A novel retinoblastoma therapy from genomic and epigenetic analyses

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Retinoblastoma is an aggressive childhood cancer of the developing retina that is initiated by the biallelic loss of RB1. Tumours progress very quickly following RB1 inactivation but the underlying mechanism is not known. Here we show that the retinoblastoma genome is stable, but that multiple cancer pathways can be epigenetically deregulated. To identify the mutations that cooperate with RB1 loss, we performed whole-genome sequencing of retinoblastomas. The overall mutational rate was very low; RB1 was the only known cancer gene mutated. We then evaluated the role of RB1 in genome stability and considered non-genetic mechanisms of cancer pathway deregulation. For example, the proto-oncogene SYK is upregulated in retinoblastoma and is required for tumour cell survival. Targeting SYK with a small-molecule inhibitor induced retinoblastoma tumour cell death in vitro and in vivo. Thus, retinoblastomas may develop quickly as a result of the epigenetic deregulation of key cancer pathways as a direct or indirect result of RB1 loss.

Retinoblastoma is a rare childhood cancer of the retina that can develop in a sporadic or a heritable form and is fatal if untreated. When the RB1 gene was first cloned, it was found to undergo biallelic inactivation in virtually all retinoblastoma tumours1. Since then, hundreds of genetic lesions have been identified in human cancer. These genetic lesions can be grouped on the basis of the signalling pathways they affect that have direct or indirect mechanistic links to many of the common cellular properties, or hallmarks, of cancer. Thus, the rate of cancer progression is related to the kinetics of acquisition of multiple genetic lesions and/or epigenetic changes that ultimately lead to activation of growth-signalling pathways, evasion of cell death and senescence, acquisition of limitless replicative potential, sustained angiogenesis, and local tissue invasion and metastasis2.

RB1 inactivation confers limitless replicative potential to retinoblasts and is rate limiting for retinoblastoma tumorigenesis3. However, the mechanisms that enable retinoblastoma cells to acquire the additional hallmarks of cancer remain unknown. Evidence from molecular, cellular and cytogenetic studies suggest that RB1 is required for maintaining chromosomal stability4,5, and that its loss leads to chromosome instability in cells maintained in culture. These data raise the possibility that RB1 inactivation may underlie the rapid acquisition of cooperating mutations in key cancer pathways through chromosome instability. Alternatively, epigenetic changes may have a more dominant role in cooperating with the loss of RB1 retinoblastoma tumorigenesis. RB1 has been implicated in regulating most major epigenetic processes, including microRNA regulation, DNA methylation, histone modification and AP-1-dependent chromatin reorganization6–10. Thus, inactivation of RB1 in retinoblasts may lead to the rapid epigenetic deregulation of cancer genes that contribute to the essential cellular properties of retinoblastoma.

In this study, the St Jude Children’s Research Hospital – Washington University Pediatric Cancer Genome Project characterized the genetic landscape of retinoblastoma. Whole-genome sequencing (WGS) of four retinoblastomas and their paired germline DNA samples showed no genetic lesions in known tumour suppressor genes or oncogenes, other than RB1 and MYCN. More importantly, an orthotopic xenograft derived from one of the primary tumours showed no evidence of clonal variation or new coding-region mutations. This finding suggests that the retinoblastoma genome is more stable than previously believed.

Unlike the genetic landscape of retinoblastoma, the epigenetic profile shows profound changes relative to that observed in normal retinoblasts. One of the most striking results was the induction of the expression of the proto-oncogene spleen tyrosine kinase (SYK) in human retinoblastoma. SYK is required for tumour cell survival, and inhibition of SYK with a small-molecule inhibitor caused the degradation of MCL1 and caspase-mediated cell death in retinoblastoma cells in culture and in vivo. These findings highlight how comprehensive genetic and epigenetic analyses of tumours can be integrated, leading to the discovery of promising new therapeutic approaches and shedding light on the mechanisms underlying the rapid progression of retinoblastoma following RB1 inactivation.

