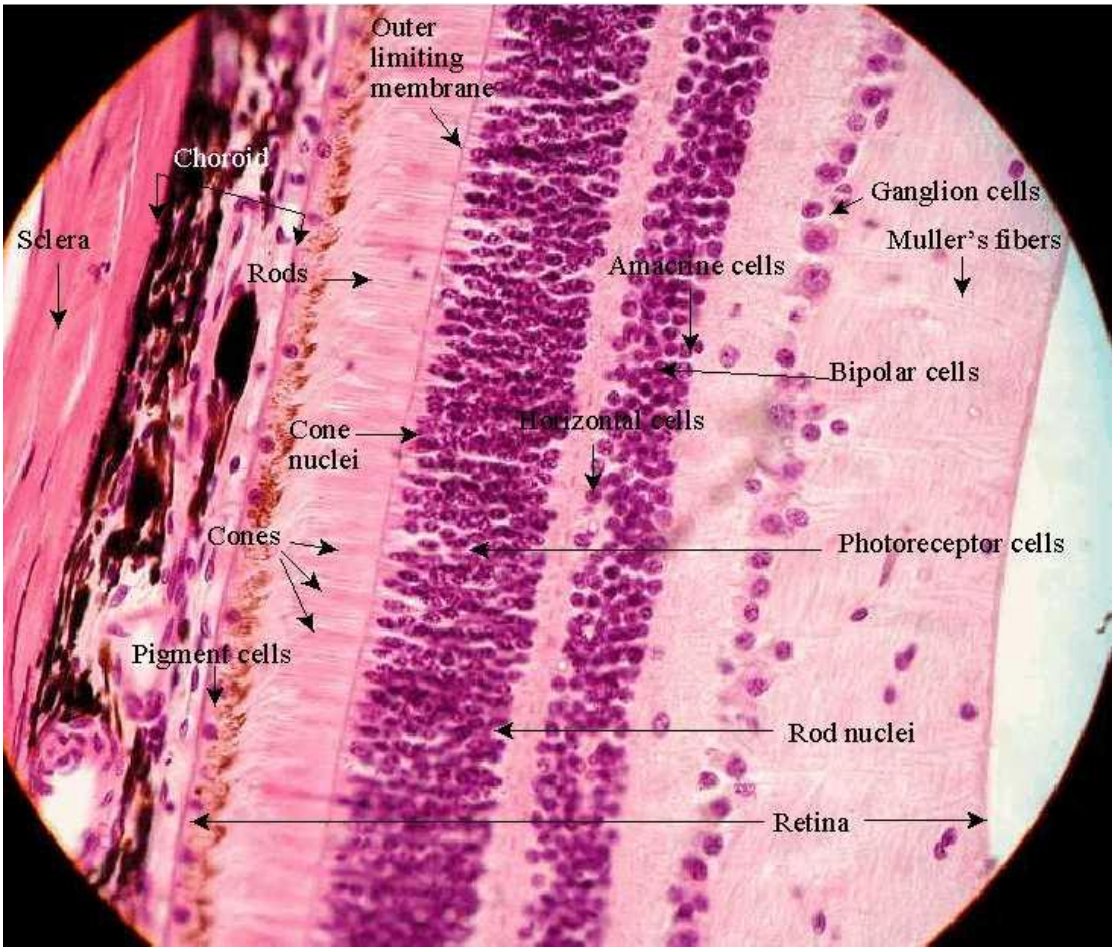

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Figure 1 shows the hematoxylin and Eosin stained cross section of normal human retinal layers.

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. Endophytic tumors grow into the vitreous cavity, obscuring the retina, which remains attached. 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).

3.2 Microscopic features of retinoblastoma tumors:

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).

3.3 Tumor differentiation patterns

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 incidental 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 contradictory 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 its degree of differentiation, that counts in predicting prognosis (Herm and Heath 1956; Carbajal 1959; 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.

4. Risk Factors for Retinoblastoma

4.1 Clinical and genetic factors

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). Bilaterality and delay in diagnosis are also important risk factors for metastatic disease (Finger et al., 2002a).

4.2 Ethnicity and Nutritional factors

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.

4.3 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).

4.4 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).

4.5 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., 2002b).

When tumor cells are detected in the optic nerve and/or choroid, it's extent of involvement should be determined by histopathology and is crucial for selecting patients for adjuvant therapy (Figure 2 A and B). 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). 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 2A and 2B

Figure 2A

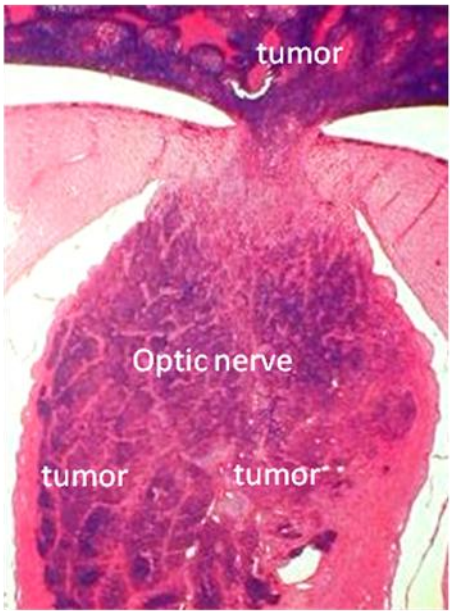

Figure 2B

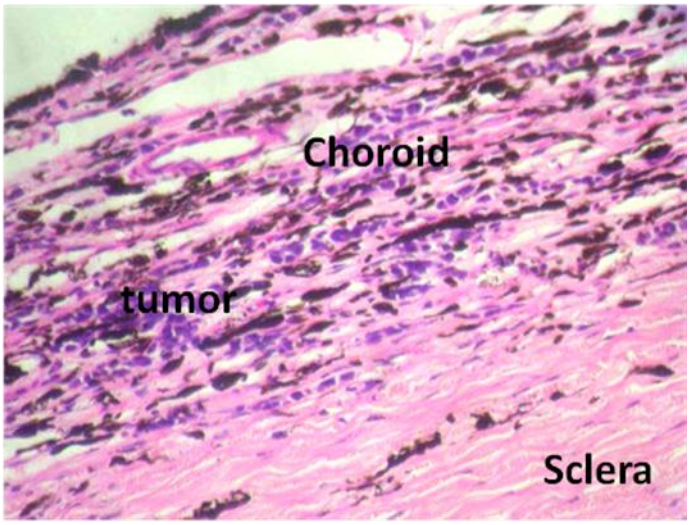

Figure 2A: Tumor invading entire optic nerve including surgical resection. Figure 2B: Tumor invading the choroidal layers.

Courtesy: Geneva foundation of medical education and research.

4.6 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 histological 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).

5. Metastatic retinoblastomas:

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).

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).

Recent studies suggest that adjuvant chemotherapy effectively reduced the incidence of metastases in patients with retinoblastoma with massive choroidal invasion or postlaminar optic nerve invasion (Uusitalo et al., 2001; Honavar et al., 2002). These statistical figures have been drawn from several retrospective studies performed in the past and are difficult to interpret because of the significant variations in the definition of high-risk features and the different treatment regimens used for each series. Therefore, to study this issue prospectively the "Childrens Oncology Group" (COG) has opened a clinical trial to study the pathology of the enucleated eyes in all unilateral patients from participating institutions in North America and assign high-risk status based on well-defined histopathologic features. These patients are then 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. Similar studies have been initiated in Europe.

6. Theory of cancer stem cells

The inability of conventional chemotherapeutic drugs and even various targeted therapies to produce complete remissions demands a more in-depth understanding of the key cellular events underlying tumor formation, maintenance, and progression, and the molecular pathways that dictate such processes. It has become increasingly apparent that the tumor, rather than consisting of a uniform population of rapidly proliferating cells, is actually composed of a heterogeneous population of cells with variable cellular and molecular characteristics (Foulds, 1965; Heppner,

1984). Therefore, one possible explanation for the failure of chemotherapy is that it cannot eliminate this entire mixed composition of tumor cells, thus necessitating multiple treatment approaches. Along these lines, it has been proposed that a rare group of cells with stem cell-like properties lies within the tumor and gives rise to the heterogeneous tumor cell population (Reya et al., 2001). The existence of these cells indicates that while our current anticancer therapeutics may be successful in debulking a tumor, they remain ineffective in targeting the minute, yet crucial, population of tumor cells that ultimately sustains the tumor (Figure 3).

Figure 3

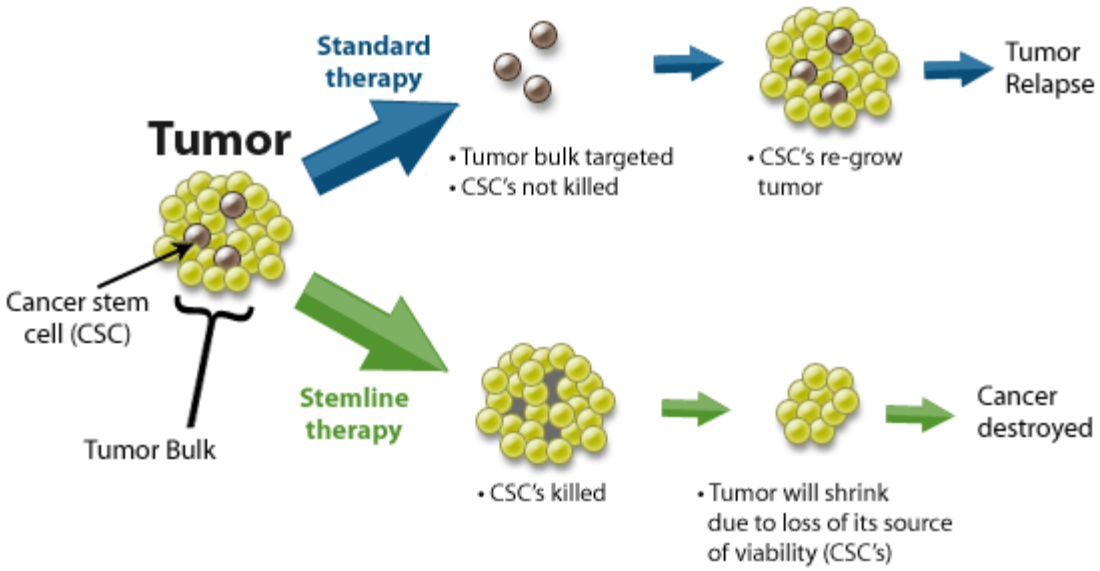

Figure 3: Courtesy: Stemline Therapeutics, Inc.New York, NY 10128

Cancer Stem Cells (CSCs) comprise a unique subpopulation of neoplastic cells within tumors that is highly tumorigenic and relatively resistant to standard therapy. Importantly, while conventional anti-cancer treatments (e.g. chemotherapy and radiation) can often transiently shrink tumors by targeting tumor bulk, these therapies fail to target and kill CSCs leading to treatment failure, relapse, and ultimately death.

Hence, there is not doubt that better understanding of the contribution of cancer stem cells to the tumor progression may help design better targeted therapies. In the first part of my thesis, I have studied the expression of cancer stem cell markers to phenotypically characterize the presence of cancer stem cells in retinoblastoma primary tumors and their association with tumor invasion.

7. Role of p53 family proteins in retinoblastoma.

While p53 has been extensively characterized as a tumor suppressor, it has been more difficult to determine whether p63 and/or p73 play a similar role. Every system in which these family members have been studied, from cells to animal models to human tissues, seems to create more questions than answers. Recently it has been shown that p53 is inactivated in retinoblastoma (Laurie et al., 2006). MDMX, an oncogene is involved in the p53 inactivation in retinoblastoma. Hence, they have proposed that MDMX could be a target molecule and using small molecule inhibitors such as nutlin-3, they have successfully inhibited MDM2 and restored functional p53 protein in retinoblastoma. However, the discoveries of p73 and p63 have both added new insights and increased complexities to the analysis of p53 function. Earlier study (Elsa et al., 2002) has shown that the combined loss of *p63* and *p73* results in the failure of cells containing functional *p53* to undergo apoptosis in response to DNA damage. Hence, it is mandatory to investigate the expression of p63 and p73 proteins in retinoblastoma before treating the retinoblastoma tumors with MDMX inhibitors for functional p53 restoration. In the second chapter of this thesis, we have studied the expression status of p63, p73 and their isoforms in retinoblastoma and correlated with clinicopathological features.

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8. Proteomics and Clinical applications:

8.1 Defining proteomics:

Proteomics is the large-scale study of proteins, usually by biochemical methods. The word proteomics has been associated traditionally with displaying a large number of proteins from a given cell line or organism on two dimensional polyacrylamide gels (Wilkins et al., 1997; Wilkins et al., 1996; Celis et al., 1996; Anderson et al., 1996). In this sense proteomics already dates back to the late 1970s when researchers started to build databases of proteins using the then newly developed technique of two-dimensional gel electrophoresis (O'Farrell, 1975). This resulted in extensive cataloguing of spots from two-dimensional gels to create databases of all expressed proteins. However, even when such gels could be run reproducibly between laboratories, determining the identity of the proteins was difficult because of a lack of sensitive and rapid analytical methods for protein characterization (such as the polymerase chain reaction and the automated sequencer for DNA analysis). In the 1990s, biological mass spectrometry emerged as a powerful analytical method that removed most of the limitations of protein analysis. This development, coupled with the availability of the entire human coding sequence in public databases, marks the beginning of a new era. Today, the term proteomics covers much of the functional analysis of gene products or 'functional genomics', including large-scale identification or localization studies of proteins and interaction studies using the yeast two-hybrid system. The more focused large-scale study of protein structure, however, is usually not included and designated 'structural genomics' instead (Burley et al., 1999). Likewise, strategies that target only genes or messenger RNA, such as large-scale mutagenesis or antisense experiments, should not be considered part of proteomics.

8.2 Why is proteomics necessary?

With the accumulation of vast amounts of DNA sequences in databases, researchers are realizing that merely having complete sequences of genomes is not sufficient to elucidate biological function. A cell is normally dependent upon a multitude of metabolic and regulatory pathways for its survival. There is no strict linear relationship between genes and the protein complement or 'proteome' of a cell. Proteomics is complementary to genomics because it focuses on the gene products, which are the active agents in cells. For this reason, proteomics directly contributes to

drug development as almost all drugs are directed against proteins. The existence of an open reading frame (ORF) in genomic data does not necessarily imply the existence of a functional gene. Despite the advances in bioinformatics, it is still difficult to predict genes accurately from genomic data (Krogh et al 1998; Dunham et al., 1999). Although the sequencing of related organisms will ease the problem of gene prediction through comparative genomics, the success rate for correct prediction of the primary structure is still low (Claverie, 1997; Pandey A & Lewitter, F, 1999). This is particularly true in the case of small genes (which can be missed entirely) or genes with little or no homology to other known genes. A recent study concluded that the error rate was as least 8% in the annotations for 340 genes from the *Mycoplasma genitalium* genome (Brenner et al., 1999). If such error rates are extrapolated to the human genome, the outcome and consequences can easily be imagined. Therefore, verification of a gene product by proteomic methods is an important first step in 'annotating the genome'. Modifications of the proteins that are not apparent from the DNA sequence, such as isoforms and post-translational modifications can be determined only by proteomic methodologies. Furthermore, it may be necessary to determine the protein expression level directly as mRNA levels may or may not correlate with the protein level (Gygi et al., 1999; Futcher et al., 1999). The localization of gene products, which is often difficult to predict from the sequence, can be determined experimentally. Mechanisms such as regulation of protein function by proteolysis, recycling and sequestration in cell compartments affect gene products and not genes. Finally, protein–protein interactions and the molecular composition of cellular structures such as organelles can be determined only at the protein level.

8.3 Identification and analysis of proteins: Protein preparation methods

One of the most crucial steps in proteomics is obtaining and handling the protein sample. Out of the entire complement of the genome of about 100,000 genes, a given cell line may express about 10,000 genes and an even higher number is expressed in tissues. Furthermore, the dynamic range of abundance of proteins in biological samples can be as high as 10^6. Because even the best two-dimensional gels can routinely resolve no more than 1,000 proteins, it is obvious that only the most abundant proteins can be visualized by gel electrophoresis if a crude protein mixture is used. The ideal solution to reduce complexity and differences in abundance is to use affinity-based protein purification strategies using the whole protein complement. For example,

the erythropoietin receptor is of medium abundance, occurring in about 1,000 copies per cell, or less than two micromoles (100 ng) in one liter of cell culture. This protein would not be visualized from whole-cell extracts but can be enriched easily by antibody-based affinity purification to yield a silver-stained band. This fact has to be borne in mind if signalling and other regulatory molecules are being studied. After obtaining the protein fraction, the method of choice for proteomic studies is one- or two-dimensional gel electrophoresis. The advantages of one-dimensional electrophoresis as a preparation method are that virtually all proteins are soluble in SDS, the range of relative molecular mass from 10,000 to 300,000 is readily covered, and extremely acidic and basic proteins are easily visualized.

8.4 Mass spectrometric identification of proteins

The most significant breakthrough in proteomics has been the mass spectrometric identification of gel-separated proteins, which extends analysis far beyond the mere display of proteins. Mass spectrometry has essentially replaced the classical technique of Edman degradation even in traditional protein chemistry, because it is much more sensitive, can deal with protein mixtures and offers much higher throughput. It relies on digestion of gel-separated proteins into peptides by a sequence-specific protease such as trypsin. The reason for analyzing peptides rather than proteins is that gel-separated proteins are difficult to elute and to analyze by mass spectrometry, and that the molecular weight of proteins is not usually sufficient for database identification. In contrast, peptides are easily eluted from gels and even a small set of peptides from a protein provides sufficient information for identification.

There are two main approaches to mass spectrometric protein identification. In the ‘peptide-mass mapping’ approach, initially suggested by Henzel and co-workers (Henzel et al., 1993), the mass spectrum of the eluted peptide mixture is acquired, which results in a ‘peptide-mass fingerprint’ of the protein being studied. This mass spectrum is obtained by a relatively simple mass spectrometric method —matrix-assisted laser desorption/ionization (MALDI) — which results in a time-of-flight distribution of the peptides comprising the mixture (Fig. 4). Advances have been made in automation of the MALDI identification procedure whereby hundreds of protein spots

Figure 4

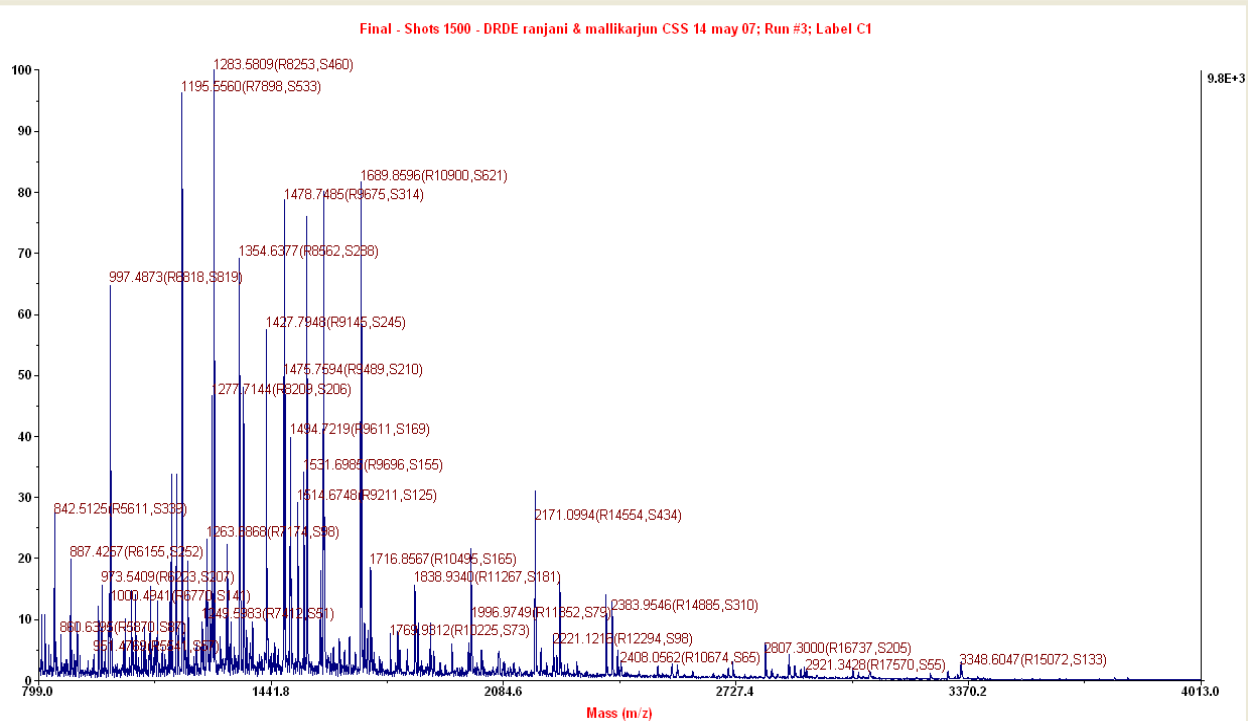

Figure 4 shows the mass spectrum obtained by a relatively simple mass spectrometric method —matrix-assisted laser desorption/ionization (MALDI) — which results in a time-of-flight distribution of the peptides comprising the mixture.

can be excised, digested enzymatically, their mass spectra obtained and automatically searched against databases (Jensen et al., 1997; berndt et al, 1999). As more full-length human genes are represented in the database, the success rate of identification by MALDI will increase further. In a two-step procedure for rapid and unambiguous protein identification, MALDI fingerprinting is the first step (Shevchenko et al., 1996). The second method for protein identification relies on fragmentation of individual peptides in the mixture to gain sequence information. In this method, the peptides are ionized by ‘electrospray ionization’ directly from the liquid phase. The peptide ions are sprayed into a ‘tandem mass spectrometer’ which has the ability to resolve peptides in a mixture, isolate one species at a time and dissociate it into amino- or carboxy-terminal-containing fragments. The tandem mass spectrometric method is technically more complex and less scalable than MALDI fingerprinting. Its main advantage is that sequence information derived from several peptides is much more specific for the identification of a protein than a list of peptide masses. The fragmentation data can not only be used to search protein sequence

databases but also nucleotide databases such as expressed sequence tag (EST) databases and more recently even raw genomic sequence databases (B. Küster, P. Mortensen, J. S. Andersen and M. Mann, unpublished data).

8.5 Differential-display proteomics: The two-dimensional gel approach

Until recently, proteomics was almost synonymous with two dimensional gel electrophoresis (Fig.5). In biomedical applications of the comparative two-dimensional gel approach, the objective is usually to identify proteins that are up- or downregulated in a disease-specific manner for use as diagnostic markers or therapeutic targets. There are several technical challenges in such experiments. First, hydrophobic and large proteins usually do not enter the second dimension of the gel. Second, the issue of dynamic range makes it difficult to visualize all but the most abundant proteins. Particularly in body fluids such as serum and cerebrospinal fluid, more than 99% of the protein complement consists of serum albumin and globulins. Third, because of the biological variation inherent in these samples, it is difficult to define normal protein-expression patterns that can be compared with the disease state. In spite of these difficulties of comparing two-dimensional gel patterns, several applications have appeared in the literature. For example, Celis and co-workers have found a putative urinary marker, psoriasin, which can be used for the follow-up of patients with bladder squamous cell carcinomas (Ostergaard et al., 1999). This marker was identified when they compared the profile of secreted proteins from normal tissue with that from cancerous tissue. In my first part of my thesis, I worked on identification of differentially expressed proteins in RB tissues using normal adult donor retinas as controls. I have used two dimensional electrophoresis (2DE) and MALDI-TOF/TOF (Matrix assisted laser desorption ionization-time of flight/time of flight).

Figure 5

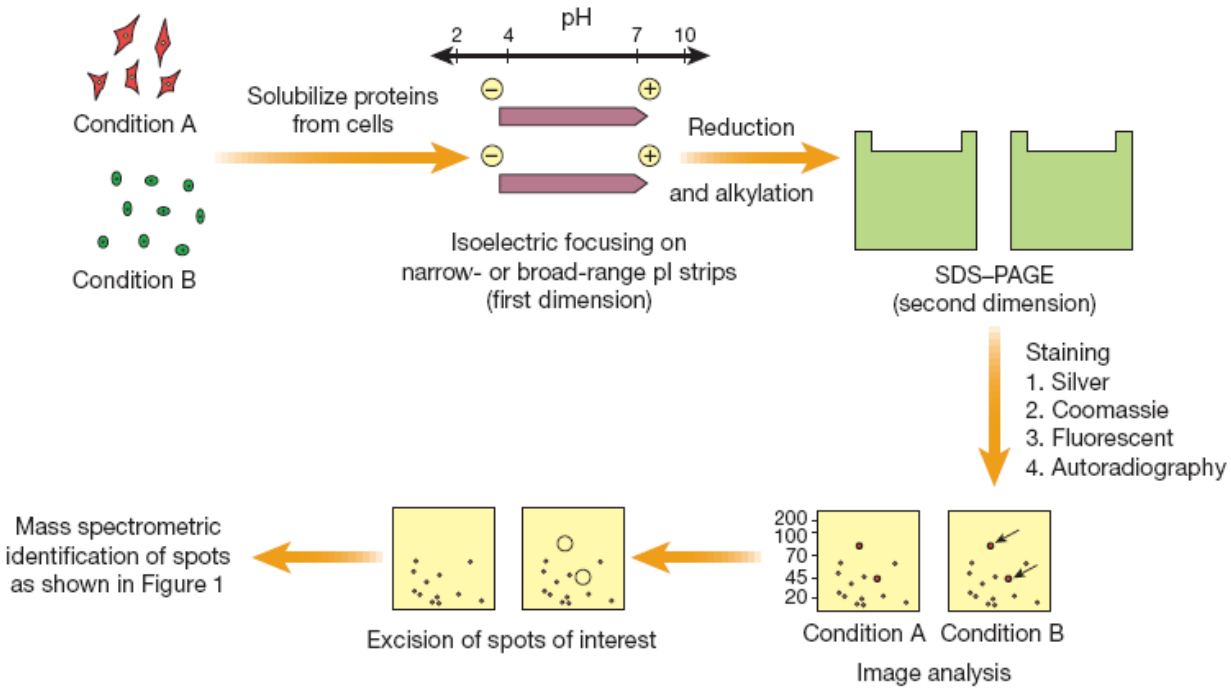

Figure 5 A schematic showing the two dimensional gel approach. Cells (or tissue) derived from two different conditions, A and B, are harvested and the proteins solubilized. The crude protein mixture is then applied to a 'first dimension' gel strip that separates the proteins based on their isoelectric points. After this step, the strip is subjected to reduction and alkylation and applied to a 'second dimension' SDS–PAGE gel where proteins are denatured and separated on the basis of size. The gels are then fixed and the proteins visualized by silver staining. Silver staining is less quantitative than Coomassie blue but more sensitive and is also compatible with mass spectrometric analysis. After staining, the resulting protein spots are recorded and quantified. Image analysis requires sophisticated software and remains one of the most labor-intensive parts of the two dimensional gel approach. The spots of interest are then excised and subjected to mass spectrometric analysis.

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CHAPTER 1

EXPRESSION OF CANCER STEM CELL MARKERS IN RETINOBLASTOMA

Introduction:

Chemotherapy has an important role in the management of retinoblastoma. To improve treatment outcome, intensive research has focused on clinically relevant mechanisms of chemotherapeutic drug resistance in retinoblastoma (Shields et al., 2004). Siegel et al (Siegel et al., 2005) observed the presence of a small subpopulation of cancer stem cells (ABCG2 positive) and neural stem cells (MCM2 positive) in tumours from transgenic mice, human retinoblastoma cell lines and a small cohort of archival human retinoblastomas. They concluded that the presence of these cells in retinoblastomas might have significant impact on future treatment strategies. ABCG2 is a half ATP binding cassette (ABC) transporter expressed on plasma membranes. Overexpression of ABCG2 in cell lines confers resistance on a wide variety of anticancer drugs including mitoxantrone, daunorubicin, doxorubicin, topotecan, and epirubicin (Allen et al., 1999; Scheffer et al., 2000; Litman et al., 2000). MCM2 is one of six members of the family of minichromosome maintenance (MCM) proteins (Kearsey et al., 1996). MCM proteins are components of the prereplicative complex, which binds to replication origins in the G1 phase of the cell cycle and is essential for the initiation of DNA replication (Maslov et al., 2004).7 MCM2 is a proved marker for detecting neural stem cells (Schlesinger et al 1981).8 Since primitive neuroectodermal cells are involved in retinoblastoma tumorigenesis (Dyer and Bremner, 2005), the presence of these neural stem cells could increase the aggressiveness of the original tumour. There are no studies on ABCG2 and MCM2 proteins in a large cohort of archival retinoblastoma tumour samples. We investigated the expression of these two proteins and correlated their expression with clinicopathological parameters such as laterality, differentiation, and tumour invasiveness.

MATERIALS AND METHODS

Thirty nine tumours were available from 39 eyes for the study. Among them were tumours from 30 males and nine females. The age ranged from 4 months to 21 years (median 1 year). There were 31 unilateral retinoblastomas and eight bilateral retinoblastomas.

Tumour specimens

The study was reviewed and approved by the local ethics committee at Vision Research Foundation, Sankara Nethralaya, and the committee deemed that it conformed to the generally

accepted principles of research, in accordance with the Helsinki Declaration. Tumours enucleated between 1997 and 2002 with a minimum follow up of at least 24 months were included in the study. Paraffin embedded blocks from 39 cases derived from enucleation of retinoblastomas was used for immunohistochemistry. For nonneoplastic retina donor eye balls, which were received in the pathology laboratory, were used. The tumours were classified into three groups: group 1 (n=18), tumour samples obtained from enucleated eyes as a part of the treatment and the patients were not subjected to either preoperative or postoperative chemotherapy ; group 2 (n=15), tumour samples obtained from enucleated eyes as a part of management and then the patients were subjected to postoperative chemotherapy because the histopathological study showed risk factors for metastasis such as invasion of choroids and optic nerve; and group 3 (n=6), tumour samples obtained from enucleated eyes of patients who received preoperative chemotherapy.

Histopathological features

All tumour slides were reviewed and examined for invasion of choroid, optic nerve, and orbital invasion. Choroidal invasion was classified as either focal invasion or diffuse invasion of the choroid. For optic nerve invasion, postlaminar invasion and invasion of the surgical end of the optic nerve were considered (Folberg et al., 2003).

Invasion of tumours:

There were 20 tumours with no invasion of the choroid or optic nerve and 19 tumours with invasion of the choroid, optic nerve, and orbit. Among the 19 tumours with invasion, eight had choroidal invasion (three diffuse and five focal choroidal invasion). There were four tumours with postlaminar optic nerve invasion. There were seven tumours with invasion of both choroid and optic nerve (five tumours with diffuse choroidal and post-laminar optic nerve invasion and two tumours with focal choroidal and post-laminar optic nerve invasion).

Differentiation of tumours:

Retinoblastomas were graded microscopically into three groups according to the predominant pattern of differentiation (Folberg et al., 2003). There were 11 well differentiated, five moderately differentiated, and 23 poorly differentiated tumours.

Treatment:

Groups 1 and 2 tumours were not subjected to preoperative chemotherapy. Group 2 consisted of patients whose tumours were enucleated and then the patients were subjected to postoperative chemotherapy, because of risk factors for metastasis. Group 3 consisted of tumours, which were subjected to preoperative chemotherapy. In bilateral retinoblastomas, the eye where the tumour was small was treated with local therapy. The focal therapies employed were external beam radiation therapy (EBRT), diode laser photocoagulation (DLP), and transconjunctival cryopexy (TCC). The chemotherapy cycles ranged from two to eight cycles and drugs used included carboplatin, vincristine, and etoposide.

Clinical outcome:

In group 1, where the tumours did not have any invasion nor had only focal choroidal invasion, there was no recurrence. In group 2, all the 15 tumours were subjected to postoperative chemotherapy because of the risk factors for metastasis. There was orbital invasion in four tumours (cases 4, 6, 8, 10), bone marrow metastasis in one tumour (case 5), and submandibular node metastasis in one tumour (case 12). In group 3, the tumours were subjected to preoperative chemotherapy and then enucleated.

Antibodies and chemicals:

Mouse monoclonal anti-ABCG2 (5D3); sc-18841 (200 mg/ml) antibody for the detection of ABCG2 protein was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and also mouse monoclonal antibody MAAB4146 Ms X BRCP (0.25 mg/ml) was obtained from Chemicon International. Primary mouse monoclonal human MCM protein MCM2 (150 mg/ml); clone: CRCT2.1) antibody was obtained from Novocastra, Newcastle, UK. Labeled streptavidin biotin (LSAB) kit and developing solution (DAB) were obtained from Dakocytomation Corp, Glostrup, Denmark.

Immunohistochemistry

Paraffin sections of 39 tumour samples of retinoblastoma were washed in phosphate buffered saline (PBS) and incubated for 1 hour with the ABCG2/MCM2 antibody or with 1% BSA-PBS as a negative control. After washing, the slides were incubated with biotinylated anti-mouse immunoglobulin (LSAB) for 30 minutes, washed again, and incubated with horseradish peroxidase conjugated streptavidin for 30 minutes. The reaction was revealed by 3, 3′-diaminobenzidine and counterstained with haematoxylin.

