MYCN amplification levels in primary retinoblastoma tumors analyzed by Multiple Ligation-Dependent Probe Amplification

Elizabeth A. Price[1], Roopal Patel[1], Irene Scheimberg[2], Esin Kotiloglu Karaa[2], Mandeep S. Sagoo[3,4], M. Ashwin Reddy[3] and Zerrin Onadim[1].

Author affiliations:

[1]Retinoblastoma Genetic Screening Unit, Barts Health NHS Trust, London E1 2ES, U.K.

[2]Department of Pathology, Barts Health NHS Trust, London E1 1FR, U.K.

[3]Retinoblastoma Service, Royal London Hospital, Barts Health NHS Trust, London E1 1FR, U.K.

[4]NIHR Biomedical Research Centre for Ophthalmology at Moorfields Eye Hospital and UCL Institute of Ophthalmology, London, UK

Corresponding Author:

Zerrin Onadim

Retinoblastoma Genetic Screening Unit

Barts Health NHS Trust

3rd Floor, Pathology & Pharmacy Building

80 Newark Street

London E1 2ES

Tel: 020 3246 0265

Fax: 020 3246 0321

E-mail: z.onadim@nhs.net

Abstract

Retinoblastoma (Rb) is a childhood tumor of the developing retina where predisposition is caused by RB1 pathogenic variants. MYCN amplification (MYCN^A) has been implicated in around 2% of sporadic unilateral Rb tumors with no detectable RB1 variants. We audited data from tumors collected between 1993-2019 to determine if this is the case for patients treated at Barts Health NHS Trust. MYCN^A screening was performed by Multiplex Ligation-dependent Probe Amplification of 149 Rb tumors and 114 matched blood samples. 10/149 (6.7%) tumors were positive for MYCN^A in a population containing a disproportionate number of cases negative for RB1 pathogenic variants. Of 65 unbiased tumors collected from 2014-2019, 2 (3.1%) had MYCN^A. All MYCN^A samples were from sporadic, unilateral patients and 3/10 (30%) had RB1 pathogenic variants. Where MYCN^A occurred alongside RB1 variants the age of diagnosis was later than for those without RB1 variants. MYCN^A was not detected in any blood sample. No MYCN^A tumor had 6p gain which is usually a common alteration in retinoblastomas.

Key Words Retinoblastoma, genetic, sporadic, somatic, *MYCN, RB1*

Introduction

There are two forms of the childhood eye cancer retinoblastoma (Rb): heritable (genetic; 45-50% cases) and non-heritable (somatic; 50-55% cases). All bilateral cases and 15-20% of unilateral sporadic cases are heritable. Most heritable Rb is due to a *de-novo* germline alteration of the tumor suppressor gene *RB1* (13q14.2) with a minority of cases having a previous family history. Inheritance is autosomal dominant with high penetrance of over 90% (1). Patients with heritable *RB1* pathogenic variants are at increased risk of second primary non – ocular tumors such as osteosarcomas and soft tissue sarcomas later in life.

A broad spectrum of *RB1* variants is distributed across the gene. The most common are single base substitutions (50-60%) giving rise to missense, nonsense, splice site changes, and small length pathogenic variants (30%) which mostly generate premature stop codons. These variants are usually associated with highly penetrant and expressive (bilateral, multifocal) Rb. Missense variants, in frame deletions/insertions, promoter and some splicing changes are associated with a low penetrance phenotype (2). About 60-70% of tumors display loss of heterozygosity (LOH) (3). Promoter hypermethylation is present in up to 15% of retinoblastomas (4). Whole genome sequencing (WGS) added chromothripsis to the list of *RB1* pathogenic variants in around 3% of Rb tumors (5). Around 2% of sporadic, unilateral cases characterised by early onset of large, invasive tumors, display over-amplification of *MYCN* ($MYCN^A$; 28-121 copies) in the absence of an *RB1* pathogenic variant (6).

Rb protein (pRB) acts as an inhibitor of the cell cycle in quiescent cells and during check point mediated cell cycle arrest. It represses proliferation by binding E2F transcription factors (TF) and blocking their DNA interactions. Mitogenic signals lead to *RB1* hyperphosphorylation, release of TF, and progression through the cell cycle. *RB1* pathogenic variants can inhibit a cell's exit from the cell cycle and predispose to tumor formation. However, other genetic alterations are required for progression to tumor formation. Such changes include recurrent copy number alterations such as gain at 1q, 2p, 6p, 19q or loss at 13q, 16q and 17p (7). Gain at 2p can lead to amplification of the *MYCN* oncogene (2p24.3).

The *MYC* proto-oncogene family (*C-MYC*, *MYCN* and *MYCL*) of TFs are involved in the initiation and progression of many human tumors. They play a role in many oncogenic processes and potentially regulate the transcription of at least 15% of the genome (8). The MYCN TF is involved in the control of embryonal development via pathways that promote

cell proliferation. The family has multiple effects on gene expression and regulates pathways controlling the cell cycle, progression, and senescence (9). pRB and MYCN have antagonistic effects on the cell cycle. pRB phosphorylation, which allows cells to enter the S-phase of the cell cycle, is performed by a cyclin E/CDK2 complex which is stimulated by MYC. Deregulation of *MYCN* is seen in various childhood tumors such as neuroblastoma, medulloblastoma and Wilms' tumor. In adults it is associated with cancers such as prostate and lung cancer (10). In neuroblastoma, $MYCN^{A}$ is associated with poor prognosis. In a subset of Rb cases with no detected *RB1* pathogenic variants $MYCN^{A}$ was linked to very early onset (median 4.5 months) tumors that were undifferentiated with aggressive histological features (6). This audit aimed to establish the percentage of Rbs with $MYCN^{A}$ in our cohort, to determine how frequently it was seen in the presence/absence of *RB1* pathogenic variants, and gain insight of the utility of *MYCN* testing in routine Rb genetic screening.

