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Phosphorylation of pRb: mechanism for RB pathway inactivation in MYCN-amplified retinoblastoma

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Keywords
Childhood-onset ocular tumor, MYCN amplification, pRb and ppRb expression in RB, pRb inactivation by phosphorylation, RB1 mutation-negative retinoblastoma

Abstract
A small, but unique subgroup of retinoblastoma has been identified with no detectable mutation in the retinoblastoma gene (RB1) and with high levels of MYCN gene amplification. This manuscript investigated alternate pathways of inactivating pRb, the encoded protein in these tumors. We analyzed the mutation status of the RB1 gene and MYCN copy number in a series of 245 unilateral retinoblastomas, and the phosphorylation status of pRb in a subset of five tumors using immunohistochemistry. There were 203 tumors with two mutations in RB1 (RB1⁻/−, 83%), 29 with one (RB1⁺/−, 12%) and 13 with no detectable mutations (RB1⁺/+). Eighteen tumors carried MYCN amplification between 29 and 110 copies: 12 had two (RB1⁻/−) or one RB1 (RB1⁺/−) mutations, while six had no mutations (RB1⁺/+). Immunohistochemical staining of tumor sections with antibodies against pRb and phosphorylated Rb (ppRb) displayed high levels of pRb and ppRb in both RB1⁺/+ and RB1⁺/− tumors with MYCN amplification compared to no expression of these proteins in a classic RB1⁻/−, MYCN-low tumor. These results establish that high MYCN amplification can be present in retinoblastoma with or without coding sequence mutations in the RB1 gene. The functional state of pRb is inferred to be inactive due to phosphorylation of pRb in the MYCN-amplified retinoblastoma without coding sequence mutations. This makes inactivation of RB1 by gene mutation or its protein product, pRb, by protein phosphorylation, a necessary condition for initiating retinoblastoma tumorigenesis, independent of MYCN amplification.

Introduction
In 1971, Alfred Knudson studied a series of cases of retinoblastoma, a childhood-onset ocular tumor, and proposed the “two-hit-model” [1]. According to this model, retinoblastoma is caused by biallelic inactivation of a single gene, retinoblastoma 1 (RB1). These two “hits” or mutations originally defined the concept of “tumor suppressor” genes. In heritable cases of retinoblastoma, the first hit is an inherited germline mutation, whereas the second hit is a somatic mutation; in sporadic cases, both hits are somatic in origin in a single retinal cell which initiates tumorigenesis [1].

Recently, a small subset of retinoblastoma with no detectable mutation in RB1, and amplification of the MYCN gene was identified [2]. It was hypothesized that MYCN amplification initiated retinoblastoma tumorigenesis in the presence of functional pRb protein [2].

It is known that pRb can be inactivated by various mechanisms including genetic mutations and phosphorylation [3]. To define which of the identified mechanisms is present in MYCN-amplified retinoblastoma, we first investigated MYCN copy number and the mutations present in RB1 in a series of 245 cases of unilateral retinoblastoma. We compared and contrasted the clinical features of the two groups of tumors classified by their MYCN...
amplification status. To explore the possibility of pRb inactivation by phosphorylation as an alternate pathway, we used immunohistochemical staining to evaluate the expression of four proteins: SKP2, a target of MYCN amplification, p27, a substrate for SKP2 ubiquitination thereby inhibiting pRb phosphorylation, total pRb, and phosphorylated pRb (ppRb).

**Materials and Methods**

**Retinoblastoma specimens**

A total of 245 unilateral retinoblastomas that had undergone complete screening for coding sequence and promoter region mutations in the RB1 gene were studied [4–6]. Ninety-four tumors were collected following enucleation at Wills Eye Hospital (CLS), Thomas Jefferson University, Philadelphia, PA. These specimens were submitted to the histopathology laboratory at Wills Eye Hospital (RCE) for routine processing, diagnosis, and descriptive analysis. Frozen or formalin-fixed paraffin-embedded samples were sent to the Genetics Diagnostic Laboratory (GDL), Perelman School of Medicine, University of Pennsylvania (AG), Philadelphia, PA for genetic testing. An additional 151 cases of unilateral retinoblastoma were submitted to the GDL for genetic testing by 62 different sites in the US, Canada, Thailand, and Chile. Tumors used in this study were collected between 1982 and 2014. A small subset of 41 tumors was included in a previous study [7] and is indicated by an asterisk in Table S1. Pathology reports were available for 111 of the retinoblastoma samples (Table S2).