Evaluation of slides:

Antigen expression was defined as the presence of membrane staining for ABCG2 and nuclear staining on the tumour cells for MCM2. All stained cells were considered positive, irrespective of staining intensity. Because ABCG2 and MCM2 were expressed heterogeneously, 20 vital tumour fields were evaluated (under 20 X magnifications) and a final mean score for each tumour was obtained. The staining was scored as the percentage of positively stained cells. Both ABCG2 and MCM2 were scored on a three tiered scale: – 0 (when ABCG2 or MCM2 negative); 1% – ≤50% cells positive and >50% cells positive for ABCG2 or MCM2. ABCG2 and MCM2 immunoreactivity was correlated with the invasiveness and differentiation of the tumours and in the three groups of tumours.

Statistical analysis:

Statistical analyses were performed using the non-parametric Mann-Whitney U test. p values <0.05 were considered statistically significant. Well differentiated and moderately differentiated tumours were grouped and compared against poorly differentiated tumours.

RESULTS:

Tables 1–3 show the clinicopathological information and immunoexpression of ABCG2 and MCM2 in the three groups of tumours. The immunoreactivity data of ABCG2 from two antibodies was concurrent. Figures 1A-B show ABCG2 positivity in the non-neoplastic retina obtained from donor cadaveric eyes and figures 1C–D show MCM2 immunoreactivity in the in the non-neoplastic retina obtained from donor cadaveric eyes. Figures 2A–B show ABCG2

positivity in the retinoblastoma tumour samples and figures 2C–D show MCM2 immunoreactivity in the retinoblastoma tumour samples.

ABCG2 and MCM2 in non-neoplastic retina:

Both ABCG2 and MCM2 were sporadically positive in the ganglion cells, inner nuclear layers, and in the photoreceptor layers in the retina obtained from donor eyes and in the nonneoplastic portion of the retina with retinoblastoma.

ABCG2 expression in retinoblastoma

Among the 20 non-invasive retinoblastomas, ABCG2 was positive in 12 tumours (two tumours with >50% cells positivity and 10 tumours with 1%–≤50% cells positivity) and negative in eight tumours. Among 19 invasive retinoblastomas, ABCG2 was positive in 15 tumours (10 tumours with >50% cells positivity and five tumours with 1 to ≤50% cells positivity) and negative in four tumours. Among the six tumours which had either orbital recurrence or metastasis, ABCG2 was positive in six tumours (all >50% cells positivity). The percentage of ABCG2 positive cells was more in the invasive tumours (Mann-Whitney U test; $p<0.01$).

MCM2 expression in retinoblastoma:

Among 20 non-invasive retinoblastomas, MCM2 was positive in 14 tumours (six tumours with >50% cells positivity and eight tumours with 1% to ≤50% cells positive) and negative in six tumours. Among the 19 invasive retinoblastomas, MCM2 was positive in 16 tumours (13 tumours with >50% cells positivity, three tumours with 1% to ≤50% cells positivity) and negative in three tumours. Among the six tumours, which had, either orbital recurrence or metastasis MCM2 was positive in five tumours (all >50% cells positivity). The percentage of MCM2 positive cells was more in the invasive tumours (Mann-Whitney U test; $p<0.01$).

ABCG2 and MCM2 expression in tumours not subjected to preoperative chemotherapy: Among 33 tumours (groups 1 and 2) that had no preoperative chemotherapy, ABCG2 was positive in 22 tumours (11tumours with >50% cells positivity and 11 tumours with 1%–50%

cells positivity) and MCM2 was positive in 28 tumours (18 tumours with >50% cells positivity and 10 with 1%–50% cells positivity).

ABCG2 and MCM2 expression in tumours subjected to preoperative chemotherapy:

Among the six tumours, ABCG2 was positive in five tumours (four tumours with 1%–0% cells positivity and one tumour with >50% cells positivity) and negative in one tumour. MCM2 was positive in one tumour (>50% cells positivity) and negative in five tumours.

ABCG2 and MCM2 expression and correlation with differentiation and laterality of tumours:

There was no correlation observed with MCM2 expression against differentiation and laterality of tumours.

Figure 1

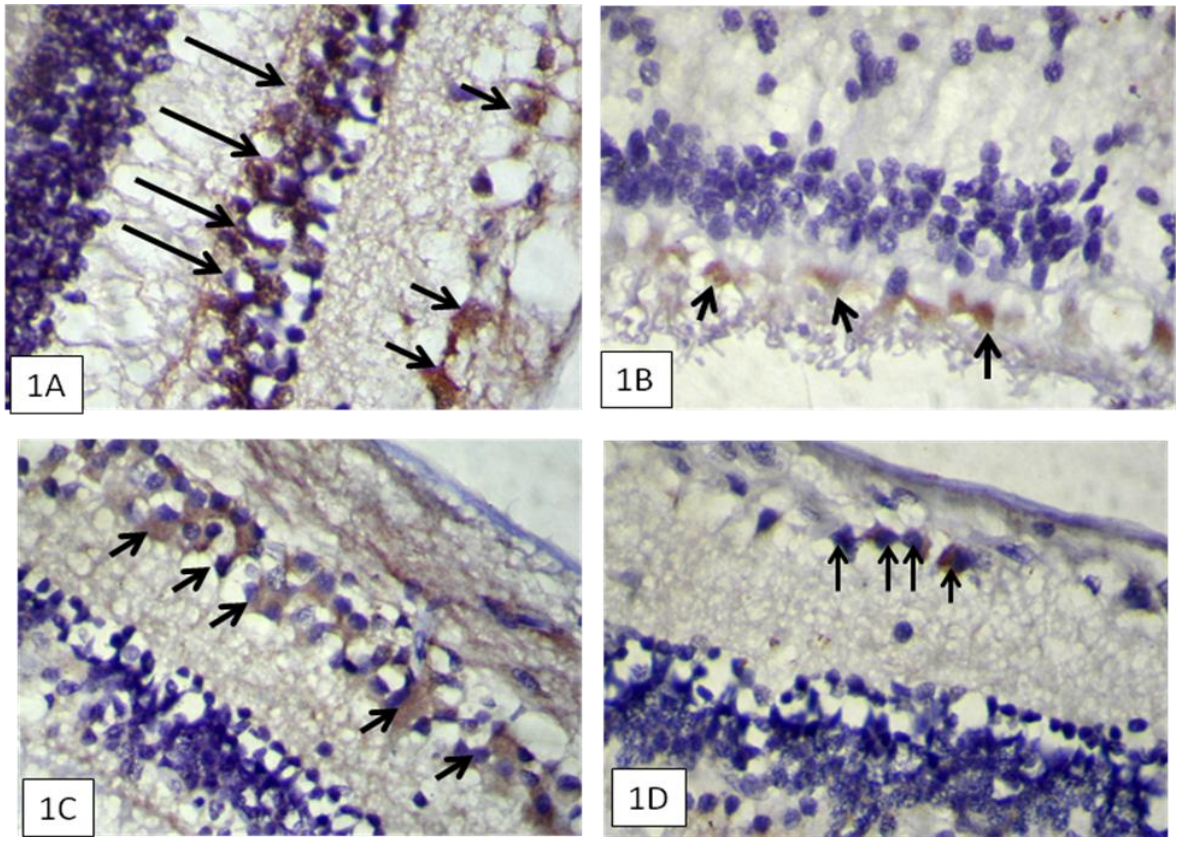

Figure 1A: Photomicrograph showing the ABCG2 positivity in the ganglion cells (small arrows) and in the inner nuclear layers (long arrows) in non-neoplastic retina obtained from donor eye tissue (DAB with haematoxylin counterstain,X100). Figure 1B: Photomicrograph showing the ABCG2 positivity in the photoreceptor cells (arrows). (DAB with haematoxylin counterstain,X100).Figure 1C: Photomicrograph showing the MCM2 positivity in the inner nuclear layers of the donor retina (arrows). (DAB with haematoxylin counterstain, X100). Figure 1D: Photomicrograph showing the MCM2 positivity in the ganglion cells (arrows) of the donor retina. (DAB with haematoxylin counterstain, X100).

Figure 2

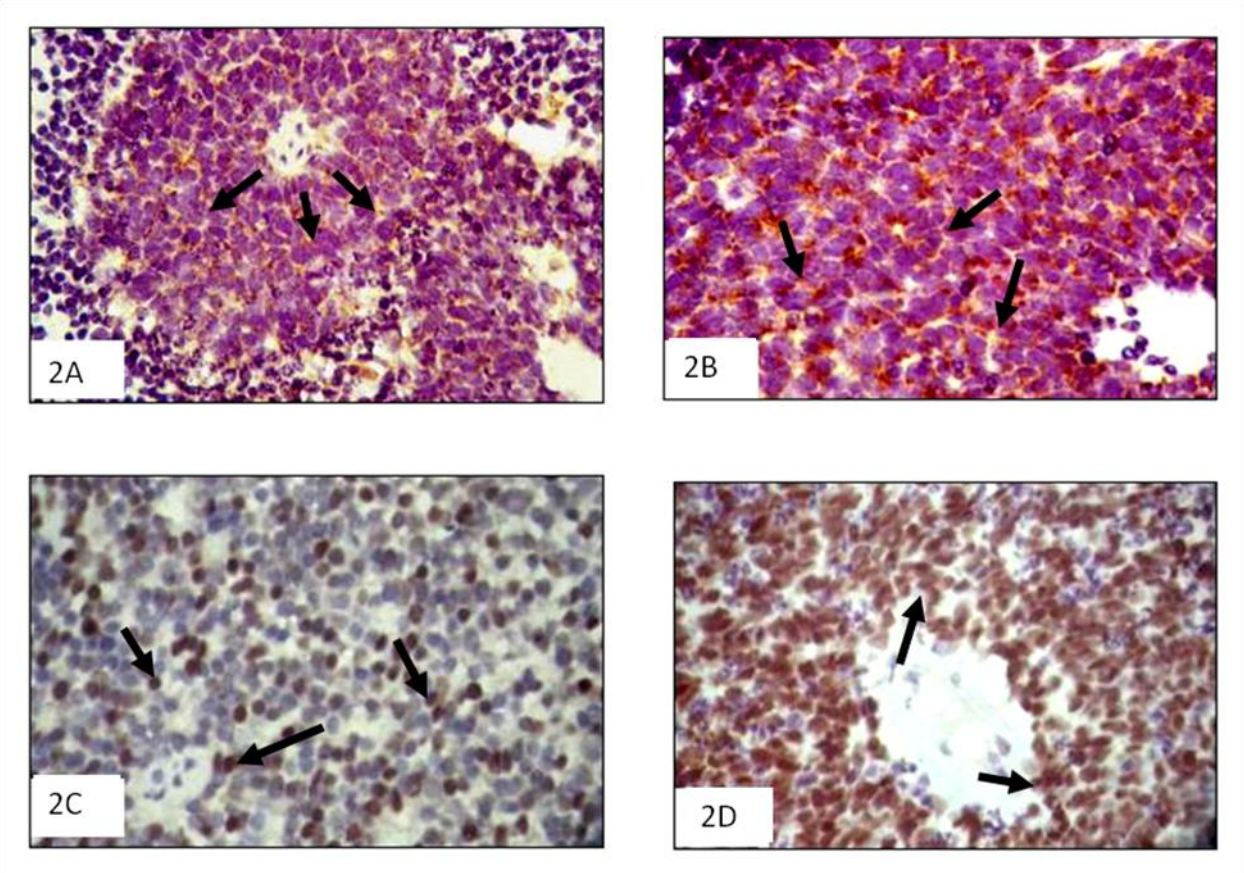

Figure 2A: Photomicrograph showing the ABCG2 in the tumours cells of a tumour lobule with tumour cells showing no differentiation arranged around a blood vessel (black arrow) (DAB with haematoxylin counterstain,X40) Figure 2B: Photomicrograph showing the ABCG2 positivity in the majority of the tumours cells showing no differentiation. (DAB with haematoxylin counterstain,X100).Figure 2C: Photomicrograph showing the 10–20% MCM2 positivity in the tumour cells of retinoblastomas showing differentiation (DAB with haematoxylin counterstain, X100). Figure 2D: Photomicrograph showing the .50% MCM2 positivity in majority of the tumours cells showing no differentiation and arranged around the blood vessel (DAB with haematoxylin counterstain, X100).

DISCUSSION

The current study compares the immunoexpression of ABCG2 and MCM2 in human archival retinoblastoma tumours with an earlier study by Siegel et al (Seigel et al., 2005) where they observed the immunoexpression of ABCG2 and MCM2 in retinoblastoma tumours from mice transgenic for the SV40 T antigen (driven by the b luteinizing hormone promoter), cell pellets of human Y79 and WERI-Rb27 retinoblastoma cell lines, and three archival human retinoblastoma pathological specimens. In their study, small numbers of retinoblastoma cells exhibited immunoreactivity to stem cell markers ABCG2 and MCM2. MCM2 positivity was observed in tumour tissue surrounding blood vessels, adjacent to the optic nerve, and in the inner nuclear layer and photoreceptor layers of the retina in eyes with retinoblastoma. Our results are concurrent with their study in that the human archival retinoblastoma tumour samples showed the immunoexpression of both ABCG2 and MCM2. Both ABCG2 and MCM2 immunoreactivity was observed in both well and poorly differentiated tumours and in tumour cells arranged around blood vessels and also in the invading portion of the tumour cells in the optic nerve and orbit. We also had the opportunity of correlating the expression of these two stem markers with invasiveness and with chemotherapy in our cohort. The percentage of ABCG2 and MCM2 positive cells was more in the invasive tumours (Mann-Whitney U test; $p<0.01$ for both). There was no correlation with respect to laterality and differentiation of the tumours. These data support the earlier observation that stem cells expressed in retinoblastoma could have a role in tumorigenesis (Seigel et al., 2005). Among the 33 tumours not subjected to preoperative chemotherapy, ABCG2 was positive in 21 tumours and MCM2 was positive in 29 tumours. This observation suggests that retinoblastoma expresses ABCG2 and MCM2 proteins even previous chemotherapy. This could have implications with regard to chemotherapy response. Among the six tumours subjected to preoperative chemotherapy (table 3) ABCG2 was positive in five tumours and MCM2 was positive in one tumour. The increased percentile of ABCG2 positive cells in tumours, which were enucleated after preoperative chemotherapy, suggests that ABCG2 positive cells could escape the chemotherapy drugs. Regarding the correlation of ABCG2 and MCM2 protein with response to chemotherapy, in the 15 tumours in group 2 (table 2) where the patients were subjected to postoperative chemotherapy, ABCG2 was positive in 12 tumours. In

the six patients who had developed orbital recurrence or developed metastasis, ABCG2 was positive in all six tumours and MCM2 was positive in two tumours. Overexpression of ABCG2 has been observed in several human cancer cell lines selected for drug resistance (Allen et al., 1999; Scheffer et al., 2000; Litman et al., 2000; Malipaard et al., 1999), as well as in tumour samples of cancer patients (Robey et al., 2001; Diestra et al., 2002). Tumorigenic cells with characteristics similar to neural stem cells with MCM2 positivity have been isolated from pediatric brain tumours (Masloy et al., 2004). Presence of neural stem cells could increase the aggressiveness of the original tumour. Thus the expression of MCM2 in retinoblastoma is similar to other tumours where MCM2 is seen more in aggressive tumours (Freeman et al., 1999; Meng et al., 2001; Rodins et al., 2002; Dudderidge et al., 2005; Kato et al., 2003). We also observed the immunoreactivity of ABCG2 and MCM2 in ganglion cells, inner nuclear layer, and the photoreceptor cells of non-neoplastic retinas obtained from donor eyes. ABCG2 and MCM2 positivity was also observed in the retinal tissue adjacent to the tumour. In normal tissue, high expression of the ABCG2 is found in stem cells, epithelial cells of small and large intestines, ducts and lobules of the breast, endothelial cells of veins and capillaries, and synchitiotrophoblastic cells of the placenta (Maliepaard et al., 2001). The localisation of ABCG2 suggests that it could have a potential role in protection against toxins.

In summary, we confirm and expand upon the findings that cancer stem cell and drug efflux protein ABCG2 and neural stem cell and proliferative marker MCM2 are expressed in archival retinoblastoma samples. However, further studies are needed to understand the role of these stem cells in retinoblastoma and their contribution to drug resistance and tumour progression.

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EXTENDED REPORT

Stem cell markers: ABCG2 and MCM2 expression in retinoblastoma

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CHAPTER 2

EXPRESSION OF p63 AND p73 (p53 FAMILY PROTEINS) IN RETINOBLASTOMA AND THEIR ASSOCIATION WITH CLINICOPATHOLOGICAL FEATURES.

1. Introduction:

p53 controls powerful stress response as it integrates upstream signals from many types of DNA damage and inappropriate oncogenic stimulation, all of which lead to p53 activation and subsequent regulation of genes involved in cell cycle arrest or apoptosis (Lane, 1992; Ko and Prives, 1996). Hence, it is not surprising that this is the most frequent site for genetic alterations found in human cancer (Hollstein et al.,1991,1996). Recently, Laurie et al. (2006) have proved that p53 pathway is inactivated in retinoblastoma and that this cancer does not originate from intrinsically death-resistant cells as previously thought. They have shown that MDMX amplification in retinoblastoma leads to p53 inactivation and supported the idea that MDMX could be a specific chemotherapeutic target for treating retinoblastoma. However, the discoveries of p73 (Kaghad et al., 1997) and p63 (Schmale and Bamberger, 1997; Yang et al., 1998) have both added new insights and increased complexities to the analysis of p53 function. This is due to the discoveries of multiple isoforms of both genes, some of which are p53 agonistic while others antagonistic, and of their p53 independent roles in neurogenesis and stem cell biology (Yang et al., 1998, 1999, 2000; Jost et al., 1997; Mills et al., 1999). p73 was the first p53-family member discovered and was initially described as a putative tumor suppressor as it was located on a region of chromosome 1, 1p36, that frequently undergoes loss of heterozygosity (LOH) in certain cancers (Kaghad et al., 1997) and because of its ability to mimic p53 in certain transcription and growth control assays (Kaghad et al., 1997; Jost et al., 1997). However, it is not universally induced by all types of DNA damage and the remaining allele in neuroblastomas that have undergone LOH are apparently wild-type, suggesting that its role is more complex than previously assumed (Kaghad et al., 1997). p63 also possesses significant homology with p53 and p73 and is widely expressed in a number of basal cells of epithelial origin of human and murine tissues (Yang et al., 1998) and encodes one class of transcripts lacking the N-terminal transactivation domain that exerts strong, inhibitory effect on p63 and p73 activity and the other with the same domain intact, that can transactivate p53 target genes (Yang et al., 1998; Irwin and Kaelin, 2001; Levrero et al., 2000). In this study, we sought to determine whether p63 and p73 are expressed in retinoblastoma, a common childhood eye tumor, and examine their correlations with tumor histopathology.

2. Materials and methods

2.1. Clinical information

The study was reviewed and approved by the local ethics committee of our institute, and the committee deemed that it conformed to the generally accepted principles of research, in accordance with the Helsinki Declaration. Forty-nine tumors were available from 49 eyes for the study. Among them, there were tumors from 35 males and 14 females. The age ranged from 1 month to 21 years. (median¼ 24 months). There were 38 unilateral retinoblastomas and 11 bilateral retinoblastomas. Ten fresh tumors were used for studying the expression of p63, p73 and their respective delta isoforms.

2.2. Histopathological information

There were 33 tumors with no massive invasion of choroid or optic nerve (lower risk to extraocular relapse) and 16 tumors with invasion of choroid/optic nerve/orbit (higher risk to extraocular relapse). Among the 16 high risk tumors, 3 tumors had diffuse choroidal invasion, 2 tumors with post-laminar optic nerve invasion. There are 5 tumors with both diffuse choroidal and post-laminar ON invasion, 2 tumors with both diffuse choroidal and pre-laminar ON invasion, 2 tumors with focal choroidal and post-laminar ON invasion, 1 tumor with post-laminar optic nerve invasion with surgical end involved. There was 1 tumor with orbital invasion. There were 31 tumors with poorly differentiated cells, 11 tumors with well differentiated and 7 tumors with moderately differentiated cells.

2.3. Immunohistochemistry:

In brief, paraffin sections (5 mm thick) were dewaxed and rehydrated. Antigen retrieval was performed by the pressure-cooker method in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% H_2O_2 in water (10 min) and the slides were incubated with the respective antibodies (p63 mouse monoclonal antibody (clone: 4A4) with a dilution of 1:50 (4 mg/ml)); Dp63 goat polyclonal (N-16) with a dilution of 1:100 (2 mg/ml); p73 mouse monoclonal (E-4) with a dilution of 1:50 (4 mg/ml); Dp73 goat polyclonal (N-16) with a dilution of 1:100 (2 mg/ml). Immunostaining was performed using Dako LSAB + system-horseradish peroxidase (Dakocytomation, Glostrup, Denmark). The reaction was revealed by 3, 3′-diaminobenzidine tetrahydrochloride (Dakocytomation, Glostrup, Denmark) and counterstained

with hematoxylin. For p63 and p73 isotype negative controls immunohistochemistry was performed using normal mouse immunoglobin with a dilution of 1:100 (0.4 mg) and normal goat immunoglobin with a dilution of 1:100 (8 mg/ml).

2.4. Immunoanalysis:

Evaluation of immunostaining in tumor cells was objectively performed by 2 investigators (MA and SK). Randomly 10 vital tumor fields were scanned for protein expression under 40X and percentage of positive tumor cells were noted for each field. Then finally the average expression was calculated from the 10 values for the entire slide. Depending on the percentage of positive cells, 4 categories were established: 0, no positive cells; 1+, positive cells in less than one third (faint); 2+, positive cells in 33–67% (heterogeneous) and 3+, positive cells in more than two thirds (positive) of total tumor cell population. Immunoreactivity of the antigens was correlated with the invasiveness and differentiation of the tumors.

2.5. Western blotting:

Tumor samples (50 mg concentration) were mixed with an equal volume of loading dye (BPB, glycerol, 2-mercaptoethanol, Tris–HCl and MQ) and heated at 100°C for 4 min and resolved on 10% SDS PAGE gels. They were then transferred onto nitrocellulose membrane (Hybond-ECL nitrocellulose, Amersham) at 100 V for 2 h. The blots were then stained with 0.1% Ponceau S to confirm uniform transfer of proteins and then blocked in 1% non-fat dry milk for overnight (for p63) or 10% non-fat dry milk for 2 h (for p73). The blots were then incubated in primary antibody for 2 h (p63 – 1:2000 dilution) or overnight (p73 – 1:2000 dilution; Dp63 – 1:1500 dilution; Dp73 – 1:1500 dilution). They were then washed in TTBS thrice for 15 min each and then incubated in HRP-tagged anti-mouse secondary antibody (Dakocytomation, Denmark) at 1:2000 dilutions for 90 min (for p63) or 1:10,000 for 2 h (for p73). Detection of positivity was by using ECL system (Amersham Pharmacia) or the Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

2.6. Statistics:

Mann–Whitney U test was used to determine association of immunoreactivity of p63, p73 and their respective isoforms with tumor invasion and differentiation. For statistical purposes, moderately differentiated and well-differentiated tumors were grouped together and were compared with poorly differentiated tumors. The analyses were performed using the SPSS software package for Windows (version 10.0; SPSS Inc., Chicago, IL).

3. Results:

3.1. p63 and delta p63 reactivity in ocular tissues and tumors

The basal cells of the corneal epithelium and limbal epithelium were seen to express p63 and served as an internal control. There was no expression of p63 in the retinal nuclear layers, ganglion cell layers, the photoreceptor layer, Retinal Pigment Epithelial (RPE) cells or the optic nerve tissues (Fig. 1A). We observed faint expression of delta p63 in ganglion cells of non-neoplastic retina (Fig. 3A).We observed strong nuclear expression of p63 (Fig. 1B–D) and strong nucleocytoplasmic expression of delta p63 (Fig. 3B) in the tumor cells.

3.2. p73 and delta p73 reactivity in ocular tissues and tumors:

p73 and delta p73 was negative in non-neoplastic retina. We observed strong nucleocytoplasmic expression of p73 (Fig. 2B–D) and delta p73 in the tumor cells (Fig. 3D).

3.3. Correlation of p63 and p73 expressions with clinicopathological features:

We observed that tumors with invasion showed significantly higher expression of p73 compared to tumors without invasion ($P < 0.05$). However, we did not observe any correlation of p63 and their isoforms and p73 isoforms with invasion/differentiation. The immunoreactivity of p63, p73 and their isoforms in high risk and low risk tumors is shown in Table 1.

3.4. Western blotting of p63 and Dp63 in tumors:

Of 10 tumors studied, p63 was strongly expressed in 6/6 high risk tumors and 4/4 low risk tumors. Of 10 tumors studied, delta p63was strongly expressed in 5/6 high risk tumors and 2/4 low risk tumors (Fig. 4A and B).

3.5. Western blotting of p73 and Dp73 in tumors:

Of the 10 tumors studied, p73 was strongly expressed in 4/6 tumors and faintly expressed in 2/4 high risk tumors and p73 was strongly expressed in 3/4 tumors and faintly expressed in 1/4 low risk tumors. Of the 10 tumors studied, delta p73 was strongly expressed in 5/6 high risk tumors and strongly expressed in all 4/4 low risk tumors. The p73 gene encodes 6 different isoforms which range in size from 47.5 to 70 kDa (deltaNp73) and 80 kDa (p73- alpha). The retinoblastoma tumors studied showed a single band of approximately 66 kDa in all the tumors. (Fig. 4C and D).

Table 1
The expression of p63 and p73 and their isoforms in high risk tumors and low risk tumors

Tumor parameters	p63			Delta p63			p73			Delta p73		
	P	H	F	P	H	F	P	H	F	P	H	F
Overall cohort ($n = 49$)	4	10	15	4	3	18	13	16	9	3	5	16
High risk tumors ($n = 16$)	2	4	3	3	2	3	6	7	2	2	1	5
Low risk tumors ($n = 33$)	2	6	12	1	1	15	7	9	7	1	4	11
P-value	NS			NS			$P < 0.05$			NS		

Abbreviations: P: positive; H: heterogeneous; F: faint; NS: not significant.

Figure 1

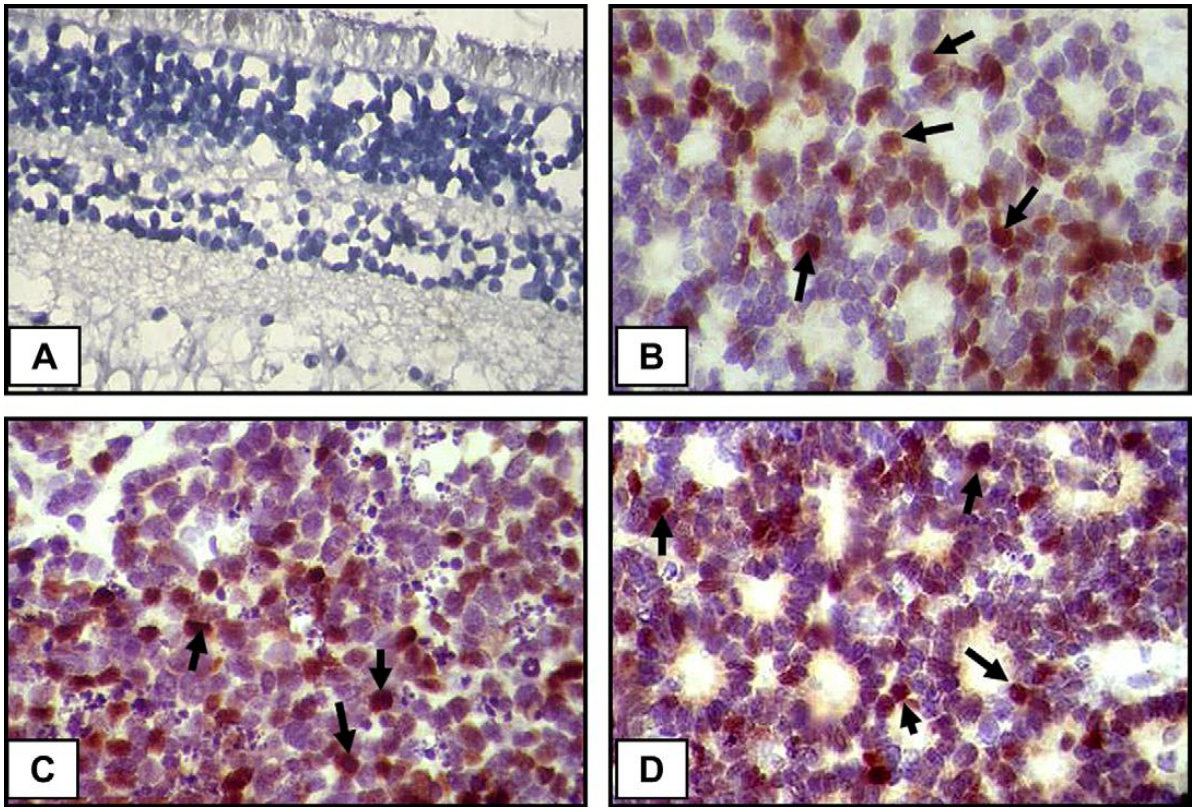

Fig. 1. (A) The photomicrograph shows the negative expression of p63 in non-neoplastic retina from donor eyeball. DAB staining with hematoxylin counter staining (40Xmagnification). (B) The photomicrograph shows the strong nuclear expression (arrows show positivity) of p63 in the low risk RB without invasion and well-differentiated tumor. DAB staining with hematoxylin counter staining (40X magnification). (C) The photomicrograph shows the strong nuclear expression (arrows show positivity) of p63 in the RB with diffuse choroidal invasion and poorly differentiated tumor. DAB staining with hematoxylin counter staining (40Xmagnification). (D) The photomicrograph shows the strong nuclear expression (arrows show positivity) of p63 in the RB without invasion and well-differentiated tumor. DAB staining with hematoxylin counter staining (40X magnification).

Figure 2

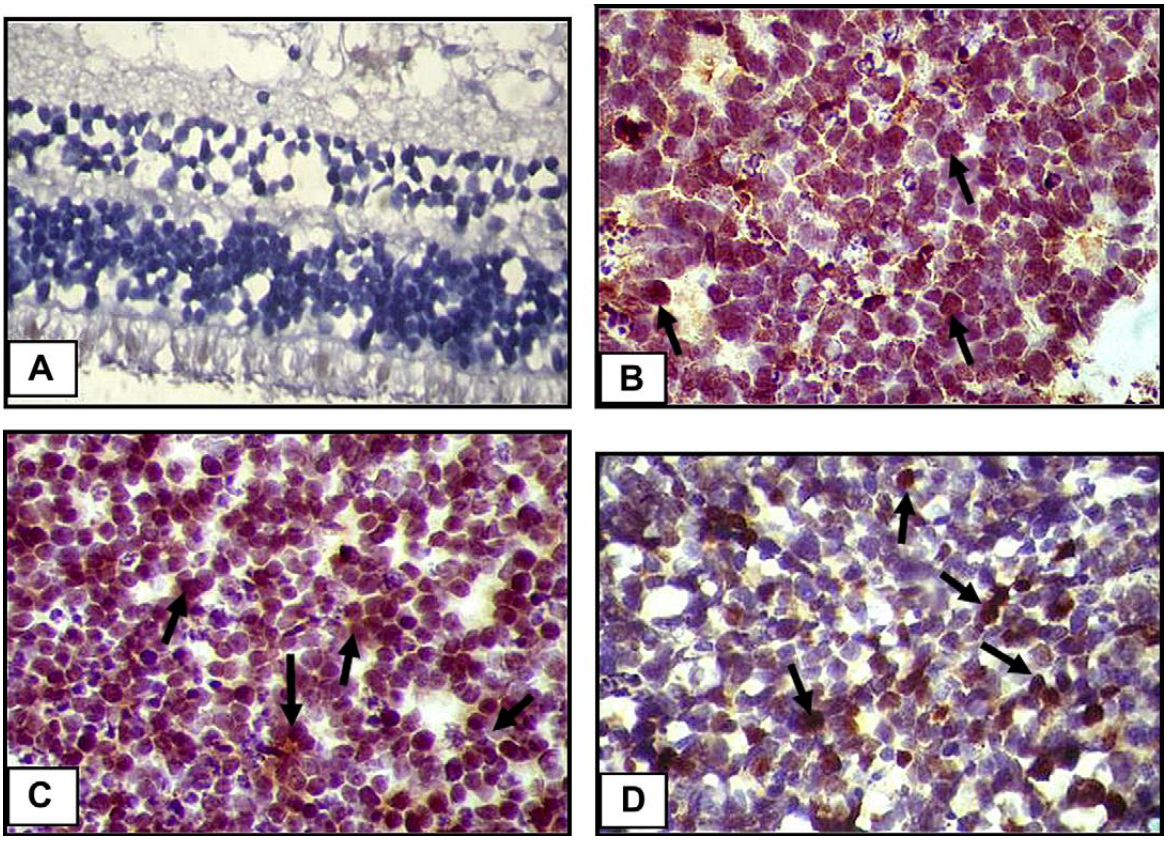

Fig. 2. (A) The photomicrograph shows the negative expression of p73 in non-neoplastic retina from donor eyeball. DAB staining with hematoxylin counter staining (40X magnification). (B) The photomicrograph shows the strong nucleocytoplasmic expression (arrows show positivity) of p73 in the RB without invasion and moderately differentiated tumor. DAB staining with hematoxylin counter staining (40X magnification). (C) The photomicrograph shows the strong nucleocytoplasmic expression (arrows show positivity) of p73 in the RB with post-laminar optic nerve invasion and poorly differentiated tumor. DAB staining with hematoxylin counter staining (40X magnification). (D) The photomicrograph shows the strong nucleocytoplasmic expression (arrows show positivity) of p73 in the RB without invasion and poorly differentiated tumor. DAB staining with hematoxylin counter staining (40X magnification).

