GENOMIC CHARACTERIZATION OF RECURRENT CHROMOSOMAL ABERRATIONS IN RETINOBLASTOMA

by

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Abstract

Retinoblastoma is the second most common childhood intraocular malignancy and development of the tumour is initiated by bi-allelic loss of the \textit{RB1} gene. Bi-allelic \textit{RB1} loss may not always directly lead to malignant retinoblastoma. Subsequent mutational events, specifically gain and loss of chromosomal regions harbouring key oncogenes and tumour suppressor genes, appear to underlie the progression of precursor lesions such as retinoma to retinoblastoma. Research thus far has implicated several cytogenetic aberrations in this sequence of molecular events, the two most recurrent and notable being the augmented copy number status of \textit{KIF14} and \textit{MDM4} on 1q and of \textit{DEK} and \textit{E2F3} on 6p. A small subset of retinoblastoma exhibits high-level amplification of the \textit{MYCN} gene on chromosome 2p and is characterized by pRb expression and aggressive histology. 1p36 deletion is also associated with \textit{MYCN} amplification in neuroblastoma. Our project is testing the hypothesis that 1q and 6p gain and 2p amplification – defined by extra copies of \textit{KIF14} and \textit{MDM4}, \textit{DEK} and \textit{E2F3} and \textit{MYCN} respectively, and 1p36 deletion – are biomarkers of retinoblastoma progression. This study reports the results of formalin-fixed paraffin-embedded fluorescence in situ hybridization (FFPE-FISH) analysis of the aforementioned genes in two pre-constructed tissue microarrays (TMAs) comprising 270 retinoblastoma patient tumours. Results show 1q gain (3-10 copies) in 136/262 (52%), 6p gain in 127/262 (48.7%), \textit{MYCN} gain in 18/265 (6.8%) and \textit{MYCN} amplification (>10 copies) in 20/265 (7.5%) patient tumours. \textit{MYCN} amplification was also observed in a sample which retained pRb protein expression. Additionally we have demonstrated statistically significant associations between 1q and 6p gain and \textit{MYCN} amplification and 1p36 deletion. The large cohort and consequent statistical power of our results has enabled a better understanding of the M3 to Mn sequence of genomic aberrations in the progression from retinoma to retinoblastoma. Statistically significant associations between 1q and 6p, indicates synergy within these two regions of gain
that would be particularly beneficial to tumours. Owing to the biological interactions among the proteins encoded by *DEK*, *E2F3*, *KIF14* and *MDM4*, tumour cells manifesting both 1q and 6p aberrations would have a selective survival and proliferative advantage.
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<th>Full Form</th>
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<tr>
<td>aCGH</td>
<td>array-based Comparative genomic hybridization</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AOF1</td>
<td>Amine oxidase (flavin containing) domain 1</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine 5’-triphosphatase</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>CAN</td>
<td>Alias of NUP214</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>CEP</td>
<td>Centromere</td>
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<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase 2</td>
</tr>
<tr>
<td>CRI</td>
<td>Cancer related inflammation</td>
</tr>
<tr>
<td>CyclD</td>
<td>Cyclin D</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-Phenylindole</td>
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<tr>
<td>DDX1</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 1</td>
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<td>DEK</td>
<td>DEK oncogene (DNA binding)</td>
</tr>
<tr>
<td>DM</td>
<td>Double minute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside 5’-triphosphate</td>
</tr>
<tr>
<td>E2F3</td>
<td>E2 transcription factor 3</td>
</tr>
<tr>
<td>EBRT</td>
<td>External beam radiation therapy</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed Paraffin embedded</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HLH</td>
<td>Helix loop helix</td>
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<tr>
<td>HSR</td>
<td>Homogenously staining region</td>
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<tr>
<td>ICRB</td>
<td>International classification of retinoblastoma</td>
</tr>
<tr>
<td>ID2</td>
<td>Inhibitor of DNA binding 2</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<td>KIF</td>
<td>Kinesin family member</td>
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<td>KIF14</td>
<td>Kinesin family member 14</td>
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<tr>
<td>KLP38B</td>
<td>Kinesin-like protein at 38B</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse double-minute 2</td>
</tr>
<tr>
<td>MDM4</td>
<td>Mouse double-minute 4</td>
</tr>
<tr>
<td>MGMT</td>
<td>O-6-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MIM</td>
<td>Mendelian inheritance in man</td>
</tr>
<tr>
<td>MiRNA</td>
<td>Micro-ribose nucleic acid</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MRG</td>
<td>Minimal region of gain</td>
</tr>
<tr>
<td>MRL</td>
<td>Minimal region of loss</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribose nucleic acid</td>
</tr>
<tr>
<td>Myc</td>
<td>v-myb-myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>MYCN</td>
<td>v-myc-myelocytomatosis viral related oncogene, neuroblastoma derived</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NHLRC1</td>
<td>NHL repeat containing 1 gene</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non small-cell lung cancer</td>
</tr>
<tr>
<td>NUP153</td>
<td>Nucleoporin 153kDa</td>
</tr>
<tr>
<td>NUP214</td>
<td>Nucleoporin 214kDa</td>
</tr>
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<td>ON</td>
<td>Ontario</td>
</tr>
<tr>
<td>PARP1</td>
<td>Polyadenosine ribose polymerase 1</td>
</tr>
<tr>
<td>PASW</td>
<td>Predictive analytic software</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>E2F</td>
<td>E2 transcription</td>
</tr>
<tr>
<td>PTM</td>
<td>Posttranslational modification</td>
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<tr>
<td>QM-PCR</td>
<td>Quantitative multiplex polymerase chain reaction</td>
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<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma tumor suppressor gene</td>
</tr>
<tr>
<td>RBKIN</td>
<td>Retinoblastoma kinesin (KIF13A)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute culture medium</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>SAF</td>
<td>Scaffold attachment factor</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMI</td>
<td>Small molecule inhibitor</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride - sodium citrate buffer</td>
</tr>
<tr>
<td>STS</td>
<td>Sequence tagged site</td>
</tr>
<tr>
<td>TCAG</td>
<td>Toronto Centre for Applied Genomics</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
</tr>
<tr>
<td>TPMT</td>
<td>Thiopurine-S-methyl transferase</td>
</tr>
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</table>
Chapter 1

Introduction

1.1 General introduction to retinoblastoma

1.1a Retinoblastoma occurrence and incidence

Retinoblastoma (MIM# 180200) is the second most common ocular childhood cancer with an incidence of 1:14,000 to 1:22,000 live births\(^1\). This rare paediatric malignancy is often described as a ‘cancer of the infant retina’ because of the tumour’s propensity to develop before or shortly after birth. The disease manifests itself in one eye (unilateral, two-thirds of cases) or in both eyes (bilateral)\(^2\). In rare instances (4%), patients exhibit an additional pinealoblastoma or supra/parasellar tumour known as trilateral retinoblastoma\(^3,4\), and this is most often associated with bilateral disease\(^5\).

Retinoblastoma is considerably more prevalent in developing countries with a mortality rate of 20%, 39% and 70% in Latin America, Asia (Japan excluded) and Africa, respectively. This is largely due to delayed detection of the tumour and a consequently worse prognosis. Retinoblastoma is the cause of approximately 3001-3376 cancer-related childhood deaths worldwide\(^6\).

As with most cancers, retinoblastoma can either be sporadic or familial. Forty percent of affected individuals have a family history of retinoblastoma and are more likely to develop bilateral disease and multifocal tumours. They are also at a greater risk for developing secondary malignancies, with osteosarcomas being the most common\(^7\). Sporadic retinoblastoma is noted in 60% of all retinoblastoma and the tumours are monofocal and confined to one eye in the majority of these cases. Individuals with unilateral tumours without a family history usually (85%) have
nonheritable retinoblastoma, but in rare cases (15%), they may have retinoblastoma that can be inherited.

1.1b Diagnosis and treatment of retinoblastoma

Retinoblastoma diagnosis is reliant on tumour visibility under certain light conditions as a white reflection in the pupil also known as ‘leukocoria’. While patients at this stage might be able to retain vision, the tumour is usually malignant and difficult to treat. Advancement in retinoblastoma genetics has paved the way for highly sensitive detection of RB1 loss or mutation as early as the foetal stage by using allele-specific PCR assays. This has spawned the inclusion of genotyping, genetic counselling and reproductive planning in retinoblastoma diagnosis and prevention.

Retinoblastoma treatment is largely determined by the age at diagnosis, histopathological characteristics of the presenting tumour and laterality. Initially, the Reese-Ellsworth classification system was adopted when surgery and external beam radiation therapy (EBRT) were the only treatment options. Since then, the international classification of retinoblastoma (ICRB) has become the gold standard and is used to stratify patients based on their suitability for newer therapies. The disease is stratified into groups A, B, C, D and E depending on tumour size, location and extravasation into the vitreous, optic nerve or subretinal space. Treatment regimens encompass different types of chemotherapy, radiotherapy, thermotherapy, cryotherapy and occasional surveillance. In general, eyes classified as group A are treated with cryotherapy or laser photocoagulation, those classified as groups B or C usually receive chemoreduction and eyes classified as group D are treated with either chemoreduction or enucleation depending on whether the condition is unilateral or bilateral. Retinoblastoma is fatal if the tumour progresses along the optic nerve and affects the central nervous system. Hence, enucleation or
surgical removal of the cancerous eye is the preferred treatment of choice with a 94% cure rate in North America. Although localized or smaller tumours that are diagnosed in a timely manner can be treated by radiation or cryotherapy, severe side-effects, including the development of secondary malignancies are often the sequela of radiation treatment in paediatric patients. Recently, there has also been considerable interest in the investigation of adenoviral vectors as tumour-specific cytotoxic agents in retinoblastoma treatment. The dual objectives of early detection and elegant treatment have spurred the search for reliable biomarkers and therapeutic targets in the clinical management of retinoblastoma.

1.1c Retinoblastoma genetics and pRb loss

As early as the 1970s it was discovered by Alfred Knudson that two mutational events, termed M1 and M2, initiate the development of retinoblastoma tumours. These events were later defined as the bi-allelic inactivation of a tumour suppressor gene, subsequently identified and cloned as the retinoblastoma-1 (RB1) gene on chromosome 13q14.2. The first mutation occurs in a single RB1 allele and runs the gamut from large deletions to single nucleotide changes. The second mutational event is typically loss of heterozygosity (LOH) resulting in two mutant RB1 alleles, either by mitotic recombination or non-disjunction followed by reduplication. Individuals that inherit the first germline mutation are acutely vulnerable to the acquisition of the second mutation in a susceptible retinal cell and often develop bilateral retinoblastoma that is 90% penetrant.

RB1 encodes retinoblastoma protein (pRb), a cell-cycle regulatory protein with roles in cell differentiation and apoptosis. pRb principally functions as a ‘gatekeeper’ of the cell-cycle by binding to and negatively regulating the heterodimeric transcription factor E2F-DP. Phosphorylation of pRb by cyclin-dependent kinases (CDKs) releases E2F-DP to stimulate
transcription of target genes that mediate progression into the S or DNA-synthesis phase of the cell-cycle (Figure 1.1)\textsuperscript{24-26}. pRb inactivation results in uncontrolled proliferation and dysregulation of apoptosis \textsuperscript{27}.

\textbf{Figure 1.1:} Mechanism of pRb/E2F regulation of the cell-cycle. pRb binds to and negatively regulates the heterodimeric transcription factor E2F-DP. Phosphorylation of pRb by cyclins and cyclin-dependent kinases (CycD/CDKs 4/6) releases E2F-DP to stimulate transcription of target genes that mediate progression into the S or DNA-synthesis phase of the cell-cycle. (Modified from \textsuperscript{28}).
The default cellular response to loss of pRb is not tumourigenesis but rather apoptosis, as observed in homozygous mouse mutants. However, pRb loss results in limited proliferation in the murine central nervous system. In the mouse, retinal tumours only result when Rb1 is conditionally lost, along with at least one other key cell cycle regulatory gene.

In humans, loss of both copies of RB1 in a susceptible retinal cell does not lead directly to retinoblastoma, but to the benign, euploid precursor lesion, retinoma, which commonly progresses to retinoblastoma. While the initiating M1 and M2 events set the stage for further genomic alterations, they are insufficient to trigger progression to malignant disease. In keeping with Knudson’s nomenclature, the subsequent genomic events required to advance retinoblastoma progression were termed M3-Mn events. Therefore, current investigations of retinoblastoma at the molecular level are geared toward the discovery of subsequent genomic alterations that instigate and promote tumour development.

1.1d Models of tumour progression

The multistep nature of carcinogenesis was first proposed by Nordling, Armitage and Doll, wherein they described an association between the number of cancer-related cellular changes and the rate of cancer-related mortality in different age groups. This theory was subsequently supported by in vitro and in vivo studies. The activation of a single oncogene in mouse embryonic fibroblasts was insufficient to cause cellular transformation. Similarly, oncogenic activation in transgenic mice led to tumour formation only after a substantial period of time, during which mutational changes in other genes were observed in clonal populations of the tumour.