Materials and methods

Sample collection

This audit was approved by the Barts Health Clinical Effectiveness Unit (audit no. 10839). It covered a sub-set of samples collected during the period 1993–March 2019. Patients were referred to the Retinoblastoma Genetic Screening Unit (RGSU; Barts Health NHS Trust) for Rb genetic analysis by clinical geneticists, genetic counsellors or ophthalmologists, and consent for screening was obtained from parents/guardians. We looked at $MYCN^{A}$ results from 149 Rb DNAs (132 fresh frozen; 17 formalin fixed, paraffin embedded [FFPE]). The patients presented as 21 sporadic bilateral and 128 sporadic unilateral (13 germline, 115 somatic). Table 1 gives an overview of patients and tumor samples. All tumors where an *RB1* pathogenic variant was missing (n=50) were included. 29 had one *RB1* change ($RB1^{+/-}$) while 21 had no identified *RB1* change ($RB1^{+/+}$) but not all were fully screened ('Not Determined'

in Table 1). Also included sporadic unilateral cases with especially early age of diagnosis (8 months or less, n=23). *MYCN*^{A} testing performed on all 65 fresh frozen tumors received from April 2014-March 2019 provides an unbiased sample set. 114 blood DNAs were tested including samples matched to *MYCN*^{A} tumors where available.

RB1 genetic screening

Peripheral blood was collected into EDTA tubes whilst fresh tumor was harvested by pathologists and frozen for storage immediately after enucleation. DNA was extracted as previously described (11, 4). *RB1* screening covered all 27 exons plus 50 bp upstream and 30 bp downstream to cover associated splice sites, as well as the promoter. Conformation analysis of transitions/transversions and small insertions/deletions was performed by single stranded conformational polymorphism and heteroduplex analysis (GE Biotech ALFexpress), and/or high resolution melt analysis (Corbett RotorGene 6000). Samples giving abnormal traces were reamplified for Sanger sequencing. Polymorphic markers within and around *RB1* on chromosome 13 were used to determine tumor LOH. Large exonic deletions were detected by in-house Quantitative Fluorescent PCR (QF-PCR) and Multiplex Ligation-dependent Probe Amplification (SALSA MLPA *RB1* probe mix P047, MRC-Holland) (11). Methylation Specific PCR (MS-PCR) of bisulphite-modified DNA was performed to detect promoter hypermethylation (4). Around 97% of expected pathogenic variants (including *MYCN*^{A} cases) were routinely detected over the last fifteen years (April 2005-March 2020).

MYCN analysis

MYCN copy number was determined by MLPA using a commercial kit (SALSA MLPA Neuroblastoma-2 probe mix P252, MRC Holland) following the manufacturer's instructions. Tumors with high levels of *MYCN*^{A} were diluted out with normal control DNA (1/5 – 1/10) for more accurate analysis. Fragments were run on an ABI3730 with the GeneScan ROX500

size standard and analyzed using GeneMarker software (SoftGenetics). Up to 1.26 copies was considered normal, up to 3.25 as triplication, and 10 or more copies as overamplified. This analysis also gave data on 6p status which has been linked to poor prognosis in Rb.

Histopathological analysis

Histologic evaluation of the 10 *MYCN*^{A} positive tumors and age-matched (at the time of diagnosis) controls was done by experienced pediatric histopathologists who were unaware of the *MYCN*^{A} results. The assessment was done by reviewing the Rb slides when possible. Otherwise, parameters in the issued reports were retrieved. Histologic evaluation of tumors was done according to RcPath Guidelines (G055-Dataset for histopathological reporting of ocular Rb; January 2018). Tumor differentiation and all core elements about the extent and site of tumor invasion / spread were assessed and compared; pathologic staging was done according to American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 8th Ed.

Results

All samples

149 Rb tumors were tested for *MYCN*^{A} by MLPA, including 50 tumors with one or both *RB1* pathogenic variants unascertained after routine screening (Table 1). 10/149 (6.7%) of all tumors were positive for *MYCN*^{A} (9 fresh tumors, 1 FFPE). Where full screening was possible 3/9 (33.3%) *MYCN*^{A} tumors were *RB1*$^{-/-}$. The remaining 6/9 (66.7%) had no detectable *RB1* variants. 123 tumors had enough intact DNA to identify both *RB1* pathogenic variants if they were detectable at the DNA level (23 bilateral/germline; 100 sporadic/somatic; Table 2). 99/123 (80.49%) had 2 *RB1* pathogenic variants (*RB1*$^{-/-}$), 12/123 had 1 variant (*RB1*$^{+/-}$ 9.76%), and 12/123 had none detected (9.76% *RB1*$^{+/+}$) (Table 2). All 12 fully screened tumors with no *RB1* alteration were from sporadic unilateral cases. 6/12 had *MYCN*^{A} (50%) and 6 had no change detected in either *RB1* or *MYCN*. *MYCN*^{A} was primarily

seen in tumors where no *RB1* variants were identified (Tables 2, 3). None of the 114 blood samples had $MYCN^A$ including 8 samples available from the $MYCN^A$ tumor patients.

Tumors from 2014-2019

Over five years from March 2014-April 2019, 65 tumor samples were tested for $MYCN^A$ (Table 1). This was a complete cohort of all tumors collected where DNA was available. 16 tumor samples were from cases where blood was initially screened (15 bilateral; 1 unilateral germline) so a full *RB1* screen was performed on only 7. The other 49 presented as sporadic, unilateral and there was enough DNA from 47 (43 somatic; 4 germline for *RB1* pathogenic variants) to perform a full *RB1* screen. Therefore, a total of 54 tumors (43 somatic; 11 germline) from this cohort underwent *MYCN* MLPA plus full *RB1* analysis. 52/54 carried *RB1* variants (96.3%). 2 unilateral sporadic Rbs had $MYCN^A$ (2/65, 3.1%) and one of those had 2 pathogenic *RB1* variants (Tables 1, 2).

RB1 variants and $MYCN^A$ in sporadic unilateral Rb

Table 3 shows which *RB1* variants were detected in 100 sporadic, unilateral tumors where no germline changes could be identified after a complete *RB1* screen. 50% (6/12) of $RB1^{+/+}$ tumors display $MYCN^A$ compared to 3.9% (3/77 in $RB1^{-/-}$ tumors). Where tumors are $RB1^{+/-}$ there is an excess of large insertions/deletions including whole exons.

Age of diagnosis

There were 115 sporadic/somatic samples, and 34 cases where an *RB1* variant was detected in blood DNA (21 bilateral and 13 sporadic unilateral referrals). Around 1% of the apparently somatic cases could still be low level mosaic carriers of *RB1* variants below the level of detection. The 115 somatic cases had a mean diagnosis age of 26.5 months (range 0.75-120; median 25; SD 20.3). The 34 germline cases, which were expected to have an earlier diagnosis age, had a mean of 15.26 months (range 1-54; median 12; SD 13). A two-tailed t-

test gave significantly different mean ages for tumors from germline cases [t(147) = -3.05, p = .003]. *RB1* cases with known germline variants were excluded from analysis of age vs *MYCN*^{A}.