Figure 3

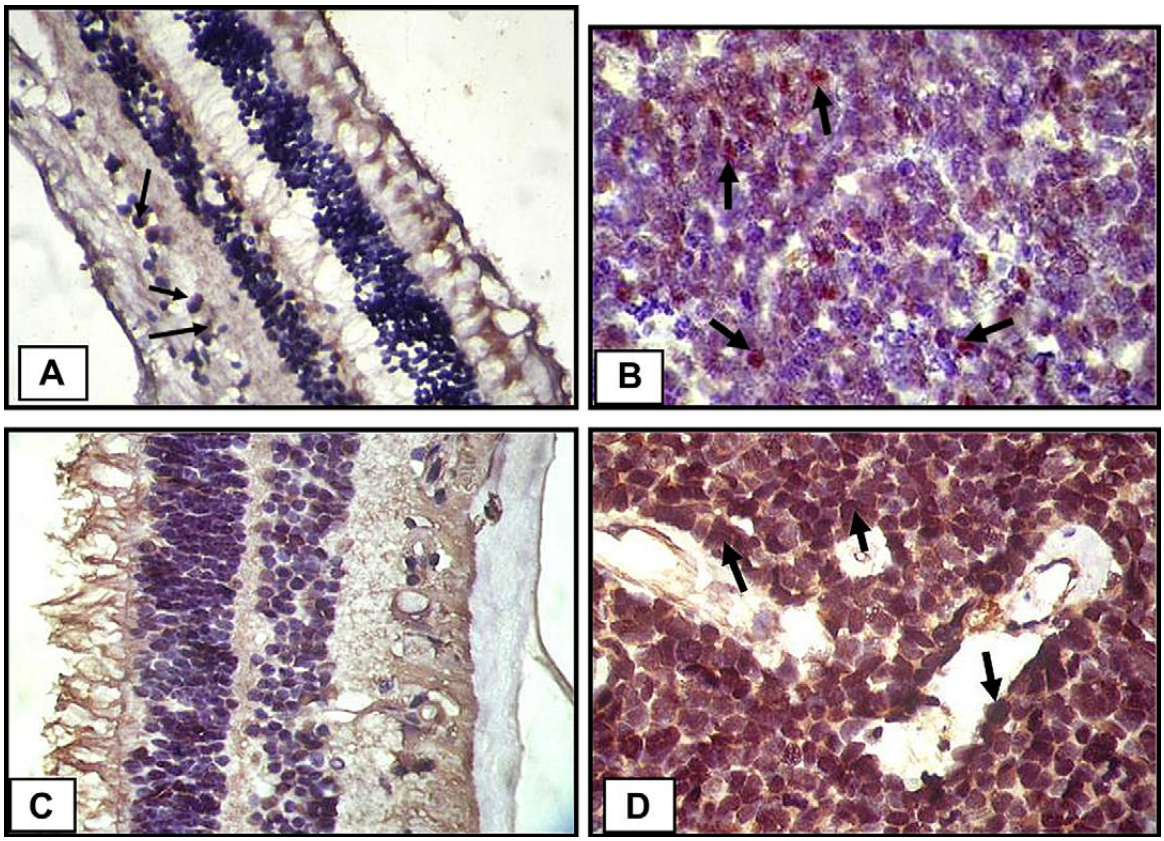

Fig. 3. (A) The photomicrograph shows the negative expression of delta p63 in the nuclear layers whereas faint expression (arrows show positivity) was observed in the ganglion cells of non-neoplastic retina from donor eyeball. DAB staining with hematoxylin counter staining (40X magnification). (B) The photomicrograph shows the nucleocytoplasmic expression (arrows show positivity) of delta p63 in the RB without invasion and poorly differentiated tumor. DAB staining with hematoxylin counter staining (40X magnification). (C) The photomicrograph shows the negative expression of delta p73 in non-neoplastic retina from donor eyeball. DAB staining with hematoxylin counter staining (40X magnification). (D) The photomicrograph shows the strong nucleocytoplasmic expression (arrows show positivity) of delta p73 in the RB without invasion and moderately differentiated tumor. DAB staining with hematoxylin counter staining (40X magnification).

Figure 4

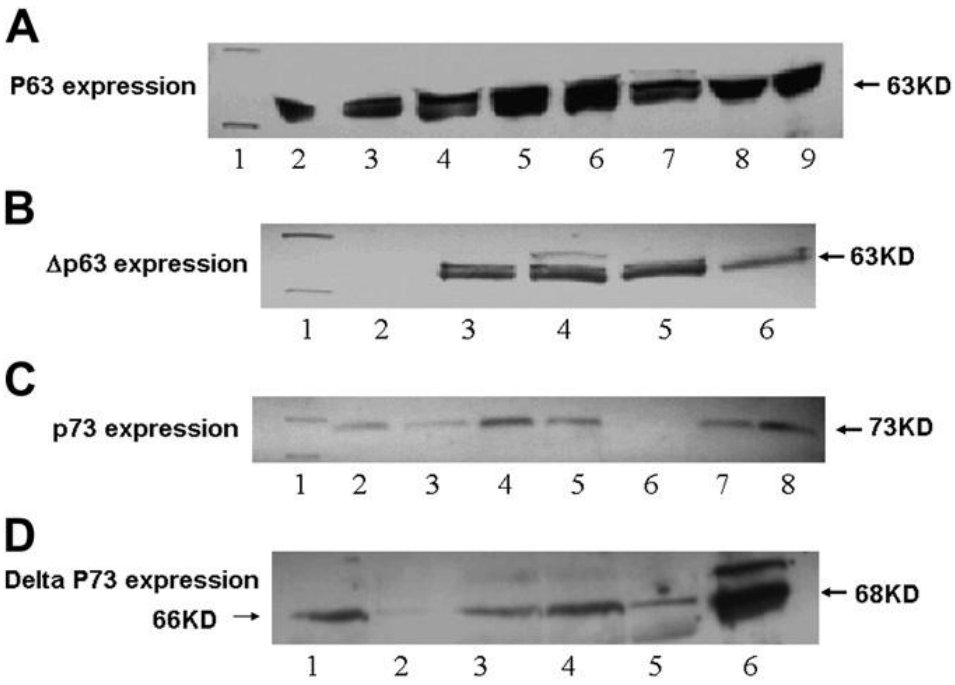

Fig. 4. (A) The photomicrograph shows the p63 positive expression in 4 tumors with invasion (lanes 2–5) and positive expression in 3 tumors without invasion (lanes 7–9). (B) The photomicrograph shows delta p63 positive expression in 2 tumors without invasion (lanes 3 and 4) and positive in 2 tumors with invasion (lanes 5 and 6) and negative in 1 tumor without invasion (lane 2). (C) The picture shows the positive p73 expression in 4 tumors with invasion (lanes 2–5) and positive in 2 tumors (lanes 7 and 8) and negative in 1 tumor (lane 6) without invasion. (D) The picture shows the positive delta p73 expression in 2 tumors with invasion (lanes 1 and 3) and positive in 2 tumors without invasion (lanes 4 and 5). Lane 6 – molecular weight markers.

4. Discussion

In our study, normal retina lacked p63 immunoreactivity, and overall p63 immunoreactivity was observed in 59% (29/49) RB specimens. Among the 33 low risk tumors, p63 was expressed in 60% (20/33) tumors and among the 16 high risk tumors, p63 was expressed in 56% (9/16) tumors. We did not observe any correlation of p63 and Dp63 expressions with invasion or differentiation. Among 49 tumors, overall p73 expression was observed in 77% (38/49) RB specimens. Among the 33 low risk tumors, p73 was expressed in 69% (23/33) tumors and among the 16 high risk tumors, p73 was expressed in 93% (15/16) tumors. Tumors with higher risk for extraocular relapse showed significantly higher expression of p73 compared to tumors with lower risk ($P < 0.05$). Earlier studies have demonstrated the p63 immunoreactivity in squamous cell carcinomas of the breast (Di Como et al., 2000), oesophagus (Glickman et al., 2001), cervix (Quade et al., 2001), adenomyoepitheliomas of the breast (Barbareschi et al., 2001), thymomas, rhabdomyosarcomas, and non-Hodgkin lymphomas (Di Como et al., 2002). Thus retinoblastoma also joins the list of tumors that expresses p63. In particular, the DNp63 is the isoform shown to be expressed in squamous cell carcinoma of the aerodigestive tract, head and neck, lung tumors and non-small cell lung cancer (NSLC) (Hibi et al., 2000; Yamaguchi et al., 2000; Crook et al., 2000; Nylander et al., 2000). The DNp63 isoform is considered to have oncogenic properties based on the following observations: first, DNp63 overexpression is often observed in and enhances oncogenic growth of squamous cell carcinomas; (Quade et al., 2001; Barbareschi et al., 2001) second, DNp63 could function as dominant negative against the p53 tumor suppressor activities by binding to specific promoter elements of genes involved in the apoptotic response, such as MDM2, Bax, NOXA, PERP etc.; third, DNp63 overexpression induces nuclear accumulation of β-catenin and activates β-catenin signaling that promotes cell proliferation (Irwin and Kaelin, 2001; Levrero et al., 2000). In our study, DNp63 was expressed in 25/49 (51%) tumors, (50% in high risk and 51% in low risk tumors) and there was no difference in expression among the 2 groups of tumors, which suggests that its expression may be an early event in tumor progression. In addition, it would be of importance to correlate the immunoreactivity of p63 and p73 with the p53 expression status in RB. Parallel to the findings of Laurie et al. (2006) that MDMX gene amplification occurs in the preneoplastic retinoblastoma

cells and inactivates p53, it would be interesting to explore the possible role of p63 in the inactivation p53 in RB.

The p73 gene is located on chromosome 1p36.2-3 and is closely related to the tumor suppressor p53. It encodes up to 6 different isoforms that are generated by the usage of 2 different promoters and alternative splicing (of exons 11–14 to produce variants with different C-termini), yielding those with p53-transactivation activity and those with dominant negative activity. Transactivation includes genes such as p21, Bax, MDM2 and GADD45 leading to induction cell cycle arrest and apoptosis and the dominant negatives (lacking the N-terminal transactivation domain) function by inhibiting TAp73 as well as other members of the p53 family. So our understanding of their biological roles will greatly depend on knowing exactly which forms get expressed under what circumstances.

While initial studies on cancers such as breast and colon cancer, neuroblastoma, oligodendroglioma and melanoma reported loss of heterozygosity of the region harboring p73 and its suggestion of role of tumor suppressor, genetic data on most other cancer types failed to demonstrate a similar loss of expression of the other allele or function during tumorigenesis (Melino et al., 2003; Sun, 2002). To date, in a total of >1100 primary tumors, loss of function mutations and imprinting of p73 (which would satisfy 1 hit of the 2 hit hypothesis) are rare. In fact, in lung, esophageal, and renal carcinoma, the second p73 allele is specifically activated in tumors (Uramoto et al., 2004; Moll and Slade, 2004). p73 positivity has also been correlated with poor prognosis in hepatocellular carcinoma (Tannapfel et al., 1999), colorectal adenocarcinoma (Sun, 2002) and ovarian carcinoma (Niyazi et al., 2003). Most of these studies have found overexpression of full length-TAp73 at the mRNA and protein levels while the tumor-specific upregulation of the DN forms has been reported in breast cancer, gynecologic cancers, hepatocellular carcinoma and neuroblastoma. Our present study showed increased expression of p73 in high risk tumors compared to low risk tumors. Hence in this respect it could be said that the functional loss or gain of p73 is tumor-specific and further study of the isoforms expressed would be crucial on shedding light onto the role of p73 in RB. Specifically, retinoblastoma expresses their N-terminal truncated forms, which are believed to have p53-antagonistic activity.

From this study we must make a note that neither p63 nor p73 could be used as a diagnostic tool for retinoblastoma since it was mostly based upon immunohistochemical analysis. Also it would be interesting to have data on the expression of p63 and p73 in other ocular malignancies occurring in children to add robustness to the use of these molecules for differential diagnosis. Hence we conclude that further studies are required to confirm our initial findings and to explore the cause for the expression of these proteins in retinoblastoma.

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Expression of p63 and p73 in retinoblastoma: A clinicopathological correlation study

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ABSTRACT

The aim of the study was to explore the expression profile of p63, p73 and their delta isoforms in the retinoblastoma tumor samples and to correlate with clinicopathological parameters. Immunohistochemistry was performed for p63, delta p63, p73 and delta p73 on the archival paraffin sections of retinoblastoma and correlated with clinicopathological features. Western blotting was performed to confirm immunoreactivity results. p63 immunoreactivity was observed in 59% (29/49) of the RB specimens. p63 was expressed in 60% (20/33) low risk tumors and in 56% (9/16) of high risk tumors. p73 was expressed in 77% (38/49) RB specimens. Among the 33 low risk tumors, p73 was expressed in 69% (23/33) tumors and among the 26 high risk tumors, p73 was expressed in 93% (15/16) tumors. High risk tumors showed significantly increased expression of p73 compared to tumors with low risk ($P < 0.05$). This is the first correlation between p63/p73 expression and histopathology in human RB tumors. Our study showed increased expression of p73 in high risk tumors ($P < 0.05$) compared to low risk tumors. Further functional studies are required to explore the role of p63, p73 and their respective isoforms in retinoblastoma.

CHAPTER 3
COMPARATIVE PROTEOMIC ANALYSIS OF PRIMARY RETINOBLASTOMA TUMORS

Introduction:

Inactivation of both copies of the *RB1* gene (located at 13q14) in a retinal cell, through mutations or epigenetic modifications, initiates the onset of RB. This event is followed, like in other cancers, by the sequential acquisition of additional genetic abnormalities that define the course leading to tumor formation and metastasis (Honavar et al 2002; Makimoto A 2004; Chantada et al., 2004; Bowles et al., 2007; Corson and Gallie, 2007; Hong et al., 1989). Genomic instability contributes to the progression of retinoma to malignant RB (Dimaras et al., 2008; Dimaras and Gallie, 2008; Dimaras et al., 2006). In humans, this progression is characterized by loss of both copies of the *RB1* gene in retinomas followed by changes in the copy number of oncogenes such as *MYCN* (2p24.3), *E2F3* and *DEK* (6p22), KLF14 (7q32), and *MDM4* (1q32) as well as tumor suppressor genes *CDH11* (16q21) and *p75NTR* (17q21). It has also been shown that when *RB1* and *TP53* are inactivated in mice, RB develops (Windle et al., 1990; Gallie et al., 1999). Collectively, these observations indicate that, beyond biallelic inactivation of *RB1*, additional "hits" are required for the development of RB tumors in humans and mice (Amare et al., 2004; Knudson AG, 1996; Knudson AG, 2001).

RB1 codes for the retinoblastoma protein, Rb, which functions as a tumor suppressor by controlling the cell cycle through complex interactions of multiple kinases and their inhibitors which, together, form the Rb pathway. In the absence of mitogenic stimuli, Rb activity is engaged to inhibit cell-cycle progression through inhibition of transcription of multiple genes required for S-phase entry (Nevins JR, 2001; Blais A and Dynlacht, 2007; Wang et al., 1994). However, Rb function can be disrupted by the overexpression of D-type cyclins (Diehl JA, 2002; Arnold A and Papanikolaou, 2005) or loss of $P16^{INK4A}$ a CDK inhibitor in various cancers (Palmero I and Peters G, 1996; Malumbres M and Barbacid M, 2001). Additionally, Rb is the target of the HPV-E7 oncoproteins involved in the etiology of cervical cancer (Munger et al., 2001). Recently, few HPV strains such as HPV 16, 18, 6a, 33, 11, 31, 35 and 51 have been described in fresh tumor tissue from RB patients also (Orjuela et al., 2000; Espinoza et al., 2005; Montoya-Fuentes et al.,2003; Palazzi et al.,2003; Mohan et al., 2009). However, it is still not clear whether HPV is the causative agent for RB development. In contrast, an earlier study has shown neither HPV nor any other pRb-inactivating human DNA tumor viruses play a role in the

development of RB, regardless of RB1 genotype (Gillison et al., 2007). Despite recent advances in the understanding of Rb function, the precise mechanism of RB development is still not clear. Hence, based on these controversial evidences on the concept of RB development, it is imperative to understand the molecular pathogenesis of malignant transformation and progression of the RB tumor in order to develop novel and specific therapeutic agents for targeted therapy.

Proteomics is the systematic study of the total proteins expressed in a cell or tissue (Jung et al., 2000). Proteomic analysis is an accurate, sensitive, and high-throughput protein identification strategy (Liska et al., 2003). In the research of the molecular mechanisms of diseases, comparative proteomic analysis has been used as an innovative method to investigate the protein expressions between cancerous and normal tissues/cells. Investigating the differential protein profiling of RB and their association with clinicopathological features will help identify target proteins for RB management/therapy.

To our knowledge there are no studies available on the differential protein profiling of RB to date. In this study we used two dimensional electrophoresis (2-DE) and MALDI-TOF-TOF (MS/MS) to identify the differentially expressed proteins using non-neoplastic retinas as control tissues. Twenty seven differentially expressed proteins were identified and six of them were validated by real time quantitative reverse transcriptase PCR (Q-RT-PCR) in the fresh tumors. Immunohistochemistry was also performed for five proteins in the formalin fixed paraffin embedded RB sections.

Materials and Methods

Clinical samples:

Control donor retinas:

The study adhered to the guidelines in the Declaration of Helsinki. This study was conducted at the Medical Research Foundation and Vision Research Foundation, Sankara Nethralaya, India, and was approved by the institutional ethics boards. Normal non-neoplastic retinas of donors were obtained from the C. U. Shah Eye Bank (Sankara Nethralaya Medical Research Foundation, Chennai). Normal retinal tissues were obtained from eye globes donated for corneal transplantation. Time from death to isolation of the retina samples varied from 4 to 6 h. Eyes were obtained from donors without known concomitant ocular disease. The lens was removed with the iris; the ciliary body and pars plana were dissected, followed by removal of the vitreous. As the vitreous was removed from the eyecup, in its entirety, the often detached retina could be stripped from the hyaloid face and remained whole within the eyecup attached to the optic nerve. The eyeballs were examined under microscope to confirm no major pathology in the retina and its vessels including old scars. The whole retina was removed en bloc by dissecting it from the optic nerve head. Retinal pigment epithelium, choroid and ciliary body remained within the eyecup. This permits selective dissection of the whole retina. The ages of the non-neoplastic retina donors ranged from 45 to 89 years (mean 75.5 years), with 11 eyes from males and 3 from females. Among 14 donors, 4 of them died of cardiac arrest, 2 of stroke, 2 of cerebral bleed, 1 in accident and the death of other donors are not known.

Tumor samples and Histopathology

Twenty nine fresh RB tumor samples were included for proteomic analysis. RB sections were graded microscopically and were divided into three groups, Well differentiated (WD), Moderately differentiated (MD) and Poorly differentiated (PD) according to the predominant pattern of differentiation (Finger et al., 2002). Tumor slides were reviewed, and the level of choroidal invasion was classified as either focal or diffuse. For the optic nerve, prelaminar invasion, post laminar invasion, and invasion of the surgical end of the optic nerve were analyzed (Sastre et al., 2009) .

Two-dimensional Gel Electrophoresis (2DE) and Image analysis:

Fresh RB tissues, retinal tissues of donor eyes were lysed in 300 mL ice-cold lysis buffer containing (50mM Tris-HCl, 5mM EDTA, 150mM NaCl, 0.1% SDS, 1% Nonidet, 1mM protease inhibitor cocktail). Then the tumor cells were disrupted by sonication (Misonix sonicator XL 2000 series) with 10 bursts in 5 sec pulses in ice and centrifuged at 12 000X*g* for 90 min. The supernatant was subjected to overnight TCA precipitation and the pellet was dissolved in rehydration buffer (6 M urea, 3 M thiourea, 2% CHAPS, 50 mM DTT, and 0.2% (w/v) Bio-Lyte 4/7 ampholytes; Bio-Rad). The concentration of the protein was determined with Bradford protein assay (BioRad, USA). The first-dimensional IEF was performed with Protean IEF cell (Bio-Rad, USA). Eleven cm prefabricated IPG strips with a linear pH range of 3–10 were used (BioRad USA). Four hundred μg of total proteins were mixed with 180 μL of rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 50mM DTT and 0.2% carrier ampholytes pH 3–10 and 0.001% bromophenol blue. After rehydration for 12 h with mineral oil covering the fluid, IEF was carried out at 20^0C with a maximum current setting of 50 μA/strip using the following conditions: 250 V for 30 min in linear mode, 8,000 V for 2 h in linear mode, 8,000 V for 5 h in rapid mode, and then 500 V with the temperature maintaining at 20^0C. After IEF, strips were equilibrated for 2X15 min at room temperature in equilibration buffers (6 M urea, 30% glycerol, 2% SDS, 375 mM Tris-HCl pH 8.8), first supplemented with 2% DTT and then with 2.5% iodoacetamide. Equilibrated IPG strips were then transferred onto 12% uniform polyacrylamide gels for SDS-PAGE. The constant voltage was first at 70 V/gel and then at 100 V/gel until bromophenol blue reached the bottom of the gels. Protein standards were added to the samples as internal markers to determine the PI and molecular weight (MW). Each sample was analyzed two times by 2-DE for reproducibility. After 2-DE, the preparative gels were stained with sensitive colloidal coomassie brilliant blue stain (CCBB). Images were acquired using Fluor-S MultiImager (Bio- Rad) using a visible light source and image analysis was carried out using PDQuest image analysis software (Bio-Rad). All images were taken under uniform settings (3.8 sec exposure time and with 2.7 numerical aperture). After acquiring the images, auto-scaling transformation was applied to all the images. Using spot detection wizard, we have selected a faint spot and a large spot cluster on the gel images for the software to detect the spots. Before proceeding for spot detection, we have applied vertical and horizontal streak removal options and speckle filter options to remove streak and speckles in the gels. Comparison

of the relative intensity of each protein spot between tumors and non-neoplastic retinas (two replicate samples for each tumor and normal tissue) was performed with the Student's *t*-test. The test compared two mean values to determine whether the two groups (tumor and control groups) from which the averages were calculated could stem from a common population. The following was computed from the observations of the two groups: the two sample sizes, the respective mean values, and the respective standard deviations.

In-gel trypsin digestion:

The protein spots were manually excised from the CCBB stained gels and transferred to eppendorf tubes. Spots were destained with 50 acetonitrile (ACN) in 25mM ammonium bicarbonate for 60 min and were dehydrated with 100% ACN for 10 min. Then, 10–20 μL digestion buffer was added and incubation was performed at 37^0C for 20 h. The digestion buffer contained 0.1 μg/μL trypsin in 25 mM ammonium bicarbonate. The peptides were extracted from the digested gel pieces three times with 0.3% trifluoro acetic acid (TFA) in 50% ACN. The peptide solutions were then dried by vacuum centrifugation. The final volumes of the extracts were about 20 μL.

MALDI-TOF-TOF:

Peptides from in-gel digest were reconstituted in 50% ACN with 0.1% TFA for peptide mass fingerprinting (PMF). Peptides were spotted with CHCA (alpha-cyano-4-hydroxy cinnamic acid) on the MALDI plate and measured on matrix-assisted desorption ionization time-of flight (4800 MALDI-TOF-TOF) system (Applied Biosystems, USA). Peptide mass calibration was performed with external mass standards (Calmix 5, Applied Biosystems). Mass fingerprints and tandem mass spectra (MS/MS) from selected peptides were searched against the human MSDB database (using Mascot; Matrix Science Ltd., London, UK). One missed cleavage site was allowed for the search program to take into account for reliable and significant hits as partial enzymatic protein cleavage often occurs with trypsin. Carbamidomethyl cysteine was taken as a fixed modification and oxidized methionine as a variable modification.

Quantitative Real Time RT-PCR

Total RNA was extracted from 10 fresh RB tumors, 10 adult donor retinas by the guanidine isothiocyanate and chloroform method (TRI Reagent; Sigma-Aldrich, Bangalore, India). The 10 fresh tumors used for RNA extraction overlap with 2DE sample cohort (Table 3 sample numbers: 1,3,4,7, 15, 18, 19, 23, 25, 27). Briefly, 1ml of TRI reagent was added to the tissues, vortexed and incubated at room temperature (RT) for 5 minutes. After incubation, 200ul of chloroform was added and shaken vigorously for 15 sec and incubated for 5 minutes at RT, centrifuged at 18,000 X *g* for 15 min at 4^0C. The aqueous phase was transferred to fresh vial; 500ul of isopropanol was added, incubated at RT for 10 min. and centrifuged at 18,000 X *g* for 10 min at 4^0C. The supernatant was decanted, 75% v/v Ethanol was added to the pellet, centrifuged at 18,000 X *g* for 5 min at 4^0C, and the supernatant was decanted. The vials were air dried for 2-3 min and RNase free water was added to reconstitute the RNA. All RNA samples (10μg each) in a 50μl reaction were treated with 1μl of TURBO DNase (2U; Ambion, Genetix Biotech Asia Pvt. Ltd., Chennai, India)) in 1X TURBO DNase buffer at 37^0C for 30 minutes. After incubation, the RNA sample was extracted with phenol/chloroform to inactivate TURBO DNase. Reverse transcription was performed using 1 μg of total RNA and 100 pmol/μl of random primers. The reaction was heated to 65°C for 10 min and put on ice prior to the addition of 1μl of SuperScript II RT (200 U), 2 mM dNTPs, 10 mM DTT, and reaction buffer (all reagents: Invitrogen, Karlsruhe, Germany) to a total volume of 20 μl. The reaction was incubated at 42°C for 1 h. cDNA synthesis was terminated by inactivation for 15 min at 70°C, and RNA degraded by RNase H treatment (Invitrogen) for 20 min at 37°C. PCR was performed using 1X SYBR Green PCR Master Mix (Applied Biosystems) on ABI HT 7900 real time PCR system. The selected primers (Parolini et al., 2003; Yaung et al., 2007; Van den Bogaerdt et al., 2004; Karlsson et al., 2002; Chowers et al., 2006) were used in this study are listed in Table 1. Cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 sec at 95°C, plus 1 minute at 60°C. Commercial software (SDS ver.2.3; ABI) was used to calculate ΔΔCt relative expression values for all the genes studied normalized to the GAPDH endogenous control.

Immunohistochemistry:

Briefly, after deparaffinization and blockage of the endogenous peroxidase with 3% hydrogen peroxide, the tissue sections in 0.01 M citrate buffer solution (pH 6.0) were heated in a water bath at 98^0C for 20 min, incubated overnight with the primary antibodies at recommended dilutions at 4^0C, and visualized using Novolink Polymer detection kit (Novocastra laboratories, UK) according to the manufacturer's instructions. Isotypic antibodies were used for negative control staining. Tissue sections were read independently by two investigators using light microscopy, each without knowledge of the results obtained by the other investigator. There was a good agreement in the interpretation of all the protein expressions with a kappa value ranging from (kappa= 0.75 to 0.85). The average expression was calculated for the entire slide by scanning 10 fields randomly under 40X magnification. All the primary antibodies were purchased from Santa Cruz Company, USA except RCVRN and TUBB1. Immunohistochemical detection of CRABP2 (goat polyclonal N-14; 200µg/ml), ApoA1 (mouse monoclonal A5.4; 200µg/ml) CRYAA (mouse monoclonal, B-2; 200µg/ml), RCVRN (rabbit polyclonal; gifted by Prof. Karl-Wilhelm Koch, Carl Von Ossietzky Universität, Oldenburg) and TUBB1 (Rabbit monoclonal; TUJ1 from Covance, California) on paraffin embedded tumor sections was performed at a dilution of 1:100 for all the antibodies.

Immunoanalysis:

First, a number from 1 to 3 was given according to staining intensity: Weak (1); moderate (2); and strong (3). Then the number of positive cells per slide was stratified into three groups based on the percentage of positive cells: group I, <33%; group 2, 33–67%; group 3, >67%. Scores ranging from 1 to 9 for specific staining for each case were obtained by multiplying the staining intensity with the number of the group that represented the percentage of positive cells as previously described (Kommoss et al., 1990). A score of zero represents no specific staining observed, the scores of 1 and 2 were accepted as grade I (1+), 3 and 4 as grade II (2+), and 6 and 9 as grade III (3+).

Statistical analysis:

The statistical difference of the data was analyzed using Independent t test. Intraclass correlation coefficient analysis was used for comparing mRNA data with protein expression data. When the *p* value was less than 0.05, the difference was regarded as statistically significant.

Results:

2-DE:

2DE of 29 RB tumors, 14 adult healthy retinas was performed over a pH range of 4-7 or 5-8. More than 450 detectable protein spots were found on each gel with the aid of image analysis software PDQuest version 8.1 (Biorad, USA). The spot matching rates for RB 2DE gels (replicates) ranged from 84-90% and for retina 2DE gels (replicates) ranged from 87-94% as determined by intraclass correlation coefficient analysis. Twenty-seven differentially expressed protein spots between the non-neoplastic retinas, and RB tumors were identified. Among 27 differentials, 16 proteins were upregulated and 11 proteins were downregulated in RB (Figure 1).

MSMS identification of differentially expressed proteins in RB

After the differentially expressed protein spots were digested in gel, the peptide fragments were identified using MSMS. Twenty three proteins were characterized by MALDI-TOF-TOF and the MASCOT search engine (http://www.matrixscience.com/, MatrixSicence Ltd, UK) was used to search the SWISSPROT protein database/*Homo sapiens*. Twenty seven differentially expressed proteins in RB when compared to normal retinas were identified (Table 2). The upregulated proteins in RB compared to non-neoplastic retina are Fatty acid binding protein 5 (FABP5), Cellular retinoic acid binding protein-2 (CRABP2), Apolipoprotein A1 (ApoA1), recoverin (RCVRN), Triose phosphate Isomerase (TPI), Alpha crystallin-A (CRYAA), Transaldolase (TALDO1), Alpha enolase (ENO1), T-complex protein zeta (CCTZ), Transferrin precursor (TF), Peroxiredoxin 6 (PRDX6), Beta-tubulin (TUBB2), Gamma enolase (ENO2), Lactate dehydrogenase B chain (LDHB), Stathmin (STMN1), Vitamin D binding protein (GC).

The following proteins were down regulated in the RB samples compared with non-neoplastic retinas: Cellular retinoic acid binding protein-1 (CRABP1), Keratin Type II cytoskelatal (KRTA), Annexin V (ANX5), Cellular retinaldehyde binding protein (CRALBP), Guanine nucleotide binding protein beta 1 subunit (GNB1), Isocitrate Dehydrogenase NAD subunit alpha (IDH3A), Cathepsin D precursor(CPSD), ATP synthase D (MYO32), Glutamine synthetase (GLNS), Vimentin (VIM), Peroxiredoxin 2 (PRDX2).

Gene ontology annotation of differentially expressed proteins

In order to better understand the 27 identified proteins in this study, Gene Ontology Annotation was performed to analyze the identified known proteins according to the Gene Ontology Convention (Ashburner et al., 2000) (Table 3). Each identified known protein was classified according to the three categories of cellular component, molecular function, and biological process. Cytoplasmic proteins were the most common proteins in the category of cellular component. Fifty percent of the cytoplasmic proteins were upregulated in RB. All proteins that belong to nuclear (n=2), cytovesicular (n=2) and microtubule (n=2) components were upregulated in RB. In contrast, all intermediate filament proteins (n=2) and proteins of mitochondrial origin (n=4) were downregulated. Proteins that have protein binding activity (7 were upregulated and 3 were downregulated) were the most common proteins in the category of molecular function. Seventy-five percent of the metal binding (n=3) and lipid biding proteins (n=3) were upregulated in RB. Proteins that are involved in metabolic process (7 were upregulated and 3 were downregulated), developmental process (4 were upregulated and 1 was downregulated) and transport activity (4 were upregulated and 4 were downregulated), cell signaling and transduction (2 were upregulated and 2 were downregulated), response to oxidative stress (1 upregulated and 2 downregulated) were the most common proteins in the category of biological process (Figure 2).

Correlation of the differentially expressed proteins with clinicopathological features:

We have attempted to correlate the differentially expressed proteins with clinical features of invasion, differentiation, and laterality. Tumors with invasion of optic nerve/choroid showed significantly higher expression compared to tumors without invasion of the following proteins;

CRABP2 (2.4±0.08; p=0.0000), ApoA1 (1.7±0.07; p=0.0000), Peroxiredoxin-6 (1.4±0.04; p=0.025), recoverin (1.68±0.03; p=0.0004). In contrast, CRABP1 was downregulated in tumors without invasion when compared to tumors with invasion (-1.45±0.07; p=0.0065) (Figure 3). We did not observe any correlation with other tumor parameters.