The multistep model of tumourigenesis has been best studied in colorectal cancers. Benign lesions (adenomas) acquire a number of mutational events like Ras activation and
alterations in 5q, 17p and 18q, which enable their progression to malignant colorectal cancer. Hanahan and Weinberg later proposed that the spectrum of alterations in the vast array of cancer-associated genes selectively impinge on common regulatory circuits in various cancers. These circuits govern six major properties of cell physiology that facilitate malignancy. They termed these physiological capabilities as ‘hallmarks of cancer’ and they are listed as follows. 1) Self-sufficiency in growth signals, 2) Insensitivity to anti-growth signals, 3) Invasion of apoptosis, 4) Limitless replicative potential, 5) Sustained angiogenesis and 6) Tissue invasion and metastasis. While all tumours display characteristics of these six hallmarks of cancer, there is considerable variation in the number of genomic events required and the order in which these events occur. More recently, Colotta et al. present another acquired cellular characteristic as the seventh hallmark of cancer. They suggest that cancer-related inflammation (CRI) in the tumour environment promotes the proliferation and survival of tumour cells and exacerbates metastases. Current data also indicate a mechanistic role for CRI in the induction of growth inhibition by inflammatory mediators and a potential role in instigating genomic instability.
1.2 Retinoblastoma oncogenomics

1.2a Karyotype of retinoblastoma tumours

Insight into the molecular mechanism leading to the development and progression of retinoblastoma was primarily drawn from karyotypic analyses. Individuals with retinoblastoma may have a normal constitutional chromosome complement but the karyotype of their retinoblastoma tumours reveal a spectrum of genomic aberrations. Retinoblastoma cell lines or tumours xenografted into nude mice, also display karyotypes that ranges from near normal to hyperploidy. Retinoblastoma patients with bilateral disease exhibit inter-tumour heterogeneity but monofocal tumours in unilaterally affected patients are derived from a single clone. The first demonstration of consistent patterns of genomic change was based on a study of 82 retinoblastoma tumours. As indicated by this study, the two most recurrent chromosomal abnormalities are 6p and 1q gain, reported in 45% and 44% of tumours respectively. 6p gain is of notable interest on account of the formation of an isochromosome i(6p), a cytogenetic anomaly that is almost unique to retinoblastoma and may have particular significance in tumourigenesis. Other less frequent changes include loss of chromosome 16, 1p gain and alterations in chromosomes X, 17 and 19 in addition to sporadic observations of homogenously staining regions (HSRs) and double minutes (DMs). Results from these rudimentary studies accentuated key regions of the genome that might drive retinoblastoma progression and these were later examined using modern molecular techniques.
1.2b Summary of CGH and aCGH findings

In humans, bi-allelic RB1 loss or mutation and consequent pRb inactivation is insufficient for the transformation of a normal retinal cell to malignant retinoblastoma but results in the formation of retinoma – a benign precursor lesion with a relatively normal karyotype. A subgroup of highly-differentiated tumours – formerly thought to be a result of spontaneously regressed retinoblastoma, have been identified and termed as ‘retinomas’. These lesions can sometimes be observed with retinoblastoma, and are generally considered benign, with a dormant capacity for malignant transformation. Comparisons among the genomic copy number status and spatial relationships of retina, retinoma and retinoblastoma led to the proposal that retinoma represents a precursor lesion, or premalignant form of retinoblastoma.

Retinoma is thought to develop into retinoblastoma through the accumulation of a series of genomic alterations termed M3 to Mn mutational events. The exact sequence of these events has yet to be delineated however research in recent years has implicated a number of chromosomal changes as potentiating factors in the progression to malignant retinoblastoma. These events typically represent gains or losses of chromosomal regions that harbour candidate oncogenes and tumour suppressor genes. While it is useful to study genes that exhibit aberrant regulation at the level of gene and protein expression, changes in gene expression as a result of genomic change present a more robust means of identifying candidate biomarkers and therapeutic targets.

The advent of comparative genomic hybridization (CGH) has enabled significantly higher resolution analysis of genomic changes that have been consistently associated with retinoblastoma by gross karyotypic studies. To date, five CGH and two array-CGH (aCGH) have reported 1q, 6p and 2p gain, 13q and 16q loss in addition to other less recurrent cytogenetic abnormalities in a total of 197 retinoblastoma patient tumours. Chromosomal
instability is defined as the excessive rate of numerical and structural genomic change in tumours. Numerical changes in copy number may arise through errors in mitotic segregation while structural changes in the genome are triggered by erroneous DNA repair mechanisms or unstable genomic regions. Retinoblastoma patients evincing gross 13q deletions often show signs of multiple facial deformities and perceptible mental impairment. Clinical characterization of four retinoblastoma patients by aCGH revealed de novo 13q14 deletions in two patients and germline mutations in 13q and 7q11.21 that may not be related to the observed phenotype. The most recent aCGH analysis reported low-level chromosomal instability in bilateral disease as compared to unilateral retinoblastoma. Furthermore, the unilateral cases that were diagnosed earlier displayed fewer chromosomal aberrations (< 4) in contrast to those diagnosed at a later stage (> 7). This underscores the necessity for studies that incorporate an assessment of potential correlations between clinical parameters and genomic change. While these experiments have been performed on primary tumour material and present compelling evidence for putative oncogenes that drive progression to malignant retinoblastoma, they lack the cohort size that allows for rigorous statistical analysis and thereby validation of these potential biomarkers and therapeutic targets.

1.2c 6p gain in retinoblastoma: DEK and E2F3

6p gain is the most frequent cytogenetic abnormality in retinoblastoma (52.8%, 104/197 tumours by CGH). Retinoblastoma is distinguished from other cancers as it harbours the formation of an isochromosome i(6p). All seven CGH studies on retinoblastoma report a common minimal region of gain (MRG) at 6p22, suggesting that tumours with 6p22 gain may be at a developmental advantage regardless of i(6p) presence (Figure 1.2).
Figure 1.2: Recurrent chromosome aberrations in chromosome 6 in retinoblastoma detected by CGH and aCGH (Modified from 62). Each vertical line represents a single tumour; lines to the right of the chromosome ideogram represent gains, lines to the left represent losses. Bold lines indicate amplification. Gray boxes identify the minimal regions of gain or loss. Numbers below figure indicate references: 1. Mairal et al. (2000); 2. Chen et al. (2001) 3. Herzog et al. (2001); 4. Lillington et al. (2003); 5. Van der Wal et al. (2003); 6. Zielinski et al. (2005); 7. Sampieri et al (2009).
However the idea of a specific 6p subregion driving the formation of i(6p) prompted a high-resolution evaluation of genomic changes succeeding *RB1* loss in retinoblastoma. Using quantitative multiplex PCR (QM-PCR) on 70 retinoblastoma tumours the previously reported MRG was narrowed down to 0.6Mbp. The most frequently gained sequence tagged site (STS) was found to be a kinesin gene, designated as *RBKIN* and concomitantly characterized as a cargo-bearing kinesin *KIF13A*. The 0.6Mbp MRG comprises 6 genes – *DEK, AOF1, TPMT, NHLRC1, KIF13A* and *NUP153*. *E2F3*, which is located 2.2Mb centromeric to the MRG was also placed under scrutiny owing to its established role as a pRb target gene in regulating cell-cycle progression. Subsequent studies disregarded *KIF13A* as a potential driver of tumourigenesis despite increased expression in some retinoblastoma as compared to normal retina.

*DEK* and *E2F3* were also high priority candidate loci to consider in this region and both genes are extensively implicated as potential drivers of oncogenesis in a variety of cancers. In an independent study using immunohistochemistry (IHC), *DEK* and *E2F3* exhibited elevated transcript abundance levels in correlation with increased copy number and protein levels in retinoblastoma tumours. In addition, they also displayed marked mRNA and protein expression downregulation in normal retina adjacent to retinoblastoma with overexpressed *DEK* and *E2F3*. FISH analysis of primary retinoblastoma tumours corroborated previous findings of augmented *DEK* and *E2F3* copy number status. *E2F3* levels were elevated in cells undergoing active proliferation whereas *DEK* expression was heightened in all tumour cells. *Dek* and *E2f3* overexpression has been noted in transgenic mice (Tag-Rb) with the murine equivalent of retinoblastoma. These genes also demonstrate differential expression in the immature retina as compared to adult retinal cells and are implicated in developmental regulation – a characteristic oncogenic trait. In normal retina, E2F3 and DEK localize to the inner nuclear layer (presumptive
DEK is a 375 amino acid (aa) nuclear phosphoprotein. Its biological function is ambiguous but it has imputed roles in transcription regulation, chromatic remodelling and mRNA splicing. It harbours two DNA-binding domains – a scaffold attachment factor (SAF) and a DEK-DEK multimerization domain and is structurally homologous to the E2F/DP family of transcription factors. It has been widely characterized as an oncogene primarily because of the common t(6;9) chromosome translocation with the nucleoporin CAN (NUP214) to form the DEK:CAN fusion protein in acute myeloid leukemia (AML) which augurs an unfavourable prognosis for patients that harbour this arrangement. DEK is also found to be overexpressed in AML, melanoma, hepatocellular carcinoma, brain tumours and other cancers. DEK expression in bladder cancer is heightened in invasive tumours as compared to early-stage tumours. While the exact biological function of DEK remains obscure, its involvement in several cancers warrants further investigation as a potential oncogene in retinoblastoma.

The eight-member E2F family of transcription factors has been categorized into ‘activators’ (E2Fs 1 and 2) and ‘repressors’ (E2Fs 4 and 5) depending on their regulation of target genes. E2F inhibition by peptides has also been shown to hinder proliferation and induce apoptosis in human tumour cells, validating E2F family members as potential therapeutic targets in cancer. E2F3 encodes a 224aa protein and plays an important role in cell-cycle regulation. It consists of a DNA-binding domain, dimerization domain, transactivation domain and a pRb binding domain. E2F3 has garnered particular interest as it is both amplified and overexpressed in several human tumours. The E2F3 locus can give rise to either the E2F3a or the E2F3b isoform through the recruitment of varying promoters and alternative 5’-coding exons. While the biological activities of these isoforms have not been well characterized in humans they are
thought to exhibit partly antagonistic functions. Evidence of oncogenic activity mediated by $E2F3$ was drawn from an anchorage-independent growth assay wherein $E2F3$ overexpression from a retroviral vector in a mouse fibroblast cell-line NIH 3T3 enabled growth of stably infected NIH 3T3 cells in soft agar. Moreover, NIH 3T3 cells overexpressing $E2F3$ and $E2F1$ exhibited increased saturation density than the control in a saturation-density growth assay.

1.2d 1q gain in retinoblastoma: KIF14 and MDM4

1q gain is the second most recurrent chromosomal anomaly observed in retinoblastoma (50%, 99 of 197 tumours by CGH). It is also a characteristic feature of several cancers and betokens the presence of one or more oncogenes in this region of gain that play a prominent role in tumourigenesis. The common minimal region of gain (MRG) identified in former CGH studies was found to be 1q31-1q32 confirming the results of a former karyotypic study (Figure 1.3).
The MRG was further concretized by identifying the most frequently gained sequence tagged site (STS) in a cohort of 55 retinoblastoma patient tumours by QM-PCR and 1q32.1 was found to be gained in 71% of the tumours. Subsequent expression analysis of various genes in
this MRG featured highly overexpressed *KIF14* as the most likely candidate for driving progression to malignancy. This was evidenced by the fact that *KIF14* manifests aberrant regulation at the gene expression level as well as copy number status in several cancers \(^93\).

*KIF14* encodes a 1648aa mitotic kinesin member of the N3 kinesin family. It consists of a kinesin-motor domain and forhead-associated domain \(^94\). It interacts with protein regulator of cytokinase 1 (PRC1) and localizes to the central spindle and midbody at cytokinesis. KIF14 has also been shown to exhibit microtubule-dependent ATPase activity and midbody formation during telophase \(^95\). In cancer cells elevated KIF14 levels may trigger unchecked and impaired mitosis, thereby facilitating rapid cell-cycle entry and anomalous ploidy changes. Gain of the *KIF14* locus is noted in retinoblastoma, breast cancer and hepatocellular carcinoma and its overexpression correlates with increased copy number (5-7 signals) in these tumours \(^37\). *KIF14* is also overexpressed in medulloblastoma cell-lines, breast tumours and non-small cell lung cancer (NSCLC) \(^96,97\) with prognostic significance in the latter two cancers. The implied oncogenic activity of *KIF14* has been functionally assessed wherein its knockdown leads to vitiated proliferation and colony formation in vitro \(^95,98\). Previous work on the precursor lesion retinoma demonstrated unchanged *KIF14* expression in retinoma as compared to its augmented copy number and overexpression in adjacent samples of retinoblastoma, implying that *KIF14* copy-number dysregulation and overexpression may be specific to malignancy and not merely an effect of pRb loss \(^36\). In light of this evidence, *KIF14* overexpression is most likely an early M3 or M4 event in the sequence of M3 to Mn mutational events from retinoma to retinoblastoma.

The *MDM4* gene is located in the 1q MRG at 1q32 and encodes a 490aa protein. It is comprised of a p53-binding domain, nuclear localization signal, RING-finger domain and a transactivation domain \(^99,101\). Former aCGH and FISH analyses have highlighted *MDM4* as a potential oncogene due to elevated *MDM4* genomic copy number and accompanying increase in
transcript abundance and protein expression in retinoblastoma as compared to normal retina\textsuperscript{67,102}. 

MDM4 overexpression portends a mechanism for p53 inactivation as it enhances p53 degradation by the E3 ubiquitin ligase MDM2\textsuperscript{103,104}. Furthermore, MDM4 has also been implicated as an oncogene in glioma\textsuperscript{105,106}, a cancer that shares common neuroectodermal origins with retinoblastoma. The small molecule Nutlin-3 that targets the p53-MDM2/4 interaction, is reported to be efficacious in exterminating retinoblastoma cell lines\textsuperscript{102,107} accentuating MDM4 as another potential therapeutic target in this region of gain.

1.2.e 2p gain and MYCN amplification in retinoblastoma

In addition to the primary 1q and 6p genomic changes, the short arm of chromosome 2 is also found to be modestly gained in 34% of retinoblastoma tumours by CGH studies\textsuperscript{55,62-68}. The MRG was found to be 2p24 in all but one of these CGH analyses\textsuperscript{62} (Figure 1.4).
Figure 1.4: Recurrent chromosome aberrations in chromosome 2 in retinoblastoma detected by CGH and aCGH (Modified from 62). Numbers below figure indicate references: 1. Mairal et al. (2000); 2. Chen et al. (2001) 3. Herzog et al. (2001); 4. Lillington et al. (2003); 5. Van der Wal et al. (2003); 6. Zielinski et al. (2005); 7. Sampieri et al. (2009).

The 2p24 chromosomal region has particular relevance in the formation and propagation of several cancers and harbours the infamous oncogene MYCN. MYCN is chiefly involved in the
development of peripheral and central nervous system tumours and is also a prognostic marker of neuroblastoma progression. It is both amplified and overexpressed in the Y79 retinoblastoma cell line. MYCN amplification is discernible as low-level (10-20) and high-level (100-200) copy number increase, and as DMs and HSRs in retinoblastoma. Its overexpression is noted in retinoblastoma tumours regardless of their amplification status. However, elevated MYCN mRNA levels have been reported to be no different from those in normal developing retinal cells and possibly unrelated to RB1 loss in retinoblastoma. In mice exhibiting the murine equivalent of retinoblastoma, Mycn is both amplified and overexpressed in 7.14% of primary tumours and 18.75% of tumours evincing metastases. CGH analyses have reported MYCN amplification in retinoblastoma tumours at frequencies ranging from 0 to 30%. However varying sample sizes and a difference in technique may be the cause of this variability.