Retinoblastoma whole-genome sequencing

We performed WGS analysis on four primary human retinoblastoma samples (Supplementary Information, section 1, and Supplementary Table 1) and on matched normal tissue. Local tumour cell invasion, genetic and epigenetic variation or new coding-region mutations in key cancer pathways can be integrated, leading to the discovery of promising new therapeutic approaches and shedding light on the mechanisms underlying the rapid progression of retinoblastoma following RB1 inactivation.

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but not metastasis, was evident in each patient (Fig. 1a–c and Supplementary Fig. 1). We generated an orthotopic xenograft, SJRB001X, of the primary tumour SJRB001 by inoculating primary tumour cells into the vitreous humour of the eyes of immunocompromised mice (Supplementary Information, section 2). SJRB001X had molecular, genetic and histopathologic features similar to those of SJRB001 (Fig. 1d–f; Supplementary Figs 2–4; Supplementary Tables 2–4; and Supplementary Information, section 3).

Using a paired-end sequencing approach, we generated 1,040.9 gigabase pairs (Gb) of sequence data for the samples described; 956.8 Gb (92%) was successfully mapped to the NCBI 36.1 reference genome (Supplementary Information, section 4, and Supplementary Table 5). The average genome coverage was $28.9$, and the average exon coverage was $23.8$ with $98.4\%$ of single nucleotide polymorphisms (SNPs) detected across all nine genomes showing concordance with their corresponding SNP array genotype calls at the same genomic positions (Supplementary Table 5). To provide additional sequence coverage, we performed transcriptome sequencing of all four primary tumours (Supplementary Information, section 5, and Supplementary Table 6).

We identified 668 validated somatic sequence mutations and 40 structural variations across the four cases (Table 1). These included 23 tier-1 mutations in genes, 35 tier-2 mutations in evolutionarily conserved regions of the genome (Supplementary Information, section 6), 309 tier-3 mutations in non-repetitive regions of the genome that are not part of tiers 1 and 2, and 301 tier-4 mutations in repetitive sequences in the genome (Table 1 and Supplementary Table 7). The average number of sequence mutations was 167 per case (range, 56–258), with only 3.25 mutations per case (range, 0–5) resulting in amino-acid changes (Table 1). The estimated mean mutation rate was $6.7 \times 10^{-8}$ per base (range, $1.03 \times 10^{-7}$–$2.17 \times 10^{-8}$), which is 15-fold less than that in adult tumours analysed by WGS, except for acute myeloid leukaemia$^{14}$. The predominant changes were C $\rightarrow$ A and G $\rightarrow$ T transitions (Supplementary Fig. 5), which is consistent with the possibility that some of the transversions result from production of 8-oxoguanine during oxidative stress. Tumour SJRB002 had no somatic sequence variations that resulted in amino-acid changes; the only structural variations were the loss of heterozygosity (LOH) at the $RB1$ locus on chromosome 13 and a gain of chromosome 6p. This suggests that very few genetic lesions are required for retinoblastoma progression after $RB1$ inactivation.

### RB1 inactivation in retinoblastoma

Both $RB1$ alleles were inactivated in each sample (Supplementary Figs 6 and 7). Tumours SJRB002 and SJRB003 had mutations in $RB1$ combined with copy-number-neutral LOH, and SJRB001 and SJRB004 had somatic sequence mutations combined with $RB1$-promoter hypermethylation (Supplementary Figs 6–8). Deep-sequence analysis of the germline sample from SJRB002 revealed that about 10% of reads contained the R445 nonsense mutation, suggesting the presence of germline chimaerism for the $RB1$ mutation (Supplementary Fig. 6). Combining the WGS data with SNP array data of an additional 42 samples, we found that tumours from patients with lower regional nucleotide diversity were much less likely to undergo LOH at the $RB1$ locus (Supplementary Tables 8 and 9 and Supplementary Information, section 7). These data show a significant association ($P = 8 \times 10^{-8}$, Fisher’s exact test) between a germline genetic variation and mechanism of biallelic $RB1$ inactivation in retinoblastoma.