Quantitative Real time PCR (Q-RT-PCR)

Quantitative Real time RT-PCR was performed to correlate mRNA levels with the protein expression data obtained by proteomic approach. Among six proteins selected for RT-PCR, proteins such as Apolipoprotein A1 and Transferrin were selected for the reason that these proteins are also abundantly expressed in the serum and vitreous samples. When the fresh tumor samples are being harvested from the grossed eyeball, there are chances of tumor cells getting vitreous contamination. Before protein extraction, we have taken precautions to avoid vitreous contamination of the tumor samples as follows: the fresh tumor samples were washed with PBS (Phosphate buffered saline; PH: 7.4) by centrifugation at 20,000 X *g* for 3 times to get rid of the vitreous material. However, by 2DE we have identified abundant proteins like Apolipoprotein A1 (APOA1) and Transferrin (TF) as upregulated proteins in tumor samples. In this context, we wanted to clarify whether APOA1 and TF were truly expressed by the tumor cells or due to vitreous contamination. All the genes studied by real time PCR were normalized against the housekeeping gene *GAPDH*. The relative mRNA (mean±SD) fold changes in 10 RB tumors compared to 10 adult retinas is shown in Figure 4. RB tumors showed increased mRNA expression of ApoA1 (401±20; p<0.001), CRABP2 (111.2±22; p<0.001), CRYAA (2±1; p<0.001) and TF (12.14±3; p<0.001) compared to adult retinas. In contrast, FABP5 mRNA levels were not higher in RB compared to adult retina (p=1.000). RB tumors showed lower mRNA levels of CRABP1 (-35±4; p<0.001) compared to adult retinas. Hence, these observations confirmed that the mRNA levels are quite abundantly present in the tumor cells and are consistent with the proteomics results.

Immunohistochemical examination:

Immunohistochemistry was performed to demonstrate the localization, tissue distribution to provide a semi-quantitative assessment of 5 proteins each in ten formalin fixed paraffin embedded RB and two non-neoplastic retina sections by immunohistochemistry. The ten tumor samples used for the immunohistochemistry were listed in the Table 3 (Sample No. 1,3,4,7, 15, 18, 19, 23, 25, 27). This included 5 tumors with invasion and 5 without invasion. The necrotic areas in the section were excluded and only viable cell clones (morphological assessment by pathologists) were considered for the immunoanalysis.

Immunoreactivity in normal donor retinas:

Grade III staining for CRABP2 was observed in the photoreceptor layers and grade II staining was observed in the ganglion cells and inner nuclear layers of non-neoplastic retina (Figure 5A). Grade III staining of ApoA1 was observed in the photoreceptor cells, whereas grade I staining in the nuclear and ganglion layers of the non-neoplastic retina (Figure 5D). Grade II staining for CRYAA was seen in photoreceptor and ganglion layers. Grade I staining was observed in inner and outer nuclear layers of non-neoplastic retina (Figure 5G). Grade III staining for RCVRN was seen in photoreceptor layer. Grade I staining in the inner and outer nuclear layers and ganglion layers of non-neoplastic retina (Figure 5J). Grade III staining of TUBB1 was seen in ganglion cells (Figure 5M).

Immunoreactivity in tumors:

All the 10 tumors studied, were immunoreactive for all 5 proteins. RB tumor cells demonstrated cytoplasmic expression for CRABP2. Among 10 tumors, Grade III staining of CRABP2 was seen in 8 tumors (Figure 5B: grade III in tumor with invasion), grade II staining in 1 tumor (Figure 5C: grade II in tumor without invasion) and grade I staining in 1 tumor. RB tumor cells demonstrated cytoplasmic expression for ApoA1. Among 10 tumors, Grade III staining for ApoA1 was seen in 7 tumors (Figure 5E: grade III in tumor with invasion), grade II in 1 tumor and grade 1 in 2 tumors (Figure 5F: grade I in tumor without invasion). RB tumor cells

demonstrated cytoplasmic expression for CRYAA. Among 10 tumors, grade II staining was observed in 2 tumors (Figure 5H: grade II in tumor with invasion) and grade III in 5 tumors (Figure 5I: grade III in tumor without invasion), and grade I in 3 tumors. RB tumor cells demonstrated cytoplasmic expression for RCVRN. Among 10 tumors, Grade III staining for RCVRN was observed in 5 tumors (Figure 5K: grade III in tumor with invasion), grade II in 2 tumors and grade I in 3 tumors (Figure 5L: grade I in tumor without invasion). RB tumor cells demonstrated cytoplasmic expression for TUBB1. Among 10 tumors, Grade III staining for TUBB1 was observed in 5 tumors (Figure 5N: grade III in tumor with invasion), grade II in 3 tumors (Figure 5O: grade II in tumor without invasion) and grade I in 2 tumors.

Correlation between mRNA levels and protein expression by 2DE and IHC:

We have attempted to correlate the protein expression data (analyzed by 2DE and IHC) with mRNA levels of the target proteins identified. Using correlation coefficient analysis by F test, we found significant correlation between mRNA expression (fold change against normal retinas) and protein expression (fold change by 2DE and IHC grades) for CRYAA ($R=0.884$; $p=0.005$), ApoA1($R=0.867$; $p=0.008$). There was a significant correlation between mRNA levels and 2DE protein expression of TF ($R=0.763$; $p=0.01$).

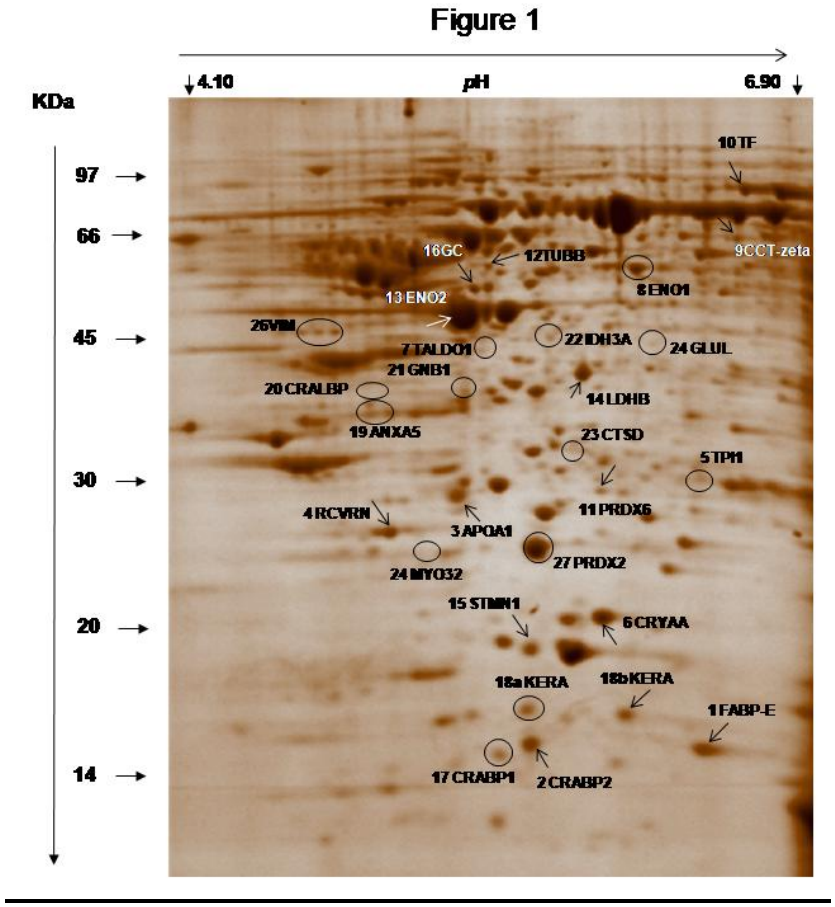

Figure 1: 2-DE (PH-4-7) gels used to identify the differentially expressed spots in RB compared to normal retinas. After 2DE, colloidal coomassie staining was performed and twenty seven differentially expressed protein spots were analyzed. The closed circles of the selected spots indicate downregulated proteins and the arrows of the selected spots indicate upregulated proteins compared to normal retina.

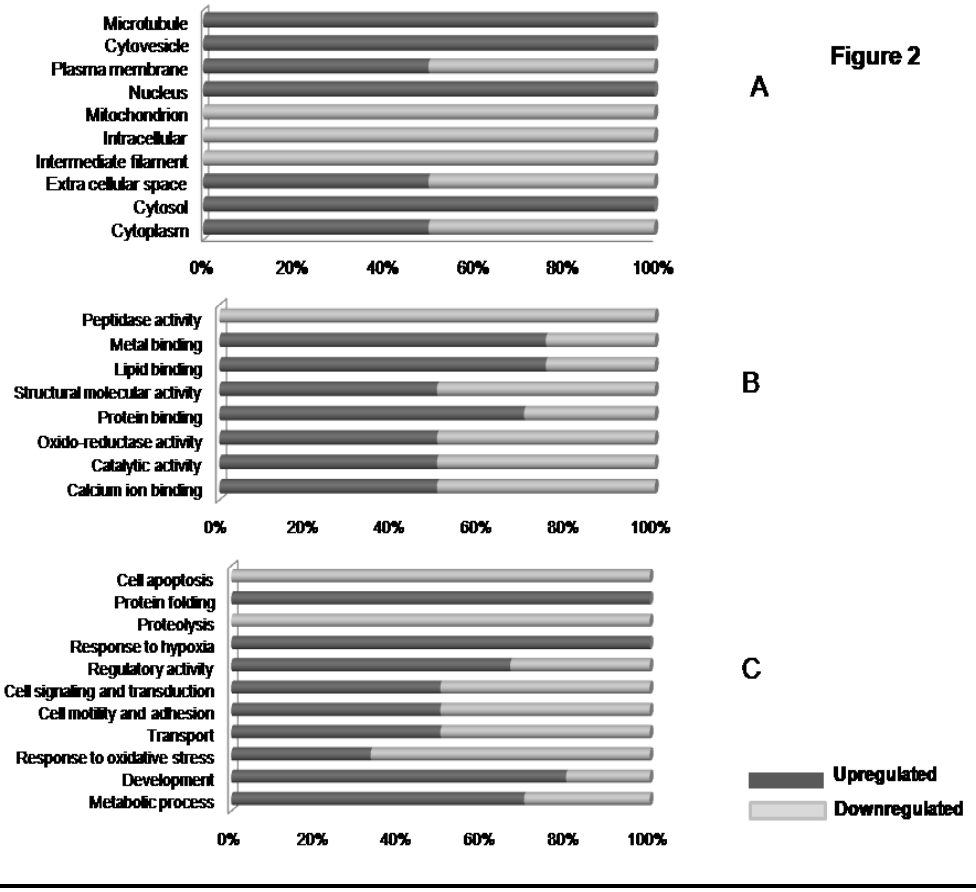

Figure 2: The identified differentially expressed proteins were classified to three ontologies of cellular component (A), molecular function (B), and biological process (C) with distribution of each sub-ontology. The graph represents the percentage of proteins either upregulated or downregulated belonging to the 3 different ontologies mentioned above.

Figure 3

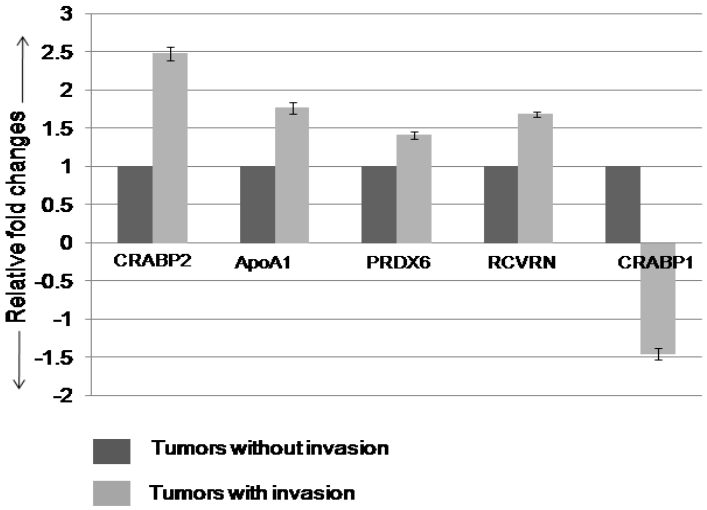

Figure 4

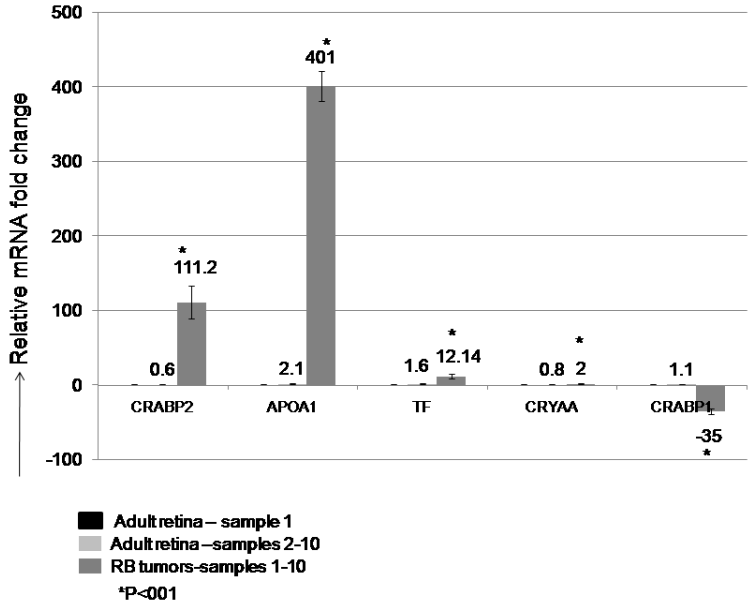

Figure 3: Fold changes of proteins analyzed by 2DE in invasive tumors (mean±SD fold values) compared to tumors without invasion.

Figure 4: Relative mRNA expression (mean±SD) of proteins in 10 RB tumors compared to 10 adult retinas.

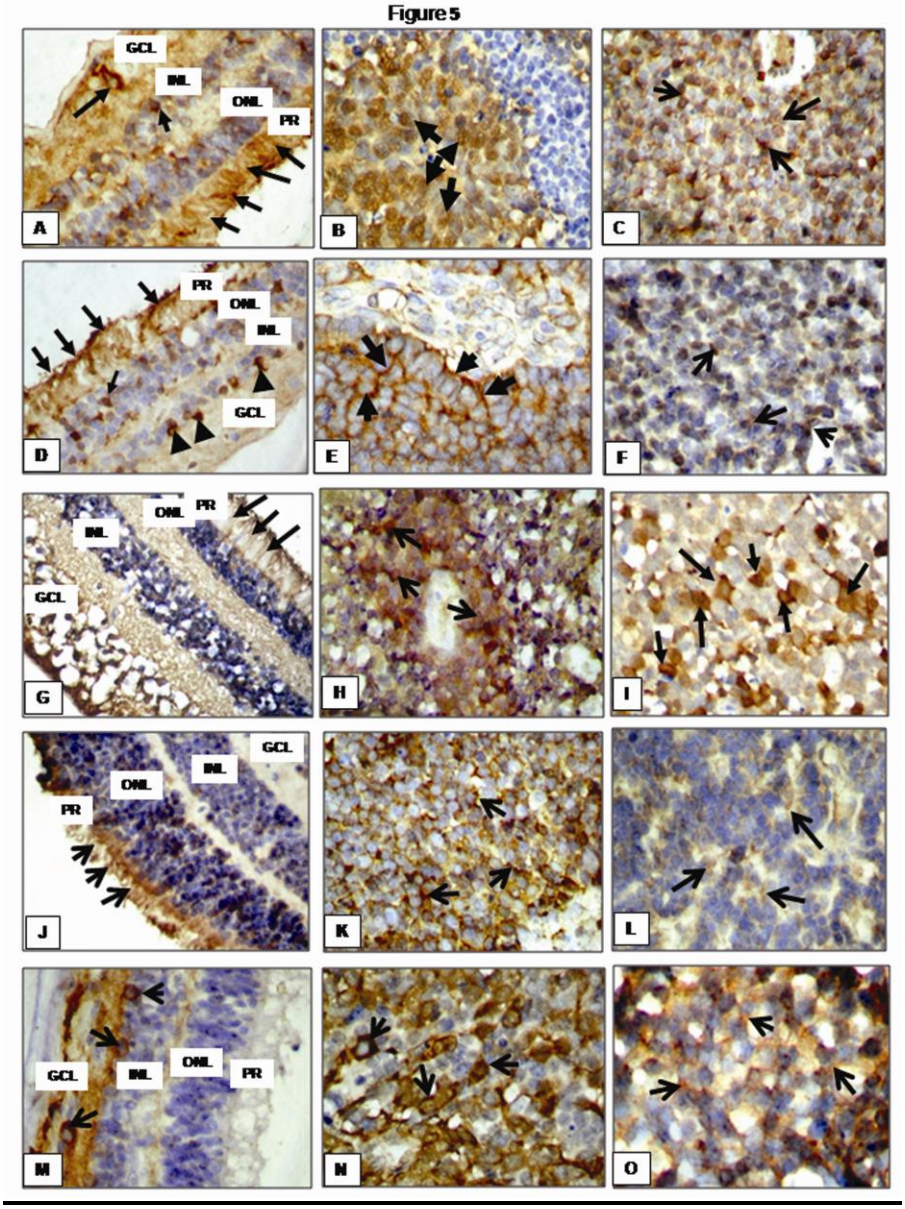

Figure 5A-O: "Immunohistochemistry for selected proteins in normal retina and RB"**(Arrows indicate positivity)**

GC: Ganglion cell layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; PR: Photoreceptor layer

A: Grade III cytoplasmic staining of CRABP2 (goat polyclonal N-14) in the photoreceptor cells and grade II staining in the nuclear layers and ganglion cells of non-neoplastic retina. DAB staining. Hematoxylin counter staining. Magnification-40X

B. Grade III cytoplasmic staining of CRABP2 in tumor with diffuse choroidal invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

C. Grade II cytoplasmic staining of CRABP2 in tumor without invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

D. Grade III cytoplasmic staining of ApoA1 (mouse monoclonal A5.4) in the photoreceptor cells and occasional grade I staining in nuclear layers and ganglion layers of normal retina. DAB staining. Hematoxylin counter staining. Magnification-40X

E. Grade III cytoplasmic staining of ApoA1 in tumor with diffuse choroidal invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

F. Grade I cytoplasmic staining of ApoA1 in tumor without invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

G. Grade II cytoplasmic staining of CRYAA (mouse monoclonal, B-2) in the photoreceptor cells and ganglion layer cells of non-neoplastic retina. Grade I staining was seen in the inner and outer nuclear layers. DAB staining. Hematoxylin counter staining. Magnification-40X

H. Grade II cytoplasmic staining of CRYAA in tumor with diffuse choroidal and post laminar optic nerve invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

I. Grade III cytoplasmic staining of CRYAA in tumor without invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

J. Grade III staining of RCVRN (rabbit polyclonal) in the photoreceptor layer and grade I staining in the inner and outer nuclear layers and ganglion cell layer. DAB staining. Hematoxylin counter staining. Magnification-40X

K. Grade III cytoplasmic staining of RCVRN in tumor with post laminar optic nerve and diffuse choroidal invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

L. Grade I cytoplasmic staining of RCVRN in tumor without invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

M. Grade III staining of TUBB1 (Rabbit monoclonal; TUJ1) in the ganglion cells. Inner and outer nuclear layers and photoreceptor layer were negatively stained. DAB staining. Hematoxylin counter staining. Magnification-40X

N. Grade III cytoplasmic staining of TUBB1 in tumor with diffuse choroidal invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

O. Grade II cytoplasmic staining of TUBB1 in tumor without invasion. DAB staining. Hematoxylin counter staining. Magnification-40X

Discussion:

Our study has demonstrated the differential proteomic profiling of RB compared to non-neoplastic retina. In this study, we have used 14 non-neoplastic retina samples as controls for proteomic analysis. In the present study, a pilot data on 2DE comparison between adult and age-matched retinas from healthy individuals has shown only few differentially expressed proteins (Table S1 and Figure S1) with 97.2% shared proteins (Figure S2). This suggests that adult retinas from healthy individuals were appropriate controls for comparative analysis in the present study.

From the 2-DE/MSMS analysis of 29 RB tumors, we have determined 27 differentially expressed protein spots when compared to non-neoplastic adult retina. All the reported proteins were consistently identified by 2DE-MSMS in all the RB specimens. The most common differentially identified proteins in the cellular component category are cytoplasmic proteins, and the others are components of cytosol, mitochondrion, plasma membrane and other cellular organs reflecting the profound changes of the cells when oncogenesis occurs. The most common differentially expressed proteins in the molecular function category have protein binding activity, catalytic activity, oxido-reductase activity and structural activity, reflecting the dynamic changes of some functional molecules in the RB tumors. The most common differentially expressed proteins in the biological process category are involved in metabolic process, transport activity, response to oxidative stress, cell signaling and transduction, cell adhesion and motility, and response to hypoxia reflecting the important role of these processes during RB oncogenic progression.

Further validation is necessary to elucidate the exact roles of these differentially expressed proteins identified in RB. Quantitative RT-PCR analysis showed that the relative mRNA of CRABP2 ($p<0.001$), ApoA1 ($p<0.001$), TF ($p<0.001$) and CRYAA ($p<0.001$) were significantly higher in RB compared to adult retinas. The mRNA levels of CRABP1 ($p<0.001$) were significantly lower in RB compared to adult retinas. Immunohistochemistry was also performed to demonstrate the localization (cytoplasmic/nuclear), tissue distribution and a semi-quantitative assessment of 5 proteins (CRABP2, APOA1, RCVRN, CRYAA and TUBB1) in paraffin sections. All the 5 proteins showed cytoplasmic positivity in the normal retina and tumor cells. Grade III immunostaining for CRABP2 was observed in 80% of the tumors, ApoA1 in 70% tumor sections and CRYAA, RCVRN & TUBB1 in 50% tumors. However,

immunohistochemistry has limitations that it provides only a semi-quantitative scoring. Correlation coefficient analysis further showed that the mRNA levels of CRYAA (R=0.884; p=0.005) and ApoA1(R=0.867; p=0.008) correlate with their 2DE and IHC protein expression status. Also, there was a significant correlation between mRNA levels and 2DE protein expression of TF (R=0.763; p=0.01).

By 2DE-MSMS approach, our study interestingly shows significantly increased expression of CRABP2 ($p<0.001$), APOA1 ($p<0.001$), PRDX6 ($p=0.025$), RCVRN ($p<0.001$) in tumors with invasion compared to tumors without invasion. In contrast, CRABP1 ($P=0.0065$) was significantly downregulated in tumors without invasion compared to tumors with invasion. Earlier, Chang et al (Chang et al., 2007) has demonstrated that upregulation of PRDX6 enhanced the *in vitro* proliferation and invasion of breast cancer cells. The enhancement was associated with increasing levels of the urokinase-type plasminogen activator receptor (uPAR), Ets-1 (E26 transformation-specific-1), matrix metalloproteinase (MMP)-9 and RhoC (ras homolog gene family, member C) expression. Peroxiredoxin functions in antioxidant defense mainly by facilitated repair of damaged cell membranes through reduction of peroxidised phospholipids. Prx-6 overexpression has been observed in malignant mesothelioma, (Kinnula et al., 2002) and human bladder cancer (Quan et al.,2006). However, the precise role of Prdx-6 is unknown in RB and hence further studies are needed. Understanding the precise functional role of these proteins in contributing tumor invasiveness will further help us to target those using novel drugs/inhibitors.

Interestingly we observed increased expression of Apo A1 in RB compared to normal retina. ApoA1 reverse transports the cholesterol from tissues to the liver. However, its precise function in cancer cells is yet to be elucidated. Earlier, Tachibana et al. has demonstrated the expression of ApoA1 as a potential marker for colonic adenocarcinoma aggressiveness (Tachibana et al., 2003). The other lipoproteins such as apo-E were frequently detected in ovarian serous carcinomas, furthermore it has been demonstrated that apo-E is required for cell proliferation and survival in ovarian cancer (Chen et al., 2005). Therefore further studies are necessary to explore the precise role ApoA1 in RB oncogenesis.

We observed increased expression of RCVRN in all RB tumors compared to normal retinas. Recoverin is a calcium-binding protein expressed in retinal photoreceptors. It appears to delay the termination of the phototransduction cascade by blocking the phosphorylation of photoexcited rhodopsin. RCVRN is expressed in more than 50% of several kinds of cancer cells (Maeda et al., 2001; Maeda et al., 2000). Earlier study has shown that recoverin may be potentially linked with caveolin-dependent regulations of tumor progression, metastasis, and drug resistance with G-protein-coupled receptor kinase (GRKs) (Miyagawa et al.,2003). Therefore the functional role of RCVRN in RB oncogenesis need to be further studied.

Our study has demonstrated increased expression of CRYAA in RB compared to non-neoplastic retina. Our results are consistent with a recent report where CRYAA expression was demonstrated in RB tumor tissues and correlated with apoptotic index (Kase et al., 2009). It appears that CRYAA may possibly play a role in preventing the apoptosis of tumor cells. Hence, it is very important to understand the molecular mechanisms involved in CRYAA mediated prevention of RB cell apoptosis. CRYAA could be an attractive therapeutic target in RB management.

Additionally, we observed higher expression of TF (Morrone et al., 1988; Vostrejs et al., 1988; Schaeffer et al., 1989) and T-complex protein zeta (CCTZ) (Yokota et al., 1999; Yokota et al., 2001) in a subset of RB which may possibly help in the RB cell proliferation. Further evaluation of the role of TF and CCTZ in RB would be helpful in informing whether or not this could be a target for development of novel RB therapy. CRABP expression has been demonstrated much earlier in RB primary tumors (Daxecker et al., 1987; Daxecker et al., 1987). Increased expression of CRABP2 in RB compared to non-neoplastic retina was observed in the current study. CRABP2 overexpression has been reported in a wide variety of human cancers like breast cancer cell lines and primary breast tumors (Bertucci et al., 2000), ovarian cancer (Hibbs et al.,2004), uterine leiomyoma (Tsibris et al., 2002), promyelocytic leukemia (Delva et al.,1993) and Wilms tumors (Li et al., 2002). In head and neck squamous cell carcinoma, CRABP2 antisense treatment led to a decrease in cell invasion (Vo and Crowe, 1998). Earlier study has identified in neuroblastoma a mechanism of feedback loop in which MycN amplification leads to CRABP2 up-regulation and subsequent Hu overexpression. The increased levels of Hu result in still higher levels of MycN protein, leading to an increase in cell migration, thereby exacerbating the effect

of MycN gene amplification (Gupta et al., 2006). Therefore, the earlier report on MycN amplification in RB (MacPherson et al., 2007) and CRABP2 upregulation in the present study suggests the possible existence of direct CRABP2 transcription regulation by MycN in RB. Unlike, CRABP2, we observed consistent down regulation of CRABP1 in all the RB compared to all the controls. Several reports have demonstrated the lack of CRABP1 in various cancers while retaining its expression in normal cells. Earlier study has proved that CRABP1 is silenced by promoter methylation mechanisms in esophageal squamous cell carcinoma (Tanaka et al., 2007) and based on several other reports it has been hypothesized that CRABP1 could be a tumor suppressor protein. However, the mechanism responsible for decreased expression of CRABP1 needs to be studied in RB. We also conclude that epigenetic mechanisms might possibly mediate the differential regulation of CRABP1 and CRABP2 to initiate or maintain the oncogenic events in RB. Further investigations are needed to reveal the roles of these differentially expressed proteins in RB as well as their use as potential biomarkers for prognostic monitoring or therapeutic use.

Table 1: Primers used for real time Quantitative PCR

Gene	Primer sequence
ApoA1	FP: 5′-AGCTTGCTGAAGGTGGAGGT-3′ RP: 5′ ATCGAGTGAAGGACCTGGC - 3′
CRYAA	FP: 5′- GAGATCCACGGAAAGCACAAC-3′ RP: 5′- GGTAGCGGCGGTGGAACT-3′
CRABP2	FP: 5′- AGCAGAAGCTCCTGAAGGGA-3′ RP: 5′- CCCATCGTTGGTCAGTTCTCT-3′
CRABP1	FP: 5′- TTCGACGAGCTGCTGAAGG-3′ RP: 5′- GCCGCTACGGCCACTTT-3′
TF	FP: 5′- CCCTTAACCAATACTTCGGCTAC-3′ FP: 5′- TTTGCCAAGTTCTCAAATATAGTCG-3′

Abbreviations: FP: Forward Primer; RP: Reverse Primer

Table 2: Differentially expressed proteins (upregulated and downregulated) in RB compared to normal adult retina.

Spot No.	Name	Swiss-prot ID	MW (kDa)/PI	MSMS score	Subcellular location	Function	Fold change compared to normal retina (Mean± SD)
1	Fatty acid binding protein epidermal (FABP-E)	Q01469	15.5/6.6	62	Cytoplasm	involved in fatty acid metabolism	1.55+0.03
2	Cellular Retinoic acid binding protein (CRABP-II)	P29373	15.7/5.43	25	Cytoplasm	Retinoic acid-mediated cell growth and differentiation	5.16+0.05
3	Apolipoprotein A1	P02647	28.6/5.27	238	Cytoplasm	Transportation of cholesterol	4.79+1.41
4	Recoverin	P35243	23/5.06	152	Cytoplasm	Involved in the inhibition of the phosphorylation of rhodopsin in a calcium-dependent manner.	2.70+0.29
5	Triose phosphate Isomerase	P60174	26.9/6.45	273	Cytoplasm	Involved in several biological processes like, fatty acid biosynthesis, glycolysis, gluconeogenesis, pentose pathway.	2.06+0.08
6	Alpha crystallin A	P02489	20.0/5.77	190	Cytoplasm	Chaperon protein	1.41+0.03
7	Transaldolase	P37837	37.6/6.36	-	Cytoplasm	Transaldolase is important for the balance of metabolites in the pentose-phosphate	1.34+0.01

						pathway.	
8	Alpha enolase	P06733	47.4/7.01	260	Cytoplasm	Binds to the c-myc promoter and acts as a transcriptional repressor. May be a tumor suppressor.	1.47+0.10
9	T-complex protein zeta (TCPZ)	P40227	58.3/6.25	25	Cytoplasm	Molecular chaperon	2.19+0.12
10	Transferrin precursor	P02787	79.2/6.81	304	Cytoplasm/ Extracellular	Transports Iron and may stimulate cell proliferation	3.00+0.04
11	Peroxiredoxin 6	P30041	25/6.02	164	Cytoplasm	Redox regulation of the cell. Protection against oxidative injury.	2.53+0.78
12	Beta tubulin	P07437	50/4.7	75	Cytoplasm	Structural supports and lines of transport within the cell, as well as serving a key role in mitosis	2.87+0.11
13	Gamma enolase	P09104	47.5/4.91	500	Cytoplasm	neurotrophic and neuroprotective properties	3.07+0.06
14	Lactate dehydrogenase B chain	P07195	36.7/5.7	75	Cytoplasm	Catalyses the interconversion of pyruvate and lactate	1.96+0.01
15	Stathmin (Oncoprotein 18)	P16949	17.1/5.77	66	Cytoplasm	Prevents assembly and promotes disassembly of microtubules	4.04+0.06
16	Vitamin-D binding protein precursor	P02774	54.5/5.4	38	Centrosome	Vitamin transporter activity	3.90+0.33

Down-regulated proteins							
17	Cellular retinoic acid binding protein I	P29762	15.5/5.3	195	Cytoplasm	retinoic acid-mediated cell proliferation and differentiation	-5.74+0.16
18a &b	Keratin Type II cytoskelatal	P48666	65.6/8.07	178	cytoskelatal	Intermediate filament involved in ectoderm development	-1.33+0.00
19	Annexin V	P08758	35.8/5.6	235	Membrane	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis.	-2.08+0.04
20	Cellular retinaldehyde binding protein	P12271	36.5/4.98	188	Cytoplasm	Carries 11-cis-retinol and 11-cis-retinaldehyde as endogenous ligands and may be a functional component of the visual cycle.	-3.27+0.11
21	Guanine nucleotide binding protein beta 1 subunit	P62873	38.1/5.6	179	Cytoplasm	Guanine nucleotide-binding proteins (G proteins) are involved as a modulator or transducer in various transmembrane signaling systems.	-2.28+0.17
22	Isocitrate Dehydrogenase NAD subunit alpha	P50213	40/6.47	42	mitochondria	Involved in oxidative decarboxylation (TCA cycle)	-1.86+0.06
23	Cathepsin D precursor	P07339	45.03/6.1	-	Lysosome	Acid protease active in intracellular protein breakdown	-2.03+0.15

24	ATP synthase D	O75947	18.4/5.2	69	Mitochondrial membrane	Produces ATP from ADP	-1.96+0.03
25	Glutamine synthetase	P15104	42.6/6.4	--	cytoplasm	Glutamine synthesis	-1.87+0.03
26	Vimentin	P08670	47.5/5.01	248	Intermediate filament	Structural constituent of cytoskeleton binds to proteins and involved in cell motion.	-2.93+0.04
27	Peroxiredoxin 2	P32119	22/5.6	342	Cytoplasm	Redox regulation of the cell. Protection against oxidative injury.	-2.06+0.04

Table 3: Classification of the identified known proteins according to the gene ontology convention

Category	Protein names
Cellular component	
Cytoplasm	CRYAA, CRABP2, CRABP1, PRDX6, RCVRN, ENO2, GLNS, PRDX2, ANX5, FABP5, ENO1, CRALBP, STMN1, VIM
Cytosol	TPI, CCTZ, LDHB, TALDO1
Extra cellular space	APOA1, TF, CPSD, GC
Intermediate filament	KRTA, VIM
Intracellular	GLNS
Mitochondrion	CPSD, IDH3A, GLNS, MY032
Nucleus	PRDX6, ENO1
Plasma membrane	ENO2, KRTA, ENO1, GNB1
Cytovesicle	APOA1, PRDX6,
Microtubule	TUBB1, STMN1
Molecular function	
Calcium ion binding	RCVRN, ANX5
Catalytic activity	TPI, GLNS, MY032, LDHB
Oxido-reductase activity	PRDX6, IDH3A, PRDX2, LDHB
Protein binding	APOA1, TUBB, CCTZ, KRTA, FABP5, ENO1, TALDO1, STMN1, VIM, GC
Structural molecular activity	CRYAA, TUBB1, KRTA, VIM
Lipid binding	APOA1, CRABP2, CRABP1, FABP5
Metal binding	TF, ENO2, IDH3A, ENO1
Peptidase activity	CPSD
Biological process	
Metabolic process	APOA1, TPI, ENO2, IDH3A, GLNS, FABP5, LDHB, ENO1, CRALBP, TALDO1
Development	TPI, KRTA, FABP5, ENO1, STMN1
Response to oxidative stress	PRDX6, KRTA, PRDX2
Transport	APOA1, CRABP2, CRABP1, TF, MY032, FABP5, CRALBP, GC
Cell motility and adhesion	TUBB1, VIM

Cell signaling and transduction	APOA1, ANX5, GNB1, STMN1
Regulatory activity	RCVRN, KRTA, ENO1
Response to hypoxia	TF
Proteolysis	CPSD
Protein folding	CCTZ
Cell apoptosis	PRDX2, ANX5

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RESEARCH ARTICLE

Comparative proteomic analysis of differentially expressed proteins in primary retinoblastoma tumors

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CONCLUSIONS FROM PART-I THESIS

The frist part of the thesis revealed the mechanisms adapted by the RB tumor cells that play a major role in inhibiting the apoptotic processes. We have identified the presence of cancer stem cells in retinoblastoma. These cancer stem cells have the characteristics of impaired apoptotic processes and higher self renewal capacity. It is well known that the tumors exhibit multiple aberrations in the signaling pathways including p53 pathway which helps them to survive against chemotherapy or radiation therapy mediated stress. P53 pathway is proven to be inactivated in retinoblastoma through MDMX mediated suppression by earlier studies. However, we have demonstrated that a subset of RB tumors express truncated isoforms of p63 and p73 which may also suppress p53 mediated apoptosis in tumor cells. Several other proteins identified by proteomics such as Peroxiredoxin 6, alpha crystallin A can make the tumor cells escape from apoptosis and lead to uncontrolled cell proliferation. The other group of proteins which are involved in increased cell proliferation such as recoverin, transferrin and T-complex protein zeta were highly expressed in RB cells than the normal retinal cells. Overall, this study has provided interesting and novel information on the mechanisms adapted by RB tumor cells to escape from apoptosis. Hence, this study gives a clue on the disease mechanism and further these proteins have to be tested for its use as target molecules or prognostic biomarkers in the clinical settings.