MYCN amplification often appears as either DMs or HSRs in retinoblastoma cell lines Y79 and RB355. The implication of the nature of MYCN amplification (DM versus HSR) in the clinical context of this disease has yet to be determined. MYCN was also observed as part of a 1p HSR in Y79 cells. However there are as yet unidentified amplicons in retinoblastoma and certain tumours without MYCN amplification also show signs of DMs. This is suggestive of other genes being involved in the formation of these structures in retinoblastoma. Out of the six retinoblastoma tumours with 2p gain (CGH analyses) only three displayed amplification of MYCN mRNA. Two other genes have been posited as likely candidates in addition to MYCN within the 2p region of gain – DDX1 and ID2. While these genes are not located within the MRG defined by CGH analyses, there is substantial evidence of their capability as prospective drivers of oncogenesis.
DDX1 encodes DDX1, one of 36 members of the DEAD-box-1 family of proteins\textsuperscript{123} with ascribed roles in differentiation, RNA transport and processing in addition to functioning as an RNA helicase\textsuperscript{124}. Its precise role in cancer has not been well characterized, however recent studies suggest it may be a prognostic determinant of early recurrence in breast cancer\textsuperscript{125}. DDX1 is co-amplified with MYCN in Y79 and RB522 cell lines\textsuperscript{126} and demonstrates both amplification and overexpression in neuroblastoma and retinoblastoma tumours\textsuperscript{127}. Additionally, it is also part of the complex 1p HSR in Y79 cells along with ATP5A1\textsuperscript{128,129}.

ID2 located at 2p25, encodes ID2, one of four members of the inhibitor of DNA (ID) binding proteins which inhibit helix loop helix (HLH) transcription factors in a dominant negative manner\textsuperscript{130,131}. In recent years this gene has emerged as a prime player in the cellular promotion of several cancers\textsuperscript{132}. ID2 has been shown to functionally impact proliferation, differentiation, angiogenesis and tumourigenesis\textsuperscript{130,131,133}. It is also reported to mediate MYCN amplification in neuroblastoma\textsuperscript{134}. ID2 interacts physically with all three members of the retinoblastoma protein family and abolishes the pRb-mediated cell-cycle checkpoint thereby driving mitosis and consequent proliferation\textsuperscript{135}. Tumourigenesis induced by RBL-loss may well be influenced by ID2 activity\textsuperscript{135-140}. In contrast to results from the above-mentioned studies, it has also been reported to perform as a tumour-suppressor in cancers of the mammary gland and intestine\textsuperscript{141,142} and a recent study of ID2 in a panel of three retinoblastoma cell lines (Y79, WERI-Rb and CHLA223) and murine retinal tumours implied that ID2 may act as a tumour suppressor in retinoblastoma. ID2 has varying roles in different tissues\textsuperscript{143} and it remains to be seen whether it acts as a tumour suppressor or an oncogene or if its overexpression in retinoblastoma is simply a passenger event to the driving mutation of MYCN amplification.

Lillington et al\textsuperscript{65} reported an absence of clinical correlation between MYCN amplification and clinical variables of progression, however their study consisted of 25
retinoblastoma tumours and MYCN amplification is one of the least recurrent (3%) genomic alterations. In order to effectively assess the clinical consequences of MYCN amplification, a larger cohort with a substantial percentage of late-stage tumours with metastasis is required. Nevertheless there have been instances in the literature where MYCN amplification is clinically relevant. Overexpression of MYCN mRNA has been linked with highly proliferative tumours. It has also been noted in the highly metastatic retinoblastoma cell line FMC-Rb1 and is more frequent in metastatic murine retinal tumours as compared to primary tumours. Given that MYCN amplification is recurrently associated with extremely aggressive retinoblastoma and in certain cases with tumours that retain positive pRb expression (Gallie et al, in press), it might represent a subtype of tumours that progress in a different and possibly more accelerated fashion than pRb-inactive tumours. While the clinical consequences of MYCN amplification have yet to be assessed in retinoblastoma, its proclivity for tumourigenic activity and multifaceted cellular functions make it a particularly interesting candidate and biomarker for metastatic disease.

1.2f 1p36 deletion in retinoblastoma

1p LOH was first observed in 21% of retinoblastoma tumours with an increased incidence in metastatic disease. It is also part of a genomic signature in conjunction with MYCN amplification in the molecular subtyping of neuroblastoma. Tumours with 1p loss exhibit aggressive histology and unfavourable clinical outcome. 1p36 monosomy is one of the most frequent terminal deletion syndromes and is associated with delayed development, mental impairment and facial dysmorphism. Interestingly, 1p36 deletion is also the underlying cause of congenital cataract, a rare paediatric disorder that renders most affected children blind or visually impaired.
On account of its association with a variety of cancers and the fact that 1p36 loss has severe implications for development, this region is alleged to harbour one or more tumour suppressor genes. Efforts in the identification and characterization of 1p36 candidate genes have been futile, however there are a number of genes (UBE4B, KIF1B, PGD, APITD1, DFFA and PEX14) within this region that are downregulated in neuroblastoma and require exploration into their potential as tumour suppressors \(^{152}\). The 1p region has not been well studied in the context of retinoblastoma, however the minimal region of loss (MRL) in neuroblastoma evincing 1p36 deletions was mapped to 1p36.31 and spans approximately 2Mb \(^{153-155}\). Twenty-three genes have been identified in this region, however only two genes (CHD5 and RNF207) appear to be likely candidates as tumour suppressors. None of the other genes show evidence of being downregulated or mutated in neuroblastoma \(^{156}\). CHD5 has materialized as the most interesting owing to its ability to severely impact clonogenicity and tumourigenicity \(^{156}\). CHD5 is preferentially expressed in the nervous system \(^{157}\) and has been the subject of former investigations in the development of neuroblastoma \(^{158-160}\). CHD5 expression is strikingly associated with clinical characteristics of neuroblastoma. Elevated CHD5 gene and protein levels are linked with favourable outcome. In an independent study, CHD5 was reported to regulate proliferation, apoptosis and senescence through interactions with the p19\(^{\text{ARF}}\)/p53 signalling pathway \(^{161}\). CHD5 expression is also inversely related to risk factors for neuroblastoma \(^{162}\).

Owing to the wide-ranging cellular consequences of CHD5 loss and its functional similarity with tumour suppressors, it remains the most promising candidate for further investigation in retinoblastoma tumours with 1p36 deletion.

1.3 Correlation between genomic changes and clinical variables of progression
Tremendous advancements in the field of genomics and cytogenetics have enabled retinoblastoma researchers to chart a molecular pathway of retinoblastoma progression. The present model of retinoblastoma development is based on an assortment of genomic changes garnered from karyotypic and CGH studies (Figure 1.5).

**Figure 1.5:** Potential molecular model of retinoblastoma progression, based on frequencies of gain and loss of genomic regions on chromosomes. Loss of each \( RB1 \) allele is shown as the M1 and M2 events, leading to the benign retinoma. 1q32.1 (\( KIF14 \) and \( MDM4 \)) and 6p22 (\( DEK \) and \( E2F3 \)) gain are the most common genomic changes, shown as M3 and M4. 16q22 loss and \( MYCN \) gain negatively associate so are shown as alternate M5 events. Note that this model only incorporates copy number changes, and not other molecular events in retinoblastoma progression (Modified from 37).

Age at diagnosis or enucleation correlates with the number of genomic alterations in retinoblastoma. Tumours in older patients show more genomic changes than those in younger
patients. Gains of 1q, 13q, 2p, chromosomes 17 and 19 and 16q loss occur at a higher frequency in tumours diagnosed at a later stage as compared to tumours detected at an early stage. Gains of 1q, 6p and 16q LOH also correlate with an older age at diagnosis. These observations are likely on account of the increased size and higher grade of tumours in older patients due to delayed detection.

Retinoblastoma progression is pathologically dictated by the degree of tumour differentiation. Well-differentiated tumours resemble retinomas and are often in the form of varying flower-like structures called Flexner-Wintersteiner rosettes, Homer-Wright or Flexner-Wintersteiner fleurettes. Tumours that are more advanced and likely to metastasize are poorly differentiated. They display a higher number of genomic alterations than well-differentiated tumours. \textit{Kras} mutations, \textit{MGMT} hypermethylation and changes in 1q and 13q occur at an increased frequency in undifferentiated tumours. Pathological parameters of clinical assessment like extraocular extension and vitreous seeding are also associated with more genomic changes than tumours without these features. LOH at 16q and 1q gain are linked to tumours with vitreous seeding and 16q LOH is also an independent prognostic marker of this clinical phenotype. 1q and 6p gained retinal tumours advance at an astonishing pace in severe combined immunodeficient (SCID) mice, and 6p gain also correlates with poor differentiation and optic nerve invasion in these tumours. Since most tumours are studied as primary lesions, the prognostic value of these changes has yet to be appraised in a statistically significant cohort of retinoblastoma tumours at different stages of disease progression. However the consistent association of certain changes like 1q and 6p with clinical phenotypes merits a thorough search for genes within these regions as drivers of retinoblastoma oncogenesis.

Tumour metastasis is also linked with recurring genomic changes in retinoblastoma. 1q trisomy, 2p gain, loss of 5q, 7q and 6p is associated with metastasis to the bone marrow.
Chromosome 17 was found to exhibit structural variation in a tumour post-radiation treatment.

1p LOH has also been noted in advanced tumours with metastasis and not in primary tumours. Three tumours with metastases post-enucleation were found to harbour 1q and 6p gain and 13q loss. Further studies are required in order to determine the underlying genomic signature of retinoblastoma tumours with metastatic potential, however 1p and 5q loss, 1q, 2p and 6p gain and changes on chromosome 17 currently present as appealing markers.

1.4 Relationships among regions of genomic alteration

The difficulty in attaining a sufficient number of retinoblastoma samples for study has precluded a thorough assessment of functional liaisons among chromosomal changes identified in this disease. Nevertheless, several reports in the literature reveal that in the series of genomic changes imputed in the promotion of retinoblastoma, some appear to be synergistically linked while others are autonomous.

1q and 6p gain are the two most striking aberrations, both by karyotypic and CGH studies and appear in a large proportion of both early and late-stage tumours. Expression analyses of a number of plausible candidate genes within this region have also been functionally assessed as to their bearing on the tumourigenic process in retinoblastoma. As potential primary events in retinoblastoma progression, they may be synergistically linked. Indeed, tumours with 1q and 6p gain show a higher number of imbalances. 1q gain appears in concert with a number of other genomic changes and is consistently associated with 13q gain and 5q and chromosome 16 loss. 1q gain is also negatively linked with 16q loss. 6p gained tumours evince a higher degree of genomic instability than tumours diploid for 6p. Furthermore, MYCN-amplified tumours do not show evidence of 1q and 6p gain reinforcing the notion that they may represent a unique
subtype of retinoblastoma that does not rely on the early alterations following pRb loss for malignant transformation.

In all five CGH and two aCGH studies, a small percentage of tumours are near-euploid. These may be primary growths that have not yet progressed to malignancy. Since retinoblastoma tumour samples used in research are derived from academic health science centres that facilitate early diagnosis, the tumours tend to be earlier lesions. While these offer valuable insight into the initiating events of retinoblastoma tumourigenesis, it is also of utmost utility to determine the subsequent genomic progression events that determine the growth rate and invasion capacity of retinoblastoma tumours and ensuing clinical consequences for prognosis.
1.5 Hypotheses and specific aims

In humans, bi-allelic \( RB1 \) inactivation and consequent loss of function of the cell-cycle regulator pRb does not directly lead to malignant retinoblastoma but results in the formation of a benign precursor lesion called retinoma \(^{36}\). Further mutational events, specifically gain and loss of significant chromosomal regions harbouring key oncogenes and tumour suppressor genes are requisite for progression to the aggressive form of this disease. The body of work in the field of retinoblastoma thus far has implicated several cytogenetic aberrations in this sequence of molecular events, the two most recurrent and notable ones being augmented copy number status of \( KIF14 \) and \( MDM4 \) on 1q and \( DEK \) and \( E2F3 \) on 6p. Focal amplification of the \( MYCN \) oncogene at 2p24 and deletions of the short arm of chromosome 1 have also emerged as noteworthy genomic changes and may have critical roles in perpetuating advanced disease. The functional roles of the proteins encoded by these genes confer tumour cells manifesting these copy number deviations with a selective survival and proliferative advantage. To our knowledge, there has been no recent study associating genomic change of the aforementioned genes with clinical parameters of progression in a sample size of statistical significance. The large cohort available in this project not only allowed for rigorous evaluation of their copy number status in numerous retinoblastoma tissue sections but also enabled correlation with clinical variables of disease progression.

This project was part of a collaboration between Dr. Jeremy Squire (Queen’s University, Kingston), Dr. Brenda Gallie (Sick Kids Hospital, Toronto) and the Retinoblastoma Research Group at the Hôpital Do Câncer (São Paulo, Brazil). My principal aim was to examine significant genomic changes in retinoblastoma progression specifically, the copy number status of \( KIF14 \) and \( MDM4 \) on 1q, \( DEK \) and \( E2F3 \) on 6p, the oncogene \( MYCN \) and the 1p36 region in 270 retinoblastoma tumours, and to evaluate correlation among the copy number statuses of these
regions of gain and deletion. The clinical evaluations and statistical comparisons of patient outcome are being performed by the group in São Paulo. Ultimately, I hope my research will contribute towards the identification of novel therapeutic targets in retinoblastoma, with the objective of adopting treatment strategies that do not necessitate surgical removal of the diseased eye in patients and a consequently better quality of life.

The following hypotheses were tested in this project –

1) 1q, 6p gain (defined by extra copies of KIF14 and MDM4 and DEK and E2F3, respectively) and MYCN amplification are genomic biomarkers of retinoblastoma progression.

2) MYCN amplification is associated with pRb protein expression and together with 1p36 deletion, constitutes a novel molecular subtype of retinoblastoma.