RB1$^{+/+}$ *MYCN*^{A} tumors are expected to have a very early age at diagnosis. In our set of fully screened *MYCN*^{A} tumors, 50% (6/12) of *RB1*$^{+/+}$ tumors had *MYCN*^{A} and the age of diagnosis ranged from 3-10 months (mean 7.2, median 8, SD 2.6). The age of diagnosis for the remaining 6 *RB1*$^{+/+}$ samples with no identified pathogenic changes ranged from 19-96 months (mean 41.1, median 34, SD 28.1). A two-tailed t-test gave significantly different mean times for those tumors with *MYCN*^{A} [t(10) = -2.95, p = .015].

Around 30-40% of tumors have triplicated (1.27-3.25 copies) *MYCN* depending on the cohort looked at. In the complete set of 149 tumors it is 32.9% (49/149). For all tumors collected over 2014-2109 the value is 41.5% (27/65). In the 100 sporadic unilateral tumors it is 36% (36/100). In the sporadic unilateral set, there appears to be a cluster of normal *MYCN* samples at the early diagnosis age of ten months or less (Fig.1). The age for samples with normal *MYCN* (n=55) ranged from 0.75-96 months (mean 25.7, median 24, SD 20.1). The age for triplicated samples (n=36) ranged from 3-120 months (mean 33, median 34, SD 19.1). A two-tailed t-test did not give significantly different mean ages at diagnosis [t(89) = -1.74, p = 0.086].

MYCN*^{A} *patients

MYCN^{A} (11-89 copies) cases occurred in both early and late age tumors. 7/10 (70%) were diagnosed at 10 months or younger; 3/10 (30%) were diagnosed at over 3 years of age (Table. 4. Fig 1). The late diagnosis age cases were all from samples where *MYCN*^{A} was present alongside two pathogenic *RB1* variants. However, for the FFPE with *MYCN*^{A} it was not possible to carry out full *RB1* screening due to poor DNA integrity. Histopathologic

parameters showed that *MYCN*^{A} tumors showed less differentiation compared to an age matched control group. 9/10 *MYCN*^{A} tumors were undifferentiated or poorly differentiated while all control group tumors were moderately or well differentiated. There was no difference in other core elements, resulting in similar pathologic tumor stages in both groups. *MYCN* MLPA did not detect any other consistent chromosomal changes and it was striking that none of the *MYCN*^{A} tumors displayed gain at 6p which is otherwise a common change in Rb tumors.

6p gain

We looked at 6p gain (triplication) as this is one of the most common chromosome gains in Rb and has been related to poor outcomes such as increased risk of enucleation and adverse histopathological features (12, 13, 14). In the total Rb cohort 51% (76/149) of tumors had 6p triplication. It was not seen in any *MYCN*^{A} samples. The proportion rose to 63.1% (41/65) in the unbiased set of tumors collected from 2014-2019. In the cohort of 100 sporadic unilateral tumors it was seen in 62% (62/100) overall, in 61.8% (34/55) of tumors with normal *MYCN*, and 77.8% (28/36) of *MYCN* triplicated tumors. For the early age cases of ≤10 months diagnosis it was seen in only 37% tumors (10/27), and at ≤ 6 months it was seen in 23.1% (3/13). (If the *MYCN*^{A} samples are excluded the levels become 47.6% (10/21) at ≤10 months and 27.3% (3/11) at ≤ 6 months).

Discussion

In the U.K., *RB1* genetic screening is part of standard management for Rb patients. Routine screening of tumor tissue where available, or blood if the eye can be saved, should include all 27 exons and associated splice sites, the promoter region for coding and hypermethylation changes, and deletion/loss of heterozygosity analysis. Cytogenetic analysis is available to detect large chromosomal deletions and rearrangements in blood samples. If these tests fail to

identify pathogenic variants, then RNA sequencing for deep intronic variants can be performed, especially for bilateral or familial cases. Around 2% of sporadic unilateral Rbs display amplification at the *MYCN* locus in the absence of *RB1* alterations (6, 15). *MYCN*^{A} has been associated with large, aggressive tumors with early age at diagnosis that carry only a few of the genomic copy-number changes that are characteristic of Rb (6). If Rb can be shown to be solely due to *MYCN*^{A},then it would be considered non-heritable. If such a tumor is truly sporadic, and aggressive, then the affected eye could be enucleated knowing that the second eye will not be at risk. The risk of Rb to other family members will be reduced to 'population' risk (1 in 15-20,000) and the patient should not have an increased risk for later primary (non-ocular) cancers. Currently, there are no best practice guidelines for the reporting and interpretation of *MYCN*^{A} in Rb patients.

This study aimed to establish the fraction of Rbs with *MYCN*^{A} in our samples, and whether these also displayed pathogenic *RB1* variants as McEvoy *et al* found that 8/94 (8.5%) of Rb tumors had *MYCN*^{A} (over 10 copies) and that 6 of those (75%) also carried at least one *RB1* variant (5). We looked at a sub-set of Rb tumors (n=149) screened over a twenty-five-year period of referrals to Barts Health NHS Trust. This cohort contained a disproportionate number of cases diagnosed at an early age and samples where *RB1* pathogenic variants were absent after a full DNA screen (not covering deep intronic changes). This full set (149 Rbs) had an excess of tumors with difficult to detect alterations (or DNA too fragmented to screen fully). In this Rb population 6.7% (10/149) had *MYCN*^{A}. However, where we tested an unbiased set of all 65 tumors collected between 2014-2019 the percentage of *MYCN*^{A} samples was 3.7% (2/65).

Of the 10 *MYCN*^{A} tumors, one was formalin fixed paraffin embedded (FFPE; 2 months) and could not be fully screened for *RB1* alterations due to degraded DNA. This sample was therefore excluded from some analysis as its' *RB1* status could not be accurately determined.