### Recurrent lesions in retinoblastoma

To determine whether any of the 11 genes with somatic mutations that caused amino-acid changes or a frameshift in the coding region (Table 1; Fig. 2a, b; Supplementary Figs 9 and 10; Supplementary Information, section 8; and Supplementary Table 10) were recurrently mutated in retinoblastoma, we sequenced all exons from the 11 genes in our recurrent screening cohort of 42 retinoblastomas (Supplementary Information, section 4). Only $BCOR$ was recurrently mutated in retinoblastoma (6 of 46, or 13%). Five of the samples had $BCOR$ mutations that resulted in truncation of the encoded protein, and one sample had a focal gene deletion (Fig. 2c, Supplementary Table 11 and Supplementary Fig. 11).

We also used the WGS data to identify somatic structural variations including whole-chromosome gains and losses, focal deletions (DEL), insertions (INS), inversions (INV), intrachromosomal rearrangements (ITX), interchromosomal rearrangements (CTX) and regions of LOH (Fig. 2a–c; Supplementary Information, section 9; Supplementary Table 12; and Supplementary Fig. 12). The average number of structural variations was 23 per case (range, 5–72), with structural variations including focal amplifications and deletions, LOH, and intrachromosomal and interchromosomal translocations.

### Table 1 | Somatic mutations and structural alterations in retinoblastoma

| Sample* | Tier 1† | Non-silent tier 1‡ | Genes | Tier 2§ | Tier 3¶ | Tier 4‖ | Total | Mutation rate | Structural variations** |
|---------|---------|--------------------|-------|---------|---------|---------|-------|---------------|-------------------------|
| SJRB001 D, G | 7 | 4 | $RB1$, $CCNC$, $AGMO$, $RB1G$ | 16 | 117 | 85 | 225 | $1.03 \times 10^{-7}$ | 4 |
| SJRB001 X, D§ | 0 | 0 | NA | 8 | 68 | 9 | 85 | $5.87 \times 10^{-10}$ | 4 |
| SJRB002 D, G | 1 | 0 | NA | 1 | 25 | 29 | 56 | $2.17 \times 10^{-10}$ | 0 |
| SJRB003 D, G | 7 | 4 | $RB1$, $HMT$, $LX8$, $SOM$ | 5 | 67 | 50 | 129 | $5.79 \times 10^{-10}$ | 24 |
| SJRB004 D, G | 8 | 5 | $RB1$, $C300L$, $SDK1$, $TX$, $DMWD$ | 13 | 100 | 137 | 258 | $8.63 \times 10^{-10}$ | 12 |

Background mutation rate was calculated on the basis of the ratio of tier-3 mutations to tier-3 bases covered at least tenfold in tumour and germline samples for each pair.

* D, diagnostic tumour sample; G, germline (blood DNA) sample; X, xenograft sample.
† Tier-1 mutations are found in genes and include exons, 5’ and 3’ untranslated regions, and splice sites. Introns are not included.
‡ Non-silent tier-1 mutations change amino acids in genes.
§ All of the somatic mutations in SJRB001 D, G were identified in SJRB001X. This row highlights the new mutations acquired in the xenograft relative to the primary tumour.
¶ Tier-2 mutations are found in regions of the genome that are conserved between humans and mice.
‖ Tier-3 mutations are found in regions of the genome that are not evolutionarily conserved.
** Structural variations include focal amplifications and deletions, LOH, and intrachromosomal and interchromosomal translocations.
number of structural variations was ten per case (range, 0–24) (Supplementary Table 1). Tumour SJRB001 had four structural variations (two DEL and ins INS) including a gain of a region of chromosome 2 spanning MYCN (Supplementary Table 12), and the only chromosomal lesion in SJRB002 was a gain of chromosome 6p, which occurs in about 40% of human retinoblastomas12 (Fig. 2b). Only a few genomic regions were affected by the structural variations in SJRB003 and SJRB004 (Supplementary Figs 10, 13 and 14 and Supplementary Table 12).