Specific aims undertaken to address these hypotheses are listed below –

**Aim 1** – To assess genomic copy number status of 1q (KIF14 and MDM4) and 6p (DEK and E2F3) regions of gain.

**Aim 2** – To assess the genomic copy number status of the MYCN oncogene and pRb protein expression.

**Aim 3** – To assess the genomic copy number status of the 1p36 region.

**Aim 4** – To evaluate statistical correlations among the copy number status of 1q (KIF14 and MDM4) and 6p (DEK and E2F3) regions of gain, MYCN amplification and 1p36 deletion.
Chapter 2

Materials and Methods

2.1 Preparation of FISH probes

2.1a Culturing single-clone bacterial colonies

Bacterial artificial chromosome (BAC) clones spanning KIF14 (RP11-92G12), MDM4 (RP11-970B14 and RP11-433N15), E2F3 (RP11-159C8) and DEK (RP11-298J15 and RP11-478E3) genes and DNA sequences close to centromere 1 (RP11-196G18 and RP11-541H15) were obtained from The Centre for Applied Genomics (TCAG, Toronto, ON) on agar plates. Each BAC clone was streaked on a separate plate and stored at 4°C overnight. A single colony was then isolated and inoculated into 25mL Luria Bertani (LB) medium [NaCl 10g/L, Bacto tryptone 10g/L, yeast extract 5g/L (All from Sigma-Aldrich, Oakville, ON)] containing 12.5µg/mL chloramphenicol (Sigma-Aldrich) in 50mL centrifuge tubes and the cultures were incubated in a 37°C shaker (225 rpm) for 15 hours or until medium was turbid prior to DNA extraction (2.1b).

Additionally, 5mL of the cultured medium was stored in the form of glycerol stocks at -80 C for future use. Prior to use, the 400µL glycerol stocks were thawed at room temperature. Using a 1000µL pipette tip, a small portion of the glycerol stock was streaked on LB agar (Sigma-Aldrich) plates containing 12.5µg/mL chloramphenicol and the above process was repeated in order to obtain single-clone bacterial colonies.
2.1b BAC DNA extraction

The cultures were grown to saturation and 1mL of the LB media from each culture was transferred to microfuge tubes (Four tubes/BAC). Cells were precipitated by centrifugation at 8000rpm for two minutes in a microcentrifuge and the supernatant was discarded. This was repeated five times to obtain a bacterial cell pellet from 20mL media. The cell pellet was then washed with 1X phosphate saline buffer (PBS) (Qiagen Sciences, Germantown, MD) by centrifugation at 8000rpm for two minutes. The pelleted bacterial cells were resuspended by pipetting up and down in 300µL of P1 resuspension buffer (50mM Tris chloride, pH = 8.0, 10mM EDTA) (Qiagen Sciences) with 5µL of 100µg/mL RNase Type III-A (Roche, Laval, QC). Three hundred µL of P2 lysis buffer (200mM NaOH, 1% SDS) (Qiagen Sciences) was then added to the cells and mixed by gently inverting the tube several times. The lysis reaction was allowed to proceed for five minutes at room temperature. Three hundred µL P3 neutralization buffer (3.0 mM potassium acetate) (Qiagen Sciences) was added to the cells drop-by-drop and mixed by inverting the tubes two times. The tubes were placed on ice for five minutes till the solution appeared viscous. The cells were then centrifuged at 13,000 rpm for five minutes at room temperature to obtain a compact white pellet on the side of the tube wall. The supernatant was transferred to new microfuge tubes and centrifuged at 13,000rpm for ten minutes at 4°C. The supernatant (~600µL) was then transferred to microfuge tubes with 700µL of ice-cold isopropanol (Fisher Scientific, Toronto, ON). The tubes were inverted forty times and incubated -80°C for an hour for DNA precipitation. The tubes were thawed at room temperature and centrifuged at 13,000 rpm for thirty minutes at 4°C. The supernatant was then discarded and the DNA pellet was washed in 500µL of 70% ethanol (Absolute ethanol obtained from Queen’s University Stores, Kingston, ON) by inverting the tube several times. The tubes were then centrifuged at 13,000rpm for ten minutes at 4°C. The DNA pellet was air dried at room
temperature for approximately an hour until it appeared translucent. The DNA pellet was then resuspended in 35-50µL of nuclease-free water (Abbott Molecular, Des Plaines, IL) by incubation in a 55°C water bath for fifteen minutes. The DNA was then left at room temperature for twenty-four hours for complete resuspension in nuclease-free water.

The quality of the DNA was assessed by gel electrophoresis. Human genomic DNA (male) (488ng/µL) (Promega, San Luis Obispo, CA) was used as a positive control and nuclease-free water as a negative control in addition to a 100bp ladder. Four µL of each DNA sample was prepared for loading with 1µL of 6X loading dye. The DNA samples and controls were electrophoresed in adjacent lanes on a 0.7% agarose (Sigma-Aldrich) gel with 2µg/50mL ethidium bromide (Fisher Scientific) at 135V for thirty minutes. The quality of extracted DNA was evaluated based on the size of the band with respect to the normal DNA and the appearance (if any) of bands indicating RNA contamination.

2.1c BAC extracted DNA quantification and PCR validation

The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The software was calibrated using nuclease-free water as a blank and the concentration of DNA was measured using the 260/280 ratio as calculated by the DNA module of the NanoDrop software.

Polymerase chain reaction (PCR) validation was carried out in order to ensure that the BACs spanned the genes of interest (KIF14, MDM4, E2F3 and DEK). PCR reactions were
performed for each extraction product as well as for the control, Human genomic DNA (Male). 

50μL reaction volumes consisted of 25μL Illustra Hot Start Master Mix, 50ng extraction product (template), up to 25μL nuclease-free water, and gene-specific forward/reverse primers for KIF14, MDM4, E2F3 and DEK. For each extraction product and the control, PCR reactions were run for each set of primers. The PCR products were then run on a 0.8% agarose gel to verify the presence of amplification products for all primer sets for the control, and the absence of bands for extraction products amplified with unmatched primer sets.

2.1d Labelling of BAC extracted DNA

Labelling of the extracted and quantified BAC DNA was carried out using a nick translation kit (Abbott-Molecular). BAC clones for KIF14 and E2F3 were directly labelled with Orange-dUTP (Enzo Life Sciences, Plymouth Meeting, PA) and those for MDM4 and DEK were directly labelled with Green-dUTP dyes (Enzo Life Sciences). BAC clones for centromere 1 were labelled with Red-dUTP (Enzo, Life Sciences). The 50µL reaction mix (One reaction per BAC clone) consisted of 10µL of 1µg extracted DNA, 2.5µL of 0.2nM Green/Orange-dUTP, 5µL of 0.1mM dTTP, 10µL of 0.3mM dNTP, 5µL nick translation buffer and 10µL nick translation enzyme in a 200µL microfuge tube. The components were added in the order described and incubated at 15°C for ten hours, followed by storage at 4°C (short-term) or at -20°C (long-term).

The labelled DNA was then precipitated in the following manner. The 50µL probes were transferred to microfuge tubes. Ten µL of Human Cot-1 DNA (Invitrogen, Burlington, ON) and 10µL of salmon sperm DNA (Invitrogen) were added to the 50µL labelled DNA probe. This was followed by adding 140µL of 100% ethanol and 7µL of 3M sodium acetate (Fisher Scientific) pH = 5.5. The tube was inverted several times to ensure proper mixing of reaction components and
the labelled DNA was allowed to precipitate at -80°C for one hour. The labelled probe was then thawed at room temperate and centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was discarded and the DNA pellet was washed with 500µL 70% ethanol. The DNA pellet was then air-dried and approximately 300ng of probe was suspended in nuclease-free water and 10µL LSI/WCP Hybridization Buffer (Abbott Molecular) by incubation in a 55°C water bath for 15 minutes. The labelled DNA probes were stored at -20°C prior to use in step 2.2 and 2.3a (Table 2).
Table 2 In house BAC and commercial probes used in the present study.

<table>
<thead>
<tr>
<th>Probe(s)</th>
<th>Probe label</th>
<th>Cytogenetic location</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEP 6</td>
<td>Aqua-dUTP</td>
<td>Centromere of chromosome 6</td>
<td>Abbott Molecular, Des Plaines, IL</td>
</tr>
<tr>
<td>CEP1</td>
<td>Red-dUTP</td>
<td>Sequences close to centromere of chromosome 1</td>
<td>BAC (TCAG, Toronto, ON)</td>
</tr>
<tr>
<td>1p36 cocktail probe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p36</td>
<td>Orange-dUTP</td>
<td>1p36</td>
<td>Abbott Molecular, Des Plaines, IL</td>
</tr>
<tr>
<td>1psubtelomeric region (st)</td>
<td>Green-dUTP</td>
<td>1pst</td>
<td></td>
</tr>
<tr>
<td>1q25</td>
<td>Aqua-dUTP</td>
<td>1q25</td>
<td></td>
</tr>
<tr>
<td>E2F3</td>
<td>Orange-dUTP</td>
<td>6p22.3</td>
<td></td>
</tr>
<tr>
<td>DEK</td>
<td>Green-dUTP</td>
<td>6p22.3</td>
<td>BAC (TCAG, Toronto, ON)</td>
</tr>
<tr>
<td>KIF14</td>
<td>Orange-dUTP</td>
<td>1q32.1</td>
<td></td>
</tr>
<tr>
<td>MDM4</td>
<td>Green-dUTP</td>
<td>1q32.1</td>
<td></td>
</tr>
</tbody>
</table>
2.2 Metaphase validation of FISH probes

2.2a Normal metaphase preparation

Fluorescence in situ hybridization (FISH) was performed on normal human lymphocytes in order to confirm the genomic location of the BACs. Peripheral blood lymphocytes from the venous blood of healthy adults were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 20% fetal bovine serum, 0.2% phytohemagglutinin (PHA, M-form), 2mM L-glutamine, 50μg/mL and 50U/mL penicillin (all from Gibco). Cells were grown to confluence at 37°C and 5% CO2 in a humidified atmosphere. For peripheral blood lymphocytes, confluent cells were harvested after treating with 10μg/mL colcemid (Gibco) overnight to arrest cells in mitosis following condensation of homologous chromosomes pairs. The cells were then harvested for cytogenetic preparations by arresting the cells in metaphase using colcemid (100μL/10mL medium) (Enzo Life Sciences). Four hours post colcemid addition, the normal blood cell cultures were transferred from the growth flasks into 15mL centrifuge tubes and the blood cells were pelleted by centrifugation at 1500rpm for five minutes. The supernatant was gently discarded using a pasteur pipette until approximately 4mm above the red cell precipitate. The pellet was homogenized using a pasteur pipette and the blood was aspirated into the pipette. The blood was then resuspended in 10 mL of pre-warmed (37°C water bath) potassium chloride (KCl) (Fisher Scientific, Toronto, ON) 1mL at a time. The centrifuge tube was then placed on a vortex in order to homogenize the blood with KCl. The sample was then incubated in a 37°C water bath for 15 minutes. Five drops of an ice-cold Carnoyl fixative (3:1 methanol:acetic acid) (both from Fisher Scientific) was added to the 15mL tube followed by vortexing immediately. This was followed by centrifugation at 1500rpm for ten minutes. The supernatant was discarded until approximately 4mm above the cell pellet. The pellet was then resuspended in 1mL fixative with a transfer pipette. This was repeated with 9mL fixative by drop-by-drop addition of the fixative. The 15mL
tube was then centrifuged at 1500rpm for ten minutes. The supernatant was discarded to just above the pellet and the pellet was resuspended in another 10mL of fixative, 1mL at a time. The tube was then vortexed and incubated at -20°C overnight. The tubes were then centrifuged at 1500rpm for ten minutes and the supernatant was discarded. The pellet was resuspended in 10mL fixative as before and the harvested cells were stored at -20°C until preparation of metaphase slides.

Slides (25 x 75 x 1mm) were pre-cooled in water at -20°C. The harvested cells were centrifuged at 1500rpm for ten minutes. The supernatant was discarded and the pellet was resuspended in ~1mL fixative. Using a transfer pipette two drops of the resuspended pellet were dropped onto the glass slide from a short distance and the slide was then air dried a few inches over a hot plate (~250°C). Following complete evaporation of the fixative the slide was observed under an inverted light microscope for the presence of metaphase chromosomes. After ten metaphase slides of normal leucocytes were prepared, they were incubated in 70% ethanol at -20°C. The harvested cell sample was refilled with 10mLs of fixative and stored at -20°C. Metaphase slides were aged for at least three weeks prior to use in 2.3.