SPECIFIC CONTRIBUTIONS AND FUTURE SCOPE OF WORK:

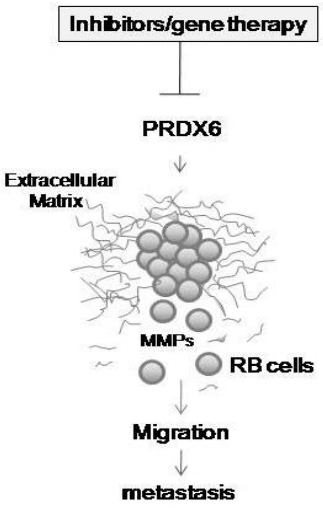
This study has significantly contributed in providing the vast information about RB disease mechanism and the pathways accustomed by the tumors that help them escape from chemotherapy or radiotherapy induced apoptosis. The concept of cancer stem cell theory, role of p53 family proteins in understanding the RB tumor biology and the differential proteins in RB identified by proteomics approach that contribute significantly to RB progression and invasion is the major accomplishment of this first part of the thesis. One of the reasons that could account for the tumor relapse may be that RB tumor harbors cancer stem cells. Hence, incorporation of specific drugs for cancer stem cells along with conventional chemotherapy would be beneficial in effective RB treatment. In another aspect pertaining to the use of MDMX inhibitors for RB treatment, the status of p63 and p73 expression also should be taken into account. This is because; truncated forms of p63 and p73 may also play a role in suppression of apoptosis mediated by active p53 protein during DNA damage. However, further studies on understanding the role of p63 and p73 in retinoblastoma should be carried out in *in vivo* RB animal models to confirm the above hypothesis.

Proteomic study has contributed significantly in understanding the biology of RB tumors by revealing the altered protein expressions. Several altered proteins belong to different categories such as apoptosis, cell proliferation, signal transduction, metabolic and active transport processes. Upon functional characterization in future, these altered proteins could be used for targeted therapy in the clinical management of retinoblastoma. Some of the important molecules identified by proteomic approach that could be promising in the better clinical management of retinoblastoma in future are discussed below.

Peroxiredoxin 6:

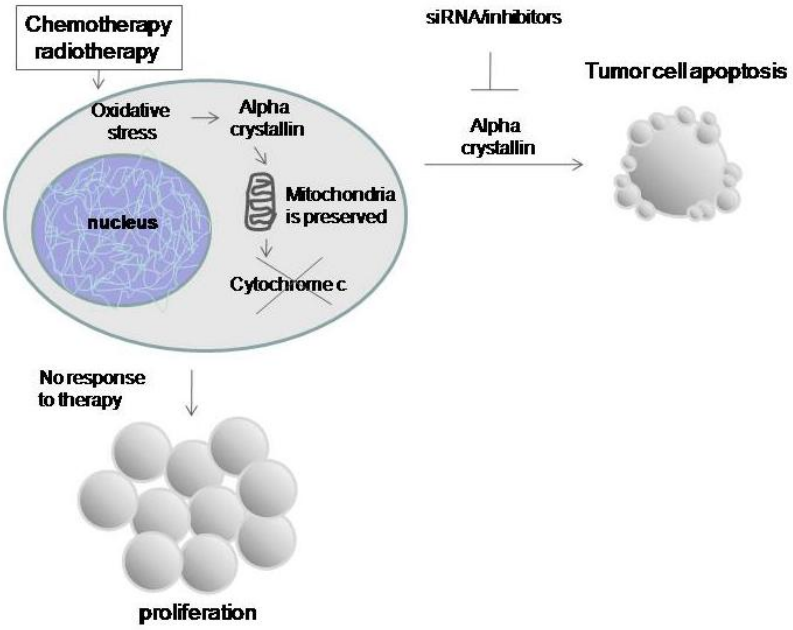
Peroxiredoxins are involved in antioxidant defense mechanisms mainly by facilitated repair of damaged cell membranes. Overexpression of Peroxiredoxin 6 leads to invasive phenotype in breast cancer cells atleast in part through regulation of the levels of uPAR, Ets-1, MMP-9, RhoC and TIMP-2 expression. Peroxiredoxin 6 knockdown breast cancer cells grew more slowly and had fewer pulmonary metastases. Higher expression of MMP-2 and 9 were demonstrated earlier in RB tumors by Adithi et al. This could relate to the increased expression of PRDX6, a possible

upstream molecule that may induce higher MMPs expression in RB. Upon proving this fact in RB, PRDX6 can be considered instead of MMPs for targeted therapy.



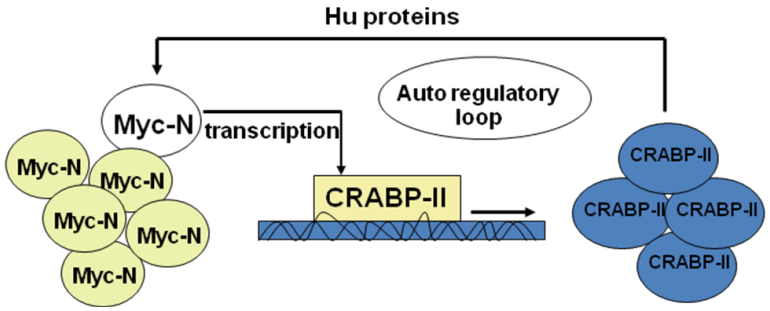
Alpha crystallin A:

The role of alpha crystallin A is not understood well in cancer biology. Increased expression of alpha crystallin A and its correlation with lower apoptotic index in RB cells were demonstrated earlier by Kase et al. Alpha crystallin expression is induced during oxidative stress in retinal photoreceptors. One of the major roles of alpha crystallin is to preserve the integrity of mitochondria and restrict the release of cytochrome c, subsequently resulting in tumor growth through escape from apoptosis.



Cellular retinoic acid binding protein 2:

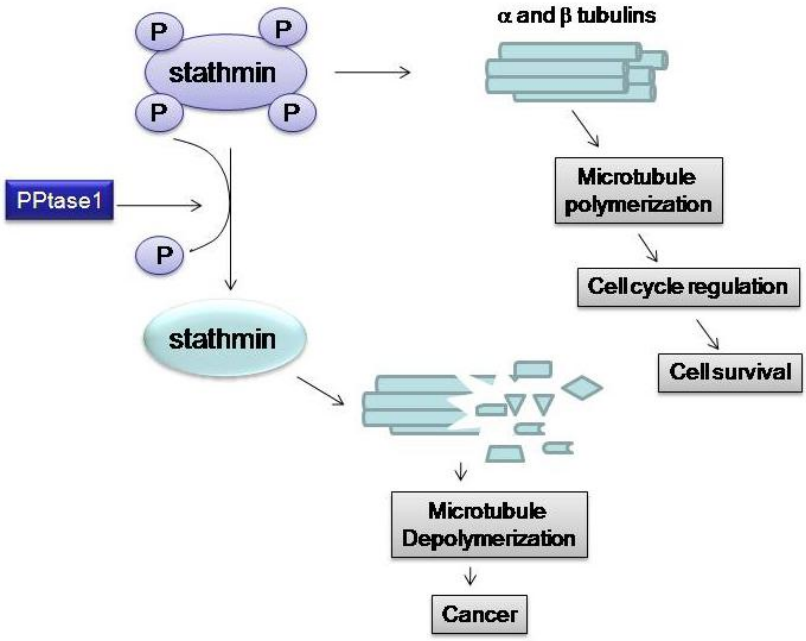
CRABP expression has been demonstrated much earlier in RB primary tumors. Increased expression of CRABP2 in RB compared to non-neoplastic retina was observed in the current study. A feedback loop mechanism by which MycN amplification leads to CRABP2 up-regulation and subsequent Hu overexpression was demonstrated in neuroblastomas. The increased levels of Hu result in still higher levels of MycN protein, leading to an increase in cell migration, thereby exacerbating the effect of MycN gene amplification. Earlier, amplification of MycN gene was reported in retinoblastoma. Therefore, the earlier report on MycN amplification in RB [83] and CRABP2 upregulation in the present study suggests the possible existence of direct CRABP2 transcription regulation by MycN in RB.



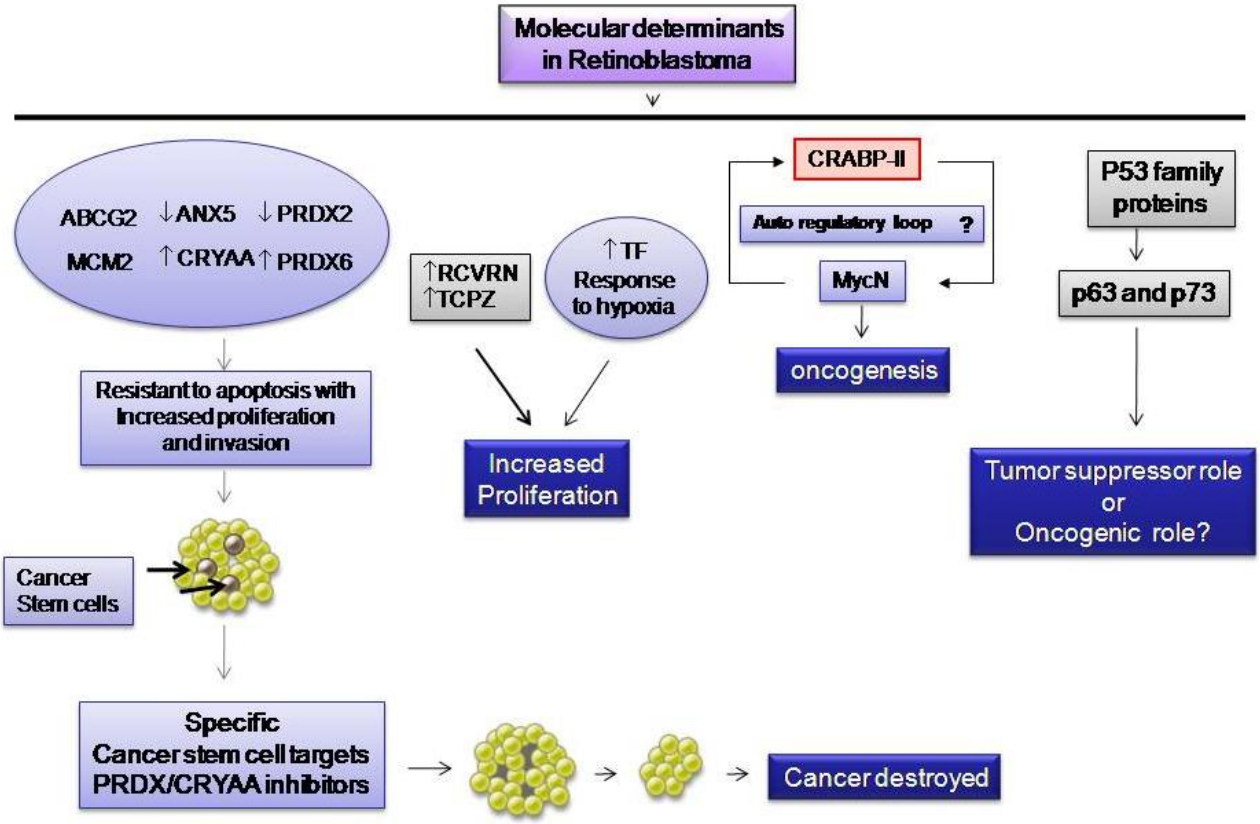
Role of Stathmin in RB:

Stathmin is a highly conserved cytosolic phosphoprotein that destabilizes microtubules. Stathmin, which has been proposed as a relay protein integrating diverse cell signalling pathways, acts in vitro as a tubulin-sequestering protein, and its activity is dramatically reduced by phosphorylation. Interestingly, stathmin expression and phosphorylation are regulated during the control of cell growth and differentiation, and there is much evidence suggesting that in vivo stathmin plays a role in the control of microtubule dynamics during mitosis. Stathmin may thus be considered as one of the key regulators of cell division. Stathmin is highly expressed in RB. Thus it is very interesting to speculate that this protein could play a role in uncontrolled proliferation and tumor cell invasion of RB. However, future studies are warranted to investigate the phosphorylation status of Stathmin which will provide the precise role in RB progression.

Regulation of cell cycle by stathmin



An illustration showing the possible role of the proteins studied in RB progression.



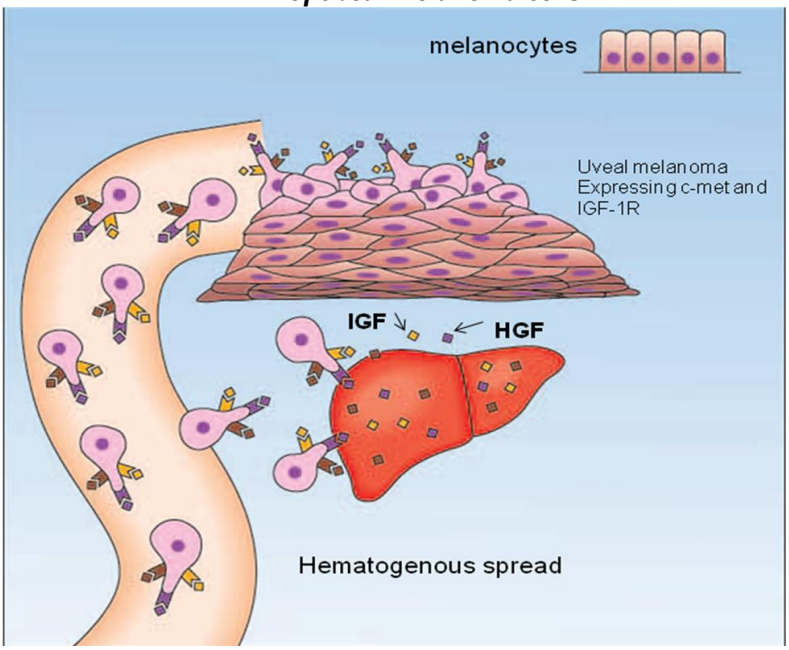
PART 2

TO UNDERSTAND THE MOLECULAR MECHANISMS INVOLVED IN UVEAL MELANOMA PROGRESSION

ABSTRACT OF THESIS – PART II

Uveal melanoma is the most common primary intraocular malignant tumor in adults, with an incidence of roughly seven cases per million. The principal target organ for metastasis of uveal melanoma tumor cells is the liver, which is involved in 71.4% to 87% of patients with metastatic disease (Kath et al., 1993). The liver is the exclusive site of systemic metastasis in ~40% of the patients and is often the first metastatic site in patients (Bedikian, 2006). Unfortunately, when liver metastases are diagnosed, treatment options are limited and life expectancy is poor. Cells that escape the primary tumor do so by hematogenous dissemination, due to the lack of lymphatics in the eye itself (Woll et al., 1999). The predominance of liver metastasis cannot be solely explained by circulation because the lungs are the first set of capillary beds that these cells would encounter. Therefore, uveal melanoma offers a unique setting in which to study the hematogenous dissemination of cells and the subsequent homing of these cells, or their preferential survival, in the liver of patients. Understanding of the mechanisms underlying this phenomenon could have vast implications for the large number of patients who are at a high risk for the development of liver metastasis from other cancers, such as colorectal or breast carcinomas (Nicolini et al., 2006; Widel MS and Widel M, 2006). Substantial questions remain regarding the specific liver metastasis in uveal melanoma patients. Is it a reflection of homing of cells to the liver or simply preferential growth and survival of these uveal melanoma cells in that microenvironment? As in many things, it may be a mixture of the two.

Illustration showing the ligand-receptor mediated liver metastasis of uveal melanoma cells



Picture was taken from "Bakalian S et al., Clin Cancer Res. 2008 ;14:951-6."

Role of HGF in uveal melanoma:

One of the molecules that have long been suspected of playing a role in specific growth of cells in the liver is hepatocyte growth factor (HGF), also known as scatter factor, and its corresponding receptor, c-Met. Studies have shown that increased levels of c-Met expression in the primary tumors of patients significantly increased the risk of those patients to develop subsequent liver metastasis. Uveal melanoma cells have also been shown to become highly motile and invasive when HGF was used as a chemoattractant. Our study shows significantly higher expression of c-met in uveal melanoma tumors from patients with liver metastasis than the tumors without liver metastasis. On activation of c-met by HGF, multiple genes are upregulated which can increase cellular proliferation, cell cycle progression, protection of apoptosis, increased cell motility, and invasive ability.

Role of IGF-1R in Uveal melanoma:

The expression of HGF alone, however, does not explain the predominance of liver-specific metastasis. An earlier study by Economou and co-workers described the interrelation of insulin like growth factor I receptor (IGF-IR) and c-Met from uveal melanoma samples. We have also

demonstrated the higher expression of IGF-1R in tumors with liver metastasis compared to tumors without liver metastasis. Similar to HGF, IGF-I is mainly produced by the liver and may help explain the preferential homing or growth of cells to the liver in uveal melanoma patients. IGF-I binds to IGF-IR, a heterotetrameric plasma membrane glycoprotein. Among the downstream pathways that can be stimulated by insulin receptor is the phosphorylation of Akt via phosphatidylinositol 3-kinase.

Role of Cripto in uveal melanoma, a potential target molecule in the treatment of uveal melanoma:

The role of Cripto-1 in tumorigenesis is not clear. However, Cripto-1 activities appear to be essential for tumor growth and/ or maintenance. Cripto activates ras/raf/extracellular signal-regulated kinase/ mitogen-activated protein kinase (MAPK) and cell survival phosphatidylinositol 3'-kinase/Akt pathways for cell survival and proliferation. Our study shows for the first time that Cripto is highly expressed in uveal melanoma primary tumors and metastatic and non-metastatic uveal melanoma cell lines. Interestingly Cripto is absent in normal uveal melanocytes. Hence, it is a potential candidate for target therapy in uveal melanoma treatment. Further functional studies on understanding the precise role of Cripto are advised before determining its use for treatment.

I. REVIEW OF LITERATURE

1. CLINICAL ASPECTS OF UVEAL MELANOMA

1.1 Epidemiology

Uveal melanoma is the most common primary intraocular tumor in adults in the Western world and represents 3% of all melanoma (Egan et al., 1988; Singh et al., 2003). The age adjusted incidence of uveal melanoma varies from 5-8 patients per 1 million per year and has remained stable over the past 25 years (Singh et al., 2003).Uveal melanoma spread almost without any exception hematogenously, predominantly to the liver. The tumor is of an aggressive type: almost 50% of the patients with uveal melanoma will ultimately die from metastatic disease (Jensen et al., 1982; McLean et al., 1993), while no effective treatment for metastases is yet available. Once the primary tumor has been diagnosed in the patient, the median survival time is 6.5 years (McLean et al., 1993). Life expectancy after metastases have been detected is only 2-9 months (Seddon et al., 1983; Albert et al., 1992; Kath et al., 1993).

Most uveal melanomas are located in the ciliary body and/or the choroid (23% and 72% respectively), whereas iris melanomas account for only a small percentage of these tumors, i.e. 5% (Yanoff and Fine, 1989). At the time of diagnosis, less than 2% of the patients have clinically detectable metastases, but many patients may have already subclinical metastases (Donoso et al., 1985). It may be that treatment at this stage with e.g. immunotherapy may be the only way to improve survival in these patients, but would only be used in very high risk individuals. This highlights the critical need to identify prognostic markers indicative of uveal melanoma invasive and metastatic potential.

1.2 Diagnosis and treatment

Like cutaneous melanoma, uveal melanoma is originally derived, from melanocytes of the neurectoderm (choroid and ciliary body, Figure 1). Two main cell types have been recognized, i.e. the spindle cell type and the epithelioid cell type. Since both cell types are present in many uveal melanomas, additionally, the mixed cell type classification has been introduced (Zimmerman 1986).

The basis of treatment is a proper diagnosis. Prior to the development of ultrasonography, differentiating between a large nevus and small melanoma was rather difficult. This changed in the nineteen-fifties, when the 32P isotope-uptake test was developed to differentiate benign from malignant lesions of the choroid and ciliary body. 32P positivity was considered to be an indicator of nuclear activity and, therefore, malignancy. The 32P test was used for more than a decade as a diagnostic method for uveal melanoma, but is no longer performed today (Thomas et al., 1952; Shields et al., 1978; Lommatzsch et al., 1984; Overkleeft et al., 2003). Nowadays, better and less invasive diagnostic methods are used, mainly ultrasonography. After the primary tumor has been diagnosed the patient will undergo screening to detect potential local spread or distant metastases. The protocol includes physical examination, liver ultrasonography and function tests, and a chest X-ray.

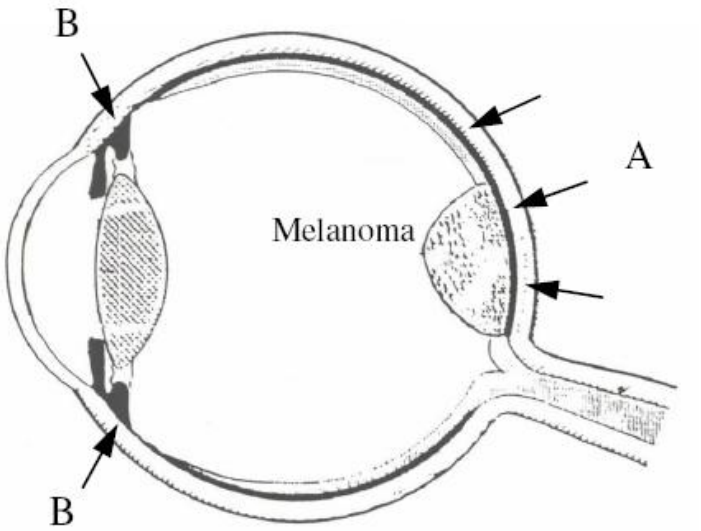


Figure 1: SCHEMATIC VIEW OF AN EYE (A). Choroid and (B) Ciliary body

In order to reduce the risk to develop metastases it is essential to treat the primary tumor as effectively as possible. For large tumors (Largest Tumor Diameter (LTD) ≤15 mm and/or thickness ≥ 5 mm), enucleation remains the first choice of treatment, as well as in case of extensive involvement of the optic disc or extensive extrascleral extension (Shields et al., 1991; Seregard and Kock 1995). For small (LTD <10 mm and thickness <3 mm) and medium-sized (LTD between 10-15 mm and/or thickness between 3-5 mm) tumors, different treatment

strategies are used (Shields et al., 1991). These include local resection of the tumor (Damato 1993), plaque radiotherapy (Lommatzsch and Kirsch 1988; Shields and Shields., 1993; Lommatzsch et al., 2000), stereotactic radiotherapy (Zehetmayer et al., 2000) and thermotherapy (Oosterhuis et al., 1995). A new trend in ocular oncology is combining different treatment modalities such as transpupillary thermotherapy (TTT) combined with plaque radiotherapy to obtain better tumor control (Journee-de Korver et al., 1997; Seregard and Landau 2001).

1.3 Predisposing factors

Several parameters that predispose to uveal melanoma have been described, including phenotypic risk factors. Race is one of the most significant host factors, as uveal melanoma is about 150 times more common in Caucasian than in African individuals (Egan et al., 1988; Singh et al., 2003) and is less common in Asians (Kuo et al., 1982; Biswas et al., 2002). People with light-colored eyes appear to be at a higher risk than dark-eyed people (Gallagher et al., 1985; Tucker et al., 1985) and subjects with brown eyes seems to be more protected against sunlight compared to those with blue eyes (Tucker et al., 1985; Holly et al., 1990). Although there is ample evidence of sunlight exposure as a risk factor for cutaneous melanoma (Elwood and Jopson 1997; Gilchrest et al., 1999), the evidence in uveal melanoma is contradictory (Dolin et al., 1994; Egan et al., 1988). In a report by Li et al. (2000), the distribution of tumor origin correlated with the dose distribution of solar radiation on the retinal hemisphere, but these findings contradicted another report, in which the distribution of tumors in the choroid was found to be randomly distributed (Schwartz et al., 1997). Although there is biologic evidence that ultraviolet radiation induces DNA damage in cutaneous melanoma (Gilchrest et al., 1999), still, no conclusive judgement about the role of sunlight in uveal melanoma can be made (reviewed by Singh 2004). Uveal melanoma usually occurs sporadically in the absence of obvious genetic predisposing factors. However, in some patients, there might be a genetic predisposition. Familial uveal melanoma most often (63) affects first-degree relatives, but rarely affects more than two persons in one family, and therefore it may be associated with a generalized inherited predisposition to cancer (Singh et al., 1996). Furthermore, there are a few clinical conditions that can predispose to or are associated with uveal melanoma, including ocular melanocytosis, neurofibromatosis type I, and familial atypical multiple mole melanoma syndrome (FAMMM). Ocular melanocytosis is a congenital condition characterized by hyperpigmentation of the uveal

tract, sclera and epissclera. When hyperpigmentation of periocular skin takes place along the trigeminal nerve distribution, it is known as oculodermal melanocytosis (Nevus of Ota) (Gonder et al., 1982). Approximately 14% of uveal melanoma patients have oculodermal melanocytosis (ODM), indicating that ODM is about 35 times more common in the uveal melanoma population (Gonder et al., 1982). The lifetime risk to develop uveal melanoma is estimated to be one of about 400 ODM patients compared to one out of 13,000 in the general population (Singh et al., 1998). Neurofibromatosis type 1 has also been linked to uveal melanoma. This combination of tumors serves to emphasize the common neuroectodermal origin of tumors in this autosomal dominant condition (Specht and Smith 1988; Antle et al., 1990). In the FAMMM or Dysplastic Nevus Syndrome, increased numbers of cutaneous nevi, cutaneous melanoma, as well as conjunctival nevi and uveal melanoma have been reported. Patients with uveal melanoma are more at risk to develop dysplastic nevi and cutaneous melanoma (Bataille et al., 1995; Van Hees et al., 1998). The p16 gene (CDKN2A) which is localized on chromosome 9p21, is inactivated in a significant number of sporadic cancers, including cutaneous melanoma. It has been demonstrated that approximately 50% of all FAMMM families show linkage to this region (Goldstein et al., 1994; Gruis et al., 1995). Whereas *p16ink4a* (*CDKN2A*) is the main target for inactivation in cutaneous melanoma, mutation screening and deletion mapping in uveal melanoma did not reveal the same results (Merbs et al., 1999). However, Van der Velden et al. (2001) demonstrated that hypermethylation of *p16ink4a* is the cause of inactivation of this gene in uveal melanoma, which interestingly was found more frequently in tumors from patients who developed metastatic disease. Germline *BRCA2* gene mutations have also been described to have an association with ocular melanoma (Easton et al., 1997; Sinilnikova et al., 1999): they occur in 3% of patients younger than 50 years (Scott et al., 2002).

1.4 Prognostic indicators, clinical, immunological and histopathological parameters

A wide variety of clinical and histopathological parameters are related to survival, however, very few are specific. Well known established factors associated with bad prognosis are old age, high tumor thickness, large tumor diameter and tumor localization in ciliary body (Mooy and De Jong 1996), the presence of epithelioid cells (Seddon et al., 1983; Coleman et al., 1993), a high mitotic activity, and vascular network formation (Folberg et al., 1993; Maniotis et al., 1999). In addition, male gender and the presence of tumor-infiltrating lymphocytes are also considered to be

significant factors. Other immunological parameters are also of prognostic relevance. The number of CD3 □ □ (T-lymphocytes) and CD4 lymphocytes are positively correlated with HLA expression (De Waard-Siebinga et al., 1996). HLA phenotype alterations is a common feature in various tumors, including cutaneous and uveal melanoma (Krishnakumar et al., 2003).

In uveal melanoma, lack of expression of HLA-A as well as HLA-B antigens was found to be correlated with better patient survival (Blom et al., 1997). Most tumors lack HLA-C and HLA-G as well, but the biological importance of this is not yet clear (Hurks et al., 2001). These findings may suggest that shedding of uveal melanoma micrometastases with downregulated HLA class I antigen expression into the systemic circulation may facilitate their removal and prevent the development of metastases. It also may suggest that Natural Killer-cell-based lysis may be more effective in destroying blood-borne uveal melanoma cells than T-cell-mediated cytotoxicity (Blom et al., 1997; Jager et al., 2002; Anastassiou et al., 2003). The expression of Epidermal Growth Factor Receptor (EGFR) has been found to be correlated with death due to metastatic disease in uveal melanoma (Hurks et al., 2000), with another report concluding that tumor-associated macrophages can express this receptor (Scholes et al., 2001). However, Ma and Niederkorn (1998) reported specific EGFR expression on uveal melanoma cell lines, indicating that these findings may not be exclusive.

Differences in invasive behaviour between uveal melanomas could be one of the most important factors for their differences in clinical outcome. Molecular markers like integrins and the involvement of cadherin-catenin adhesion complexes in the invasive potential of uveal melanoma cells has been the subject of several studies and have provided more insight in these complicated processes (Elshaw et al., 2001; Woodward et al., 2002; Seftor et al., 2002; Conway et al., 2003). Cytogenetic and genetic markers as prognostic indicators will be discussed in the next paragraph.

2. CHROMOSOMAL ABERRATIONS

Cytogenetic and comparative genomic hybridization analyses have revealed the involvement of several chromosomal aberrations in uveal melanoma. Sisley et al. (1997) identified a correlation with abnormalities in chromosomes 3, 6 and 8 and prognostic outcome. Monosomy 3 in uveal melanoma has been proven to be an important determinant to predict metastatic potential (Prescher et al., 1996) and gain of chromosomal arm 8q, which includes the C-Myc oncogene locus, has been found in 65% of uveal melanomas (Speicher et al., 1994; Ghazvini et al., 1996). C-Myc proto-oncogene, located at chromosome 8q24, is commonly expressed in uveal melanoma as demonstrated by immunohistochemistry and flow cytometry (Chana et al., 1999; Royds et al., 1992). In addition, fluorescent in situ hybridization analysis showed an association of C-Myc amplification with increased tumor size (Parrella et al., 2001).

The subsequent paragraphs describe the potential involvement of genes and genetic pathways in uveal melanoma.

3. AKT and MAPK pathways

Another potential role in uveal melanogenesis involved the *PTEN* (Phosphatase and tensin homologue) gene. The *PTEN* gene encodes a dual specific phosphatase, which plays a major role in the inhibition of cell migration and the formation of focal adhesions (Tamura et al., 1998). *PTEN* counteracts phosphatidylinositol 3-OH kinase (PI3-kinases) functions, which are associated with cell growth and survival (Di Cristofano and Pandolfi 2000; Vazquez and Sellers 2000). Somatic mutations of the *PTEN* gene have been detected in about 40% of cutaneous melanoma and loss of this gene contributes to tumor development (Guldberg et al., 1997, Stahl et al., 2003). Furthermore, it has been recently found that there is a potential cooperation between the tumor suppressor gene *PTEN* and two other components of the RAS signaling network, NRAS and BRAF, suggesting that the MAPK (RAS) and the AKT pathways (PTEN) are frequently activated in parallel in melanogenesis (Tsao et al., 2004). In this thesis, we have studied the expression of CRIPTO which is a non-EGFR receptor that activates the MAPK pathway in uveal melanoma. This second part of the thesis will discuss about CRIPTO correlation with clinicopathological features and also its expression status in primary uveal melanocytes and malignant uveal melanoma cell lines.