Orthotopic retinoblastoma xenograft

The genomic landscape of the orthotopic xenograft was remarkably similar to that of the primary tumour, despite continuous growth and multiple passages in vivo over nine months (Fig. 2 and Supplementary Table 1). All of the sequence mutations and structural variations detected in SJRB001 were retained in SJRB001X. Only 67 new SNVs and four structural variations were identified in the xenograft, and none affected annotated genes (Fig. 2 and Supplementary Table 1). Moreover, each mutation was identified at a subclonal level (range, 20–30%) and the mutant allele frequency for the lesions in tiers 1–4 in SJRB001 was retained in SJRB001X (Supplementary Fig. 15).

This result was surprising because several studies in mice and cell cultures have linked RB1 inactivation to defects in chromosome segregation that result in aneuploidy4,5,13–15 and chromosome instability6. We measured the distance between sister chromatids, the distance between kinetochores and the proportion of lagging chromatids in two RB1-deficient human retinoblastoma orthotopic xenografts16 (SJRB001X and SJRB002X). Consistent with results from RB1-deficient retinal pigmented epithelium cells8, the distances between sister chromatids and between kinetochores were increased, and there was evidence of lagging chromosomes (Supplementary Figs 16 and 17). However, less variation in ploidy was observed during spectral karyotype analysis of SJRB001X and SJRB002X, which was more consistent with the ploidy of wild-type cells (Fig. 3a, b and Supplementary Table 13). Moreover, copy number variations were much lower in our cohort of 46 retinoblastomas than in tumours with known genome instability such as ovarian cancer (Fig. 3c). Together, the cytogenetic data and WGS data suggest that the genome is stable and that newly acquired lesions do not provide a selective growth advantage and are thus probably passenger mutations (Supplementary Information, section 9).

Identifying deregulated cancer pathways

There are many examples over the past several decades of epigenomic changes such as DNA methylation contributing to tumorigenesis17–19. Indeed, a recent study demonstrated changes in DNA methylation in Wilms’ tumours20, which tend, like retinoblastomas, to have stable genomes. To explore whether epigenetic deregulation of genes or pathways promotes tumorigenesis in retinoblastoma, we carried out an integrative analysis of chromatin immunoprecipitation (ChIP) data, DNA methylation data and gene expression data using order statistics. The SJRB001X sample was used for ChIP assay (Supplementary Figs 18–20), and primary tumour and xenograft samples were used for both DNA methylation and gene expression assays. In all three analyses, experimental results in retinoblastoma tumours were compared with those from human fetal retinae. In this comparison, a total of 104 genes, including 15 known cancer genes (Fig. 4a and Supplementary Tables 14 and 15), were found to have significant differences, indicating that several key cancer genes were epigenetically deregulated.

SYK is a novel therapeutic target

SYK is the fifth most significant gene identified by the integrative analysis and the only upregulated kinase gene (Supplementary Table 15 and Fig. 4a). SYK is expressed throughout the haematopoietic system, regulates immunomodulatory signalling and has been implicated in several haematologic malignancies21–24. Small-molecule inhibitors of SYK have been developed to treat autoimmune disorders25, and two of those agents, BAY 61-3606 and R406, have shown efficacy in preclinical leukaemia studies26–28.

ChIP-on-chip analysis showed increased activating histone modifications (H3K4me3 and K3K9/14Ac) at SYK’s promoter, and the repressive histone marker (H3k9me3) was unchanged. Binding of
RNA polymerase II to the SYK promoter was also increased (Fig. 4b and Supplementary Tables 14 and 15). These ChIP-on-chip results were validated in independent samples by real-time PCR analysis with reverse transcription (Fig. 4c), and we confirmed the increase in SYK gene expression (Fig. 4d). SYK protein levels were higher in human retinoblastoma orthotopic xenografts and cell lines than in human fetal retinae (Fig. 4e). To determine whether SYK is expressed in primary human retinoblastomas, we performed immunohistochemistry on a retinoblastoma tissue microarray or whole-eye sections. In total, to 153 high-grade serous ovarian cancer (Ov) cases from The Cancer Genome Atlas. The median percentage of the genome involved in copy number variations (CNVs) was 1.5% for retinoblastoma and 27.7% for ovarian cancer. Red circles, samples used for WGS.