2.2b Metaphase-FISH

The slides were dehydrated in a series of 75%, 90% and 100% ethanol for 2 minutes each and air dried. The prepared BAC probes for the genes of interest and centromere 1, and the commercial probe for centromere 6 were thawed at room temperature. Two normal slides were used to verify the genomic location of the BAC probes and the prepared probes were added to the slides in the following cocktails:

Slide A: 2µL KIF14 + 2µL MDM4 + 2µL CEP1
Slide B: 2µL E2F3+ 2µL DEK) + 1µL CEP6
A coverslip was placed directly above the areas on the slide containing metaphase chromosomes and the normal human metaphase slides were co-denatured with the added probes in a Thermobrite System (Abbott Molecular) for 1 minute at 80°C and hybridized at 37°C for ~15 hrs. The slides were then subjected to a rapid-wash protocol of 0.4X sodium chloride (NaCl) and sodium citrate (SSC) solution (Sigma-Aldrich, Oakville, ON) and 0.3% Igepal CA-630 (Sigma-Aldrich) in a 72°C water bath for two minutes, then 2X SSC for five minutes at room temperature. The slides were allowed to air dry and counterstained for DNA content using Vectashield DAPI/Antifade (Vector Laboratories).
Figure 2.1: Metaphase validation of BAC probes for *KIF14* (RP11-92G12), *MDM4* (RP11-433N15 and RP11-970B14) and CEP1 (RP11-196G18 and RP11-541H15). The orange signals represent the location of the BAC probe for *KIF14* and the green signals represent the location of the BAC probes for *MDM4*. The red signals (masked by the green and orange signals in this figure) represent the location of the BAC probes for centromere 1. Original magnification x100.
Figure 2.2: Metaphase validation of BAC probes for *E2F3* (RP11-159C3) and *DEK* (RP11-298J15 and RP11-478E3). The orange signals represent the location of the BAC probe for *E2F3* and the green signals represent the location of the BAC probes for *DEK*. The blue signals represent the location of the commercial probe for centromere 6. Original magnification x100.
2.3 FISH Analysis

2.3a FFPE-FISH of retinoblastoma TMAs

The two tissue microarrays (TMAs) with 270 retinoblastoma samples were obtained from the Retinoblastoma Research Group at the Hôpital Do Câncer (São Paulo, Brazil). Two unstained 5µm formalin fixed paraffin embedded (FFPE) TMAs consisting of 270 retinoblastoma patient tumours were incubated at 56°C overnight. The TMAs were then dewaxed in 100% xylene (Fisher Scientific) for fifteen minutes three times. This was followed by dehydration in 100% ethanol two times and the TMAs were air dried. The TMAs were then immersed in sodium citrate, pH 6.0 (Invitrogen) at 80°C for forty-five minutes. The TMAs were then passed through an ethanol dehydration series (70%, 90% and 100%) for two minutes each and allowed to air dry. The TMAs were then incubated in 0.01N hydrochloric acid (HCl) (Fisher Scientific), with 500µL of 75,000U/mL pepsin (Sigma Aldrich) in a 37°C water bath for fifteen minutes. The TMAs were then passed through an ethanol dehydration series (70%, 90% and 100%) for two minutes each and air-dried. The prepared BAC probes for KIF14 and MDM4 and CEP1 were added to the processed TMAs and co-denatured using a Thermobrite System for ten minutes at 80°C and hybridized at 37°C for ~fifteen hrs. The TMAs were then subjected to a rapid- wash protocol of 0.4X SSC and 0.3% Igepal in a 72°C water bath for two minutes, then 2X SSC for five minutes at room temperature. The TMAs were passed through an ethanol dehydration series (70%, 90% and 100%) for two minutes each and allowed to air dry. They were then mounted in 10µL Vectashield DAPI/Antifade (Vector Laboratories), incubated at -20°C for thirty minutes and visualized with a Zeiss Axioskop 2 plus microscope equipped with a fluorescent light source and Isis imaging software (V. 3.4.0).
The FFPE-FISH was repeated for six more sections of the TMA blocks (three from each) with 6p probes (DEK/E2F3 and CEP6), a commercial probe for MYCN and 1p36 in three separate FISH experiments.

2.3b Manual scoring of retinoblastoma TMAs

Enumeration of signals was performed at 100x magnification using a Zeiss Axioskop 2 plus microscope equipped with a fluorescent light source and Isis imaging software (V. 3.4.0). A total of 100 non-overlapping intact nuclei per core were evaluated at various focal planes so as to enable detection of all probe signals for each interphase FISH experiment. Tumour nuclei were distinguished from normal and necrotic nuclei based on DAPI staining and annotated areas on corresponding Hematoxylin and Eosin (H and E) stained images of the TMAs previously scanned using the Aperio imaging software. Copy number changes of the aforementioned genes were enumerated by the number of probe and centromeric signals found within a nucleus. Physically linked doublet or triplet spots (with a gap smaller than the diameter of the largest signal), were counted as one signal only. However, spots that were adjacent but separated by at least the diameter of the largest signal were counted as separate signals. A copy number of 2 was considered normal, 3-7 signals as gain and greater than 10 signals as amplification. Images were captured using the MetaSystems Isis FISH Imaging System v5.3 (MetaSystems, Altlussheim, Germany).
2.4 IHC Analysis

The Y79 and WERI-Rb retinoblastoma cell lines were obtained from the laboratory of Dr. Brenda Gallie (Princess Margaret Hospital, Toronto, ON) and were maintained in RPMI 1640 (Gibco,) supplemented with 20% FBS, 0.2%PHA (M-form), 2mM L-glutamine, 50 µg/mL and 50 U/mL penicillin (all from Gibco). Cells were grown to confluence at 37°C and 5% CO₂ in a humidified atmosphere. Confluent cells were harvested after treating with 10µg/mL colcemid overnight to arrest cells in mitosis following condensation of homologous chromosomes pairs. Briefly, 1xTrypsin was added to cell culture flasks and suspended cells were washed out into tubes. WERI-Rb and Y79 cells were formalin-fixed and paraffin-embedded into a cell block according to a previously published procedure 172, following by sectioning by microtome into 5µm slices which were mounted on slides.

The pRb antibody used in this study was a mouse polyclonal from BD Pharmingen (clone G3-245; PharMingen, San Diego, CA; dilution, 1:500) and the method of detection was 3,3’-Diaminobenzidine (DAB) staining. FFPE tissue sections of cell pellets of the retinoblastoma cell lines WERI-Rb were used as the positive control because it is known to express pRb at the protein level. IHC staining of the two retinoblastoma TMAs and corresponding controls was performed using the Ventana Molecular Discovery system (Tuscon, USA). The FFPE Y79 cell section was used as a negative control, since these cells are known not to express pRb 173.
2.5 Statistical analysis

All data analysis was carried out PASW Statistics version 18.0.2 (http://www.spss.com/software/statistics/). Pearson’s chi-squared test of significance was used to determine associations between regions of genomic alternation. A p-value of 0.05 was used as the criterion for statistical significance.
Chapter 3

Results

3.1 Interphase FISH analysis of 1q and 6p regions of gain in retinoblastoma

3.1a 1q (KIF14 and MDM4) is gained in retinoblastoma

Three-colour interphase FISH for the two candidate genes on chromosome 1q was performed on 270 FFPE retinoblastoma patient samples using three in house bacterial artificial chromosome (BAC) clones spanning the regions harbouring KIF14 (RP11-92G12, labelled with Orange-dUTP) and MDM4 (RP11-970B14 and RP11-433N15, labelled with Green-dUTP) on 1q and a two BAC clones spanning DNA sequences close to centromere 1 (Labelled with Red-dUTP). Eight samples could not be scored for a variety of reasons – weak hybridization of the probe to the sample, inadequate signal intensity, missing cores on the TMAs and/or poor tissue quality. The copy number of KIF14, MDM4 and centromere 1 was enumerated in a total of 100 non-overlapping tumour nuclei per sample. A ‘threshold for gain’ was calculated using five samples (Tables 3.1a and 3.1b) with a high percentage of normal cells. The threshold was based on the equation:

\[
\text{Average (Number of nuclei with gain) + 2* Standard Deviation.}
\]

Table 3.1a: Five normal samples used in the calculation of the threshold for gain.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Normal</th>
<th>Gained (1q region)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB7</td>
<td>91</td>
<td>6</td>
</tr>
<tr>
<td>RB9</td>
<td>87</td>
<td>11</td>
</tr>
<tr>
<td>RB11</td>
<td>83</td>
<td>12</td>
</tr>
<tr>
<td>RB23</td>
<td>92</td>
<td>7</td>
</tr>
<tr>
<td>RB125</td>
<td>89</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 3.1b: Threshold for gain calculation.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Average (Gained nuclei)</td>
<td>9</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2.54</td>
</tr>
<tr>
<td>Threshold for gain</td>
<td>14.099</td>
</tr>
</tbody>
</table>

For the 1q analysis, samples with greater than 2 copies of either locus or all loci in greater than 15 nuclei were labelled as ‘gained’. Those with greater than 10 copies of either locus or all loci were labelled as ‘amplified’\(^{171}\). Normal cells within the samples served as internal negative controls. The results of the scoring are shown in Table 3.2.

Table 3.2: Percentage of samples with copy number changes in 1q. Each gene is represented by the colour of the respective probe used in the FISH experiment prior to analysis.

<table>
<thead>
<tr>
<th>Copy Number change</th>
<th>Frequency of samples with genomic aberration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gain</td>
<td>KIF14: 6.5, MDM4: 2.3, KIF14/MDM4: 27.5, KIF14/MDM4/CEP1: 52</td>
</tr>
<tr>
<td>Amplification</td>
<td>KIF14: 1.15, MDM4: -</td>
</tr>
</tbody>
</table>

The most consistent genomic change among the samples was ploidy change – gain of \(KIF14\), \(MDM4\) and centromere 1, with 52% (136/262) of the samples exhibiting greater than two copies in at least 15% of all scored cells (Figure 3.1).
Figure 3.1: 1q (KIF14, MDM4 and centromere 1) is gained in retinoblastoma. FISH analysis on FFPE retinoblastoma (Sample RB33). The copy number of KIF14 is indicated by orange signals and that of MDM4 is indicated by green signals. The copy number of centromere 1 is indicated by red signals (appears dark pink in the image). Nuclei are counter stained with DAPI. Truncation of the cells due to sectioning, results in loss of signals in some cells. Retinoblastoma cells show additional copies of KIF14, MDM4 and centromere 1. Original magnification x100.
The lowest copy number was three and highest, seven for each of the loci in samples with 1q gain. Gain of *KIF14* and *MDM4* but not centromere 1 was noted in 27.5% (72/262) samples (Figure 3.2).

**Figure 3.2**: *KIF14* and *MDM4* are gained in retinoblastoma. FISH analysis on FFPE retinoblastoma (Sample RB49). The copy number of *KIF14* is indicated by orange signals and that of *MDM4* is indicated by green signals. The copy number of centromere 1 is indicated by red signals (appears dark pink in the image). Retinoblastoma cells show additional copies of *KIF14* and *MDM4*. Original magnification x100.
The copy number for both genes ranged from three to eight while centromere 1 was diploid in all 72 samples. In all samples greater than 15% of cells had both KIF14 and MDM4 gain, however the copy number for KIF14 was usually higher (5-8 signals) than the copy number change for MDM4 (3-4 signals). Additionally, in two samples more than 15% of cells displayed amplification of KIF14. The copy number in each amplified sample ranged from 10 to 27. The copy numbers for MDM4 and centromere 1 were normal in these samples.

KIF14 and MDM4 gain without a change in the copy number of centromere 1 were noted at a lower frequency. Seventeen samples possessed greater than 15% of cells with KIF14 gain and normal MDM4 and centromere 1. Only six samples had greater than 15% cells with MDM4 gain and normal KIF14 and centromere 1. Copy number changes for both KIF14 and MDM4 ranged from three to six in these samples. Twenty-nine samples displayed two copies for KIF14, MDM4 and centromere 1. Copy numbers of less than two signals was noted in less than ten percent of cells in all of the samples, and as such these cells were not included in the scoring.

3.1b 6p (DEK and E2F3) is gained in retinoblastoma

Similar to the 1q analysis, the copy number of DEK, E2F3 and CEP6 was evaluated by three-colour interphase FISH on 270 FFPE retinoblastoma samples. In house BAC clones were used for DEK (RP11-298J15 and RP11-478E3, labelled with Green-dUTP) and E2F3 (RP11-159C8, labelled with Orange-dUTP) while a commercial probe was used for centromere 6 (Pre-labelled Aqua-dUTP). The copy number of DEK, E2F3 and centromere 6 were enumerated in a total of 100 non-overlapping, intact tumour nuclei per sample. A ‘threshold for gain’ was calculated using five samples (Tables 3.3a and 3.3b) with a high percentage of normal cells.
Table 3.3a: The five samples used in the calculation of the threshold for gain.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Normal</th>
<th>Gained (6p region)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB7</td>
<td>91</td>
<td>7</td>
</tr>
<tr>
<td>RB9</td>
<td>87</td>
<td>11</td>
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<td>RB11</td>
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<td>13</td>
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<tr>
<td>RB23</td>
<td>92</td>
<td>6</td>
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<tr>
<td>RB125</td>
<td>89</td>
<td>10</td>
</tr>
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</table>

Table 3.3b: Threshold for gain calculation.

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Average (Gained nuclei)</td>
<td>9.4</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2.88</td>
</tr>
<tr>
<td>Threshold for gain</td>
<td>15.16</td>
</tr>
</tbody>
</table>

As with the 1q analysis, samples were considered ‘gained’ if they had more than 2 copies of either locus or all loci in greater than 15 nuclei and ‘amplified’ if they had greater than 10 copies of either locus or all loci. Normal cells within the samples served as internal negative controls. The results of the scoring are shown in Table 3.4.

Table 3.4: Frequency of samples with copy number changes in 6p. Each gene is represented by the colour of the respective probe used in the FISH experiment prior to analysis.

<table>
<thead>
<tr>
<th>Copy Number change</th>
<th>DEK</th>
<th>E2F3</th>
<th>DEK/E2F3</th>
<th>DEK/E2F3/CEP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gain</td>
<td>5</td>
<td>3</td>
<td>25.3</td>
<td>48.7</td>
</tr>
<tr>
<td>Amplification</td>
<td>0.38</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Gain of all three loci, DEK, E2F3 and CEP6 was the most recurrent genomic change on 6p. Greater than 2 copies of these loci in more that 15% of all scored cells were noted in 48.7% of the cohort (127/262 samples). (Figure 3.3).
Figure 3.3: 6p (DEK, E2F3 and centromere 6) is gained in retinoblastoma. FISH analysis on FFPE retinoblastoma (Sample RB58). The copy number of E2F3 is indicated by orange signals and that of DEK is indicated by green signals. The copy number of centromere 6 is indicated by blue signals. Nuclei are counter stained with DAPI however the colour has been changed to gray in order to view signals for centromere 6. Retinoblastoma cells show additional copies of DEK, E2F3 and centromere 6. Original magnification x100.

The copy number changes for DEK, E2F3 and CEP6 ranged from three and eight in each of the samples with 6p gain. More than 2 copies of DEK and E2F3 but not CEP6 in at least 15% of all scored cells was observed in 25.3% of the cohort (66/262 samples) (Figure 3.4).
Figure 3.4: *DEK* and *E2F3* are gained in retinoblastoma. FISH analysis on FFPE retinoblastoma (Sample RB67). The copy number of *E2F3* is indicated by orange signals and that of *DEK* is indicated by green signals. The copy number of centromere 6 is indicated by blue signals. Nuclei are counter with DAPI however the colour has been changed to gray in order to view signals for centromere 6. Retinoblastoma cells show additional copies of *DEK* and *E2F3*. Original magnification x100.
The lowest copy number was three and the highest seven among the 66 samples with gain of \textit{DEK} and \textit{E2F3} but not CEP6. While all 66 samples evinced gain of both genes in greater than 15\% nuclei, \textit{DEK} exhibited copy changes to a greater extent (5-8 signals) than \textit{E2F3}(3-4 signals). Additionally, \textit{DEK} was also found to be amplified in two samples with the copy number ranging from 7 to 14 while \textit{E2F3} and CEP6 were gained in these two samples.