4. Rb and p53 pathways

Mutational deregulation of the cell-cycle is a hallmark of tumorigenesis (Hanahan and Weinberg 2000). The protein product of the Retinoblastoma gene (Rb) plays a central role as inhibitor of cellular proliferation (Bartek et al., 1997). The Rb gene is frequently mutated in certain cancers such as retinoblastoma, osteosarcoma, and small-cell lung cancer (Friend et al., 1986; Harbour et al., 1988), but no Rb mutations have been reported in uveal melanoma. This stimulated research into alterations in components of the interconnecting signaling pathways of the Rb gene and the p53 transcription factor (reviewed in Sherr and McCormick 2002). P53 is the most commonly mutated tumor suppressor in human cancer (Harris and Hollstein 1993), but no evidence exists for p53 mutations in either cutaneous or uveal melanoma (Chana et al., 1999; Kishore et al., 1996; Florenes et al., 1994). However, there is now evidence that functional abnormalities in both the Rb and p53 pathways are playing a role (Brantley and Harbour 2000a). HDM2 (human homologue of murine double minute MDM2)) is an inhibitor of p53 that targets p53 for degradation (Haupt et al., 1997). Although high expression of HDM2 in uveal melanoma cells has been related to poor clinical outcome (Coupland et al., 2000) and blocking experiments of HDM2 have led to rapid onset of apoptosis (Harbour et al., 2002), the mechanism for this increased expression remains unclear. Increased expression of Cyclin D1, which activates the Rb kinase CDK4, has been associated with poor prognosis as well (Brantley and Harbour 2000a; Coupland et al., 2000), and was shown to block the active repressor function of Rb by maintaining it in a phosphorylated state (Brantley and Harbour 2000b). Ras-dependent signaling involving Raf, MEK and ERK activates transcription factors that upregulate cyclin D1 transcription (reviewed in Sherr and McCormick 2002).

5. AIMS AND SCOPE OF PART-II THESIS

The prognosis of patients with uveal melanoma is poor. Therefore, the importance to identify indicators of metastatic potential of uveal melanomas is essential. First of all, biological mechanisms that determine the level of aggressiveness of uveal melanoma are still unclear and secondly development of metastases is a complex mechanism in which many interrelated, but yet to identify, steps are involved. The main outline of the second part of my thesis is to define prognostic markers and/or profiles to categorize uveal melanoma in groups in order to identify the biological mechanism of metastatic disease of uveal melanomas. In my second part of thesis, I have studied the expression of growth factors, their receptors and correlated with clinicopathological features. This is important with respect to Asian Indian Population as there are reports seldom available on the study of molecular pathways and their association with uveal melanoma clinical and pathological features.

6. DECRIPTION CONTENTS PER CHAPTER

In chapter 1, we have investigated the expression status of Epidermal Growth Factor receptor (EGFR), Hepatocyte growth factor (HGF), and its receptor c-met using immunohistochemistry technique on archival uveal melanoma paraffin sections. We also studied the expression of a cytoskeletal molecule, Ezrin and correlated with clinicopathological features of uveal melanoma primary tumors from Asian Indian population. **In chapter 2**, we studied the expression of Insulin like growth factor (IGFR), c-Fos and c-Jun (transcription factors) in archival uveal melanoma paraffin sections by immunohistochemistry and correlated with clinicopathological features of the tumors. **In chapter 3**, we studied the expression of CRIPTO in archival uveal melanoma paraffin sections by immunohistochemistry and correlated with clinicopathological features of the tumors

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CHAPTER 1

EXPRESSION OF EGFR, EZRIN, HGF AND C-MET IN PRIMARY UVEAL MELANOMA TUMORS

INTRODUCTION:

The importance of cell surface molecules in determining the organ-specific metastasis of tumors has been well documented since the seminal study of Fidler (Fidler 1973). Earlier experiments in different animal models suggest that paracrine stimulation of tumor cells by organ-derived growth factors contribute to organ-specific metastasis. TGF-α (transforming growth factor) and hepatocyte growth factor (HGF) are produced in the liver and stimulate the proliferation of various tumor cells by interacting with epidermal growth factor receptor (EGFR), which is expressed on a wide variety of normal and malignant cells (Radinsky 1995; Khazaie et al., 1993). Importantly, EGFR expression has been correlated with the growth and metastasis of various neoplasms, including hepatic carcinoma (Carlin et al., (1988) renal carcinoma (Atlas et al.,1992), laryngeal cancer (Maurizi et al., 1992), esophageal tumors (Ozawa et al., 1987), colon carcinoma (Radinsky 1995), and lung cancer (Damstrup et al., 1992). Although functional effects of HGF on uveal melanoma cells were studied earlier (Woodward et al., 2002), there are no studies available correlating the expression of HGF with clinicopathologic parameters of uveal melanoma. Earlier, Economou et al. (Economou et al., 2005) have shown the expression of c-Met (hepatocyte growth factor receptor) significantly associated with melanoma-specific mortality in the patients with uveal melanoma. Thus, we studied the expression of EGFR, HGF, and c-Met proteins in a large cohort of uveal melanomas from an Asian-Indian population and correlated this with clinicopathologic parameters. We also evaluated the expression of ezrin in uveal melanomas. Ezrin, radixin, and moesin form the ERM protein family, which mediates interaction between actin microfilaments and cell membranes (Berryman et al., 1993; Vaheri et al., 1997; Tsukita et al., 1997). Because ezrin is a protein that participates in cell migration and cell recognition by the immune system, it may have an impact on tumor progression and development of metastasis (Hiscoz et al., 1999; Helander et al., 1996; Ohtani et al., 1999; Akisawa et al., 1999).

In another dimension of the current study, it is of interest to know whether the differential expression of these proteins (EGFR, ezrin, HGF, and c-Met) would possibly classify uveal melanomas into two groups as shown earlier (Krishnakumar et al., 2004) (class I melanomas with favorable outcomes had decreased to absent human leukocyte antigen and beta-2 microglobulin, and class II melanomas with liver metastasis had strong expression of HLA and

β2m). Thus, the purpose of this study was to evaluate the expression of EGFR, ezrin, HGF, and c-Met in uveal melanoma and to correlate this with clinicopathologic features and metastatic death of the uveal melanoma patients.

MATERIALS AND METHODS

Patients

All the patients were evaluated at the ocular oncology clinic of our hospital between 1996 and 2002. Sixty uveal melanoma lesions were obtained from 38 males and 22 female patients from age 9 years to 74 years, with a median age of 45 years. Survival was defined as the elapsed interval from date of eye removal to date of last follow-up or melanoma-related death.

Inclusion and Exclusion Criteria

Patients who underwent enucleation for uveal melanoma at our institute were included in the study. Iris melanomas and metastatic melanoma to the uvea were excluded. Patients with follow-up of at least 3 years were included in the survival analysis. The study was reviewed and approved by the local ethics committee at the Vision Research Foundation, Sankara Nethralaya, and the committee deemed that it conformed to the generally accepted principles of ethics in research, in accordance with the Declaration of Helsinki.

Specimens

Each sample was processed for conventional histopathologic diagnosis. Histologic sections were prepared from tissues fixed in 10% buffered neutral formalin for 48 hr and embedded in paraffin. Hematoxylin and eosin-stained, 6-μmsections were prepared through the central region of the tumor and reviewed for cell type, location, tumor base size, extension of the tumor, nuclear grade, mitosis, and TIL (tumor infiltrating lymphocytes) according to the recommendations for the reporting of tissues removed as part of the surgical treatment of common malignancies of the eye and its adnexa (Folberg et al., 2003).

Extension

The extension of the tumor was based on the histologic sections as well as clinical and radiologic examination. The tumors were divided into three groups based on the invasion: group A, tumors of choroidal and ciliary body melanomas with no extrascleral extension; group B, tumors with extrascleral extension only; and group C, tumors with liver metastasis.

Cell Types

The cell types included spindle A, spindle B, and epithelioid cell types. For analysis in this report, they were simplified into three groups as spindle, mixed, and epithelioid cell melanoma.

Tumor Base Size

Tumor base size (TBS) was measured in millimeters from histologic sections under the microscope. Patients were divided in two groups according to the dimensions TBS < 10 mm and TBS > 10 mm.

Nuclear Grade

We defined nuclear grades as grade 1 to 3 according to the regularity versus pleomorphism of the nuclear outline and the presence of the prominent nucleoli, grade 3 being pleomorphic and anaplastic with extreme prominent nucleoli. In this study, we compared nuclear grade 1 and 2 with nuclear grade 3.

Mitotic Figures

The presence or absence of mitotic figures was examined in 10 microscopic high-power fields. The presence of mitosis was considered as ‘+’ (presence of mitosis) and ‘-‘ (absence of mitosis).

Tumor Infiltrating Lymphocytes

For judging TIL, a dense infiltrate was accepted as positive, and either no infiltration or sparse infiltration was defined as negative.

Antibody Details

Mouse monoclonal EGFR (R-1; 200 μl/ml), rabbit polyclonal ezrin (H276; 200 μl/ml), rabbit polyclonal HGFα (H145; 200 μl/ml), and mouse monoclonal c-Met (DQ-13; 100 μg/ml) were used in this study. All the anitbodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The streptavidinbiotin kit (LSAB) was purchased from Dakocytomation (Dakocytomation, Via Real, Carpinteria, CA, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Immunohistochemistry

The immunostaining procedures were then performed. In brief, 4-μm formalin-fixed paraffin sections were deparaffinized, rehydrated, and bleached before the immunohistochemical procedure. Antigen retrieval by the pressure cooker method using citrate buffer was performed before antibody incubation. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min at room temperature. Tissue sections were then rinsed in Tris-buffered saline (pH 7.6) and the slides were incubated with 10% normal goat serum for 30 min for blocking nonspecific binding sites. Then, the blocking agent was drained, and the slides were incubated overnight with respective primary antibody: EGFR (1:25 dilution), ezrin (1:25 dilution), HGF (1:25 dilution), and c-Met (1:25). This was followed by sequential 50-min incubation with biotinylated secondary antibody and streptavidin labeled to horseradish peroxidase (Dakocytomation, Via Real, Carpinteria, CA, USA). Sections were washed with Tris-buffered saline between incubation. The reaction was developed for 20 min using commercially available AEC (3-amino-9-ethyl carbazole) and counterstained with Mayer's hematoxylin. The paraffin sections incubated with bovine serum albumin omitting primary antibody were used as negative controls in the study.

Evaluation of Immunoreactivity

Tissue sections were read independently by two investigators (S.K. and K.M.) without the knowledge of the results obtained by the other investigator. There was significantly better agreement in the interpretation of expression of all the four proteins in the three groups with a kappa value $\kappa = 1$ ($p < 0.0001$) and in the no invasion group with kappa value $\kappa = 0.91$ ($p < 0.0001$). Furthermore, each investigator read all of the slides twice without the knowledge of the

results obtained in the previous reading. The expression was semiquantitatively assessed by estimating both the staining intensity and the percentage of positively stained cells (Krishnakumar et al., 2004). The staining intensity was graded as + for bright staining, ± for dull staining, and − for absent staining. Three staining categories were established: negative (absent staining), heterogeneous (bright staining in ≤10% of tumor cells and dull staining in >10% tumor cells), and positive (bright staining in >10% of the tumor cells). Although both the percentage of cells and the intensity were scored in the analysis, we have considered only percentage positivity in the statistical analysis.

Statistical Analysis of the Data

Survival analysis was done on 22 patients with follow-up of at least 3 years using Kaplan-Meier life table analysis (MedCalc Software, version 7.2.0.2). The log rank test was carried out to correlate the protein expressions with the survival of the patients. We did the correlation coefficients among the proteins studied from the overall cohort using Spearman's rho correlation test. Nonparametric Man Whitney *U*-test was used to correlate expression of all four proteins among the three groups. We also correlated the individual protein expression with each tumor parameter like cell type, TBS, TIL, nuclear grade (NG), and mitosis using Mann-Whitney *U* -test from the overall cohort.

RESULTS

Histopathology

Among the 60 uveal melanomas, 45 involved the choroid, 11 tumors involved both ciliary body and choroid, and 5 were diffuse uveal melanoma. Forty-five tumors had no extrascleral invasion, nine tumors had extrascleral extension with no liver metastasis, and six tumors had liver metastasis. Tables 1a, 2, and 3 show the histopathologic and immunoexpression in the three groups of tumors.

Histopathology of Group A (n = 45)

Tumors with No Extrascleral Extension and No Liver Metastasis

There were 18 spindle cell melanomas, 22 mixed and 5 epithelioid melanomas. There were 18 tumors with TBS ≤10 mm and 27 tumors with TBS >10 mm in size. TIL was absent in 38 tumors, mitosis was negative in 42 tumors, and nuclear grade less than 3 was seen in 42 tumors.

Histopathology of Group B (n = 9)

Tumors with Extrascleral Extension and No Liver Metastasis

There were four spindle and five mixed melanomas. There were three tumors with TBS ≤10 mm, and six tumors with TBS >10 mm in size. TIL was negative in eight tumors, mitosis was positive in two tumors, and nuclear grade 3 was seen in one tumor.

Histopathology of Group C (n = 6)

Tumors with Liver Metastasis

There were five mixed and one epitheloid melanomas. There was one tumor with TBS ≤10 mm, and five tumors with TBS >10 mm in size. TIL was positive in three tumors, mitosis was positive in two tumors, and nuclear grade 3 was seen in one tumor.

Correlation among Histopathologic Parameters

Spindle cell melanomas are absent in group C tumors (liver metastasis). Non spindle melanomas are found in all three groups. In group A, there was no correlation with TBS as both tumors with TBS ≤10 mm and tumors with TBS >10 mm were observed. When TIL, mitosis, and nuclear grade were compared among the three groups, we observed that tumors in group A had predominately negative TIL, absent mitosis, and lower nuclear grades (nuclear grades 1 and 2). However, in tumors in groups B and C, there was no correlation with TIL, mitosis, and nuclear grade.

Expression of EGFR, Ezrin, HGF, and c-Met in the Non-Neoplastic Retinal Tissues and Tumor Cells

EGFR was positive in inner and outer nuclear layers and ganglion cell layers (Fig. 1A) of non-neoplastic retina (donor eye ball). HGF was sporadically positive in nuclear layers and positive in ganglion cell layer of the retina (Fig. 2B). c-Met was positive in inner and outer nuclear layers and ganglion cell layers of non-neoplastic retina (Fig. 2D). The tumor cells showed dull membrane to cytoplasmic staining for EGFR (Fig. 1B), strong cytoplasmic staining for ezrin (Fig. 1C), cytoplasmic positivity or HGF and strong membrane positivity for c-Met (Fig. 2C).

Immunoreactivity of EGFR, Ezrin, HGF, and c-Met in Group A Tumors

In group A (n = 45) tumors with no extrascleral extension and liver metastasis, EGFR was negative in 29 tumors, heterogeneous in 5 tumors, and positive in 11 tumors. Ezrin was negative in 22 tumors, heterogeneous in 9 tumors, and positive in 14 tumors. HGF was negative in 25 tumors, heterogeneous in 7 tumors, and positive in 13 tumors. c-Met was negative in 20 tumors, heterogeneous in 12 tumors, and positive in 13 tumors.

Immunoreactivity of EGFR, Ezrin, HGF, and c-Met in Group B Tumors

In group B (n = 9) tumors with extrascleral extension but no liver metastasis, EGFR was positive in one tumor and negative in eight tumors. Ezrin was positive in three tumors and heterogeneous in one tumor and negative in five tumors. HGF was positive in two tumors, heterogeneous in two tumors, and negative in five tumors. c-Met was positive in one tumor, heterogeneous in two tumors, and negative in six tumors.

Immunoreactivity of EGFR, Ezrin, HGF, and c-Met in Group C Tumors

In group C (n = 6) tumors with liver metastasis, EGFR was positive in one tumor and negative in five tumors. Ezrin was positive in two tumors, heterogeneous in one tumor, and negative in three tumors. HGF was negative in all the six tumors. c-Met was positive in four tumors and heterogeneous in one tumor.

Survival Analysis

The Cox proportional hazards survival regression analysis showed 6.5 years of median survival period of uveal melanoma patients (Fig. 3). To determine whether immunostaining of all proteins may correlate with metastatic death, we studied the survival of 22 patients with follow-up of at least 3 years. Using Kaplan- Meier statistical analysis, we found a significant association between high c-Met expression and death due to uveal melanoma ($p < 0.03$) (Fig. 4).

Statistical Correlation of EGFR, Ezrin, HGF, and c-Met Immunoreactivity between Group A and Group B or Group B and Group C

We did not observe any significant correlation of any of the proteins between group A and group B. Group C tumors (with liver metastasis) showed higher expression of c-Met ($p = 0.009$) compared with groups A and B combined (without liver metastasis). There was a significant decrease of HGF in group C tumors compared with the tumors without liver metastasis (groups A and B). However, we did not find significant p value by non-parametric Kruskal-Wallis test.

Correlation Coefficient of All the Proteins from the Overall Cohort

There was a significant linear correlation between EGFR and HGF ($p = 0.001$); between EGFR and c-Met ($p = 0.004$), and between HGF and c-Met ($p = 0.024$) using Spearman's rho correlation coefficient test.

Correlation of EGFR, Ezrin, HGF, and c-Met with Tumor Parameters from the Overall Cohort

We did not observe any correlation of EGFR, ezrin, HGF, and c-Met with any of the tumor parameters.

Figure 1

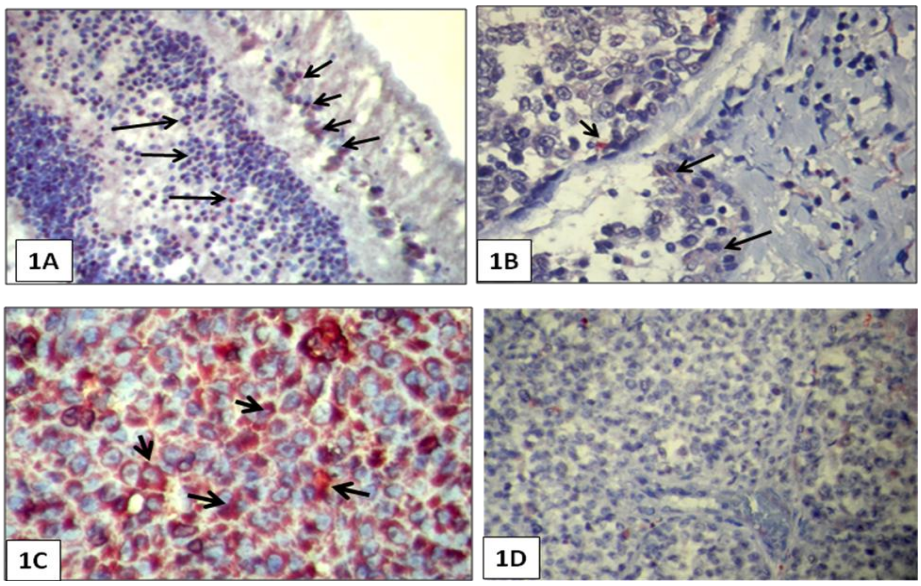

FIGURE 1 (A) Photomicrograph shows the EGFR positivity in the nuclear layers (upward arrows) and the ganglion cell layers (downward arrow) of non-neoplastic retina (donor eyeballs) (amino ethyl carbazole with hematoxylin counterstain, original magnification ×40). (B) Photomicrograph shows dull immunoreactivity (arrows show dull cytoplasmic staining) of EGFR in melanoma, which had metastasized to the liver (amino ethyl carbazole with hematoxylin counterstain, originalmagnification×40). (C) Photomicrograph shows the strong positive immunoreactivity of ezrin in the epithelioid cell melanoma (amino ethyl carbazole with hematoxylin counterstain, original magnification ×40). (D) Photomicrograph shows negative immunoreactivity (negative control, without primary antibody) in the spindle cell melanoma (amino ethyl carbazole with hematoxylin counterstain, original magnification ×40).

Figure 2

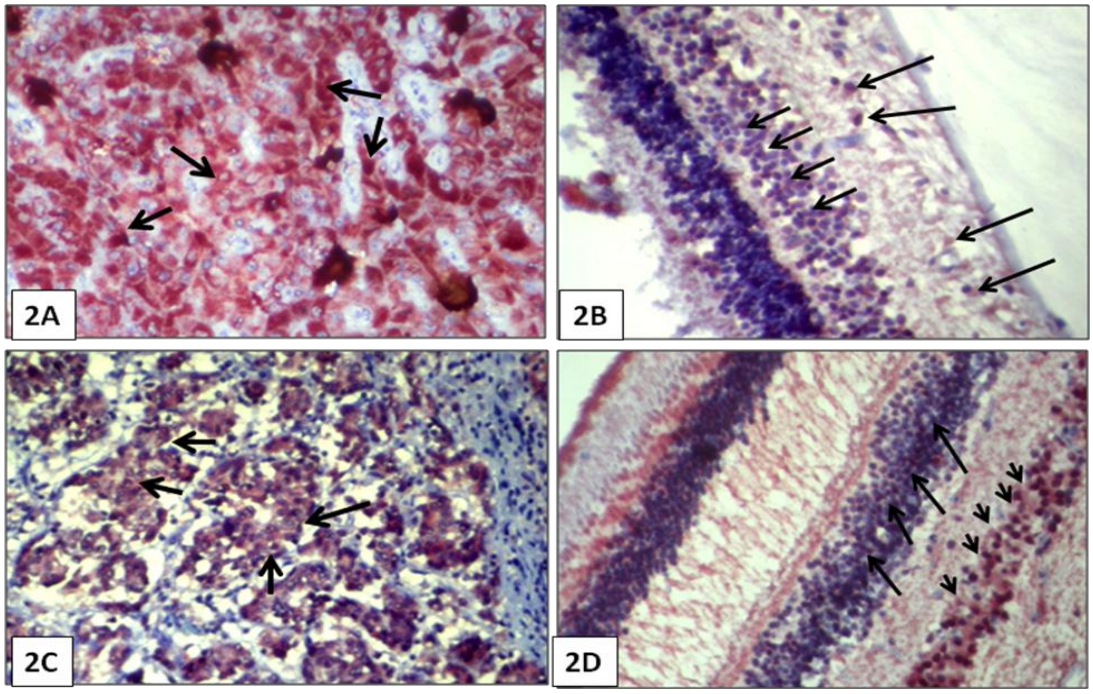

FIGURE 2 (A) Photomicrograph shows the negative immunoreactivity of HGF in melanoma with which there was liver metastasis (amino ethyl carbazole with hematoxylin counterstain, original magnification ×40). (B) Photomicrograph shows the positive immunoreactivity of HGF in the ganglion cell layer (arrows show cytoplasmic positivity) and sporadically positive in nuclear layers of non-neoplastic retina (donor eyeballs) (amino ethyl carbazole with hematoxylin counterstain, originalmagnification×40). (C) Photomicrograph shows the strong immunoreactivity of c-Met in melanoma with which occured liver metastasis (amino ethyl carbazole with hematoxylin counterstain, original magnification×40). (D) Photomicrograph shows the positive immunoreactivity of c-Met in the nuclear layers (upward arrows) and ganglion cells (downward arrows) of non-neoplastic retina (donor eyeballs) melanoma, which had metastasized to the liver (amino ethyl carbazole with hematoxylin counterstain, original magnification ×40).

Figure 3

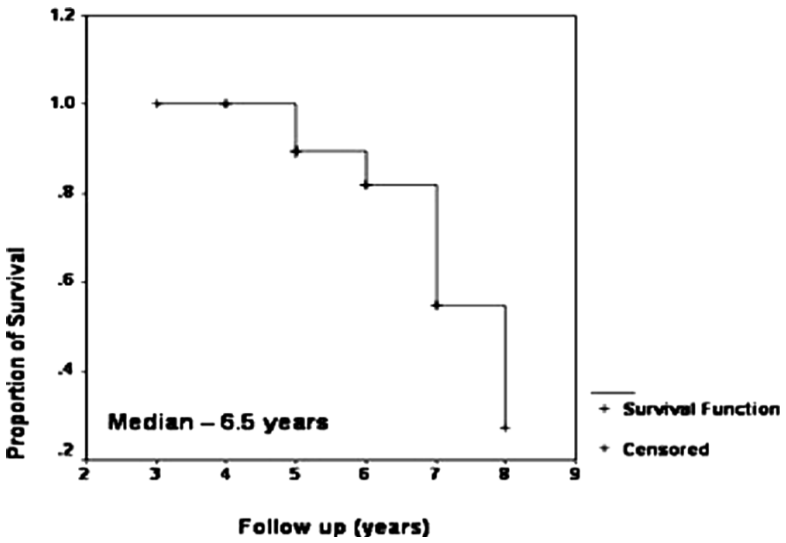

FIGURE 3 The Kaplan-Meier graph shows the survival data of patients with uveal melanoma.

Figure 4

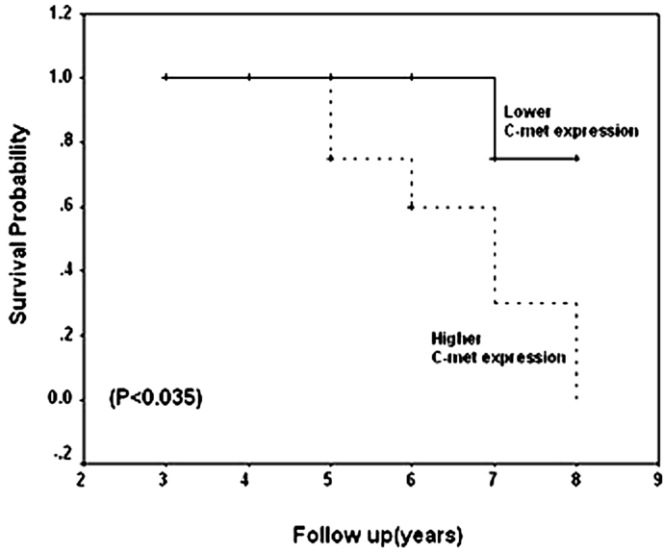

FIGURE 4 The graph shows the significant association between the higher c-Met expression and death due to uveal melanoma using the log rank test.

DISCUSSION

In this work, we studied the expression of EGFR, ezrin, HGF, and c-Met in uveal melanomas. Among 60 tumors (groups A, B, and C), EGFR was expressed in 18 (30%) tumors, ezrin was expressed in 30 (50%) tumors, HGF was expressed in 24 (40%) tumors, and c-Met was expressed in 33 (55%) tumors. Among six tumors that had undergone liver metastasis (group C), EGFR was positive in one tumor and negative in five tumors. We did not observe any statistical correlation of EGFR between any of the three groups. Earlier, Ma and Niederkorn (Ma D and Niederkorn, 1998) observed in murine studies that expression of epidermal growth factor receptor (EGFR) by uveal melanoma cells was significantly correlated with the development of liver metastases and decreased survival. In addition, Hurks et al. (Hurks et al., 2000) have correlated EGFR expression with death due to liver metastasis. However, a later study by Scholes et al. (Scholes et al., 2001) did not support the use of EGFR expression as a prognostic indicator in patients with uveal melanoma due to the macrophage-restricted expression of EGFR in uveal melanoma paraffin sections. In our study, in addition to the tumor cells that expressed EGFR, we did observe that macrophage- like cells stained positive for EGFR, confirmed by CD65 staining for macrophages. Thus, the EGFR immunoreactivity at very low frequency in the melanoma cells indicates that it could not serve as a prognostic indicator in patients with uveal melanoma.

In our study, we did not observe any association between ezrin and metastatic death. However, earlier Makitie et al. (Makitie et al., 2001) have shown the expression of ezrin in uveal melanoma patients significantly correlated with higher mortality in uveal melanoma patients. We did not observe any correlation of EGFR, ezrin, HGF, and c-Met with any of the tumor parameters. In group C, c-Met was strongly positive in four tumors, heterogeneous in one tumor, and completely negative in one tumor. Using Kaplan-Meier analysis, we found a significant association between high c-Met expression and death due to uveal melanoma ($p < 0.03$). Statistically, c-Met is significantly expressed in tumors with liver metastasis (group C) when compared with tumors without liver metastasis (groups A and B) ($p = 0.009$). Surprisingly, HGF was negative in all the six tumors that had undergone liver metastasis (group C). Hendrix et al. (Hendrix et al., 1998) have studied HGF expression in primary and metastatic uveal melanomas,

which has been correlated with tumor angiogenesis. When we correlated expression of all four proteins with metastatic death using Kaplan-Meier analysis, we did not find any significant association between other proteins (EGFR, ezrin, and HGF) and metastatic death. However, our study suffers from limitations due to the limited follow up cases for the analysis. Thus, we speculate that the concept of ligand independent activation of c-Met may be involved in uveal melanomas. c-Met activation can occur through alternative mechanisms including (1) selected Met mutations; (2) constitutive dimerization in the absence of ligand associated with overexpression (Wang et al., 2001); (3) c-Met truncation (Wallenius et al., 2000; Prat et al., 1991); (4) gene translocation/rearrangement (Park et al.,1986); (5) pathway activation under hypoxic conditions (Pennacchietti et al., 2003); (6) transactivation by other receptors including EGFR and RON (macrophage stimulating protein receptor) (Follenzi et al., 2000; Jo et al., 2000); and (7) loss of negative regulators such as VHL (von Hippel–Lindau) in renal carcinomas (Oh et al., (2002). These mechanisms may either partially or completely eliminate the dependence of c-Met on HGF for activation. The implications of this broad array of ligand-dependent versus ligand-independent mechanisms in activation of c-Met in the context of human tumors are not well understood. Thus to conclude, our study shows the significantly higher expression of c-Met and negative expression of HGF in melanomas with liver metastasis, which prompts us to speculate a mechanism of ligand independent activation of c-Met in uveal melanomas. In addition, the overexpression of c-Met in tumors with liver metastasis could classify uveal melanomas into two classes: class I (low grade, no liver metastasis) with decreased expression of c-Met, and class II melanomas (high grade, liver metastasis) with increased expression of c-Met. However, further mutational studies are required to understand the mechanism of constitutive c- Met activation in uveal melanomas.

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Expression of Epidermal Growth Factor Receptor, Ezrin, Hepatocyte Growth Factor, and c-Met in Uveal Melanoma: An Immunohistochemical Study

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ABSTRACT The immunoreactivity of epidermal growth factor receptor (EGFR) ezrin, hepatocyte growth factor receptor (HGF), and c-Met was studied in 60 uveal melanomas and was correlated with clinicopathologic parameters. Metastases were diagnosed in the patients with uveal melanoma between 5 years and 8 years (median, 6.5 years) after enucleation. Using Kaplan-Meier statistical analysis, we found a significant association between high c-Met expression and death due to uveal melanoma ($p < 0.03$). EGFR was expressed in 18 of 60 (30%) tumors; ezrin was expressed in 30 of 60 (50%) tumors. Tumors with liver metastasis ($n = 6$) showed higher expression of c-Met ($p = 0.0009$) compared with the tumors with no extension/extrascleral extension without liver metastasis (groups A-45 and B-9). HGF was negative in all the six tumors that had liver metastasis. Further studies are required to understand the possible mechanism of ligand-independent c-Met activation in patients with uveal melanoma.

KEYWORDS choroidal melanoma; c-met; epidermal growth factor receptor; ezrin; hepatocyte growth factor; immunohistochemistry; metastasis

CHAPTER 2
EXPRESSION OF INSULIN-LIKE GROWTH FACTOR RECEPTOR (IGF-1R), C-FOS, AND C-JUN IN PRIMARY UVEAL MELANOMA TUMORS

INTRODUCTION

Studies of oncogenic signaling pathways in human cancers have identified molecular targets for developing rationally designed cancer therapeutics. In particular, many receptor and nonreceptor tyrosine kinases, including EGFR (epidermal growth factor receptor) (Tang et al., 2000), IGF-1R (insulin-like growth factor receptor type I) (Pietrzkowski et al., 1992), Her-2/Neu (Nathanson et al., 2006), JAK (Song et al., 2004), and Src kinase (Niu et al., 2002), are found to be constitutively activated in various cancers. In this work, we have studied and correlated clinicopathologically the immunoreactivity of IGF-1R in uveal melanomas. IGF-1R belongs to the type 1 receptor of insulin-like growth factor receptor. In its mature form, IGF-1R is a heterotetrameric receptor (two extracellular 125-kDa α chains and two transmembrane 95-kDa β chains) that autophosphorylates after ligand binding and activates several downstream signaling routes, including the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Signaling through IGF-1R stimulates proliferation, promotes angiogenesis and metastasis, and inhibits apoptosis (Khandwala et al., 2000; Pollak 2004a; Pollak et al.,2004b).Overexpression of the IGF-1R induces growth, neoplastic transformation, and tumorigenesis (Surmacz 2003). Compared with equivalent normal tissues, the IGF-1R is overexpressed by tumors including colorectal cancer and melanoma (Hakam et al., 1993; Kanter-Lewensohn et al., 2000; Khandwala et al., 2000) and IGF-1R overexpression has been linked to radioresistance in breast cancer (Turner et al., 1997). Furthermore, a higher expression of IGF-1R has been significantly associated with death due to liver metastasis (Ericsson et al., 2002) and with melanoma-specific mortality (Economou et al., 2005) in uveal melanoma patients. In addition, IGF-1R expression was studied in uveal melanoma cell lines OCM-1, OCM-3, and 92-1 by Ericsson et al. (Ericsson et al., 2002) and proved that the growth and survival of these cell lines are strongly dependent on IGF-1R expression and activation through glycosylation inhibition and αIR-3 antibody mediated IGF-1R neutralization studies. In the current study, in addition to IGF-1R expression we have studied and clinicopathologically correlated the immunoreactivity of c-Fos and c-Jun, which belong to the activating protein 1 (AP-1) family, in uveal melanomas. The control of expression of certain oncogenes is inextricably associated with proteins that can initiate or inhibit gene transcription. Specific genes contain within their promoter regions DNA sequences that permit the binding of transactivating and transinhibiting proteins that can initiate or suppress transcription, respectively. AP-1, a dimeric complex

consisting of proteins encoded by the Jun and Fos gene families, is a transcription factor induced by a variety of signals, including those eliciting proliferation, differentiation, and programmed cell death (apoptosis) (Kang et al., 1998). In another dimension of the current study, it is of interest to know whether the differential expression of these proteins (IGF-1R, c-Fos, and c-Jun) would possibly classify uveal melanomas into two groups as shown earlier (Krishnakumar et al., 2004) (class I melanomas with favorable outcomes had decreased to absent human leukocyte antigen and beta 2 microglobulin, and class II melanomas with liver metastasis had strong expression of HLA and β2m). Recently, consistent with our findings, the Harbour et al.(Onken et al., 2004) group has classified uveal melanomas into two groups, class 1 (low grade), and class 2 (high grade), based on the gene expression profile, which could strongly predict metastatic death in uveal melanoma patients.