The Institutional Review Board of the University of Pennsylvania approved this research in accordance with an assurance filed with and approved by the U.S. Department of Health and Human Services. Since all patients were under the age of 18 years, written informed consent for use of tissues and data for research was obtained from a parent or legal guardian of all patients prior to genetic testing.

**DNA isolation and screening of the RB1 gene**

DNA was isolated from frozen and formalin-fixed, paraffin-embedded retinoblastoma specimens using Qiagen DNeasy Blood and Tissue kits following manufacturer’s protocols (Valencia, CA).

Mutation analysis of all 27 coding exons of the RB1 gene, plus promoter and flanking intronic regions was performed by Sanger sequencing as previously described [4, 8]. Methylation status of the promoter region, estimation of exonic copy number, and loss of heterozygosity (LOH) were carried out as previously described [4, 8]. Mutations were annotated based on Genbank accession L11910.1 and compared against the LOVD-RB1 database [9].

**MYCN copy number determination and amplicon size**

MYCN copy number was determined using an Applied Biosystems Taqman copy number assay (Hs00824796_cn, Life Technologies) using qPCR of tumor DNA following manufacturer’s protocols. To characterize genome-wide chromosomal changes and to determine the size of the MYCN amplicons, 38 retinoblastoma samples were genotyped using CytoScan HD SNP arrays following the manufacturer’s instruction (Affymetrix, Santa Clara, CA). Array specific CEL files were generated in GeneChip Command Console Software. The CytoScan HD array data were imported into Affymetrix Chromosome Analysis Suite 3.0 (ChAS) software for analysis using filters for marker count = 50 and amplicon size = 100KB.

**Immunohistochemistry**

Routinely processed formalin-fixed and paraffin-embedded tumor sections were immunostained with antibodies against Skp2/p45 (SC- 7164, rabbit anti-human polyclonal [H- 435], Santa Cruz Biotechnology Inc, Dallas, TX), p27Kip1 (M 7203, mouse anti-human monoclonal, Dako, Carpinteria, CA), and pRb (4H1 mAB 9309, mouse anti-human, Cell Signaling, Danvers, MA) according to manufacturer’s protocols using an automated immunostainer (Bond Max with Leica Bond Refine polymer, Leica Microsystems, Buffalo Grove, IL) with E1 (Skp2/p45) and E2 (p27Kip1 and pRb) retrieval systems at dilutions of 1:100, 1:200 and 1:300, respectively, followed by incubation at room temperature for one hour. Antibody to ppRb (S608) detects endogenous levels of pRb only when phosphorylated at Serine 608. Immunolocalization for ppRb was performed using a rabbit anti-human polyclonal antibody (92181, Cell Signaling, Danvers, MA) and a nonautomated protocol as previously described [10].

**Statistical analysis**

Statistical tests were carried out using the Fisher’s exact probability or chi-square test for contingency table analysis. Wilcoxon Mann–Whitney U-test or Kruskal–Wallis test was used to compare the ages of patients in the different mutation categories. All analyses were two-tailed and were carried out using IBM SPSS Statistics, version 23, vassarstats (http://vassarstats.net/) or SISA (http://www.quantitativeskills.com/sisa/).
Results

**RB1 gene mutations and MYCN copy number changes in 245 unilateral retinoblastomas**

The RB1 gene was scanned for the presence of mutations in the coding exons and in the gene promoter region, exonic copy number changes, and methylation status of the RB1 promoter sequence (Table 1 and Table S1). None of the tumors carried germline mutations. There were 203 tumors with two mutations in RB1 (RB1−/−, 83%), 29 with one (RB1+/−, 12%) and 13 with no detectable mutations (RB1+/-, 5%). A total of 435 somatic mutations were detected in 203 RB1−/− and 29 RB1+/− tumors. These included 199 (46%) point mutations, 136 (31%) instances of LOH, 57 (13%) with promoter methylation, 36 (8%) exon deletions or duplications, and 7 (2%) complex rearrangements. There was no significant difference in the distribution of the types of mutations among tumors carrying one or two mutations (P = 0.11, Table 1).

We next estimated the copy number status of the MYCN oncogene in the 245 retinoblastomas, which ranged from 1 to 128 copies (Fig. 1). There were 227 (93%) tumors with less than 19 copies of MYCN (range 1–19), and 18 (7.3%) with copy number greater than 29 (range 30–128), eight of which were RB1−/− (Fig. 1 and Table S1). There were no tumors with MYCN copy number between 19 and 29.