In thirteen samples more than 2 copies of \textit{DEK} were noted in at least 15\% of all scored cells while CEP6 and \textit{E2F3} were observed to be diploid. Similarly, \textit{E2F3} gain was noted in at least 15\% of all scored cells in eight samples with normal \textit{DEK} and CEP6. The lowest copy number was three and the highest eight for \textit{DEK} and E2F3 in the samples with gain of these genes.

Forty-seven samples were scored as normal for \textit{DEK}, \textit{E2F3} and centromere 6.
3.2 Interphase FISH analysis of MYCN and 1p36 copy number, and IHC analysis of pRb protein expression in retinoblastoma.

3.2a MYCN (2p24) is gained and amplified in retinoblastoma

Single-colour interphase FISH for the MYCN oncogene on 2p24 was performed on 270 FFPE retinoblastoma patient samples using a pre-labelled commercial probe for MYCN (Orange-dUTP). Five samples could not be scored for the aforementioned reasons and a total of 265 patient samples were assessed. The copy number of MYCN was enumerated in a total of 100 non-overlapping tumour nuclei per sample. A ‘threshold for gain’ was calculated using five samples (Tables 3.5a and 3.5b) with a high percentage of normal cells. The threshold was based on the equation –

Table 3.5a: The five samples used in the calculation of the threshold for gain.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Normal</th>
<th>Gained (MYCN)</th>
</tr>
</thead>
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<td>91</td>
<td>11</td>
</tr>
<tr>
<td>RB12</td>
<td>87</td>
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<tr>
<td>RB32</td>
<td>83</td>
<td>14</td>
</tr>
<tr>
<td>RB24</td>
<td>92</td>
<td>7</td>
</tr>
<tr>
<td>RB125</td>
<td>89</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 3.5b: Threshold for gain calculation.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Average (Gained nuclei)</td>
<td>10.6</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2.7</td>
</tr>
<tr>
<td>Threshold for gain</td>
<td>16</td>
</tr>
</tbody>
</table>
Similar to the 1q and 6p analysis samples were considered to be ‘gained’ if more than 2 signals were noted for the *MYCN* probe and ‘amplified’ if more than ten signals were observed. The maximum number of signals counted in a single cluster was 15, if signals were greater than 15, the cluster was considered innumerable and reported as ‘>15’. Based on the pattern of amplification, two cytological forms were observed – DMs and HSRs. The results of the scoring are shown in Table 3.6.

**Table 3.6**: Percentage of samples with copy number changes in *MYCN*. Each gene is represented by the colour of the respective probe used in the FISH experiment prior to analysis.

<table>
<thead>
<tr>
<th>Copy Number change</th>
<th>Frequency of samples with genomic aberration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gain</td>
<td><em>MYCN</em></td>
</tr>
<tr>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>Amplification</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Based on former CGH studies, *MYCN* amplification is fairly rare (1-2%) but 2p gain has been observed in 34% of retinoblastoma. *MYCN* was found to be both gained and amplified in this cohort, with 6.8% (18/265) of the samples exhibiting greater than two copies in at least 15% of all scored cells and 7.5% of the samples exhibiting greater than ten copies in at least 15% of all scored cells (Figure 3.5).
Figure 3.5: $MYCN$ is amplified in retinoblastoma. FISH analysis on FFPE retinoblastoma (Sample RB73). The copy number of $MYCN$ is indicated by orange signals. Nuclei are counter stained with DAPI. Retinoblastoma cells show extensive $MYCN$ amplification. Original magnification x100.
The lowest copy number was three and highest, five for each of the loci in the gained samples and the copy number for \( MYCN \)-amplified samples ranged from ten to greater than thirty. Two distinct kinds of amplification were observed – DMs and HSRs (Figure 3.6a and 3.6b).

Figure 3.6: \( MYCN \) is amplified in retinoblastoma. FISH analysis on FFPE retinoblastoma (Samples RB81 and RB156). The copy number of \( MYCN \) is indicated by orange signals. Nuclei are counter stained with DAPI. Types of \( MYCN \) amplification in retinoblastoma. (A) \( MYCN \) amplification in the form of double-minutes. (B) \( MYCN \) amplification in the form of heterogeneously staining regions. Original magnification x100

Twelve samples (4.5%) displayed \( MYCN \) amplification in the form of DMs with copy numbers ranging from ten to thirty and seven samples (3.0%) possessed amplification in the form of HSRs with over thirty copies of the gene in these samples. No samples showed evidence of both DMs and HSRs. In all amplified samples, there were at least 15 cells with greater than thirty copies of \( MYCN \). The \( MYCN \)-amplified samples also exhibited aggressive histology by H and E staining (Figures 3.7a and 3.7b).
Figure 3.7: *MYCN* amplification is associated with aggressive histology in retinoblastoma. (A) Image of a Haematoxylin and Eosin stained retinoblastoma sample (RB57). (B) Select DAPI-stained nuclei within this sample (RB117) with *MYCN* amplification. Original magnification x100

3.2b *pRb is not commonly expressed in retinoblastoma*

*pRb* IHC staining was performed on 270 FFPE retinoblastoma patient samples using the automated Ventana system. A section of WERI-Rb cell line pellet was stained as the positive control and a section of Y79 cell line pellet was stained as the negative control. All sections were scored and analyzed using the Aperio scanning and imaging software. The Y79 negative control lacked immunoreactivity in 100% of the section but there was strong positive nuclear staining in 100% of the WERI-Rb section (Figures 3.8a and 3.8b).
Only four of the 270 retinoblastoma samples exhibited pRb staining. The extent of staining was assessed using the H-score. The H-score is a method of assessing the extent of nuclear immunoreactivity. The score is obtained by the formula:

\[ 3 \times \text{percentage of strongly staining nuclei} + 2 \times \text{percentage of moderately staining nuclei} + \text{percentage of weakly staining nuclei}. \]

The H-scores for these samples are shown in Table 3.7.

### Table 3.7 Results of pRb immunohistochemical staining in 4 retinoblastoma samples.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Percent Nuclei (3+)</th>
<th>Percent Nuclei (2+)</th>
<th>Percent Nuclei (1+)</th>
<th>H-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB17</td>
<td>0.34</td>
<td>1.098</td>
<td>1.54</td>
<td>4.77</td>
</tr>
<tr>
<td>RB221</td>
<td>1.31</td>
<td>1.45</td>
<td>1.53</td>
<td>8.37</td>
</tr>
<tr>
<td>RB35</td>
<td>0.29</td>
<td>2.9</td>
<td>2.53</td>
<td>9.19</td>
</tr>
<tr>
<td>RB74</td>
<td>0.6</td>
<td>1.0</td>
<td>0.68</td>
<td>4.49</td>
</tr>
</tbody>
</table>

pRb staining was weak in all four of the retinoblastoma samples with immunoreactivity. The H-scores for these samples was relatively low and ranged from four to nine. There was little
to no pRb staining in the remainder of the cohort and H-scores for these samples ranged from 0.02 to 2.98 (Figure 3.9). The H-scores reflect the extremely low percentage of positively stained nuclei in these samples. Of the four samples with pRb staining, one sample was scored as MYCN-amplified.
Figure 3.9: pRb is not expressed in retinoblastoma. A) Snapshot of fifteen samples containing four RB samples with weak pRb staining. B) Higher resolution image of highlighted sample with pRb positivity.

3.2c 1p36 is deleted in retinoblastoma

Three- colour interphase FISH for the 1p36 region was performed on 270 FFPE retinoblastoma patient samples using a pre-labelled commercial probe cocktail for 1p36 (Orange-dUTP), 1q25 (Aqua-dUTP) and the sub-telomeric region (1pst) of 1p (Green-dUTP) (Figure 3.10).
Figure 3.10: Location of the pre-labelled probes in the commercial cocktail probe for 1p36.
Five samples could not be scored for a variety of reasons – weak hybridization of the probe to the sample, inadequate signal intensity, missing cores on the TMAs and/or poor tissue quality. A total of 265 patient samples were assessed. The copy number of 1p36, 1pst and 1q25 were enumerated in a total of 100 non-overlapping tumour nuclei per sample. A ‘threshold for deletion’ was calculated using five samples (Tables 3.8a and 3.8b) with a high percentage of normal cells. This was done to determine the rate of false positivity. Due to truncation effects, several nuclei can appear to contain a single copy of a gene that is not deleted. The threshold was based on the equation –

\[
\text{Average (Number of nuclei with gain) } + 3 \times \text{Standard Deviation.}
\]

**Table 3.8a: Five normal samples used in the calculation of the threshold of deletion.**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Normal</th>
<th>Single copy (1p36 region)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB11</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>RB14</td>
<td>83</td>
<td>14</td>
</tr>
<tr>
<td>RB25</td>
<td>83</td>
<td>9</td>
</tr>
<tr>
<td>RB38</td>
<td>92</td>
<td>16</td>
</tr>
<tr>
<td>RB46</td>
<td>89</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 3.8b: Threshold for deletion calculation.**

<table>
<thead>
<tr>
<th>Average (Single-copy nuclei)</th>
<th>10.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Deviation</td>
<td>5.07</td>
</tr>
<tr>
<td>Threshold for gain</td>
<td>26</td>
</tr>
</tbody>
</table>

For the 1p36 analysis, samples with fewer than 2 copies of either locus or all loci in greater than 26 nuclei were labelled as ‘deleted’. Normal cells within the samples served as internal negative controls. The results of the scoring are shown in Table 3.9.
Table 3.9: Percentage of samples with copy number changes in 1p36. Each region is represented by the colour of the respective probe used in the FISH experiment prior to analysis.

<table>
<thead>
<tr>
<th>Copy Number change</th>
<th>Frequency of samples with genomic aberration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deletion</td>
<td>18.5</td>
</tr>
<tr>
<td>Gain</td>
<td>0.75</td>
</tr>
</tbody>
</table>

The most consistent genomic change among the samples was deletion of 1p36 and 1pST, with 18.5% (49/265) of the samples exhibiting fewer than two copies in at least 26% of all scored cells (Figure 3.11).
Figure 3.11: 1p36 is deleted in retinoblastoma. FISH analysis on a FFPE retinoblastoma (Sample RB115). The copy number of 1p36 is indicated by orange signals and that of 1p unstable is indicated by green signals. The copy number of 1q25 is indicated by blue signals. Nuclei are counter with DAPI however the colour has been changed to gray in order to view signals for 1q25. Retinoblastoma cells show single copies of 1p36. Original magnification x100.
In all deleted samples, greater than 26% of scored cells had a single copy of the 1p36 and 1pst loci. Furthermore, in all samples both 1p36 and 1pst were deleted and fewer than 26 cells in each sample exhibited deletion of either 1p36 or 1pst. Homozygous deletion (no discernible copies of either or all loci) was observed in fewer than 26% of all scored cells. 1q25 was found to be gained in 46% (122/265) of all scored samples, however this was a control locus and only the samples with deletion were considered in the subsequent statistical analysis. The copy number for 1q25 ranged from three to six in samples with gain.
3.3 Statistical correlations among the copy number status of 1q (*KIF14* and *MDM4*) and 6p (*DEK* and *E2F3*) regions of gain, *MYCN* amplification and 1p36 deletion.

The chi-squared test was used to assess correlations among the 1q and 6p regions of gain in retinoblastoma. Additionally, potential associations among 1q and 6p gain, *MYCN* amplification and 1p36 deletion were also evaluated. The results of the analysis are reported in Table 3.10.

**Table 3.10**: Statistical correlations among 1q and 6p regions of gain, *MYCN* amplification and 1p36 deletion.

<table>
<thead>
<tr>
<th>Regions of genomic alteration</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1q and 6p</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><em>MYCN</em> and 1p36</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>6p and 1p36</td>
<td>0.998</td>
</tr>
<tr>
<td>1q and 1p36</td>
<td>0.444</td>
</tr>
<tr>
<td><em>MYCN</em> and 1q</td>
<td>0.420</td>
</tr>
<tr>
<td><em>MYCN</em> and 6p</td>
<td>0.671</td>
</tr>
</tbody>
</table>

1q gain (*KIF14* and *MDM4*) is positively associated with 6p (*DEK* and *E2F3*) gain in retinoblastoma (p < 0.0001). Additionally, *MYCN* is also strongly associated with 1p36 deletion in retinoblastoma (p < 0.0001). No correlations were observed between 1q gain and *MYCN* amplification (p-value 0.42) or 6p gain and *MYCN* amplification (p-value 0.671). Similarly, 1q gain and 6p gain is not associated with 1p36 deletion (p-values 0.444 and 0.998).
Chapter 4

Discussion

The objective of this project was to perform a retrospective study of the 1q and 6p regions of gain, MYCN amplification and 1p36 deletion in 270 FFPE retinoblastoma patient samples by FISH. Additionally, we also examined the copy number changes for statistically significant associations among these regions of genomic alteration. Despite its rarity, retinoblastoma research has yielded important insight into the molecular mechanism of carcinogenesis by highlighting regions of the genome that are biologically significant in a variety of cancers. Potential candidate tumour suppressors and oncogenes within these regions of genomic alteration are currently under investigation as putative biomarkers and therapeutic targets.

Bi-allelic inactivation of the classical tumour suppressor RB1 is the first step towards retinoblastoma and primes the cell for subsequent genomic events that lead to malignancy. Genomic and gene expression studies have presented key regions of the genome that are recurrently altered in retinoblastoma and putative candidates within these regions have been identified and functionally examined as potential biomarkers of retinoblastoma progression. However, collectively only 197 tumours have been studied by aCGH and FISH. Our study presents the results of a thorough assessment of copy number changes in six candidate loci – KIF14 and MDM4 on 1q, DEK and E2F3 on 6p, MYCN on 2p and 1p36 in a very large cohort of 270 retinoblastoma patient samples collected by our clinical collaborator Dr Celia Antoneli in Sao Paulo, Brazil. Furthermore, we have also demonstrated associations between these regions of genomic aberration.
In summary we have established the following:-

1) 1q gain (Defined by extra copies of KIF14, MDM4 and CEP1) is the most consistent genomic alteration in retinoblastoma, closely followed by 6p gain (Defined by extra copies of DEK, E2F3 and CEP6).

2) MYCN amplification and 1p36 deletion are observed at higher frequencies (7.5% and 18.5%) than previously documented in retinoblastoma.

3) MYCN amplification is associated with pRb protein expression.