The purpose of this study was to evaluate the expression of IGF-1R, c-Fos, and c-Jun in uveal melanoma and to correlate with clinicopathological features.

MATERIALS AND METHODS

Patients

All the patients were evaluated at the ocular oncology clinic of our hospital between 1996 and 2002. Sixty uveal melanoma lesions were obtained from 38 male and 22 female patients from ages 9 years to 74 years, with a median age of 45 years.

Inclusion and Exclusion Criteria

Patients who underwent enucleation for uveal melanoma at our institute were included in the study. Iris melanomas and metastatic melanoma to the uvea were excluded. The study was reviewed and approved by the local ethics committee at the Vision Research Foundation, Sankara Nethralaya, and the committee deemed that it conformed to the generally accepted principles of ethics in research, in accordance with the Helsinki Declaration.

Specimens

Each sample was processed for conventional histopathological diagnosis. Histological sections were prepared from tissues fixed in 10% buffered neutral formalin for 48 hr and embedded in paraffin. Hematoxylin and eosin–stained 6-μm sections were prepared through the central region of the tumor and reviewed for cell type, location, largest tumor diameter (LTD), extension of the tumor, nuclear grade (NG), mitosis, and tumor infiltrating lymphocytes (TILs) according to the recommendations for the reporting of tissues removed as part of the surgical treatment of common malignancies of the eye and its adnexa.

Extension

The extension of the tumor was based on the histological sections as well as clinical and radiological examination. The tumors were divided into three groups based on the invasion: group A, tumors of choroidal and ciliary body melanomas with no extrascleral extension; group B, with extrascleral extension only; group C, tumors with liver metastasis.

Cell Types

The cell types included spindle A, spindle B, epithelioid and mixed cell melanoma. For analysis in this report, they were simplified into three groups as spindle, mixed, and epithelioid cell types and the immunoreactivity of IGF-1R, c-Fos, and c-Jun was correlated between the spindle cell melanomas and non–spindle cell melanomas (epithelioid cell type and mixed).

Largest Tumor Diameter

Tumor size was measured as the largest tumor dimension (LTD) in millimeters from histological sections under the microscope. Patients were divided in two groups according to the dimensions LTD $\leq$ 10 mm and LTD > 10-mm and correlated with IGF-1R, c-Fos, and c-Jun immunoreactivity.

Nuclear Grade

We defined nuclear grades as grades 1 to 3 according to the regularity versus pleomorphism of the nuclear outline and the presence of the prominent nucleoli, grade 3 being pleomorphic and

anaplastic with extreme prominent nucleoli. In this study, we compared nuclear grades 1 and 2 with the nuclear grade 3 and correlated IGF-1R, c-Fos, and c-Jun immunoreactivity.

Mitotic Figures

The presence or absence of mitotic figures was examined in 10 microscopic high-power fields. The presence of mitosis was considered as positive (+; presence of mitosis) and negative (−; absence of mitosis) and correlated with IGF-1R, c-Fos, and c-Jun immunoreactivity.

Tumor-Infiltrating Lymphocytes

For judging TILs, we used the criteria of the presence or absence of 10 tumor-infiltrating lymphocytes per 20 high-power fields. A dense infiltrate was accepted as positive and either no infiltration or sparse infiltration was defined as negative and correlated with IGF-1R, c-Fos, and c-Jun immunoreactivity.

Antibody Details

IGF-1R clone; rabbit polyclonal, c-Fos (4); and rabbit polyclonal, c-Jun (N) were used in this study. All the anitbodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The streptavidinbiotin kit (LSAB) was purchased from Dakocytomation (Denmark). All other chemicals were from Sigma- Aldrich (St. Louis, MO, USA).

Immunohistochemistry

The immunostaining procedures were then performed. In brief, 4-μm formalin-fixed paraffin sections were deparaffinized, rehydrated, and bleached before the immunohistochemical procedure. Antigen retrieval by the pressure cooker method using citrate buffer was performed before antibody incubation. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min at room temperature. Tissue sections were then rinsed in Tris-buffered saline (pH 7.6) and incubated overnight with respective primary antibody: IGF-1R (1:25 dilution), c-Fos (1:25 dilution), and c- Jun (1:25 dilution). This was followed by sequential 50-min incubation with biotinylated secondary antibody and streptavidin labeled to horseradish peroxidase (DAKO, Glostrup, Denmark). Sections were washed with Tris-buffered saline between incubation. The

reaction was developed for 20 min using commercially available AEC (3-amino-9-ethyl carbazole) and counterstained with Mayer's hematoxylin. The paraffin sections incubated with bovine serum albumin omitting primary antibody were used as negative controls in the study.

Evaluation of Immunoreactivity

Tissue sections were read independently by two investigators (S.K. and K.M.) 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. The expression was semi quantitatively assessed by estimating both the staining intensity and the percentage of positively stained cells (Krishnakumar et al., 2004). The staining intensity was graded as + for bright staining, ± for dull staining, and – for absent staining. Three staining categories were established: negative (absent staining), heterogeneous (bright staining in ≤10% of tumor cells, and dull staining in >10% tumor cells), and positive (bright staining in >10% of the tumor cells). Although both the percentage of cells and the intensity were scored in the analysis, we have considered only percentage positivity in the statistical analysis.

Statistical Analysis of the Data

We did the correlation coefficients between the three proteins from the overall cohort using Spearman's rho correlation test. Non-parametric Kruskal-Wallis test was used to correlate expression of all three proteins among the three groups. We also correlated the individual protein expression with each tumor parameter like cell type, LTD, TILs, NG, mitosis from the overall cohort using non-parametric Kruskal-Wallis test, and Mann-Whitney *U* test.

RESULTS

Histopathology

Among the 60 uveal melanomas, 45 involved the choroid, 11 tumors involved both ciliary body and choroid, and 5 were diffuse uveal melanoma. Forty-five tumors had no extrascleral invasion, nine tumors had extrascleral extension with no liver metastasis, and six tumors had liver metastasis. Tables 1, 2, and 3 show the clinicopathological features and immunoexpression in the three groups of tumors. Table 4 summarizes the results of immunohistochemistry and the correlation with clinicopathological features.

Histopathology of Group A (n = 45)

Tumors with No Extrascleral Extension and No Liver Metastasis

There were 18 spindle cell melanomas, 22 mixed and 5 epithelioid melanomas. There were 18 tumors with LTD ≤ 10 mm, and 27 tumors with LTD > 10 mm in size. TILs were absent in 38 tumors, mitosis was negative in 42 tumors, and nuclear grade less than 3 was seen in 42 tumors.

Histopathology of Group B (n = 9)

Tumors with Extrascleral Extension and No Liver Metastasis

There were four spindle and five mixed melanomas. There were three tumors with LTD ≤ 10 mm and six tumors with LTD > 10 mm in size. TIL was negative in eight tumors, mitosis was positive in two tumors, and nuclear grade 3 was seen in one tumor.

Histopathology of Group C (n = 6)

Tumors with Liver Metastasis

There were five mixed and one epitheloid melanomas. There was one tumor with LTD ≤ 10 mm and five tumors with LTD > 10 mm in size. TIL was positive in three tumors, mitosis was positive in two tumors, and nuclear grade 3 was seen in one tumor.

Correlation among Histopathological Parameters

Spindle cell melanomas are absent in group C tumors (liver metastasis). Non–spindle melanomas were found in all three groups. In group A there was no correlation with LTD as both tumors with

LTD ≤ 10 mm and tumors with LTD > 10 mm were observed. When TIL, mitosis, and nuclear grade was compared between the three groups, we observed that tumors in group A had predominantly negative TIL, absent mitosis, and lower nuclear grades (nuclear grades 1 and 2). However, for tumors in groups B and C there was no correlation with TIL, mitosis, and nuclear grade.

Expression of IGF-1R, c-Fos, and c-Jun in the Retinal Tissues and Tumor Cells

The inner and outer nuclear layers of retina showed dull staining for IGF-1R (Fig. 1A), and focal positivity of c-Fos and c-Jun was observed in the retinal tissues. The tumor cells showed membrane positivity for IGF- 1R (Fig. 1B) and nucleocytoplasmic positivity for c-Fos and c-Jun (Figs. 2B and 3B).

Immunoreactivity of IGF-1R, c-Fos, and c-Jun in Group A Tumors

In group A (n = 45) tumors with no extrascleral extension and liver metastasis, IGF-1R was negative in 25 tumors, heterogeneous in 10 tumors, and positive in 10 tumors. c-Fos was negative in 29 tumors, heterogeneous in 4 tumors, and positive in 12 tumors. c-Jun was negative in 15 tumors, heterogeneous in 14 tumors, and positive in 15 tumors.

Immunoreactivity of IGF-1R, c-Fos, and c-Jun in Group B Tumors

In group B (n = 9) tumors with extrascleral extension but no liver metastasis, IGF-1R was positive in four tumors, heterogeneous in one tumor, and negative in four tumors. c-Fos was positive in one tumor and heterogeneous in one tumor and negative in seven tumors. c-Jun was positive in five tumors and negative in four tumors.

Immunoreactivity of IGF-1R, c-Fos, and c-Jun in Group C Tumors

In group C (n = 6) tumors with liver metastasis, IGF-1R was positive in five tumors and heterogeneous in one tumor. c-Fos was positive in four tumors, heterogeneous in one tumor, and negative in one tumor. c-Jun was positive in five tumors and heterogeneous in one tumor.

Statistical Correlation of IGF-1R, c-Fos, and c-Jun Immunoreactivity between the Melanomas with and without Liver Metastasis

Tumors with liver metastasis showed higher expression of IGF-1R ($p = 0.000$), c-Fos ($p = 0.004$), and c-Jun ($p < 0.018$) compared with the tumors without liver metastasis by non-parametric Kruskal-Wallis test.

Correlation of IGF-1R, c-Fos, and c-Jun with Tumor Parameters from the Overall Cohort

IGF-1R

The tumors with LTD > 10 mm showed higher expression of IGF-1R ($p = 0.027$) by non-parametric Mann-Whitney *U* test. However, there was no correlation observed with the other tumor parameters.

c-Jun

The non–spindle cell type tumors showed higher expression of c-Jun ($p = 0.033$) by non-parametric Kruskal-Wallis test. However, there was no correlation observed with other tumor parameters.

Figure 1

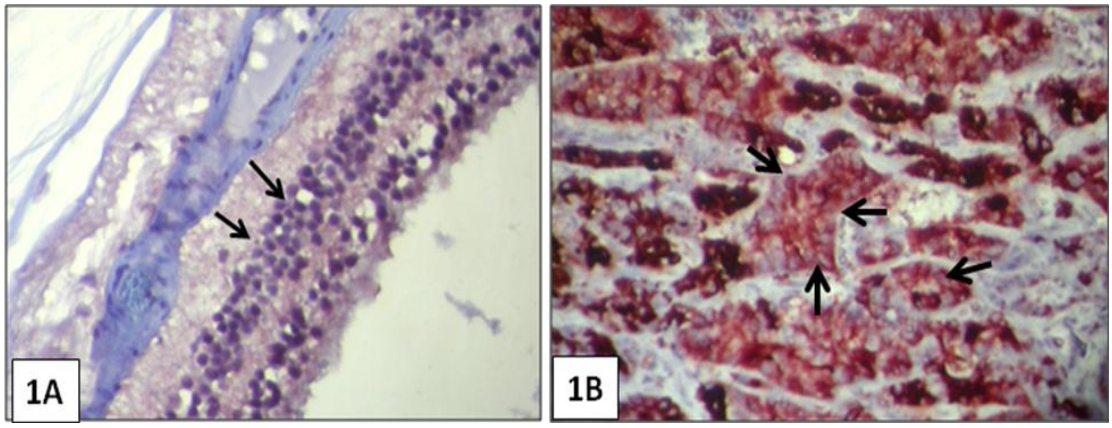

FIGURE 1 (A) Photomicrograph shows the dull membrane immunoreactivity of IGF-1R in the inner and outer nuclear layers of retina (white arrows). (Amino ethyl carbazole with hematoxylin counterstain; original magnification, ×40). (B) Photomicrograph shows the positive immunoreactivity of IGF-1R in melanoma, which had metastasized to the liver. (Amino ethyl carbazole with hematoxylin counterstain; original magnification, ×40).

Figure 2

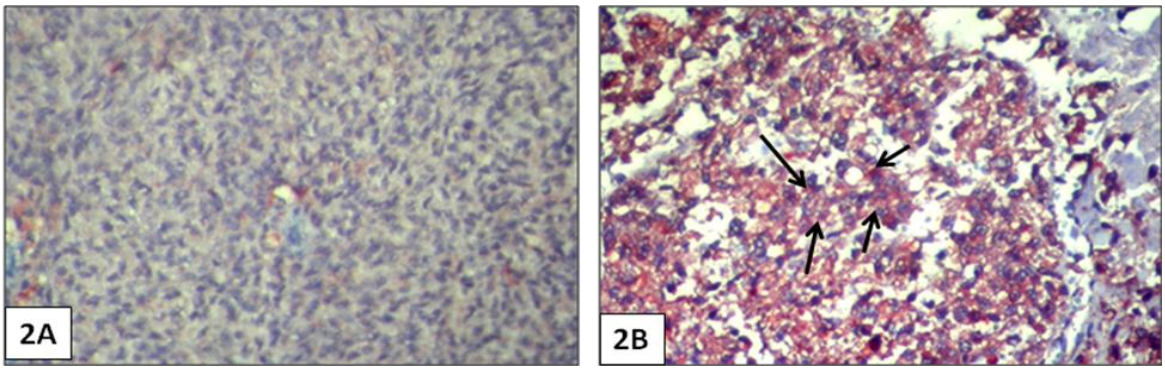

FIGURE 2 (A) Photomicrograph shows the negative immunoreactivity of c-Fos in melanoma with no extension and without liver metastasis. (Amino ethyl carbazole with hematoxylin counterstain; original magnification, ×40). (B) Photomicrograph shows the positive immunoreactivity of c-Fos in melanoma, which had metastasized to the liver. (Amino ethyl carbazole with hematoxylin counterstain; original magnification, ×40).

Figure 3

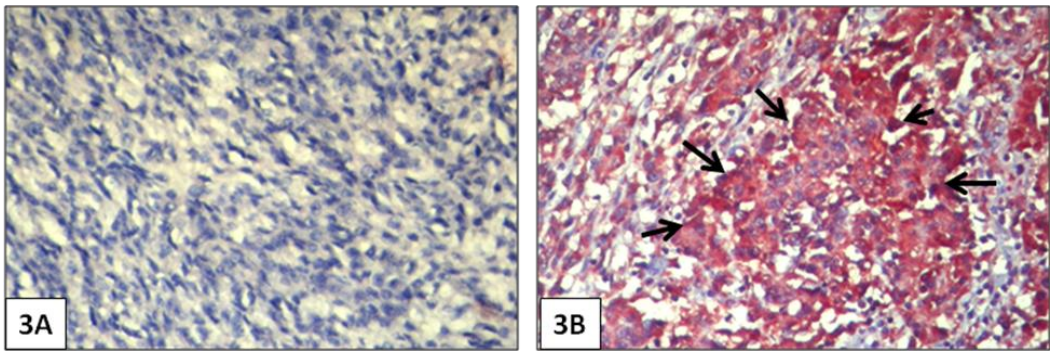

FIGURE 3 (A) Photomicrograph shows the negative immunoreactivity of c-Jun in melanoma, with no extension and without liver metastasis. (Amino ethyl carbazole with hematoxylin counterstain; original magnification, ×40). (B) Photomicrograph shows the positive immunoreactivity of c-Jun in melanoma, which had metastasized to the liver. (Amino ethyl carbazole with hematoxylin counterstain; original magnification, ×40).

DISCUSSION

In this work, we studied the expression of IGF-1R and the nuclear transcription factors c-Fos and c-Jun in uveal melanomas. Among six tumors that had undergone liver metastasis, IGF-1R was positive in five tumors. Although IGF-1R expression was seen in tumors with no extension as well, the difference in the expression between the tumors with no liver metastasis and the tumors with liver metastasis was statistically significant ($p = 0.0001$). The significant association between high IGF-1R expression and death due to metastatic disease may be explained by the fact that IGF-1 is mainly produced in the liver, which is the preferential site for uveal melanoma metastases. In addition, this study showed the expression of c-Fos and c-Jun, members of the AP-1 family of nuclear transcription factors that play a role in regulation of transcription. Among six tumors with liver metastasis, c-Fos was positive in four tumors and c-Jun was positive in five tumors. The higher expression of c-Fos ($p = 0.004$) and c-Jun ($p < 0.018$) was significant in the tumors with liver metastasis compared with that of the tumors without metastasis. Hence, it appears that c-Fos and c-Jun were also overexpressed concurrently with IGF-1R overexpression in the tumors that had undergone liver metastasis. However, DNA binding assays are essential to

demonstrate the regulatory transcriptional activity of c-Fos and c-Jun in uveal melanomas. In our study, the higher expression of these proteins in tumors with liver metastasis could classify uveal melanomas into two classes: class I (low grade, no liver metastasis) with decreased expressions of all three proteins and class II melanomas (high grade, liver metastasis) with increased expressions of IGF-1R, c-Fos, and c-Jun. However, the current study suffers from limitations due to the smaller sample size of the tumors with liver metastasis. When we correlated the immunoreactivity of all three proteins against various tumor parameters, the tumors with LTD > 10 mm showed higher expression of IGF-1R ($p = 0.027$) and the non–spindle cell type tumors showed higher expression of c-Jun ($p = 0.033$) from the overall cohort. Recent preclinical work has shown that IGF-1R could be used as a successful cotarget with EGFR in primary human glioblastoma cells (Chakravarti et al., 2002; Steinbach et al., 2004) with c-kit in small-cell lung cancer (Warshamana-Greene et al.,2004) and with HER2/erbB2 in breast cancer cells (Lu et al., 2001; Camirand et al., 2002). However, the interactions between IGF-1R and c-Fos/c-Jun proteins have not been elucidated in uveal melanoma. Thus, it gives an insight into the use of IGF-1R as target molecule for targeted therapy. Antisense-mediated downregulation of IGF-1R could enhance apoptosis by reducing the accumulation of p53 in radioresistant tumors. Thus, the concept of targeted IGF-1R downregulation should serve a potential treatment for malignant melanoma and other radioresistant tumors. Also, the current study is the first one to show the c-Fos and c- Jun expression in uveal melanoma, and further studies are required to elucidate the transcriptional activity of these proteins in uveal melanoma.

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Expression of Insulin-Like Growth Factor Receptor (IGF-1R), c-Fos, and c-Jun in Uveal Melanoma: An Immunohistochemical Study

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ABSTRACT The immunoreactivity of insulin-like growth factor receptor type 1 (IGF-1R), c-Fos, and c-Jun by immunohistochemistry was studied in three groups of uveal melanomas and was correlated clinicopathologically. Immunoanalysis was correlated with cell types, largest tumor diameter, tumor-infiltrating lymphocytes, mitosis, nuclear grade, and extrascleral extension/liver metastasis. In group C ($n = 6$), tumors with liver metastasis showed higher expressions of IGF-1R ($p = 0.0001$), c-Fos ($p = 0.004$), and c-Jun ($p = 0.018$) compared with

CHAPTER 3
CRIPTO-1 EXPRESSION IN PRIMARY UVEAL MELANOMA TUMORS AND ITS ASSOCIATION WITH CLINICOPATHOLOGICAL FEATURES.

1. Introduction

Over the past two decades, a number of molecules have been identified and used as targets in cancer immunotherapy (Gibbs, 2000). The spectacular therapeutic success of antibodies to Her2/neu in certain patients with breast cancer has encouraged the search for key molecules that are involved in other cancers and that could be targeted by specific antibodies. Recently Michele C. Madigan and co-workers (Li et al., 2005) demonstrated NG2 antigen as a suitable target for alpha immunotherapy in uveal melanoma. However, identification of newer antigens that are expressed only on the tumor cells and not expressed on the non-neoplastic ocular cells is needed. Recently, it has been shown that the EGF-CFC protein Cripto-1 could be a potential value in the immunotherapeutic treatment of various cancers and could be even superior to other molecules that are targets in current clinical trials(Hu and Xing, 2005; Xing et al., 2004). Human Cripto-1 is a Mr 36,000 molecule, classified in the epidermal growth factor- Cripto-FRL-Criptic (EGFCFC) family, caused by the conservation of six cysteines in the central region (amino acids 77e113); however, there is little resemblance to EGF, and Cripto-1 does not bind to any EGF receptors (Saloman et al., 2000). The EGF-CFC family includes human Cripto, mouse Cripto-1, cryptic Xenopus FRL-1, zebrafish one-eyed pinhead, and chick Cripto. Cripto-1 plays an important role in embryonic development in zebrafish and xenopus; in the mouse, a germ line knockout of the mouse Cripto-1 gene was lethal, and in zebrafish, injection of recombinant Cripto protein into late blastulae rescued a mutant phenotype (Shen and Schier, 2000). Cripto-1 also is an oncogenic growth factor involving tumorigenesis and cancer cell proliferation and survival, because transfection of Cripto-1 cDNA induces cell transformation. Cripto-1 is absent or in low levels on normal tissues and is expressed in most malignant tumors, including colon, breast, lung, ovarian, and pancreatic carcinomas (Ciardiello et al., 1991; Qi et al., 1994; Friess et al., 1994; Panico et al., 1996; Fontanini et al., 1998; D'Antonio et al., 2002; Ertoy et al., 2000). There is no information available on the expression of Cripto-1 in uveal melanoma. The purpose of this study was to investigate the potential of targeting Cripto-1 in the treatment of uveal melanoma. We first determined whether Cripto-1 is expressed uveal melanoma. Moreover, correlation of Cripto-1 expression with tumor aggressiveness was determined.

2. Methods

2.1 Patients and tumors

Thirty-six formalin-fixed, paraffin-embedded enucleated globes from 36 patients with uveal melanoma were available for this study. The eyeballs were from 15 female and 21 male patients with an average age of 41.5 years (range: 9 years to 74 years). The pathological information on the location of the melanomas in the uveal tract, cell types, largest tumor diameter (LTD), and extrascleral extension and the clinical information available were obtained from the ocular oncology files of our institute. There were 18 tumors with no evidence of intrascleral or extrascleral extension based on light microscopy and information obtained from the oncology files of the patients. There were 18 tumors, which had either intrascleral/extrascleral extension. Of these, 6 tumors had metastasized to the liver. The study was reviewed and approved by the local ethics committee at the Vision Research Foundation, Sankara Nethralaya and the committee deemed that it conformed to the generally accepted principles of ethics in research, in accordance with the Helsinki Declaration.

2.2. Cell lines

The uveal melanoma cell lines OCM-1 (spindle cell morphology, non-aggressive type), OCM-8 (epithelioid cell morphology), and 92-1 (spindle morphology, aggressive type), OMM-1 (metastatic uveal melanoma) were used in the study. All the 4 cell lines were initially derived from primary uveal melanomas and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). OCM-8 and 92-1 cells were cultured in RPMI 1640 supplemented with10% FBS and 3 mM L-glutamine at 37 _C in 5% CO2 humified incubator. Primary uveal melanocyte cultures obtained from donor eye balls were used in the study. (The uveal melanoma cell lines and primary uveal melanocyte culture were a generous gift from Dr. Michele C. Madigan Discipline of Clinical Ophthalmology, Save Sight Institute, University of Sydney, Sydney, NSW, Australia).

2.3. Antibody and chemicals

The uveal melanoma tumor samples were examined immunohistochemically for the expression of Cripto-1 protein using the Cripto (V-17) goat polyclonal antibody (Santa Cruz Biotechnology,

Inc.). The antibodies are target specific and exhibit no cross reactivity. The other chemicals are obtained from Dakocytomation (Denmark).

2.4. Immunohistochemistry

Immunostaining of tissue sections was performed using labeled streptavidin by indirect immunoperoxidase technique. Briefly, 4-mm formalin-fixed paraffin sections were used for the study. Tissue sections were then deparaffinised, rehydrated and bleached before immunohistochemical procedure. Antigen retrieval by pressure cooker method using citrate buffer was performed before antibody incubation. Endogenous peroxidase was blocked with hydrogen peroxide (3% H2O2) for 10 min at room temperature. Tissue sections were then rinsed in Tris-buffered saline (pH 7.6) and incubated with primary antibody (1:75 dilution) for 2 h. This was followed by sequential 50-min incubation with biotinylated secondary antibody and streptavidin labeled to horseradish peroxidase (DAKO). Sections were washed with Tris-buffered saline between incubation. The reaction was developed for 20 min using commercially available AEC (3-Amino-9-ethyl carbazole) and counterstained with Harris hematoxylin. Breast carcinoma, which expresses Cripto-1, was used as a positive control. Similarly, cellblocks were made from the primary uveal melanocyte cultures and uveal melanoma cell lines and then studied for Cripto-1 immunohistochemically. For negative control, the primary antibody was omitted, and non-immune serum was used in the immunostaining.

2.5. Evaluation of immunoperoxidase staining

Tissue sections were read independently by two investigators (SK, MK) using light microscopy without knowledge of the results obtained by the other investigator. Furthermore, each investigator read all the slides twice without knowledge of the results obtained in the previous reading. Cripto-1 is known to show cell surface and cytoplasmic staining of cancer cells and no staining of the normal tissues. Specific staining with primary antibody was graded from I to III taking into account both the intensity and extent of positive staining. First, a number from 1 to 3 was given according to staining intensity: Weak (1); moderate (2); and strong (3). Then the number of positive cells per slide was stratified into three groups based on the percentage of positive cells: group I, <33%; group 2, 33-67%; group 3, >67%. Scores ranging from 1 to 9 for specific staining for each case were obtained by multiplying the staining intensity by the number

of the group that represented the percentage of positive cells as previously described (Kommoss et al., 1990). A score of zero represents no specific staining observed, the scores of 1 and 2 were accepted as grade I (1+), 3 and 4 as grade II (2+), and 6 and 9 as grade III (3+).

2.6. Statistics

The Cripto-1 immunoreactivity was correlated with extrascleral extension and cell type of the tumors using non-parametric Mann-Whitney U-test. Values were considered significantly different with p-value of ($p < 0.05$).

3. Results

Fig. 1 shows a spotted graph of percentage of Cripto-1 positive cells in tumors with and without extrascleral extension.

3.1. Cripto-1 reactivity in normal ocular tissues and neoplastic cells

Cripto-1 expression was nucleocytoplasmic in the neoplastic cells. The retinal tissues (Fig. 1A), optic nerve (Fig. 1B), corneal epithelium (Fig. 1C) and choroidal melanocytes (Fig. 1D) were negative for Cripto-1. The ciliary epithelium was positive for Cripto-1.

3.2. Cripto-1 reactivity in primary uveal melanocyte culture and uveal melanoma cell lines

Cytoplasmic expression of Cripto-1 was observed in the 4 uveal melanoma cell lines OCM-1, OCM-8, 92-1 and OMM-1 (Fig. 2D). Primary uveal melanocyte culture did not show Cripto-1 immunoreactivity (Fig. 2C).

3.3. Cripto-1 reactivity in tumors with no intra or extrascleral extension

Among 18 uveal melanomas with no intra or extrascleral extension, 17 (94%) tumors were positive for Cripto-1. Specific staining was graded as (1+) in 11 (64%), as (2+) in 6 (35%) and (3+) in 1 tumors.

3.4. Cripto-1 reactivity in tumors with intrascleral extension/extrascleral extension/liver metastasis

Among 18 tumors with intrascleral extension/extrascleral extension/liver metastasis, 14 tumors (77.7%) were positive for Cripto-1. Specific staining was graded as (2+) in 1 tumor and as (3+) in 17 (94%) tumors. The immunoreactivity of Cripto-1 was uniform throughout the tumor cells. Cripto-1 was positive in the spindle cell melanomas, epithelioid cell melanomas and mixed melanomas. The expression of Cripto-1 was significantly higher in melanomas with extrascleral extension/liver metastasis compared to melanomas with no extension ($p < 0.001$). Fig. 2A shows the Cripto-1 expression in non-invasive spindle cell uveal melanoma. Fig. 2B shows the Cripto-1 expression in uveal melanoma, which had spread to the orbit.

3.5. Correlation of Cripto-1 immunoreactivity with cell type

Among 5 melanomas with spindle cell type, 2 tumors showed group 3+ staining and 3 tumors showed group 2+ staining. Among 6 epithelioid cell type melanomas, 4 tumors showed group 3+ staining and 2 tumors showed group 2+ staining. Among 25 mixed cell type melanomas, 9 tumors showed group 3+ staining and 16 tumors showed group 2+ staining. There was no correlation of the Cripto-1 immunoreactivity with the cell type of the tumors.

4. Discussion

In our study, we observed Cripto-1 reactivity in all the uveal melanomas (Fig. 3). Among the 36 tumors included in the study, Cripto-1 reactivity was observed in 100% (36/36) tumors and in all the melanoma cells. Cripto-1 immunoreactivity was observed in spindle, epithelioid and in mixed cell melanomas. Cripto-1 was positive in all the 4 uveal melanoma cell lines supporting our data obtained from archival paraffin embedded tumor samples. The primary uveal melanocyte culture did not express Cripto-1. The normal non-neoplastic retinal tissue, corneal epithelium, choroidal melanocytes and the optic nerve did not express Cripto-1. However, the ciliary epithelium showed Cripto-1 reactivity. This could be due to the fact that the ciliary epithelium (CE) in the adult mammalian eye harbors a mitotic quiescent population of neural stem cells and hence Cripto-1 expression could be expected (Arnhold et al., 2004). Thus, uveal melanoma joins the list of the tumors that express Cripto-1. The role of Cripto-1 in tumorigenesis

is not clear. However, Cripto-1 activities appear to be essential for tumor growth and/or maintenance. There have been some difficulties in defining the Cripto-1 receptor, but Cripto-1 was found recently to interact with Glypican-1 and indirectly with erbB-4 and fibroblast growth factor receptor-2 to promote activation of cell proliferation, Ras/Raf/Extracellular signal-regulated kinase/ Mitogen-activated protein kinase (MAPK) and cell survival phosphatidylinositol 30-kinase/Akt pathways. In addition, Cripto-1 forms a complex with activin and type II activin receptors and inhibits activin-signaling (Shen, 2003). Activin, similar to transforming growth factor ß, is a potent inhibitor of cell growth in various target tissues. Disruption of activin signaling is associated with carcinogenesis. Thus, most of the uveal melanomas express Cripto-1 protein; it could serve as a target for antibody-based therapies. Cripto-1 has not been studied in other melanocytic tumors and to our knowledge the present study is the first one to investigate the expression of Cripto-1 in uveal melanoma. Earlier, in search of target receptor molecules, Charlotta All- Ericsson et al. (2002) studied the Insulin like growth factor receptor (IGF1R) in uveal melanoma and suggested the possibility of targeting IGF-1R as a form of treatment in patients with uveal melanoma. Later, the same group (Charlotta All- Ericsson et al., 2004) has shown the expression of proto-oncogene c-kit receptor molecule in uveal melanoma and pointed its use as a target for therapy. Although, Cripto is overexpressed in a wide range of epithelial cancers, yet little is known about potential mechanisms by which Cripto-1 expression might enhance tumorigenesis. The role of Cripto-1 in uveal melanoma progression remains to be studied. However, from the findings of our fundamental study, one could target Cripto-1 using anti-Cripto-1 monoclonal antibodies (mAbs), which may interfere with the Cripto-1 function by competing with binding to its receptor, inhibiting Cripto-1-mediated MAPK/Akt pathways, or by releasing the block of activin signaling. Also, RNA interference for Cripto-1 gene should be studied to validate the importance of Cripto-1 in the biology of uveal melanoma. While much more analysis is needed to decode the molecular mechanisms of Cripto-1 function, our finding of the expression of Cripto-1 in uveal melanoma needs to be further studied, as it appears that Cripto-1 could be a unique targeting molecule in uveal melanoma.