### Table 1. RB1 mutation status in MYCN-amplified (MYCN-amp) and MYCN-low retinoblastomas.

<table>
<thead>
<tr>
<th>RB1 mutation status</th>
<th>Total (frequency)</th>
<th>MYCN-amp (frequency)</th>
<th>MYCN-low (frequency)</th>
<th>RB1−/−</th>
<th>RB1+/−</th>
<th>RB1+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N= 435 mutations</td>
<td>N= 227</td>
<td>N= 415</td>
<td>N= 16</td>
<td>N= 390</td>
<td>N= 4</td>
</tr>
<tr>
<td>RB1−/−</td>
<td>199 (46%)</td>
<td>190 (47%)</td>
<td>9 (33%)</td>
<td>10 (50%)</td>
<td>189 (46%)</td>
<td>9 (50%)</td>
</tr>
<tr>
<td>RB1+/−</td>
<td>136 (31%)</td>
<td>125 (31%)</td>
<td>11 (41%)</td>
<td>5 (24%)</td>
<td>131 (32%)</td>
<td>4 (28%)</td>
</tr>
<tr>
<td>RB1+/-</td>
<td>52 (13%)</td>
<td>5 (18%)</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
<td>55 (13%)</td>
<td>1 (11%)</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
<td>0.11</td>
<td>0.14</td>
<td>0.36</td>
<td>0.58</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LOH, loss of heterozygosity.

1P-values comparing number of tumors in RB1+/− with RB1−/−, and MYCN-amp with MYCN-low categories were determined by Fisher’s exact test (vassarstats) or Fisher 5*2 exact test (SISA).

### Characteristic features of MYCN-amplified tumors

Of the 18 MYCN-amplified tumors, eight (44%) were RB1−/−, four (22%) were RB1+/−, and six (33%) were RB1+/- . Thus, twice as many tumors (66%) carried one or two RB1 mutations compared to 33% of tumors with no mutations. This difference in the number of MYCN-amplified retinoblastomas carrying two, one, or zero RB1 mutations (eight, four, and six, respectively) compared to MYCN-low tumors (195, 25, and seven, respectively) was significant (P < 0.001, Table 1). It can be seen in Table 1, however, that there was no significant difference in the distribution of the five categories of RB1 mutations between the MYCN-amplified and MYCN-low tumors (P = 0.14), nor between MYCN-amplified and MYCN-low tumors within the RB1−/− (P = 0.36) or RB1+/− (P = 0.58) categories.

The age at which retinoblastoma was diagnosed was available for 239 cases (range = 0.5–136 months). There was no significant difference in the age of diagnosis and the presence of zero, one, or two RB1 gene mutations.
The median age at diagnosis for 13 $RB1^{+/+}$ was 15.0 months ($mean \pm SD = 18.7 \pm 17.5$, range = 1–69), 24 months ($25.9 \pm 17.4$, 0.5–77) for 27 $RB1^{+/−}$, and 24 months ($26.7 \pm 19.1$, 1–136) for 199 $RB1^{−/−}$ ($P = 0.12$). However, the 18 MYCN-amplified tumors were diagnosed at a significantly earlier age: median of 9.5 months ($16.1 \pm 12.9$, 1–40) compared 24.0 months ($27.0 \pm 19.0$, 0.5–136) for 221 MYCN-low tumors ($P = 0.012$, Fig. 2).

Genome architecture, including whole genome chromosome copy number gains and losses, were determined for 38 retinoblastomas: 14 MYCN-amplified and 24 MYCN-low tumors. Classic retinoblastoma-related chromosome copy number changes, including 1q gain, 6p gain, and 16q loss [2,11–16], were observed in 14 (37%), 15 (40%), and seven (18%) tumors (Fig. 3A), respectively; however, there was no significant difference in the fraction of tumors with these copy number changes between MYCN-amplified and MYCN-low retinoblastomas (Table 2). Figure 3B depicts copy number changes in the MYCN region on chromosome 2, showing the length of the MYCN amplicon in 14 tumors with MYCN amplification. The size ranged between 1 and 5 MB in 13 tumors, and was 11 MB in one tumor. The size of the minimum overlapping region that included the MYCN gene was 948KB and included the MYCNOS (noncoding anti-sense RNA) gene and FAM49A exons 2-11. Differences in copy number gains or losses for all other chromosomes were not significant.