4) 1q and 6p gain are linked, as are MYCN amplification and 1p36 deletion.

4.1 1q and 6p are early genomic changes in retinoblastoma development

4.1a 1q (KIF14 and MDM4) gain in retinoblastoma

The long arm of chromosome 1 is recurrently associated with a variety of cancers. Based on the Progenetix database of CGH, aCGH profiles and over 10,000 tumour karyotypes \(^{175}\), 1q31-1q32 gain or amplification has been reported in a large proportion of solid tumours in addition to a number of smaller cancers. In accordance with a previously published study of 1q gain in retinoblastoma \(^{63}\) we report 1q gain as the most frequent genomic change in retinoblastoma (52%). This is also consistent with the frequency of 1q gain in the literature, which was reported cumulatively in 99/197 tumours (50%) across seven published studies \(^{55,63-68}\). Owing to the prevalence of 1q31-1q32 gain in a wide variety of cancers, this region likely harbours one or more genes that may be broadly relevant in cancer biology and in particular, retinoblastoma tumourigenesis. Indeed, two potential candidates KIF14 and MDM4 have been recurrently associated with oncogenic activity based on their copy number status and mRNA, gene and protein expression in retinoblastoma \(^{62,93,96,102}\). Furthermore, these genes have also been functionally validated in retinoblastoma cell lines \(^{93,102}\).
How does increased copy number and consequently elevated expression of these two genes affect retinoblastoma development?

Originally cloned as a 6586bp cDNA, *KIF14* encodes a mitotic kinesin that belongs to the N3 family of kinesins. The kinesin (KIF) superfamily consists of 45 members with a shared kinesin motor domain and a microtubule-dependent ATPase that functions as an intracellular transport motor. KIFs transport a variety of intracellular organelles, mRNA protein complexes and chromatin. The drosophila ortholog of KIF14 (KLP38B) is implicated in the organization of the bipolar spindle, tethering the chromosomes to the spindle during mitosis and in cytokinesis. There is evidence to indicate that human KIF14 executes similar functions in normal cells. *KIF14* is selectively expressed at higher levels at the G2-M transition of the cell-cycle, further confirming its mitotic role. KIF14 is also reported to play a role in the alignment of chromosomes at the metaphase plate and interacts with PRC1 in the localization of the central spindle to the midbody during mitoses. Disrupted KIF14 expression results in polyploidy as a consequence of multinucleation.

Successful cytokinesis is requisite not only for mitotic consistency but also in the maintenance of genomic stability and is heavily dependent on microtubule organization. Gain of the *KIF14* locus at 1q32.1 and consequent overexpression may well lead to accelerated and defective mitoses. Moreover, the *KIF14* promoter is reported to harbour several E2F-consensus binding sequences and is possibly regulated by pRb/E2F. In pRb-inactive cells, the availability of E2F3 to regulate KIF14 levels could also be responsible for its overexpression independent of genomic gain. The genomic instability triggered by
impaired cytokinesis as a consequence of KIF14 overexpression could also prime the cell for the subsequent genomic alterations observed in malignant retinoblastoma.

KIF14-specific inhibition has yet to be explored, however given its ATPase activity it remains an excellent target for pharmalogical inhibition. In vitro studies have already reported successful results with chromokinesin inhibitors and small molecule inhibitors of the kinesin Eg5 are emerging as promising contenders in cancer chemotherapeutics. High-throughput screens for KIF inhibitors are also underway and offer compelling opportunities to explore KIF14 inhibition and its implementation in future clinical trials.

Structurally homologous to MDM2, MDM4 is overexpressed and the MDM4 locus is amplified, in 10-20% of several cancers including colon, stomach breast and lung cancer and 65% of retinoblastoma. The MDM2 locus is amplified in ten percent of retinoblastoma. MDM4 regulates p53 at the transcriptional and protein level and the exact nature of this regulation and its biological implications are still under investigation. Its most established function is in the inhibition of p53 activity by enhancing p53-degradation by the E3 ubiquitin ligase MDM2. It is also reported to independently inhibit p53 during embryonic development. p53 – which is mutated in several cancers, is noted to be intact in retinoblastoma. However, amplification of the MDM4 locus and consequent overexpression may be responsible for compromising p53 function in retinoblastoma cells. Abrogation of p53 activity enables cancer cells to bypass p53-mediated apoptosis or DNA damage repair signalling and actively proliferate.

Several studies are currently undertaking efforts to selectively target the p53-MDM2/MDM4 interaction with a number of small-molecule inhibitors and novel peptides. Nutlin 3A currently shows the most promise as an anti-MDM4 SMI, with demonstrated capability
to reduce proliferation and increase apoptosis in retinoblastoma cells \(^{102,107,191}\). More recently, it is also reported to decrease tumour size and the rate of tumour progression in mice with the murine equivalent of retinoblastoma \(^{199,191}\).

We have shown evidence for 1q gain in 52\% (136/262) FFPE retinoblastoma samples. Since all three loci – KIF14, MDM4 and centromere 1 were gained in these samples, the gain most likely spans the long arm of chromosome 1. As the probe used for the interphase FISH scoring bind the sequences flanking centromere 1, gain of this locus may not necessarily indicate a change in ploidy. Moreover, it cannot be ruled out that smaller regions of gain may be present along 1q that were not detected in this assay. FISH of multiple markers on chromosome 1q would be useful in understanding the spectrum of changes on chromosome 1q. In 125 tumour samples KIF14 copy number was higher than that of MDM4, despite the fact that both genes are located within the 1q32 MRG. KIF14 amplification and overexpression is also observed in a larger number of samples than MDM4 in retinoblastoma \(^{36,93,191}\). Based on changes in copy number and expression profiles, KIF14 may be the target gene within this region of gain. However, further studies are required to establish the importance of KIF14 versus MDM4 in retinoblastoma as both genes have been shown to be functionally significant and apposite markers of retinoblastoma progression. Additionally, the protein products of these genes would confer tumour cells with KIF14 and/or MDM4 gain with a mutually beneficial selective survival and proliferative advantage. While KIF14 overexpression would result in destabilized mitoses and polyploidy; MDM4 overexpression would enable the cells to circumvent p53-mediated apoptotic induction, thereby increasing their survival. Owing to the frequency of KIF14 and MDM4 gain in this large cohort they are most likely early events in retinoblastoma tumourigenesis. Consistent with the current hypothesized model of retinoblastoma development \(^{37}\), we posit 1q gain to be the M3 mutational event.
4.1b 6p (DEK and E2F3) gain in retinoblastoma

Similar to 1q, the short arm of chromosome 6 is also associated with a variety of cancers. Based on the Progenetix database of CGH, aCGH profiles and over 10,000 tumour karyotypes\(^{175}\), 6p22 gain or amplification has been reported in several solid tumours in addition to a number of smaller cancers. In accordance with a previously published study of 6p gain in retinoblastoma we report 6p gain as the second most frequent genomic change in retinoblastoma (48.7\%) \(^{63}\). In the literature 6p gain is the most recurrent genomic alteration and is reported cumulatively in 104/197 tumours (52.8\%) across seven published studies \(^{55,63-68}\). Given the limited number of studies that have examined the occurrence of 1q and 6p gain in retinoblastoma, the order of these events is still subject to speculation. As 6p22 gain is seen in a wide variety of cancers, this region likely harbours one or more genes that may be relevant in cancer biology and in particular, retinoblastoma tumourigenesis. DEK is the potential candidate gene within the 6p region of gain and E2F3 – which is 2Mb removed from the MRG are strong contenders, based on their copy number status and mRNA, gene and protein expression in retinoblastoma \(^{36,75}\). Furthermore, these genes have also been functionally validated in retinoblastoma cell lines \(^{75}\).

DEK encodes a nuclear phosphoprotein that is implicated in cancer and autoimmune diseases. While its biological function is as yet uncharacterized, it has imputed roles in transcriptional regulation \(^{200}\), modulating chromatin architecture \(^{79,201,202}\) and mRNA splicing \(^{203}\). The protein harbours two DNA binding regions – a SAF box and a multimerization domain \(^{78,204}\). Furthermore, its C-terminus is structurally homologous to the E2F/DP family of transcription factors \(^{204}\). Depending on the microenvironment and cell-type DEK can either induce or repress transcription \(^{205}\). The best elucidated protumourigenic features of DEK are related to DNA binding and 90\% of the protein is preferentially bound to euchromatic regions of chromatin \(^{77,79,206}\). Its DNA binding activity is largely influenced by posttranslational modifications (PTMs),
- chiefly phosphorylation, ADP-ribosylation, or acetylation. Phosphorylation by protein kinase CK2 and acetylation of its N-terminus are reported to affect the varying quality of DEK activity in the nucleus, by decreasing its affinity to DNA. DEK is poly (ADP-ribosyl)ated in cells that are affected by genotoxic stress and this modification is presumably mediated by DEK interactions with PARP1, a critical mediator of DNA-damage signalling. Poly (ADP)ation of DEK serves to release it from bound chromatin and form reactive auto-antibodies in the extracellular space. DEK auto-antibody accumulation is observed in the synovial fluid of arthritic patients and suggests that dysregulation of DEK activity may be closely tied to the inflammatory process.

In addition to these posttranslational modifications, DEK-DNA interaction is also altered by histone deacetylases, histone variants and methyltransferases. The significance of PTM of the DEK protein and its role in the inflammatory process, are yet to be mechanistically elucidated in the context of cancer. DEK expression is also regulated to some extent by members of the pRb protein family and by E2F binding to consensus E2F binding sites in the DEK promoter. Additionally, DEK is also implicated in inhibiting apoptosis through interfering with p53 activity.

Notwithstanding the lack of understanding behind the mechanism of DEK regulation and function in cancer cells, it shows ample evidence as an oncogene. In addition to its importance in clinically unfavourable AML, as part of the DEK:CAN translocation, DEK is overexpressed in AML; retinoblastoma; glioblastoma; hepatocellular carcinoma; melanoma; and in a number of other smaller tumour types. Overexpression of DEK mRNA and protein in HCC correlates with histological grade. DEK is also specifically overexpressed in invasive bladder cancer as compared to early stage tumours. The functional significance of DEK in retinoblastoma was evident from shRNA mediated knockdown in the retinoblastoma cell line...
RB247AC which normally overexpresses DEK. Its knockdown resulted in decreased cell growth and increased apoptosis.

As a ubiquitous factor in chromatin remodelling with supplementary roles in transcriptional regulation and mRNA splicing, DEK-inhibition could result in disrupting the activity of normal cells. However, in a recent study, DEK-deficiency in mice did not cause lethality whereas DEK depletion in tumour cells directly resulted in senescence or cell death. DEK is also known to modulate tumour resistance to genotoxic agents in melanoma. This particular characteristic could be exploited by using DEK inhibitors in concert with genotoxic agents to circumvent tumour resistance. This also suggests that gain of the 6p22 locus and consequent DEK overexpression might be associated with recurring disease in retinoblastoma patients. Large-scale genomic profiling of recurring retinal tumours will provide better insight into the mechanisms of tumour resistance in retinoblastoma. DEK-inhibition is not well studied in retinoblastoma, but its wide-ranging effects on apoptosis, senescence and DNA-binding activity make it a particularly compelling target for therapeutic intervention.

E2F3 is one of eight members of the E2F family of transcription factors. It consists of a DNA-binding domain, dimerization domain, transactivation domain and a pRb binding domain. E2F3 has garnered particular interest as it is both amplified and overexpressed in several human tumours including lung, bladder and prostate. The E2F3 locus can give rise to either the E2F3a or the E2F3b isoform through the recruitment of varying promoters and alternative 5’-coding exons. While the biological activities of these isoforms have not been well characterized in humans they are thought to exhibit partly antagonistic functions. Recently, E2F3a was reported to instigate proliferation in ovarian cancer cells through selective stimulation by the epidermal growth factor receptor (EGFR). E2F3 is negatively regulated by pRb during the G0 and G1 phases of the cell cycle. Following pRb phosphorylation by cyclin D and CDK4/6, E2F3
is released to stimulate transcription of target S-phase genes and enable the G1-S transition. Moreover, E2F3 is also involved in facilitating cell-cycle progression through the G2-M phase by direct activation of the Aurora kinase A. Aurora kinase A plays a pivotal role in genomic instability and is recently reported to induce tumourigenesis through cell-cycle disruption and BRCA2 repression in ovarian cancer. While the roles of aurora kinases in retinoblastoma are unstudied, E2F3 overexpression might have additional effects on the G2-M transition and possible suppression of BRCA2 tumour suppressor activity in retinoblastoma tumourigenesis. E2F3 is also implicated in driving the expression of Oncomir-1 – a cluster of oncogenic miRNAs located on chromosome 13, that exert a significant impact on proliferation both in vitro and in vivo in Wilm’s tumours. MiRNA regulation in retinoblastoma is not well characterized, but E2F3 overexpression could also tangentially induce the expression of oncogenic miRNA clusters in addition to its principal effects on cell-cycle progression.

In retinoblastoma, owing to its direct regulation by pRb, it is expected that pRb-inactive cells will exhibit elevated levels of E2F3 and consequent progression through the cell cycle. However, E2F3 overexpression is reported to selectively benefit pRb-inactive prostate cancer cells. Similar to DEK, shRNA knockdown of E2F3 also resulted in negative growth and overexpression of cyclin B1, which indicates cell-cycle arrest at the G2/M transition.