Of the 9 fully screened *MYCN*^{A} samples, 3/9 carried two pathogenic *RB1* variants (33.3% RB$^{-/-}$) and had late ages at diagnosis (over three years old). The 6 *MYCN*^{A} samples with no detectable *RB1* changes (6/9; 66.7% *RB1*$^{+/+}$) were all from patients diagnosed at 10 months or less. Rushlow *et al* estimated that 18% of children diagnosed with non-familial, unilateral Rb diagnosed at 6 months or younger will have *RB1*$^{+/+}$ *MYCN*^{A} tumors (6). We obtained numbers in that region although the contribution of *MYCN*^{A} drops steadily as you look at younger ages of diagnosis. In our population of 100 sporadic, non-germline cases we found 6/27 (22.2%) *MYCN*^{A} cases in patients diagnosed at 10 months or younger, and all those *MYCN*^{A} cases were *RB1*$^{+/+}$. From 8 months or younger *MYCN*^{A} cases made up 4/23 (17.4%) of patients, and at 6 months or younger they comprised 2/13 (15.4%). This effect may be due to the small numbers involved and the exclusion of the *RB1*$^{+/+}$ *MYCN*^{A} FFPE sample from the analysis.

The *MYCN*^{A} tumors were 1-1.8 cm in size and showed less differentiation compared to an age matched control group. 9/10 *MYCN*^{A} tumors were undifferentiated or poorly differentiated while all control group tumors were moderately or well differentiated (Table 4). This is similar to *MYCN* amplified neuroblastoma and is not surprising given the potential involvement of *MYCN* in embryonic development and pluripotency (10). There was no difference in other core elements, resulting in similar pathologic tumor stages in both groups. So far as we know none of the *MYCN*^{A} patients had extraocular relapse or developed tumors in the second eye (all are now old enough to be unlikely to develop further Rb tumors). It was striking that all three *MYCN*^{A} *RB1*$^{-/-}$ tumors had late ages at diagnosis (38 months or more). Mairal *et al* (12), and Lillington *et al* (16), previously reported that *MYCN*^{A} is not necessarily linked to adverse outcome in Rb, and Ewens *et al* found no difference in high risk features between tumors with or without *MYCN*^{A} (15), although there was earlier diagnosis age in the amplified set. The first report of two aggressive, metastatic *RB1*$^{+/+}$ *MYCN*^{A} tumors was recently published with both cases occurring children with late diagnosis (17 and 30 months)

(17). The metastasis pattern was unusual giving rise to massive orbital invasion with subsequent systemic spread rather than invading the central nervous system and bone marrow. It was suggested that this poor outcome could be due to delayed diagnosis, and delayed enucleation due to attempts to salvage the eyes.

All the *MYCN*^{A} tumors were from sporadic unilateral patients, and 9 had been fully screened for *RB1* variants with 6 having no *RB1* variant detected. However, deep intronic changes, chromothripsis and complex chromosomal rearrangements cannot be excluded. Ewens *et al* found no correlation between the type of *RB1* variant present in RB$^{-/-}$ or RB$^{+/-}$ tumors, or between tumors with normal or amplified *MYCN* (15). The *RB1* variants in our late diagnosis age *MYCN*^{A} cases involve deletions/breakpoints, or affect splicing, and could act as low penetrance changes where *MYCN*^{A} could provide a boost to progression. We also found that of 100 sporadic unilateral tumors where no germline changes were found, those missing one *RB1* variant had a high proportion of exonic insertions/deletions (7/22 expected variants in 11 Rbs, 31.8%) compared to *RB1*$^{-/-}$ tumors (10/154 expected variants in 77 Rbs, 6.5%) (Table 3). It is probable that these are complex rearrangements (chromoanagenesis) which constitute two *RB1* pathogenic variants, but which cannot be resolved using our routine screening techniques. For instance, chromothripsis at the *RB1* locus has been found in 3% of Rbs (5). A minority of tumors may be disrupted via other mechanisms such as changes in gene expression, post-translational regulation or modification, or protein stability. For example, Ewens *et al* inferred that pRb was functionally inactive, even in *MYCN*^{A} Rb tumors, due to phosphorylation (15).

A small set of the Rb tumors were analyzed by Whole Genome Sequencing (WGS), including 10 where one or two pathogenic variants were missing after routine screening (18). This set included two *MYCN*^{A} tumors, both with late onset (over 35 months). In 9/10 cases WGS identified simple rearrangements, complex intra-chromosomal and inter-chromosomal

rearrangements, and one case of potential chromothripsis. Despite the increased resolution of WGS, where the rearrangement patterns were complicated (6/10 cases) it was not possible to confirm whether both copies of *RB1* were affected. Even after a complete *RB1* screen and WGS, 1 of the 10 samples was left with no second pathogenic variant identified, suggesting that a minority of tumors maybe disrupted via other mechanisms These could be epigenetic mechanisms (e.g. noncodingRNA regulation) which may be influenced by *MYCN* amplification.

6p gain is one of the most common copy number alterations in Rb. It has been linked to adverse histopathological features and poor prognosis and has been suggested as a prognostic biomarker for eye salvage or enucleation (14). In our total Rb cohort 51% (76/149) had 6p gain, with 63.1% (41/65) in the unbiased set of tumors collected from 2014-2019. These levels match those seen in other studies which found 6p gain in 50-70% tumors (12, 14, 16, 18). Although 6p gain is a common copy number alteration, none of the 10 $MYCN^{A}$ tumors displayed this change (Table 4). These Rbs do appear to be undifferentiated, fast growing tumors, but this may not necessarily lead to poor prognosis due to lack of other adverse features.Francis *et al* found 6p gain in 59% of 83 enucleated samples but did not associate it with aggressive features (19). One of the two patients reported by Zugbi *et al* to have late diagnosed metastatic $RB1^{+/+}$ $MYCN^{A}$ did have 6p gain (17). Rushlow *et al* reported less of the characteristic genomic copy number changes in $RB1^{+/+}$ $MYCN^{A}$ tumors although they had aggressive growth (6). Ewens *et al*, however, found no significant difference in the fraction of tumors with 6p gain with or without $MYCN^{A}$, although none of his $RB1^{+/+}$ $MYCN^{A}$ tumors (n=7) had 6p gain (15). Xu *et al* also had 3 tumors (6%) with 'focal' *MYCN* gain and no other copy number changes (14) and all 3 were $RB1^{-/-}$ with 2 carrying an *RB1* pathogenic variant in the blood.