Copy number gains of $MDM4$ and $OTX2$, and loss of $BCOR$ in retinoblastoma have been reported previously [7, 12, 17]. In our collection of retinoblastomas, $MDM4$ and $OTX2$ copy number gains were found in 16 (42%) and five (13%) of 38 retinoblastoma, respectively. Loss of $BCOR$ was seen in three of 34 tumors (7.9%) (Table 2); all three carried two or one $RB1$ mutations. No significant association was found between the presence of these copy number gains or losses, and MYCN amplification. In the previous study of MYCN-amplified retinoblastoma, a lower frequency of copy number changes in four genes characteristic of retinoblastoma ($KIF14$, $P = 0.33$, Table S1, compiled data not shown). The median age at diagnosis for $13 RB1^{+/+}$ was 15.0 months ($mean \pm SD = 18.7 \pm 17.5$, range = 1–69), 24 months ($25.9 \pm 17.4$, 0.5–77) for $27 RB1^{+/−}$, and 24 months ($26.7 \pm 19.1$, 1–136) for $199 RB1^{−/−}$ ($P = 0.12$). However, the 18 MYCN-amplified tumors were diagnosed at a significantly earlier age: median of 9.5 months ($16.1 \pm 12.9$, 1–40) compared 24.0 months ($27.0 \pm 19.0$, 0.5–136) for 221 MYCN-low tumors ($P = 0.012$, Fig. 2).
Figure 3. Copy number changes on chromosomes 1, 2, 6, 13, and 16 in 38 retinoblastomas. Copy number gains (vertical blue bars), or losses (vertical red bars) were visualized using Affymetrix ChAS software. RB1 mutation status is indicated by −/−, +/-, and +/+ notation separately in the MYCN-amplified (-amp) and MYCN-low tumors. (A) Copy number changes on chromosomes 1, 6, 13, and 16 are indicated. The loss of the RB1 gene region is marked by a horizontal green line. (B) Chromosome 2 with the MYCN-amplified region is indicated by a horizontal dashed green line; blue bars indicate tumors with MYCN amplification. The figure on the right is an enlargement of the MYCN gene region, 2p24.3-p24.1, with the darker blue lines denoting the region of MYCN amplification. The vertical dashed green line indicates the location of the MYCN gene, and the vertical solid green lines denote the minimum amplified region (chr2:15,891,962-16,839,842). Among the MYCN-low tumors, two had MYCN amplification of 3 which are indicated by lighter blue bars. The positions of two genes that map within the MYCN minimum amplified region, MYCNOS and FAM49A, are marked.
**Table 2.** Chromosome and gene gains and losses in 38 retinoblastomas assessed using Affymetrix Cytoscan HD SNP arrays and analyzed with ChAS software.

<table>
<thead>
<tr>
<th>Chromosome gain or loss</th>
<th>Number with chromosome gain or loss (percent of total)</th>
<th>MYCN-amplified (CN&gt;29)</th>
<th>MYCN-low (N = 24)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1q gain</td>
<td>14 (37%)</td>
<td>4 (29%)</td>
<td>10 (42%)</td>
<td>0.50</td>
</tr>
<tr>
<td>6p gain</td>
<td>15 (40%)</td>
<td>3 (21%)</td>
<td>12 (50%)</td>
<td>0.10</td>
</tr>
<tr>
<td>16q loss</td>
<td>7 (18%)</td>
<td>1 (7.1%)</td>
<td>6 (25%)</td>
<td>0.23</td>
</tr>
<tr>
<td>Gene gain or loss</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDM4 gain⁴</td>
<td>Chr:1:204,485,507-204,527,248</td>
<td>16 (42%)</td>
<td>6 (43%)</td>
<td>1.00</td>
</tr>
<tr>
<td>OTX2 gain²</td>
<td>Chr:14:57,267,425-57,272,381</td>
<td>5 (13%)</td>
<td>2 (14%)</td>
<td>1.00</td>
</tr>
<tr>
<td>BCO1 loss¹</td>
<td>ChrX:39,910,499-39,956,719</td>
<td>3 (7.8%)</td>
<td>2 (14%)</td>
<td>0.54</td>
</tr>
<tr>
<td>KIF14 gain⁴</td>
<td>Chr:1:200,520,625-200,589,862</td>
<td>11 (29%)</td>
<td>3 (21%)</td>
<td>0.49</td>
</tr>
<tr>
<td>DEK gain⁴</td>
<td>Chr:6:18,224,400-18,264,799</td>
<td>18 (47%)</td>
<td>4 (29%)</td>
<td>0.10</td>
</tr>
<tr>
<td>E2F3 gain¹</td>
<td>Chr:6:20,402,137-20,493,945</td>
<td>17 (45%)</td>
<td>3 (21%)</td>
<td>0.04</td>
</tr>
<tr>
<td>CDH11 loss⁴</td>
<td>Chr:16:64,980,683-65,155,919</td>
<td>7 (18%)</td>
<td>1 (7.1%)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