Figure 1

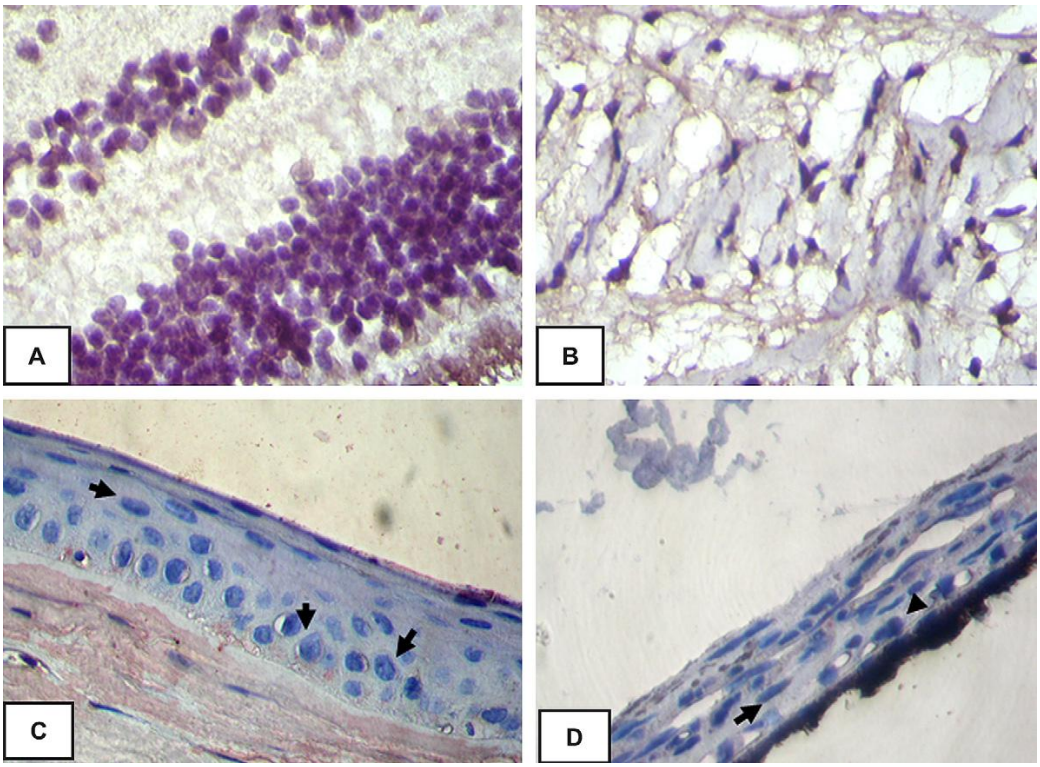

Fig. 1. Cripto Immunoreactivity in non-neoplastic Ocular tissues. A: Photomicrograph showing the negative Cripto-1 expression in retina. (AEC immunostain with hematoxylin counterstain, magnification X100). B: Photomicrograph showing the Cripto-1 negativity in the optic nerve. (DAB immunostain with hematoxylin counterstain, magnification X100). C: Photomicrograph showing the negative Cripto-1 expression in corneal epithelium (arrow shows negative cells) of a nonneoplastic donor eyeball (AEC immunostain with hematoxylin counterstain, magnification X100). D: Photomicrograph showing the negative Cripto-1 expression in choroidal melanocytes (arrow shows negative cells) (DAB immunostain with hematoxylin counterstain, magnification X100).

Figure 2

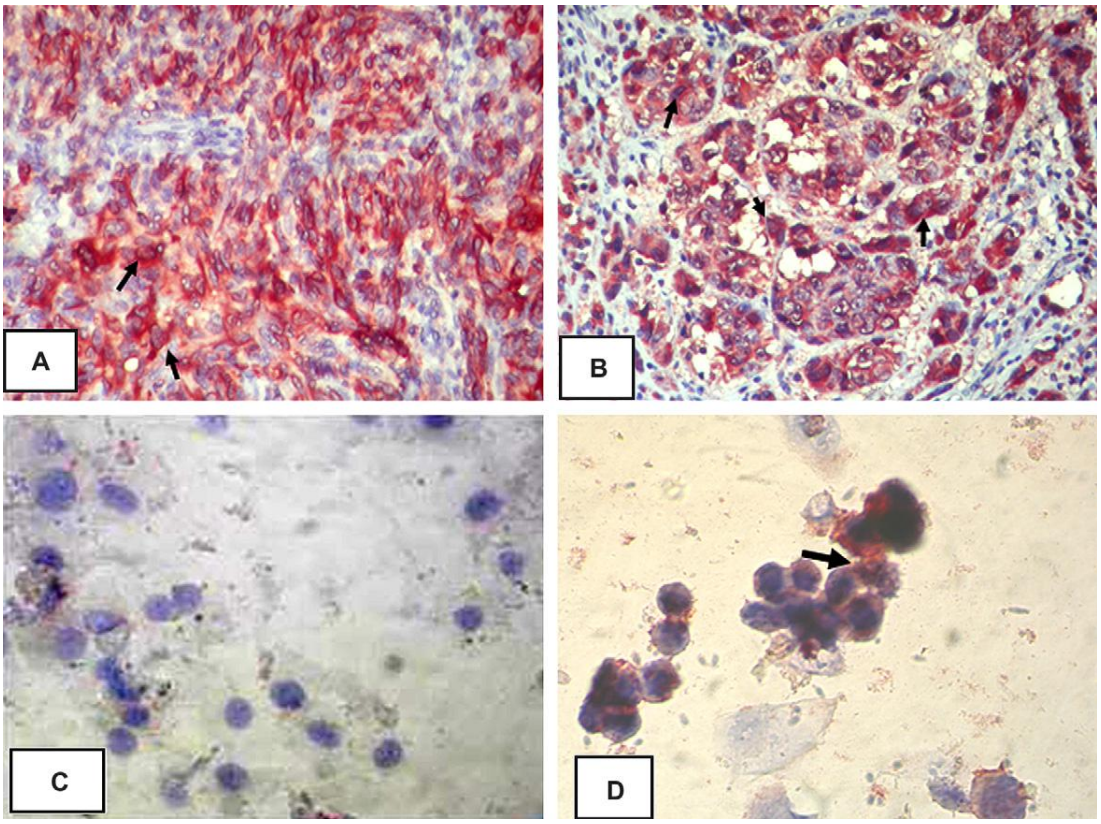

Fig. 2. Cripto immunoreactivity in uveal melanomas. A: Photomicrograph showing the Cripto-1 expression with strong intensity (group 3+) (arrow shows positivity) in non-invasive spindle cell uveal melanoma. (AEC immunostain with hematoxylin counterstain, magnification X40). B: Photomicrograph showing the Cripto-1 expression with strong intensity (group 3+) (arrow shows positivity) in uveal melanoma, which had spread to the orbit. (AEC immunostain with hematoxylin counterstain, magnification X40). C: Photomicrograph showing the negative Cripto-1 expression in primary uveal melanocyte culture cells. (AEC immunostain with hematoxylin counterstain, magnification X40). D: Photomicrograph showing the positive Cripto-1 expression (arrow shows positivity) in metastatic uveal melanoma cell line (OMM1). (AEC immunostain with hematoxylin counterstain, magnification X40).

Figure 3

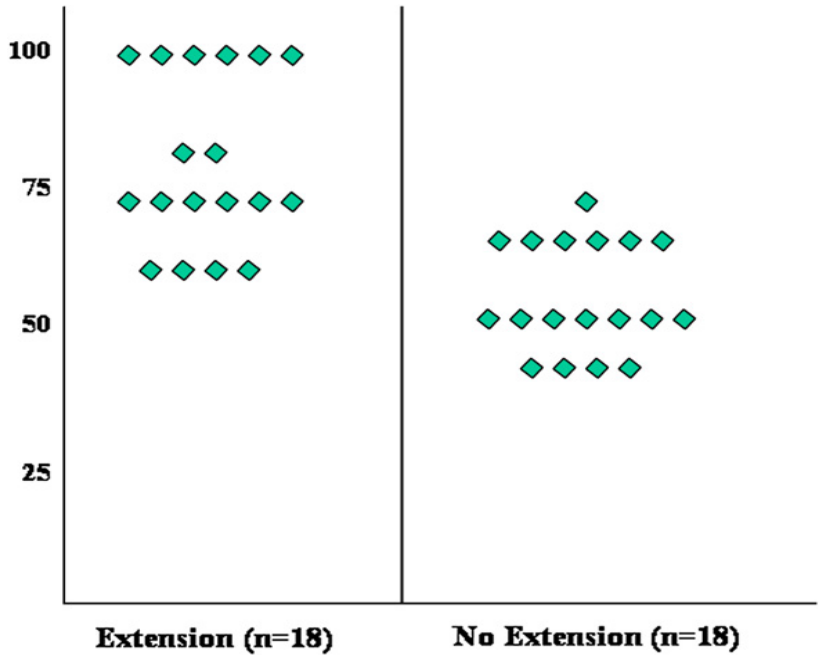

Fig. 3. Spotted Graph: Dot Plot of the percentage of Cripto-positive cells in non-aggressive and aggressive uveal melanoma.

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Cripto-1 expression in uveal melanoma: An immunohistochemical study

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Abstract

Human Cripto, the founder member of the epidermal growth factor-Cripto-FRL1-Cryptic (EGF-CFC) family, plays an important role during early embryonic development and in particular in carcinogenesis and the development of cancer metastases. Cripto-1 is over-expressed in most cancers, but is absent or only weakly expressed in normal cells. For this reason, Cripto-1 could be of potential value in the targeted treatment. There is no information on the expression of Cripto-1 in human uveal melanoma. Cripto-1 reactivity was evaluated by immunohistochemistry on 36 archival uveal melanomas using the polyclonal antibody to Cripto-1. The tumors were divided in to 2 groups. There were 18 uveal melanomas with no intrascleral or extrascleral extension and 18 uveal melanomas with intrascleral/extrascleral extension/liver metastasis. Cripto-1 reactivity was correlated with tumor aggressiveness and cell type. Furthermore, we studied the immunolocalization of Cripto-1 in 4 uveal melanoma cell lines OCM-1, OCM-8, and 92-1, and OMM-1 and in 2 primary uveal melanocyte cultures. Cripto-1 was expressed in both the non-invasive and aggressive uveal melanomas. Cripto-1 was positive in the 4 uveal melanoma cell lines and absent in the primary uveal melanocyte cultures. Retinal tissue did not express Cripto-1. The results suggest that Cripto-1 is expressed in uveal melanoma, negative in the non-neoplastic ocular tissue and point to its use as a target for therapy.

CONCLUSIONS FROM PART-II THESIS

The second part of this thesis was to investigate the expression of growth factor receptors and to identify the target molecule that would help in the clinical management of uveal melanoma. This study has significantly contributed to the understanding of uveal melanoma biology. We have observed that tumors from patients who had liver metastasis expressed higher c-met (receptor for HGF) and IGF-1R receptors (receptor for IGF). In contrast, EGFR receptors are less frequently expressed in uveal melanoma tumors and the molecule that was expected to play a role in cell motility, Ezrin was also sinficantly correlated with tumors liver metastasis. This clearly indicates and gives us an idea that the metastatic tumors have active signaling pathways mediated by ligand-receptor complexes especially IGF-IGF-1R and HGF-c-met complexes. It is well known that liver is the source for IGF and HGF and this facilitates the tumor cells to migrate specifically to liver through hematogenous route. Activation of IGF-IR has been shown to play a role in cellular proliferation, protection from apoptosis, migration, integrin mediated adhesion to the extracellular matrix, and invasion of basement membranes. Hence specific inhibitors to block c-met and IGF-1R should be considered for clinical trial assessment with long term follow up of the patients.

SPECIFIC CONTRIBUTIONS AND FUTURE SCOPE OF WORK:

In the second part of the thesis, we attempted to understand the biology of uveal melanoma. It is required because, about 50% patients with uveal melanoma die of liver metastasis. Uveal melanomas are chemoresistant and radioresistant. Hence, there is a need to understand its biology and to identify target molecules which can be targeted to inhibit the pathways that contribute to uveal melanoma progression. This study has revealed significant correlation of higher expression of IGF-1R and c-met with uveal melanoma with liver metastasis. Also the transcription factors which are downstream to several growth factor mediated pathways (figure 1) were also highly expressed in melanoma tumors and there was a significant correlation with some of the clinicopathological risk factors of uveal melanoma. Another important information revealed from this study is that CRIPTO is highly expressed in uveal melanoma tumors whereas absent in normal uveal melanocytes. Hence Cripto could be a promising target molecule that can be used in uveal melanoma treatment. Blocking crypto will inhibit the MAPK pathways which are involved in melanoma tumor progression.

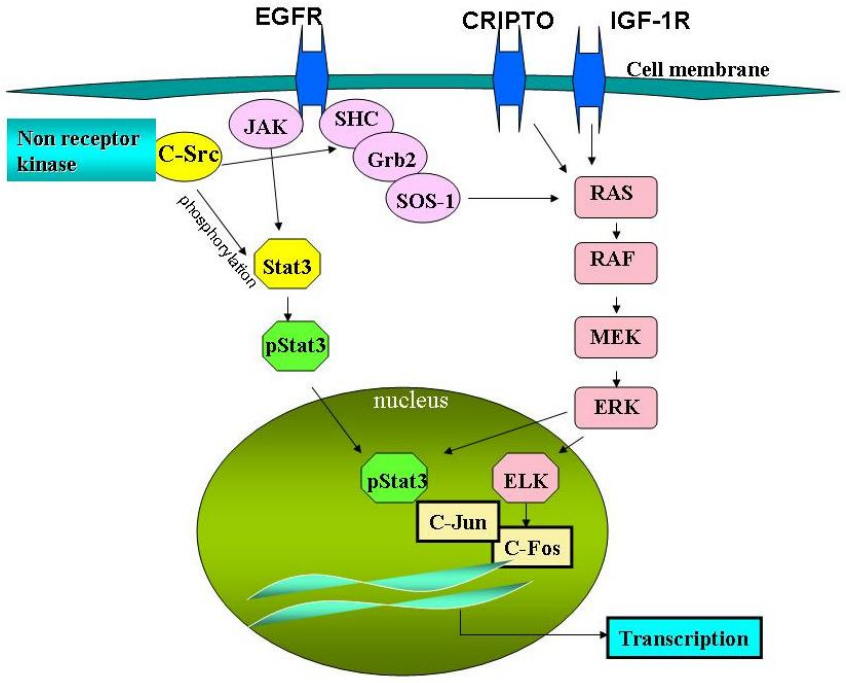

Figure 1: The illustrative pathway shows the Interaction of growth factor receptors with cytoplasmic proteins and transcription factors in the nucleus to promote proliferation and invasion of uveal melanoma tumors.

Targeting of IGF-1R mediated pathways by using a specific inhibitor of IGF-IR tyrosine phosphorylation called cyclolignan picropodophyllin was shown to cause tumor regression in a xenograft mouse model (Girnita et al.,2006). Targeting this receptor pathway may be a good choice for preventing or at least decreasing the chance of metastasis in patients. Although treatment of the primary tumor is well established and usually results in local control, treating metastatic disease is a much more daunting task.

Clinical trials assessing potential therapeutic agents, such as dacarbazine, treosulfan in combination with gemcitabine, thalidomide with IFN a2b, temozolomide, and 9-nitrocamtothecin, have also been similarly disappointing with only minor improvements in some patients (O'Neill et al., 2006). It seems unlikely, from the evidence given, that targeting only one of these molecular pathways would be sufficient to improve patient mortality. It is far more likely that it will take a combined approach, targeting at least two of these pathways that may induce liver-specific metastasis, to lead to an improvement in patient mortality rates. Several inhibitors of these specific pathways are in development, with a handful already in phase II clinical trials for other diseases. Table 1 shows the status of several of these molecules that are specific for the pathways that have been discussed above, including inhibitors targeting c-Met, IGF-IR, phosphatidylinositol 3- kinase, Akt. It would be of great importance to test these molecular targets in the context of uveal melanoma and study the effects that they would have not only on liver metastasis but also on hematogenous dissemination of tumor cells.

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Publications, Presentations and Awards:

Publications:

1. **Mallikarjuna K**, Moutushy M, Biswas J, Krishnakumar S. Molecular Pathology of Retinoblastoma. *Middle East Afr J Ophthalmol. 2010*; 17:217-23. Review
2. Madhavan J, **Mallikarjuna K**, Vikas K, George R, Bremner R, Kumaramanickavel G. CDKN1C (p57KIP2) mRNA expression in human retinoblastomas. Ophthalmic Genet. 2010;31:141-6
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Presentations and Awards

1. Received travel fellowship at IERG (Indian Eye Research Group) conference held in L V Prasad Eye Institute, Hyderabad in 2005

   Abstract: Mallikarjuna K, Vaijayanthi P, Gopal L, Krishnakumar S. Molecular pathways in Uveal Melanoma

2. Received travel fellowship at IERG (Indian Eye Research Group) conference held in L V Prasad Eye Institute, Hyderabad in 2006

   Abstract: **K Mallikarjuna,** Mohan A, , Ramkumar HL, Gopal L, Krishnakumar S. Stem cell markers: ABCG2 and MCM2 expression in retinoblastoma.

3. **Current Trends in Proteomics and Brainstorming Discussion on Plasma Proteomics**

   Title: Proteomics in Retinoblastoma: A pilot study

4. **Best Scientific Paper Award** at ASIA ARVO (Association for Research in Vision and Ophthalmology), Hyderabad, 2009. Abstract title: Comparative proteomics analysis of differentially expressed proteins in primary retinoblastoma tumors.
5. **International travel award** from Department of Biotechnology (DBT), Govt. of India to attend US ARVO, 2008 for abstract presentation on 'proteomics of retinoblastoma'
6. **International travel award** from Department of Science and Technology (DST), Govt. of India to attend HUPO congress 2008. for abstract presentation on 'proteomics of retinoblastoma'
7. **International travel award** from Centre for Co-operation in Science & Technology Among Developing Societies (**CCSTDS**), Chennai for attending HUPO congress 2008. for abstract presentation on 'proteomics of retinoblastoma'

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Research: Proteomic profiling of retinoblastoma

2006-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 the molecular mechanisms involved in intraocular tumor progression.

2005-2006: Senior Research Fellow, ICMR, Department of Ocular Pathology, Sankara Nethralaya; PI: Dr S Krishnakumar.
Project: To study the expression of Signal transduction molecules (growth factors and receptors) in uveal melanoma primary tumors.

2004-2005: Junior Scientist, Clinical Laboratory, Medical Research Foundation, Sankara Nethralaya; **Supervision: Dr S B Vasanthi, Director of Lab services and Dr S Krishnakumar.**

Publications

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12. **Mallikarjuna K**, Pushparaj V, Biswas J, Krishnakumar S. Expression of epidermal growth factor receptor, ezrin, hepatocyte growth factor, and c-Met in uveal melanoma: an immunohistochemical study. *Curr Eye Res*. 2007 Mar;32(3):281-90.

13. **Mallikarjuna K**, Vaijayanthi P, Krishnakumar S. Cripto-1 expression in uveal melanoma: an immunohistochemical study. *Exp Eye Res*. 2007 Jun;84(6):1060-6. Epub 2007 Feb 7.

14. 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.

15. Krishnakumar S, Mohan A, **Mallikarjuna K**, Ramkumar HL, Venkatesan N, Das RR. SRPK1: a cisplatin sensitive protein expressed in retinoblastoma. *Pediatr Blood Cancer*. 2008 Feb;50(2):402-6.

16. **Mallikarjuna K**, Pushparaj V, Biswas J, Krishnakumar S. Expression of insulin-like growth factor receptor (IGF-1R), c-Fos, and c-Jun in uveal melanoma: an immunohistochemical study. *Curr Eye Res*. 2006 Oct;31(10):875-83.

17. Adithi M, Venkatesan N, **Mallikarjuna K**, Biswas J, Krishnakumar S. Expressions of Rac1, Tiam1 and Cdc42 in retinoblastoma. *Exp Eye Res*. 2006 Dec;83(6):1446-52. Epub 2006 Oct 5.

18. Adithi M, **Mallikarjuna K**, Ramkumar HL, Subramanian A, Venkatesan N,Krishnakumar S. Retinoblastoma: expression of HLA-G. *Ocul Immunol Inflamm*. 2006 Aug;14(4):207-13.

19. 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. Epub 2006 May 22.

20. 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.

21. Krishnakumar S, **Mallikarjuna K**, Mohan A, Iyer A, Venkatesan N, Biswas J, Shanmugam MP. Expression of Fas ligand in retinoblastoma. *Cancer*. 2004 Oct 1;101(7):1672-6.

22. 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.

23. Mohan A, **Mallikarjuna K**, Ramkumar HL, Gopal L, Krishnakumar S. Stem cell markers: ABCG2 and MCM2 expression in retinoblastoma. *Br J Ophthalmol.* 2006 Jul;90(7):889-93.

Presentations and Awards

1. Received travel fellowship at IERG (Indian Eye Research Group) conference held in L V Prasad Eye Institute, Hyderabad in 2005

   Abstract: Mallikarjuna K, Vaijayanthi P, Gopal L, Krishnakumar S. Molecular pathways in Uveal Melanoma

2. Received travel fellowship at IERG (Indian Eye Research Group) conference held in L V Prasad Eye Institute, Hyderabad in 2006

   Abstract: **K Mallikarjuna,** Mohan A, , Ramkumar HL, Gopal L, Krishnakumar S. Stem cell markers: ABCG2 and MCM2 expression in retinoblastoma.

3. **Current Trends in Proteomics and Brainstorming Discussion on Plasma Proteomics**

   Title: Proteomics in Retinoblastoma: A pilot study

4. **Best Scientific Paper Award** at ASIA ARVO (Association for Research in Vision and Ophthalmology), Hyderabad, 2009. Abstract title: Comparative proteomics analysis of differentially expressed proteins in primary retinoblastoma tumors.
5. **International travel award** from Department of Biotechnology (DBT), Govt. of India to attend US ARVO, 2008 for abstract presentation on ‘proteomics of retinoblastoma’
6. **International travel award** from Department of Science and Technology (DST), Govt. of India to attend HUPO congress 2008. for abstract presentation on ‘proteomics of retinoblastoma’
7. **International travel award** from Centre for Co-operation in Science & Technology Among Developing Societies (**CCSTDS**), Chennai for attending HUPO congress 2008. for abstract presentation on ‘proteomics of retinoblastoma’

BIO-DATA OF THE SUPERVISOR

Name: Dr. S.Krishnakumar, MD
Designation: Professor and HOD

Department/Institute/University: L& T Ocular Pathology Department,

In charge Stem cell Laboratory; & HOD Department of Nanobiotechnology

Vision Research Foundation, Sankara Nethralaya, 18, College Road, Chennai, 600 006, Tamil Nadu, India; Phone: 91-44-28271616, Fax: 91-44-28254180,

Email: drkk@snmail.org and dockris_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	Kilpauk 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, Hematology, clinical pathology and 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
	Retinoblastoma Center			

6	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. Department of Pharmaceutical Scien ces 986025 Nebraska Medical Center,O maha, NE 68198-6025	Visiting Scholar	2005	Nanoparticles and PLGA Scaffold for 3D growth of tumor cells
9	UCSF Ocular Oncology Lab (Joan M. O'Brien, PI) Koret Vision Research Center,10 Koret Way, K240 San Francisco, CA 94143	Visiting Scholar	2005	Protein truncation tests for the diagnostics in Retinoblastoma
10	Dr Uday Kompella's Lab Durham's Research Center Nebraska school of Medicine Omaha, USA	Visiting Scholar 3 months ICMR Young Scientist award	2006	Ocular Drug delivery
11	Prof Kannan Raghuraman and Prof Kattesk Katti Lab, University of Missouri, Columbia, USA by Department of Bio-Technology,	DBT Associate and 6 months fellowship in cutting edge technology	2006-2007	Cancer Nanotechnology and targeted therapy

A. Position and Honors

Position and Employment (Starting with the most recent employment)

Sl No	Institution Place	Position	From (Date)	To (date)
1	Vision Research Foundation, Sankara Nethralaya	Post Doc Fellow	April 1998	March 1999
2		Assistant Pathologist and Lecturer	April 1999	March 2005
3		Reader and Pathologist	April 2005	January 2008
4		Professor and Pathologist In charge Stem Cell Laboratory and HOD Nanobiotechnology	January 2008	Till date

A. Honors/Awards

Year	Awards
1994	University first in Ophthalmology in MBBS securing 95%
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	ICMR Young Biomedical Scientist
2007	DBT fellowship for nanotechnology and DBT associate

Professional Experience and Training relevant to the Project

B. Publications (Numbers only) 70

Books : 1 Chapters: 2; Research Papers: 62 Reports : 8 General articles.....

Patents : ...nil.....Others (Please specify.....

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

1. Krishnakumar S, Khetan V, Shanmugam MP, Biswas J. Multi drug resistance protein in retinoblastoma. Br j ophthalmol 2004; 88:1521- 1526.
2. Adithi M, Nalini V, Kandalam M, Krishnakumar S. Expression of p63 and p73 in retinoblastoma: A clinicopathological correlation study. Exp Eye Res. 2008 Jun 21. [Epub ahead of print]
3. Coral K, Narayanasamy A, Madhavan J, Bharathselvi M, Ramakrishnan S, Nandi K, Rishi P, Kasinathan N, Krishnakumar S. Lysyl oxidase (LOX) activity in the ocular tissues and the role of LOX in proliferative diabetic retinopathy and rhegmatogenous retinal detachment. Invest Ophthalmol Vis Sci. 2008 Jun 19.
4. Sahoo SK, Dilnawaz F, Krishnakumar S. Nanotechnology in ocular drug delivery. Drug Discov Today. 2008 Feb; 13(3-4):144-51. Review.]
5. Adithi M, Nalini V, Kandalam M, Krishnakumar S. Isolation of Human Papilloma DNA Virus from human retinoblastoma tumor samples from Asian Indian Children: A Pilot study. [Accepted in Journal of Pediatric Hematology and Oncology]
6. 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. Epub 2007 Jan 9.
7. 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. Epub 2006 Oct 5
8. 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.
9. Krishnakumar S, Abhyankar D, Lakshmi SA, Pushparaj V, Shanmugam MP, Biswas J. HLA expression in choroidal melanomas: correlation with clinicopathological features. Curr Eye Res. 2004 Jun;28(6):409-16.
10. 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

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

Corneal Stem cells

- Sudha B, Sitalakshmi G, Iyer GK, Krishnakumar S. Putative stem cell markers in limbal epithelial cells cultured on intact & denuded human amniotic membrane. Indian J Med Res. 2008 Aug;128(2):149-56
- Sudha B. Sasirekha K. Sreelatha, Krishnakumar sSignal Transduction Pathway Involved in the Ex-vivo expansion of limbal epithelial cells cultured on various substrates.Indian journal Medical research, accepted for publication
- H. N. Madhavan, B. Sudha, G. Sitalakshmi, S. KrishnaKumar, Y. Mori, H. Yoshioka, and S. Abraham. **Comparative Study on Growth Characteristics of Cadaveric Human Corneal Limbal Stem Cells in Mebiol Gel (a Synthetic Polymer) and on Human Amniotic Membrane.** Invest. Ophthalmol. Vis. Sci. 2006 47: E-Abstract 3033

Retinal Stem cells

- Retinal Stem/Progenitor Properties of Ciliary Epithelial and Iris pigment Epithelial Cells From the Adult Human Cadaveric Eye. S. Jasty, S. Krishnakumar, P. Gunisha, N. Archana, G. Suganeswari, and L. Gopal. Invest. Ophthalmol. Vis. Sci. 2008 49: E-Abstract 5030.

Epigenetic mechanisms in retinoblastoma

- Moutushy Mitra**,** Mallikarjuna Kandalam, Krishnakumar Subramanian. Methylation status of EpCAM in y79 retinoblastoma cell line: Proteomic analysis shows modification of various other proteins. **HGM2008** Workshop Abstracts, 5. Disease Proteomics, Presentation 32

Tissue engineering

- G. Sitalakshmi, B. Sudha H.N. Madhavan S. Vinay, S. Krishnakumar, Yuichi Mori, Yoshioka Hiroshi, and Abraham Samuel . Ex Vivo Cultivation of Corneal Limbal Epithelial Cells in a Thermoreversible Polymer (Mebiol Gel) and Their Transplantation in Rabbits—An Animal Model TISSUE ENGINEERING: Part A Volume 14, Number 00, 2008

Retinoblastoma and targeted therapy

- M. Kandalam, S. C. S. Sundaram, Y. Sharma, P. R. Deepa, K. Vikas, T. Sharma, J. Biswas, L. Gopal, Krishna kumar, Comparative Proteomic analysis of differentially expressed proteins in primary Retinoblastoma: Proteomics Clin. Appl. 2010 (In press)

- 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.

Nanotechnology

Sahoo SK, Dilnawaz F, Krishnakumar S. Nanotechnology in ocular drug delivery. Drug Discov Today. 2008 Feb;13(3-4):144-51

Sara Baratchi[1], Rupinder Kaur Kanwar[1], Khashayar Khoshmanesh[2], Punj Vasu[3], Chauhan Ashok[4], Matta Hittu[3], Andrew. Parratt[1], Krishnakumar S[5] , Xu Sun[6] and Jagat Rakesh Kanwar[1*] Drug Nano-Shuttles to Cross the Blood Brain Barrier: Promises of Nanotechnology for Drug Delivery to the Brain". Accpeted in Current Nanoscience

Research Support
Ongoing or completed last three years: Past

Sl No.	Title of Project	Funding Agency	Date of sanction and Duration
1.	Prognostic markers in uveal melanoma (Co-PI)	ICMR	1st January 2001 – 31st January 2001
2.	To study the expression of oxidative stress in human corneal diseases by immunohistochemistry (PI)	ICMR	1st January 2004 – 31st December 2004
3.	Role of Tetraspanin, p53 family proteins, Rac 1-Tiam 1 signal transduction, Cadherins, Catenins, Proteases and its inhibitors in Retinoblastoma and correlation with aggression (PI)	ICMR Ref No: 5/4/6/12/2003-NCD II & Iris code: 2002-03970)	January 2004 – December 2005 (2 years)
4.	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 (PI)	ICMR (5/4/6/3/03-NCD-II)	January 2005 – December 2005 (1 year)

Present: ICMR

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
1	To understand the Biology of Corneal Stem cells (PI)	ICMR	Rs.47,228/- For 3 years	August 2005– August 2009
2	To study the modulation of multidrug resistant proteins expression, function and in vitro antitumor effect on Y79 retinoblastoma cells by curcumin longa derivative (PI)	ICMR	Rs.47,300/- for 3 years	1st November 2006 – 30th October 2009
3	To study the effect of FAS inhibitors on Y79 cell line (Co-PI)	ICMR	Rs.42,257.00/- for 3 years	March 2007 – February 2010

Department of Biotechnology (DBT)

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
1	Proteomic profiling for Retinoblastoma progression: (PI)	DBT	$72,334.00/- for 3 years	1st March 2007 – February 2010
2.	Efficacy of a novel adhesion molecule -conjugated drug loaded biodegradable nanoparticles for drug therapy in Retinoblastoma (Co-PI)	DBT	$238,627.00/- for 3 years	1st August 2007 – July 2010
	To identify and characterize the retinal stem/progenitor			

3.	properties of Human Iris Pigment Epithelial (IPE) cells and Ciliary Epithelial (CE) cells in vitro (PI)	DBT	$173,999.00/ for 3 years	5th June 2007 – May 2010

Department of Science and Technology (DST)

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
1	To study the effect of small interfering RNA (siRNA) on the novel adhesion molecule expression of Y 79 retinoblastoma cell line and its correlation with invasion and migration properties of cell line (PI)	DST	$62,142.00/- for 3 years	5th February 2007 - 4th January 2010
2.	Developing Molecularly Targeted Polymer nanoparticles for cancer therapeutics and diagnostics (Co-PI)	DST	$298,284.00/ for 3 years	September 2007- October 2010 [approved]
3.	Identification of monosomy 3 in choroidal melanoma by chromosome in situ hybridization and correlate clinicopathologically (PI)	DST	$23,614.00/ for 1 year	completed
4	Fabrication of cross linked substrates for culture of corneal stem cells [co-pi]	DST	$72,640.00/ for 3 years	On going
5	Fabrication of Self assembling Peptide Nanofiber Scaffolds for retinal stem cells	DST		Approved from Indian side
6	Phase 1 Clinical Trial: Subconjunctival Carboplatin in Fibrin Sealant in patients with advanced Retinoblastoma	DST		Favorable comments

Vision Research Foundation (VRF)

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
1	To study the presence of Human Papilloma Virus 16 and 18 Genome in Retinoblastoma from Indian Children with Sporadic RB	VRF, Chennai	$12,428.00 for 3 years	August 2004 – August 2007 Completed

United Kingdom (UK)

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
1.	To study the expression of HMGA2 in retinoblastoma [PI}	Collaborative project with Santa Jeremy On PhD FAAAAI	$1988.00/ for 8 months	1.12.2006 – 31.8.2006 Completed

Completed Research Projects - 6

List of students under his Supervision

Registered Ph.D. guided in Birla Institute of Technology and Science, Pilani, Rajasthan, India and Shanmugha Arts, Science, Technology and Research Academy, (SASTRA) Thanjavur, India.

PhD. Thesis Submitted:- 2 students
Presently enrolled:- 7 students.