Studies of cell free DNA (cfDNA) in aqueous humor from enucleated vs salvaged eyes found a significant correlation between 6p gain, aggressive disease and the risk of enucleation, and that age was positively associated with the frequency of somatic copy number alterations (14, 20). 2p gain (*MYCN* is at 2p24.3) was only marginally predictive of enucleation when testing cfDNA (14). It appears that the older the age at Rb diagnosis the more somatic copy number changes are present (21). This suggests that $RB1^{+/+}$ $MYCN^{A}$ tumors with a very early age of diagnosis have less time to accumulate extra copy number changes, or they have less need to acquire such changes to progress.

Accurate copy number analysis of $MYCN^{A}$ could prove difficult due to tumor heterogeneity and the presence of normal cells. However, Rbs with lower levels of *MYCN* may still overexpress this gene with it having a role in progression. In Davies *et al* RNAseq data from five tumors showed a dramatic increase in expression in the one high $MYCN^{A}$ tumor, but also to a lesser extent in samples with 2 and 3 copies (18). Ganguly and Shields observed a 9-fold increase in *MYCN* expression in tumors with *RB1* variants (n=6) compared to matched normal retina (22). Ten human Rb cell lines with a range of *RB1* genetic and epigenetic variants, including $RB1^{+/+}$ $MYCN^{A}$, were analyzed at the mRNA level by Schwermer *et al* (23). All had increased *MYCN* expression regardless of gene amplification, but expression was on average 12-fold higher in the $MYCN^{A}$ lines and protein levels increased only in those with $MYCN^{A}$. We found that 49/149 Rbs (32.9%) had triplicated *MYCN* but we cannot say how many tumors had elevated *MYCN* expression.

Tran *et al* looked at 10 high-*MYCN*-expressing cell lines including five Rbs (24). They found that *MYCN* promoted *MDM2* expression in the Rb cell of origin and *MDM2* promoted *MYCN* expression in Rb cells, suggesting that *MYCN* and *MDM2* comprise a positive feedback loop.This gave rise to rapidly proliferating *MDM2*-dependent cone-precursor-derived masses

in a cultured Rb model and the suggestion that high MYCN protein levels were sufficient to induce *MDM2* and initiate tumorigenesis in the absence of other changes. Although *MDM2* can be a negative regulator of p53, it was shown that high level *MDM2* expression can drive *MYCN* overexpression and promote Rb proliferation in a p53 independent manner(25).

MYCN can be a target for treatment in cancer patients (10, 26). 'MYCN opposite strand' (*MYCNOS*) is found on the DNA strand opposite *MYCN* so that it is also amplified in *MYCN*^{A} Rb. *MYCNOS* RNA variants can function as long noncoding RNA or coding RNA to facilitate *MYCN* expression and enable the stabilization of MYCN protein. It was reported that *MYCNOS1* supported *MYCN* amplification in *MYCN*-driven retinoblastoma and that its' inhibition might aid in therapy for *RB1*$^{+/+}$ *MYCN*^{A} Rb by suppressing *MYCN* activity. *MYCNOS1* knockdown caused MYCN protein instability leading to cell cycle arrest and impaired proliferation with an enhanced response to topotecan (27). However, a mouse model of Rb showed that *MYCN*^{A} tumors can evolve to survive without *MYCN* overexpression, which could have adverse implications for such treatments (28). The mouse model displayed no tumors solely due to *MYCN*^{A} but there was strong co-operation with *RB1* loss to drive tumor formation.

In sporadic cases with early diagnosis, Rbs with no *RB1* pathogenic variant identified after a complete *RB1* screen should be tested for *MYCN* amplification. Conversely, tumors with *MYCN*^{A} should still be screened for *RB1* pathogenic variants. Testing for promoter methylation, chromosomal rearrangements and deep intronic changes is required to exclude *RB1* involvement. Rb patients are currently referred for cytogenetic analysis of blood but this is not routinely performed for tumor cells. In the future, routine WGS (and some Next Generation Sequencing panels) of tumors should be able to identify small coding changes, large scale rearrangements and deep intronic changes in *RB1*, whilst also determining copy

number in *MYCN*, thus providing a more complete ‘one stop’ screen in difficult to analyze samples. This approach should also detect heterogeneity in multifocal tumors, as well as changes in other regions linked to progression, aggressive phenotype, and prognosis (14, 18, 19, 20, 29). Aqueous humor cfDNA from Rb obtained during intravitreal chemotherapy could provide prognostic information and would prove especially useful in cases where enucleation is not required (14, 30), allowing more accurate genetic counselling for families. Additionally, the analysis of Rb tumor cfDNA which can be detectable in plasma, shows promise in genetic screening (31). This would avoid invasive intraocular biopsies and repeat testing could possibly be used to monitor treatment efficacy, relapse, or metastasis.

Acknowledgements

The authors gratefully acknowledge the contributions of Mr. John Hungerford (Consultant Ophthalmologist) and the late Dr. Judith Kingston (Consultant Pediatric Oncologist) for the referral, provision of clinical information and material for analysis. We also thank the Barts Health Rb clinical nurse specialists and pathologists for their help in collecting samples, and RGSU genetic technologists for sample processing and testing. Thanks to Dr. Gerald Draper for his advice on statistical analysis.

Declaration of interest

The authors declare no conflicts of interest.

Ethics approval

Barts Health Clinical Effectiveness Unit (audit no. 10839).

Notes on contributors

EAP, RP, and ZO performed *RB1* screening, and analyzed the variants. EAP and ZO interpreted the data and drafted the article. EKK and IS carried out histopathological analysis of tumors. MSS and MAR referred cases, provided clinical information and material for analysis, and revised/approved the article. All authors critically read, revised, and approved the final article.

ORCID

Zerrin Onadim http://orcid.org/0000-0002-8594-9586

Mandeep S. Sagoo https://orcid.org/0000-0003-1530-3824

References

1. Vogel W. The genetics of retinoblastoma. Hum Genet. 1979;52:1-54.

2. Valverde JR, Alonso J, Palacios I, Pestaña A. *RB1* gene mutation up-date, a meta-analysis based on 932 reported mutations available in a searchable database. BMC Genet. 2005;6:53.

3.Hogg A, Bia B, Onadim Z and Cowell JK. Molecular mechanisms of oncogenic mutations in tumours from patients with bilateral and unilateral retinoblastoma. Proc Natl Acad Sci USA 1993;90:7351-7355.

4. Price EA, Kolkiewicz K, Patel R, Hashim S, Karaa E, Scheimberg I, Sagoo MS, Reddy MA, Onadim Z. Detection and reporting of *RB1* promoter hypermethylation in diagnostic screening. Ophthalmic Genet. 2018;39(4):526-531.