¹GRCh37/hg19 assembly.  
²Fisher’s exact, two-tailed, P-value (http://vassarstats.net/tab2x2.html).  
³McEvoy, et al.[7]  
⁴Rushlow, et al.[2]

DEK, E2F3, and CDH11) was observed in RB1+/+ MYCN-amplified compared to RB1−/− tumors [2]. We found no significant difference in the number of copies of KIF14, DEK, and CDH11 between MYCN-amplified and MYCN-low tumors; however, there was a significant difference in copy number gains in E2F3 where a smaller fraction of MYCN-amplified tumors showed a gain (3/14, 21%) compared to MYCN-low (14/24, 58%, P = 0.04) tumors (Table 2).

Pathology reports were available for 111 retinoblastomas and are summarized in Table S2: 12 were MYCN amplified and 99 were MYCN low. There was no significant association between the tumors carrying zero, one, or two RB1 mutations and the presence of high-risk features defined as invasion of the optic nerve to the level of the retrolamina, massive uveal/choroidal invasion and/or invasion of the anterior chamber, iris or ciliary body (P = 0.73) [18, 19]. In addition, there was no significant difference between the degree of tumor differentiation and MYCN amplification status (P = 0.16) or tumors carrying zero, one, or two RB1 mutations (P = 0.77).

Among the 12 MYCN-amplified tumors with pathology reports, four (33%) had high-risk features compared to 23 of 99 MYCN-low tumors (23%, P = 0.48). One of the four MYCN-amplified, high-risk tumors, (UPENN-RB-175) was RB1+/+ and was diagnosed at 10 months, by which time the tumor had invaded both the retrolaminar portion of the optic nerve and showed massive choroidal invasion. However, overall, the age of diagnosis was not significantly different between the four patients whose tumors had high-risk features and MYCN amplification and the 23 high-risk, but MYCN-low tumors (P = 0.16).

In terms of histological differentiation, 11 (92%) MYCN-amplified tumors were poorly differentiated consisting of neuroblastic cells and few, if any rosettes; one tumor was described as mixed with respect to differentiation (Table S2 and S3). This distribution of the degree of differentiation was not significantly different from that found in MYCN-low retinoblastoma: 63 (68%) undifferentiated/poorly differentiated, 21 (23%) well or moderately well differentiated with numerous Flexner–Wintersteiner rosettes, and seven (7.6%) with mixed status (P = 0.16, compiled data not shown).

**Immunohistochemical staining pattern**

We compared the relative levels of expression of SKP2, p27, pRb, and ppRb proteins in four MYCN-amplified and one MYCN-low retinoblastomas by immunohistochemistry (IHC) staining of tumor sections with antibodies specific for these proteins (Table 3 and Fig. 4, Figure S1 and Table S4).

Staining with anti-SKP2 antibody was essentially negative in all five retinoblastomas, although a few scattered positive cells were present in the four MYCN-amplified tumors (Fig. S1, panels A,C,E,G,H) and Table S4). The p27 staining pattern in UPENN-RB-175 was faint and diffuse (Fig. S1 B; RB1+/+), while in UPENN-RB-93 and UPENN-RB-40 (Fig. S1, D,F; both RB1+/−), it was patchy and focally strong, especially in UPENN-RB-40. Over 90% of tumor cells in UPENN-RB-201 (RB1−/−) were strongly...
Table 3. Tumor characteristics of five retinoblastoma immunostained with antibodies against pRb and ppRb.