To determine whether SYK expression is required for retinoblastoma growth, survival or both, we generated a short hairpin RNA (shRNA)
against SYK and cloned it into the lentiviral vector Lenti-SYK-9. Lenti-SYK-9 efficiently knocked down SYK in retinoblastoma cell lines (Supplementary Fig. 21) and drastically increased apoptosis in retinoblastoma cells (Fig. 4h and Supplementary Fig. 21). Similar results were obtained in vivo using SJRB001X (data not shown). We used an empty lentiviral vector and a lentiviral vector encoding an SYK shRNA that less effectively reduced SYK expression (Lenti-SYK-6) as controls. Cell lines that do not express SYK (BJ, 293T and uninduced Jurkat cells) were used as controls and the Lenti-SYK-9 lentivirus had no effect on the growth or apoptosis of the control cells.

We exposed retinoblastoma cell lines that express high levels of SYK (Wer1 and RB355) to various concentrations of the SYK inhibitors BAY 61-3606 and R406 for 72 h and then measured cell viability. Jurkat (uninduced) and 293T cells were used as negative controls. Wer1 and RB355 cells were sensitive to both SYK inhibitors, but the Jurkat and 293T cells were unaffected (Fig. 5a). Transmission electron microscopy revealed reduced SYK expression (Lenti-SYK-6) as controls. Cell lines that do not express SYK (BJ, 293T and uninduced Jurkat cells) were used as controls and the Lenti-SYK-9 lentivirus had no effect on the growth or apoptosis of the control cells.

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The proportions of cells from each line that incorporated EdU were determined (Fig. 5b–e). The proportions of cells from each line that incorporated EdU were determined (Fig. 5b–e). The proportions of cells from each line that incorporated EdU were determined (Fig. 5b–e). The proportions of cells from each line that incorporated EdU were determined (Fig. 5b–e). The proportions of cells from each line that incorporated EdU were determined (Fig. 5b–e).

Genome sequencing reveals that retinoblastomas have a relatively stable genome. The mutational rate and number of structural variations per case that we assessed were among the lowest reported in human cancer to date. Moreover, in one example (SJRB002) the only non-silent mutation found was in RB1, and only two structural variations were detected. The minimal increase in passenger mutations in SJRB001X cells, despite prolonged passage, was also consistent with a relatively stable genome. These results are surprising because previous studies have shown that the functional inactivation of RB1 can cause...

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Figure 5 | Retinoblastoma cells are sensitive to SYK inhibitors. a, Dose response for SYK inhibitors R406 (red) and BAY 61-3606 (black) in RB355 retinoblastoma cells and a negative control (Jurkat). Each data point is mean ± s.d. of triplicate samples. b–e, Immunofluorescence of activated caspase 3 (b, c) and EdU (d, e) (red) before and after treatment of RB355 cells with R406 or BAY 61-3606. A total of 250 cells were scored in duplicate for each sample and each treatment condition to derive the mean and s.d. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue).

f, Treatment of stimulated Jurkat or RB355 cells with 5 μM BAY 61-3606 for 24 h reduced MCL1 expression. g, Schematic of the treatment schedule for mice with SJRB001X tumours. h, Representative magnetic resonance images of a mouse whose tumour responded after four courses of treatment with BAY 61-3606 (left) and another whose disease progressed during treatment (right). i, Survival curves show that treatment with BAY 61-3606 plus TPT improved outcome. j, Immunostaining for activated caspase 3 (arrows) in eyes either untreated or treated with BAY 61-3606. k, Immunoblot showing reduction in MCL1 after treatment with BAY 61-3606 (BAY) or BAY 61-3606 plus TPT. C, control. Scale bars, 5 μm (b, d); 10 μm (j).
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