5. McEvoy J, Nagahawatte P, Finkelstein D, Richards-Yutz J, Valentine M, Ma J, Mullighan C, Song G, Chen X, Wilson M, et al. *RB1* gene inactivation by chromothripsis in human retinoblastoma. Oncotarget. 2014;5:438-50.

6. Rushlow DE, Mol BM, Kennett JY, Yee S, Pajovic S, Thériault BL, Prigoda-Lee NL, Spencer C, Dimaras H, Corson TW, et al. Characterisation of retinoblastomas without *RB1* mutations: genomic, gene expression and clinical studies. Lancet Oncol. 2013;14:327-34.

7. Kooi IE, Mol BM, Massink MP, de Jong MC, de Graaf P, van der Valk P, Meijers-Heijboer H, Kaspers GJ, Moll AC, Te Riele H, et al. A Meta-Analysis of Retinoblastoma Copy Numbers Refines the List of Possible Driver Genes Involved in Tumor Progression. PLoS One. 2016;11(4):e0153323.

8. Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. Semin Cancer Biol. 2006;16(4):253-64.

9. Hydbring P, Castell A, Larsson LG. MYC Modulation around the CDK2/p27/SKP2 Axis. Genes (Basel). 2017 Jun 30;8(7):174.

10. Ruiz-Pérez MV, Henley AB, Arsenian-Henriksson M. The MYCN Protein in Health and Disease. Genes. 2017;8(4), 113; doi:10.3390/genes8040113.

11. Price EA, Price K, Kolkiewicz K, Hack S, Reddy MA, Hungerford JL, Kingston JE, Onadim Z. Spectrum of *RB1* mutations identified in 403 retinoblastoma patients. J Med Genet. 2014;51:208-14.

12. Mairal A, Pinglier E, Gilbert E, Peter M, Validire P, Desjardins L, Doz F, Aurias A, Couturier J. Detection of chromosome imbalances in retinoblastoma by parallel karyotype and CGH analyses. Genes Chromosomes Cancer. 2000;28(4):370-9.

13. Lillington DM, Kingston JE, Coen PG, Price E, Hungerford J, Domizio P, Young BD, Onadim Z. Comparative genomic hybridization of 49 primary retinoblastoma tumours identifies chromosomal regions associated with histopathology, progression, and patient outcome. Genes Chromosomes Cancer. 2003;36:121-28.

14. Xu L, Polski A, Prabakar RK, Reid MW, Chevez-Barrios P, Jubran R, Kim JW, Kuhn P, Cobrinik D, Hicks J, et al. Chromosome 6p Amplification in Aqueous Humor Cell-Free DNA Is a Prognostic Biomarker for Retinoblastoma Ocular Survival. Mol Cancer Res. 2020;18(8):1166-1175.

15. Ewens KG, Bhatti TR, Moran KA, Richards-Yutz J, Shields CL, Eagle RC, Ganguly A.

Phosphorylation of pRb: mechanism for RB pathway inactivation in *MYCN*-amplified retinoblastoma. Cancer Med. 2017;6(3):619-630.

16. Lillington DM, Goff LK, Kingston JE, Onadim Z, Price E, Domizio P, Young BD. High level amplification of N-MYC is not associated with adverse histology or outcome in primary retinoblastoma tumours. Br J Cancer. 2002;87:779-82.

17. Zugbi S, Ganiewich D, Bhattacharyya A, Aschero R, Ottaviani D, Sampor C, Cafferata EG, Mena M, Sgroi M, Winter U, et al. Clinical, Genomic, and Pharmacological Study of MYCN-Amplified *RB1* Wild-Type Metastatic Retinoblastoma. Cancers (Basel). 2020;12(9):2714.

18. Davies HR, Broad KD, Onadim Z, Price EA, Zou X, Sheriff I, Karaa E, Scheimberg I, Reddy MA, Sagoo MS, et al. Whole-genome sequencing of retinoblastoma reveals the diversity of rearrangements disrupting *RB1* and uncovers a treatment-related mutational signature. Cancers. *In Press*.

19. Francis JH, Richards AL, Mandelker DL, Berger MF, Walsh MF, Dunkel IJ, Donoghue MTA, Abramson DH. Molecular Changes in Retinoblastoma beyond *RB1*: Findings from Next-Generation Sequencing. Cancers (Basel). 2021;13(1):149.

20. Berry JL, Xu L, Kooi I, Murphree AL, Prabakar RK, Reid M, Stachelek K, Le BHA, Welter L, Reiser BJ, et al. Genomic cfDNA Analysis of Aqueous Humor in Retinoblastoma Predicts Eye Salvage: The Surrogate Tumor Biopsy for Retinoblastoma.Mol Cancer Res. 2018;16(11):1701-1712.

21. Polski A, Xu L, Prabakar RK, Gai X, Kim JW, Shah R, Jubran R, Kuhn P, Cobrinik D, Hicks J, Berry JL. Variability in retinoblastoma genome stability is driven by age and not heritability. Genes Chromosomes Cancer. 2020;59(10):584-590.

22.Ganguly A, Shields CL. Differential gene expression profile of retinoblastoma compared to normal retina. Mol Vis. 2010;16:1292-303.

23. Schwermer M, Hiber M, Dreesmann S, Rieb A, Theißen J, Herold T, Schramm A, Temming P, Steenpass L. Comprehensive characterization of *RB1* mutant and *MYCN* amplified retinoblastoma cell lines. Exp Cell Res. 2019;375(2):92-99.

24. Tran HN, Singh HP, Guo W, Cambier L, Riggan L, Shackleford GM, Thornton ME, Grubbs BH, Erdreich-Epstein A, Qi D-L*, et al*. Reciprocal Induction of *MDM2* and *MYCN* in Neural and Neuroendocrine Cancers. Front. Oncol. 2020;10:563156.

25. Qi D-L, Cobrinik D. *MDM2* but not *MDM4* promotes retinoblastoma cell proliferation through p53-independent regulation of *MYCN* translation. Oncogene. 2017;36(13):1760-1769.

26. Chen H, Liu H, Qing G. Targeting oncogenic Myc as a strategy for cancer treatment. Signal Transduct Target Ther. 2018 Feb 23;3:5.