<table>
<thead>
<tr>
<th>UPEN-RB ID</th>
<th>MYCN Copy Number</th>
<th>RB1 Mutations</th>
<th>Histopathological Features(^1)</th>
<th>High Risk Features</th>
<th>Immunohistochemical Staining Patterns</th>
<th>Normal Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tumor</td>
<td>ONL(^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>INL(^3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tumor</td>
<td>ONL(^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>INL(^3)</td>
</tr>
</tbody>
</table>

| UPEN-RB-175 | 112              | \(RB1^{+/-}\) | PD-few HW rosettes; large cells with nucleoli, 50% necrotic | Invasion of optic nerve and choroid | Most cells strongly positive | Most cells moderately to strongly positive | About 50% of cells moderately positive | About 25% strongly positive cells | Very few positive cells | 30-40% moderately positive cells |
| UPEN-RB-93  | 101              | \(RB1^{+/-}\) | PD-no rosettes; cells with prominent nucleoli | None | Most cells strongly positive | Most cells positive about 30% strongly stained | About 25% strongly positive cells | About 50% moderately positive cells | 30-40% moderately positive cells | About 50% weakly positive cells | 30-40% moderately positive cells |
| UPEN-RB-40  | 59               | \(RB1^{+/-}\) | PD-no rosettes; extensively necrotic | None | Most cells strongly positive | Most cells weakly staining but with a very few strongly stained | <25% strongly positive cells | About 50% moderately positive cells | Few moderately positive cells | 30-40% moderately positive cells |
| UPEN-RB-201 | 30               | \(RB1^{+/+}\) | Mixed tumor differentiation; many FW rosettes | None | Rosettes: mostly negative but with a few strongly staining cells; PD areas: scattered positive cells | Rosettes: mostly negative; PD areas: scattered positive cells | >90% strongly positive | >90% strongly positive | Very few positive cells | >50% moderately positive cells |
| UPEN-RB-112 | 3                | \(RB1^{-/-}\) | WD with many FW rosettes; extensively necrotic | None | Negative | Negative | Negative | About 25% weakly positive cells | Few weakly staining cells | Negative |

\(^1\)Histopathological feature: WD, well-differentiated tumor; PD, poorly differentiated tumor; FW, Flexner–Wintersteiner rosettes; HW, Homer–Wright rosettes.

\(^2\)ONL, outer layer of retina.

\(^3\)INL, inner layer of retina.
Figure 4. Expression of pRb and ppRb in the normal retina and in the tumor of four MYCN-amplified and one MYCN-low retinoblastoma identified by immunostaining with antibodies against the two proteins. The anti-ppRb stain is specifically targeted against phosphorylated S608. In the retina images, the ONL is positioned on the left and the INL on the right. A moderate level of pRb and ppRb reactivity is seen in less than half of the cells in both the ONL and INL of MYCN-amplified tumors UPEN-RB-175 (RB1+/+, A, C) and the two RB1+/− tumors, UPEN-RB-93 (E, G) and UPEN-RB-40 (I, K). Most cells in both retinal layers were strongly positive for pRb (M), but not ppRb (P) in UPEN-RB-201 (MYCN-amplified, but RB1+/−). The INL of UPEN-RB-112 (MYCN-low, RB1−/−) shows weak reactivity for pRb in about 25% of cells and even fewer are positive for ppRb, the ONL is essentially negative for both proteins (S, U). The MYCN-amplified tumors with zero (B) or one RB1 mutation (F, J) are poorly differentiated with most cells strongly stained with pRb. Many cells are also positive with ppRb antibody (D, H, L) but the staining tends to be weaker. The regions of UPEN-RN-201 (MYCN-amp and RB1−/−) that are well differentiated with many FW rosettes show very little reactivity with pRb or ppRb (O, R), while scattered positive cells are present in the poorly differentiated regions (N, Q). Similarly, the well-differentiated RB1+/−, but MYCN-low tumor, UPEN-RB-112, was essentially negative for pRb and ppRb (T, V). Abbreviations: inner (INL) and outer (ONL) nuclear layer of the retina; T\_rosettes region of tumor containing numerous rosettes; T\_PD poorly differentiated region of the tumor. (Original magnification ×40).
positive (Fig. S1, I,J). UPEN-RB-112 (MYCN-low, RB1−/−) was negative for SKP2 (Fig. S1, K), but showed very weak reactivity for p27 in some areas (Fig. S1, L) (Table S4).