E2F3-specific inhibitors have not been well studied, however E2F inhibition by peptides is reported to successfully hinder proliferation and induce apoptosis in human tumour cells, validating E2F family members as potential therapeutic targets in cancer. Recently, miR-125b, which is commonly downregulated in bladder cancer is noted to inhibit E2F3 and specifically impede the G1-S transition.
DEK and E2F3 overexpression have demonstrated abilities to induce anchorage independent growth. Elevated DEK and E2F3 levels are also noted in pRb-active cells and reportedly impart these cells with proliferative advantage. ShRNA-mediated knockdown of DEK and E2F3, results in decreased proliferation and increased expression of the apoptotic marker, caspase 3.

pRb-inactive cells proliferate to a certain extent and are maintained in a quiescent state by p16 overexpression and induction of the senescence barrier. Indefinite cycling of these cells is most likely facilitated by overexpression of DEK and E2F3. DEK overexpression as a consequence of 6p22 gain would enable retinoma cells to bypass apoptosis and senescence and drive progression to malignant retinoblastoma. Coupled with E2F3 overexpression, these cells would then be able to sustain active proliferation through rapid G1-S transitions. Both DEK and E2F3 manifest extensive oncogenic potential and are recurrently gained together in our cohort. DEK gain was noted at higher levels (5-8 copies) and in more samples (thirteen) than E2F3 gain (3-4 copies, eight samples). Based on copy number alone DEK could be the target gene within the 6p22 MRG and E2F3 gain an additional mutational event that would be beneficial to cells during progression to malignancy. However, further studies are required to establish the importance of DEK versus E2F3 in retinoblastoma as both genes have been shown to have functional relevance in retinoblastoma progression. Similar to the frequency of 1q gain in this large cohort, 6p gain is also an early event in retinoblastoma tumourigenesis. Based on the frequency of this genomic alteration in our cohort (48.7%) and consistent with the current hypothesized model of retinoblastoma development, we posit 6p gain to be the M4 mutational event.
4.2 MYCN amplification and 1p36 deletion constitute a novel molecular subtype of retinoblastoma

4.2a MYCN amplification is associated with pRb expression in retinoblastoma

We report MYCN amplification at a higher frequency (7.5%) than previously documented in retinoblastoma. MYCN amplification is associated with pRb-positive cells and aggressive histology in retinoblastoma (Gallie et al, in press). MYCN-amplified samples demonstrated a higher histological grade in corresponding H and E stained sections. As expected, pRb expression was largely negative in this cohort. Interestingly, four samples showed evidence of nuclear pRb staining and one sample also displayed MYCN amplification and 1p36. This supports results by Gallie et al, where positive pRb expression is consistently associated with MYCN amplification. IHC staining of these tumours should be repeated in a large cohort of retinoblastoma samples with better tissue quality. As the samples used in the present study were collected over several years the immunoreactivity of the tissue may have been compromised. Further studies have to be done with a substantial number of samples in order to determine the relationship between pRb expression and MYCN amplification.

4.2b Role of MYCN amplification in retinoblastoma

MYCN is a member of the Myc family of b-HLH leucine zipper transcription factors that mediate a plethora of cellular processes including proliferation, differentiation, apoptosis and oncogenesis\(^{229-231}\). Members of the Myc family heterodimerize with Max and specifically induce the transcription of a variety of target genes that have oncogenic effects\(^{232}\). MYCN amplification is discernible as low-level (10-20) and high-level (100-200) copy number increase\(^{113}\), and as DMs and HSRs\(^{114}\) in retinoblastoma. Its overexpression is noted in retinoblastoma tumours regardless of their amplification status\(^{114,116,122}\). CGH analyses have reported MYCN
amplification in retinoblastoma tumours at frequencies ranging from 0 to 30% \(^{62}\). *MYCN* amplification often appears as either DMs or HSRs in retinoblastoma cell lines Y79 \(^{118}\) and RB355 \(^{126}\). The implication of the nature of *MYCN* amplification (DM versus HSR) in the clinical context of this disease has yet to be determined. We report two distinct types of *MYCN* amplification in retinoblastoma – DMs (4.5%) and HSRs (3%). Further studies are required to establish whether these represent two subtypes of retinoblastoma tumours or if they are merely a consequence of different mechanisms of *MYCN* amplification in the same tumour.

*MYCN* amplification is best characterized in neuroblastoma. The frequency of *MYCN* amplification in neuroblastoma is 25% but increased (40%) in advanced disease. It is also recurrently associated with hyperproliferative tumours and clinically unfavourable outcome \(^{109,111,113}\). Unlike c-Myc which is expressed in several embryonic and adult tissues, MYCN expression is commonly observed in the developing nervous system \(^{111}\). While c-myc regulation is well characterized \(^{233}\), little is known about the targets of *MYCN* amplification and consequent overexpression. Ectopic *MYCN* expression results in rapid cell-cycle progression \(^{234,235}\) and apoptosis \(^{236}\). As in retinoblastoma, the p53 protein is found to be intact and functional in neuroblastoma. *MYCN* amplification and consequent overexpression is speculated to contribute towards the abrogation of p53 function in *MYCN*-amplified neuroblastoma \(^{237}\).

In addition to its effects on cellular proliferation, *MYCN* is also implicated the induction of aneuploidy and chromosomal alterations. Overexpression of *MYCN* significantly alters the chromosome complement of neuroblastoma tumours that are treated with ionizing radiation \(^{238}\). While the mechanisms behind its regulation remain unclear, *MYCN* is implicated in triggering centrosome aneuploidy \(^{238}\). In *MYCN*-amplified neuroblastoma, *MYCN* overexpression could serve to instigate aberrant mitoses through centrosome aneuploidy. The role of centrosome amplification is unstudied in retinoblastoma, but given its importance in driving tumourigenesis
and reliance on MYCN amplification, it might be an interesting characteristic of aggressive retinoblastoma tumours.

MYCN inhibition has been of considerable interest in neuroblastoma as it is observed in 40% of advanced cases and closely linked to drug resistance and unfavourable outcome. Interferon-γ is noted to enhance retinoic acid-mediated differentiation and growth suppression in neuroblastoma in vitro and in vivo. The synergistic effects of interferon-γ and retinoic acid could also be harnessed in the treatment of MYCN-amplified retinoblastoma patients with particularly aggressive disease. Trichostatin A (histone deacetylase inhibitor) and epoxomycin (proteosome inhibitor) are also reported to cause growth suppression and apoptosis in MYCN-amplified neuroblastoma cell lines. While trichostatin A effectively downregulated MYCN expression, epoxomycin resulted in increased MYCN expression. The resultant cellular effect in both cases was induced differentiation and apoptosis. More recently, it has been discovered that cyclin dependent kinase 2 (CDK2) inhibition results in synthetic lethality in MYCN amplified neuroblastoma cells. Given the overexpression of cyclins in retinoblastoma, this might also be an effective therapeutic strategy in treating MYCN-amplified aggressive retinoblastoma.

4.2c MYCN amplification and 1p36 deletion in retinoblastoma

We report 1p36 deletion at a higher frequency (18.5%) than previously documented in retinoblastoma. Interestingly, both the 1p36 probe and 1pst probe were deleted in the majority of samples suggesting that the region of deletion may span 1p36 and 1pst. Gain of the control probe at 1q25 in 46% of the cohort confirmed results of the frequency of 1q gain (52%). This also suggests that the earlier event might be gain at the 1q31-1q32 MRG as it is seen in a larger proportion of samples. However, the order of genomic events would be best determined by
association of these observations with laterality of the disease and evidence of family history. Additionally, we report a strong association between \textit{MYCN} amplification and 1p36 deletion in our cohort (p< 0.0001), this suggests that \textit{MYCN}-amplified and 1p36-deleted retinoblastoma constitute a novel molecular subtype of this disease and warrant further study of the clinical relevance of these genomic aberrations.

1p LOH was first observed in 21\% of retinoblastoma tumours\textsuperscript{146} with an increased incidence in metastatic disease. It is also part of a genomic signature in conjunction with \textit{MYCN} amplification in the molecular subtyping of neuroblastoma\textsuperscript{147}. Tumours with 1p loss exhibit aggressive histology and unfavourable clinical outcome\textsuperscript{148}. 1p36 monosomy is one of the most frequent terminal deletion syndromes\textsuperscript{149} and is associated with delayed development, mental impairment and facial dysmorphism. Interestingly, 1p36 deletion is also the underlying cause of congenital cataract, a rare paediatric disorder that renders most affected children blind or visually impaired\textsuperscript{151}.

In our cohort 1p36 deletion was usually observed as a single copy. Homozygous deletion of the 1p36 region was not observed in a sufficient number of cells (26) within any sample to be considered significant. Additionally, because the probe mixture only included probes for the 1p36 region and the distal subtelomeric sequences, the question of whether this constitutes 1p loss cannot be determined from these results. Further FISH analysis with multiple probes for various regions on the short arm of chromosome 1 would enable determination of whether the deletion is specific to 1p36 and the subtelomeric region or if it spans 1p.

Loss of genetic material on 1p occurs in several cancers. In a study of 683 solid tumors of various types, the prevalence of loss of heterozygosity (LOH) on 1p ranged from 30\% to 64\% depending on tumour site\textsuperscript{241}. 1p LOH was reported in breast, lung, endometrial, ovarian, and
colorectal carcinomas. LOH was reported at 1p36.3, 1p36.1, 1p35-34.3, 1p32, and 1p31 regions and varied with the type of tumour. Genes on 1p previously subjected to mutation analysis as candidate tumour suppressors for neuroblastoma or glioma have included *TP73, RAD54L, KIF1B, HKR3, UBE4B/UFD2, EXTL1*, and *CHD5*. However, no strong candidate for the 1p tumour suppressor gene in either neuroblastomas or gliomas has thus far been identified.

4.3 **1q, 6p, MYCN amplification and 1p36 deletion are biomarkers of retinoblastoma**

4.3a **Potential model of retinoblastoma tumour development**

1q gain and 6p gain were the two most frequent genomic alterations in our cohort. These two regions appear to be synergistically linked as there is strong association between 1q and 6p gain (P <0.0001). Similarly, *MYCN* amplification was also strongly associated with 1p36 deletion (p<0.0001) and potentially represents a novel subtype of retinoblastoma.

This project represents the largest series to date of retinoblastoma characterized for genomic changes beyond *RB1*. We examined 270 FFPE retinoblastoma for gain and loss of key regions of the genome based on former karyotypic, CGH and aCGH analyses. These changes reflect the subsequent mutational events necessary for retinoblastoma progression (M3 to Mn). Based on the frequency of the genomic changes and patterns of association between genomic regions in individual tumours, we propose that the most common genomic changes in retinoblastoma, which may represent the M3 and M4 events, are chromosome 1q32.1 and 6p22 gain (Figure 4.1). These gains commonly occur together, further supporting their shared role as early genomic events in progression. It is not possible to determine conclusively which event occurs earlier from these results but based on frequency of these changes alone 1q gain is posited as the M3 mutational event and 6p gain as the M4 mutational event. Given the frequency of
MYCN gain (6.8%) in this cohort and in previously published studies, it might the M5 mutational event. 16q22 loss is negatively associated with MYCN gain \(^\text{37}\), suggesting that these might be alternate M5 events. Based on our results as 1p36 deletion is closely linked with MYCN amplification, it may represent a different subtype of retinoblastoma tumours that do not rely on the earlier genomic alterations (M3-M4) for progression to malignancy. However, the frequency of 1p36 deletion in our cohort is higher than previously documented (18.5%). It is also considerably higher than the frequency of MYCN amplification (7.5%). Given that the 1p36 region is hypothesized to harbour one or more tumour suppressor genes it may well be an alternate M5 event in the currently proposed model of retinoblastoma progression (Figure 4.1) \(^\text{37}\).

\[\text{Figure 4.1: Potential molecular model of retinoblastoma progression, based on frequencies of gain and loss of genomic regions on chromosomes. Loss of each RB1 allele is shown as the M1 and M2 events, leading to the benign retinoma. 1q32.1 (KIF14 and MDM4) and 6p22 (DEK and E2F3) gain are the most common genomic changes, shown as M3 and M4. 16q22 loss and MYCN gain negatively associate so are shown as alternate M5 events. In addition, 1p36 deletion is also posited as a potential M5 event. Note that this model only incorporates copy number changes, and not other molecular events in retinoblastoma progression (Modified from}\ ^{\text{37}}\text{).}\]
4.3b Interaction between 1q and 6p regions of gain

The four candidate genes in the 1q and 6p regions of gain are *KIF14, MDM4, DEK* and *E2F3*. A synergistic interaction among these two regions of gain might be particularly beneficial to tumour cells evincing gain and consequent overexpression of these genes. As previously described, KIF14 overexpression results in dysregulated cytokinesis and polyploidy as a consequence of multinucleation, this could be the initial destabilizing event following pRb-inactivation as cytokinesis is important in the maintenance of genomic stability. MDM4 overexpression enhances p53-degradation by MDM2, and this could enable the tumour cells to evade apoptosis. With 1q gain alone, the tumour is conferred with two hallmarks of cancer, namely 1) Insensitivity to anti-growth signals (p53 and pRb) and 2) Evading apoptosis. Tumours that also have 6p gain are mutually benefited by *DEK* and *E2F3* overexpression. DEK has a variety of roles in the evasion of senescence and apoptosis, chromatin remodeling, transcriptional activation and mRNA splicing. Similar to KIF14, DEK is also directly regulated by E2F3. Overexpression of DEK and E2F3 would confer these tumour cells with the ability to bypass apoptotic and senescence barriers. In tumours with both 1q and 6p gain, this effect would be enhanced by E2F3 regulation of DEK and KIF14 expression, thereby enabling the tumour cells to capitalize on the oncogenic properties of these proteins (Figure 4.2)
Figure 4.2: Synergistic interactions between 1q and 6p gain (Modified from 44)
4.3c A novel molecular subtype of retinoblastoma characterized by MYCN amplification and 1p36 deletion

As mentioned previously, MYCN amplification is seen at a higher frequency in this cohort and shows a strong association with 1p36 deletion. Based on the frequency of these two genomic events and the overriding proliferative advantage of MYCN amplification, our second model of retinoblastoma progression for this particular subset of tumours is presented below (Figure 4.3). There are two possibilities within this model. Based on our IHC data, pRb expression is absent and hence the allele is probably inactivated by the initiating M1 and M2 events and this is closely followed by MYCN amplification and 1p36 deletion as M3 mutational events. However, given that we used archived samples where the quality of the tissue may be compromised it is likely that pRb is expressed in these samples but was undetected by IHC. Based on previous studies (Gallie et al, in press) the pRb protein is retained in MYCN-amplified tumours. In this case, the initiating M1 event may be MYCN amplification and possibly, 1p36 deletion. Note that this figure only incorporates early events in this particular subtype of retinoblastoma based on the probes used in this study. Additionally, owing to the preliminary nature of this project, this model is also largely speculative. Future studies of other regions will provide a better picture of the progression model of this subtype of retinoblastoma.
Figure 4.3: Potential molecular model of retinoblastoma progression in MYCN-amplified retinoblastoma. MYCN amplification and 1p36 are one of the mutational events, possibly the initiating mutations in pRb-positive tumours (Modified from 37).
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