27. Saengwimol D, Chittavanich P, Laosillapacharoen N, Srimongkol A, Chaitankar V, Rojanaporn D, Aroonroch R, Suktitipat B, Saisawang C, Svasti S, et al. Silencing of the Long Noncoding RNA MYCNOS1 Suppresses Activity of *MYCN*-Amplified Retinoblastoma Without *RB1* Mutation. Invest Ophthalmol Vis Sci. 2020;61(14):8.

28. Wu N, Jia D, Bates B, Basom R, Eberhart CG, MacPherson D. A mouse model of *MYCN*-driven retinoblastoma reveals *MYCN*-independent tumour re-emergence. J Clin Invest. 2017;127(3):888-98.

29. Afshar AR, Pekmezci M, Bloomer MM, Cadenas NJ, Stevers M, Banerjee A, Roy R, Olshen AB, Van Ziffle J, Onodera C, et al. Next-Generation Sequencing of Retinoblastoma Identifies Pathogenic Alterations beyond *RB1* Inactivation That Correlate with Aggressive Histopathologic Features. Ophthalmology. 2020;127(6):804-813.

30. Gerrish A, Stone E, Clokie S, Ainsworth JR, Jenkinson H, McCalla M, Hitchcott C, Colmenero I, Allen S, Parulekar M, et al. Non-invasive diagnosis of retinoblastoma using cell-free DNA from aqueous humour. Br J Ophthalmol. 2019;103(5):721-724.

31. Kothari P, Marass F, Yang JL, Stewart CM, Stephens D, Patel J, Hasan M, Jing X, Meng F, Enriquez J, et al. Cell-free DNA profiling in retinoblastoma patients with advanced intraocular disease: An MSKCC experience. Cancer Med. 2020;9(17):6093-6101.

1993-Mar 2014 cohort	**49 *RB1*$^{-/-}$**	**10 *RB1*$^{+/-}$**	**10 *RB1*$^{+/+}$**	**15 ND**	**84 Total**
Unilateral sporadic	37 (1 *MYCN*^{A})	10 (1 *MYCN*^{A})	10 (5 *MYCN*^{A})	14 (1*MYCN*^{A})	71 (8 *MYCN*^{A})
Unilateral germline	7	0	0	0	7
Bilateral	5	0	0	1	6
Apr 2014-Mar 2019	**50 *RB1*$^{-/-}$**	**2 *RB1*$^{+/-}$**	**2 *RB1*$^{+/+}$**	**11 ND**	**65 Total**
Unilateral sporadic	40 (1 *MYCN*^{A})	1	2 (1 *MYCN*^{A})	1	44 (2 *MYCN*^{A})
Unilateral germline	4+1	0	0	1	6
Bilateral	5	1	0	9	15
Total Tumors	**99 *RB1*$^{-/-}$**	**12 *RB1*$^{+/-}$**	**12 *RB1*$^{+/+}$**	**26 ND**	**149 Total**
Unilateral sporadic	77 (2 *MYCN*^{A})	11 (1 *MYCN*^{A})	12 (6 *MYCN*^{A})	15 (1*MYCN*^{A})	115 (10 *MYCN*^{A})
Unilateral germline	12	0	0	1	13
Bilateral	10	1	0	10	21

Table 1: Populations of Rb tumors collected from 1993 to 2014 and their results for *RB1*variant and *MYCN*^{A} analysis. Not Determined (ND) applies to tumors where *RB1* status was not completely determined either due to lack of DNA or because only blood DNA was fully screened for *RB1* variants.

	123 Tumors; all fully screened No (%)	100 sporadic unilateral tumors; excludes known germline cases. No (%)	54 tumors from 2014-2019; somatic and germline No (%)
RB1$^{-/-}$ *MYCN*^{A}	99 (80.49%) *2 (1.63% total; 2.02% of RB1*$^{-/-}$*)*	77 (77%) *2 (2% total; 2.6% of RB1*$^{-/-}$)	50 (92.6%) *1 (1.9% total; 2% of RB1*$^{-/-}$*)*
RB1$^{+/-}$ *MYCN*^{A}	12 (9.76%) *1 (0.81% total; 8.33% of RB1*$^{+/-}$*)*	11 (11%) *1 (1% total; 9.09% of RB1*$^{+/-}$*)*	2 (3.7%) *0*
RB1$^{+/+}$ *MYCN*^{A}	12 (9.76%) *6 (4.88% total; 50% of RB1*$^{+/+}$*)*	12 (12%) *6 (6% total; 50% of RB1*$^{+/+}$*)*	2 (3.7%) *1 (1.9% total; 50% of RB1*$^{+/+}$*)*
Overall *MYCN*^{A}	9/123 (7.32%)	9/100 (9%)	2/54 (3.7%)
Rb status	100 sporadic unilateral 12 unilateral germline 11 bilateral	100 sporadic unilateral 0 unilateral germline 0 bilateral	43 sporadic unilateral 5 sporadic unilateral germline 6 bilateral

Table 2: Levels of *MYCN*^{A} in fresh tumors which had been sufficiently screened for complete *RB1* pathogenic variant detection. Note: it is still possible that the population of 100 sporadic unilateral cases includes germline *RB1* variants as not all possible pathogenic variants were identified in the tumor to allow checking of blood.

	100 Rb Total	**77 *RB1* $^{-/-}$**	**11 *RB1*$^{+/-}$**	**12 *RB1*$^{+/+}$**
Variant Type	Found (% of 200 expected variants)	Found (% of 154 expected variants)	Found (% of 22 expected variants)	Found (% of 24 expected variants)
LOH/Del *RB1*	58 (29%)	55 (35.7%)	3 (13.6%)	0
Point and small in/del	52 (26%)	51 (33.1%)	1 (4.6%)	0
Splice	17 (8.5%)	17 (11%)	0	0
Missense	2 (1%)	2 (1.3%)	0	0
Methylation	15 (7.5%)	15 (9.7%)	0	0
Exonic in/del	17 (8.5%)	10 (6.5%)	7 (31.8%)	0
Chromothripsis/ Rearrangement	4 (2%)	4 (2.6%)	0	0
Total Identified	165 (82.5%)	154 (100%)	11 (50%)	0 (0%)
RB1 variants missing	35 (17.5%)	0	11 (50%)	24 (100%)
MYCN^{A}	9/100 (9% of all sporadic Rb)	3/77 (3.9% *RB1*$^{-/-}$)	0/11 (0% *RB1*$^{+/-}$)	6/12 (50% *RB1*$^{+/+}$)

Table 3: *RB1* pathogenic variants and *MYCN*^{A} in 100 sporadic, unilateral tumors where no germline changes could be identified after a complete *RB1* screen. 2 *RB1* pathogenic variants are expected in each tumor.