The histopathological features, as well as pRb and ppRb (Ser608) IHC staining pattern in five retinoblastoma tumor sections and matched normal retina (defined as retinal tissue not in proximity to the tumor) are visualized in Figure 4 and described in Table 3. About 25–50% of cells in the inner (INL) and outer (ONL) nuclear layers of the normal retina were moderately to strongly stained with both pRb and ppRb antibodies in the MYCN-amplified tumors UPEN-RB-175 (Fig. 4A,C), UPEN-RB-93 (Fig. 4E,G), and UPEN-RB-40 (Fig. 4I,K) carrying zero or one RB1 mutations. This would indicate that the pRb protein that is expressed is phosphorylated. Over 90% of cells in the INL and ONL of UPEN-RB-201 (MYCN-amplified but RB1−/−) were strongly positive for pRb; however, only about 70% of cells in the INL (Fig. 4M), and less than 5% in the ONL were ppRb immunoreactive (Fig. 4N). The INL of UPEN-RB-112 (MYCN-low and RB1−/−) had about 25% pRb-positive cells, but only very few ppRb-positive cells (Fig. 4S), and the ONL was essentially negative for both proteins (Fig. 4U) (Table 3).

Tumor sections of UPEN-RB-175 (MYCN-amplified, RB1+/−) stained strongly for pRb and ppRb (Fig. 4B,D), while the two MYCN-amplified RB1−/− stained strongly with pRb antibody (Fig. 4F,J), but showed weaker staining of fewer cells with ppRb antibody (Fig. 4H,L). Interestingly, the well-differentiated region of UPEN-RB-201 with many Flexner–Wintersteiner (FW) rosettes was mostly negative for both pRb and ppRb (Fig. 4O,R); however, the poorly differentiated section had scattered positive cells (Fig. 4N,Q). UPEN-RB-112 tumor was well differentiated with many FW rosettes and essentially negative for pRb and ppRb (Table S3 and Fig. 4). In all tumors, the immunoreactive pRb and ppRb proteins were primarily concentrated in the nucleus with very little staining of the cytoplasm.

Figure 5 shows staining of the retinal section that seeds the retinoblastoma in two tumors with MYCN amplification, UPEN-RB-93 and UPEN-RB-40. With respect to pRb expression, there are relatively few scattered positive cells in both the ONL and INL; however, numerous darkly stained tumor cells can be seen originating from the adjacent weakly stained INL. Both tumors carry only one RB1 mutation; so, some expression of pRb is to be expected. In contrast, there is very little expression of ppRb in the INL of either tumor but significant expression is seen in the adjacent tumor section for both tumors. These results appear to show that tumor cells expressing both pRb and ppRb proteins may originate in the retinal INL, and not from the ONL. However, it is also possible that these cells may originate elsewhere and migrate into the INL, or preferentially invade the INL at the edges of a large tumor.
Discussion

Rushlow et al. reported a class of novel $RB1^{+/+}$ retinoblastoma with no genetic mutations in $RB1$, but having a high level of $MYCN$ amplification (>29 copies) [2]. This led to the hypothesis that high level $MYCN$ amplification is sufficient for initiation of tumorigenesis of $RB1^{+/+}$ retinoblastoma. In this manuscript, we investigated the characteristics of $MYCN$-amplified sporadic unilateral retinoblastoma and the mechanisms of inactivation of the $RB1$ gene in these tumors.

Newly diagnosed, sporadic unilateral retinoblastoma is caused by somatic mutations in over 80% of cases, and by low penetrance germline mutations in the $RB1$ gene in 15% of cases [4]. The sensitivity of mutation detection in coding sequences of $RB1$ is high, around 94% but not 100% [4]. The current methods of mutation screening of $RB1$ are limited to the promoter region, coding exons, and immediate flanking intronic regions, but not the entire introns which are known to harbor mutations [20, 21] and/or rearrangements including chromothripsis [7] that can also inactivate the $RB1$ locus. Thus, based on current sensitivity of DNA-based genetic testing, the presence of a subset of retinoblastoma without a detectable mutation in the $RB1$ gene is not unexpected. Assuming a sensitivity for finding a coding sequence $RB1$ mutation to be 94% [4], one would expect to observe only one wild-type $RB1^{+/+}$ retinoblastoma among the 245 tested. However, we identified 13 (5.3%, $P < 0.001$) tumors without any $RB1$ mutation.