Sample	Age at diagnosis Tumour differentiation Size Pathologic Stage	*MYCN* Copy No. Other Changes	*RB1* Pathogenic Variants
Case 1 FFPE (Fixed)	**2 months** Poorly differentiated Size 1.5 cm pT1	14 copies No other consistent changes.	$RB1^{+/+}$ Full *RB1* variant screen not possible in FFPE.
Case 2 Fresh Tumor	**3 months** Undifferentiated Size 1.6 cm pT2a	25 copies No other chromosomal changes.	$RB1^{+/+}$
Case 3 Fresh Tumor	**5 months** Undifferentiated Size 1.8 cm pT2a	35 copies 16q13 hemizygous	$RB1^{+/+}$
Case 4 Fresh Tumor	**8 months** Poorly differentiated Size 1.5 cm pT3a	23 copies 10p11 hemizygous	$RB1^{+/+}$
Case 5 Fresh Tumor	**8 months** Poor differentiation pT2a	12 copies TP53 hemizygous	$RB1^{+/+}$
Case 6 Fresh Tumor	**9 months** Undifferentiated Size 1.7 cm pT2a	14 copies TP53 hemizygous NF1 triplicated 16q13 hemizygous	$RB1^{+/+}$ *RB1* RNA screen also negative after c.DNA sequencing.
Case 7 Fresh Tumor	**10 months** Poorly differentiated	24 copies No other consistent	$RB1^{+/+}$

	Size 1.5 cm pT2a	changes.	
Case 8 Fresh Tumor	**38 months** Moderate differentiation Size 1.5 cm pT2b	11 copies No other chromosomal changes.	del *RB1*. Probable that missing *RB1* variant is due to complex re-arrangements.
Case 9 Fresh Tumor	**40 months** Poorly differentiated Size 1.3 cm pT1	89 copies 18q21 triplicated	g.76919_76950del32 c.1420_1421+30del Exon/intron 15 heterozygous. Exon 15 skip to STOP predicted. c.(1695+1_16961)_(*1815_?)del Heterozygous deletion from exon 18 to beyond *RB1* gene.
Case 10 Fresh Tumor	**61 months** Undifferentiated tumor. Size 1 cm pT2a	28 copies TP53 hemizygous NF1 triplicated SCN1a hemizygous	g.59793G>T ; c.861G>T p.(Glu287Asp)/splice g.170346A>T ; c.2490-26A>T Intron 23 branch point.

Table 4: Overview of *MYCN*^{A} samples (all sporadic unilateral Rb). MLPA kits shared control probes for 15q21; 16q13; 19q13; 20q13; 21q22; 5p13; 6p12 and 6p22. No *MYCN*^{A} sample had 6p gain. All enucleations occurred without prior treatments, and no extraocular relapse was reported. Pathologic tumor-node-metastasis (TNM) staging of Rb was performed according to the AJCC Cancer Staging Manual, 8th edition.

pT1 Tumor confined to eye with no optic nerve or choroidal invasion.

pT2a Focal choroidal invasion and pre- or intra-laminar invasion of the optic nerve head.

pT2b Tumor invasion of stroma of iris and /or trabecular meshwork and/or Schlemm's canal.

pT3a Choroidal invasion larger than 3mm in diameter or multiple foci of invasion totaling more than 3 mm or any full-thickness involvement.

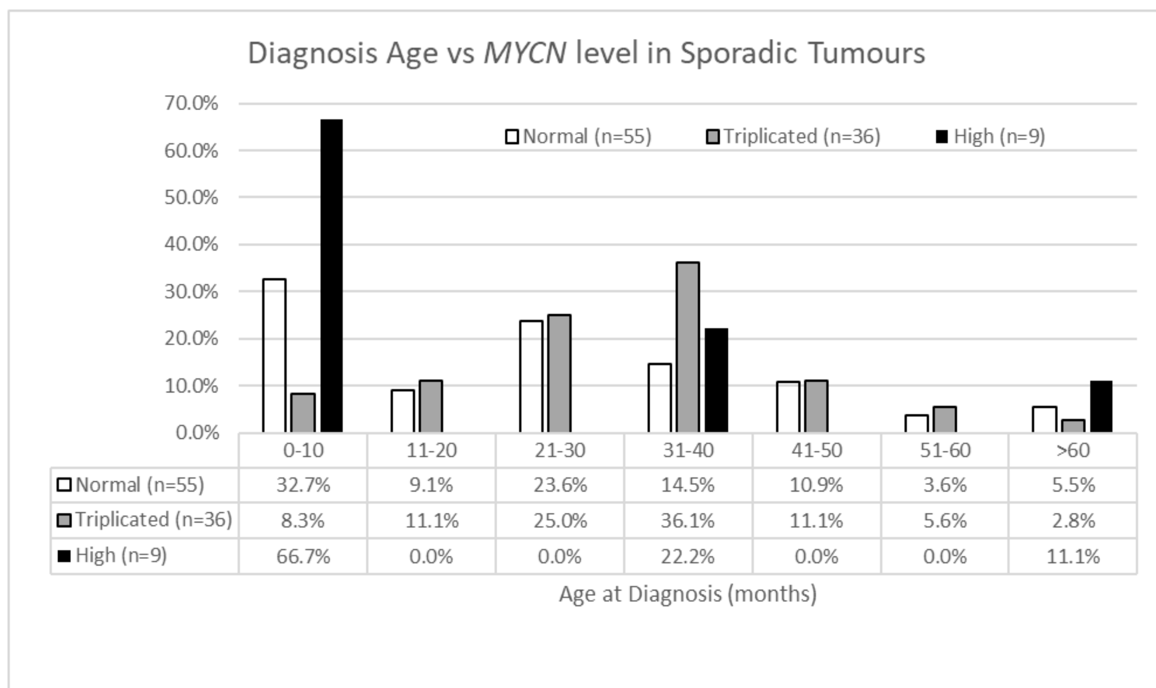

Figure 1: *MYCN* copy number according to age at sporadic Rb diagnosis (n=100 after exclusion of cases where *RB1* pathogenic variants were detected in matched blood samples e.g. known germline cases). Normal is up to 1.26 copies. Triplicated is 1.27-3.26. High is ≥10.