We identified 18 of 245 retinoblastomas (7%) with a high $MYCN$ copy number between 29 and 110 copies (Fig. 1). Of these, eight tumors carried two mutations in $RB1$, four were $RB1^{+/−}$, and six were $RB1^{+−}$. This finding that 44% of $MYCN$-amplified tumors carried two $RB1$ mutations directly contrasts the observation by Rushlow et al. [2] who found no retinoblastomas with mutations directly contrasts the observation by Rushlow et al. [28] found in a study of 25 primary retinoblastomas, that three tumors with $MYCN$ amplification (>30 fold) were not associated with high-risk histological features. In our series, retinoblastoma cases with high $MYCN$ copy number were associated with an earlier age of onset that was significantly different from that of retinoblastoma without $MYCN$ amplification (Fig. 2). However, there was no difference in the presence of high-risk features between the two groups of tumors with and without the amplification.

In many cancers, the regulation of pRb becomes unbalanced, and elevated cyclin-dependent kinase (CDK) activity prevents pRb from being an effective brake on cell cycle control. One of the potent CDK inhibitors is p27, a protein which is degraded by the protein, SKP2. In addition, SKP2 is a known target of $MYCN$ amplification in neuroblastoma [29]. The IHC staining results in our tumors indicate the absence of SKP2 protein in any significant amount and could signal high expression of p27 protein. However, the weak p27 immunoreactivity suggests that it is expressed in low levels in all $MYCN$-amplified tumors, except the $RB1^{-−}$ tumor, UPEN-RB-201, where it is highly expressed in both well and poorly differentiated areas of the tumor (Fig. S1, I, and J). Further comparison of p27 expression in UPEN-RB-201 and UPEN-RB-112 which is also $RB1^{-−}$, but $MYCN$-low suggests that p27 is up-regulated in the presence of $MYCN$ amplification, but not in $MYCN$-low tumors in the absence of pRb. In essence, these observations rule out the activation of the $MYCN$-SKP2-p27 axis in three out of four $MYCN$-amplified retinoblastomas. Since it is known that p27 is degraded upon progression into S-phase when both pRb and p27 are phosphorylated [29], we investigated the phosphorylation status of pRb.

Alterations in pRb phosphorylation during late G1 phase allow the activation of E2F-dependent transcription that drives the cell cycle and is needed for DNA replication. It has been shown that the alteration in the structure of pRb by phosphorylation at multiple residues, including S608/S612 [30, 31] causes specific conformational changes and are sufficient for inhibition of interaction with the transactivating domain of E2F, while phosphorylation at S795 inhibits binding to the marked box (MB) domain of E2F1 [29].

The observation that pRb is phosphorylated at S608 and/or S795 residue in the $MYCN$-amplified retinoblastoma provides an important insight into how retinoblastoma develops without mutational knock-out of the $RB1$ gene.
which has conventionally been known as the rate-limiting step in this process [1]. In a recent paper, Liu et al. [32] have shown that following pRb loss, there is evidence for Myc-dependent E2F3 accumulation and “rampant cell proliferation”. In commenting on these findings, Osorio [33] suggests that when functional pRb is not present in the cell, factors such as MYCN and E2Fs are “repurposed” resulting in unregulated cell proliferation.

Thus, in conclusion, in our series of 18 MYCN-amplified retinoblastomas, 12 carry inactivating coding sequence mutations in the RB1 gene and six do not. While inactivation of RB1 by mutations has been classically associated with retinoblastoma, deregulation of the pRb pathway is very common in most types of human cancer [23, 24]. Very few of these cancers carry mutations in the RB1 gene, and pRb, is inactivated by phosphorylation in these nonretinoblastoma cancers. Similarly, in the retinoblastoma without coding sequence mutations, pRb, is inactivated by phosphorylation at various sites, including S608 and S795, which are two of the key residues that determine the interaction with E2F family of transcription factors [34]. Hence, pRb inactivation, through mutation, phosphorylation or some other as yet unknown mechanism, is likely to be the initiating event in retinoblastoma tumorigenesis with or without MYCN amplification.

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Conflict of Interest

None declared.

References


Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Expression of SKP2 and p27 in four MYCN-amplified and one MYCN-low retinoblastomas detected by immunohistological staining with anti-SKP2 and anti-p27<sup>Kip1</sup> antibodies.

Table S1. MYCN copy number and RB1 mutations found in 245 retinoblastomas.

Table S2. Histopathological features of 111 retinoblastomas.

Table S3. RB1 gene mutations and high-risk histological features in 18 tumors with MYCN amplification.

Table S4. IHC staining of five retinoblastoma tumors with antibodies specific for SKP2 and p27.