CHAPTER

RB1 MUTATION SPECTRUM IN A COMPREHENSIVE NATIONWIDE COHORT OF RETINOBLASTOMA PATIENTS

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ABSTRACT

Background

Retinoblastoma (Rb) is a childhood cancer of the retina, commonly initiated by biallelic inactivation of the *RB1* gene. Knowledge of the presence of a heritable *RB1* mutation can help in risk management and reproductive decision making. We report here on *RB1* mutation scanning in a unique nation-wide cohort of Rb patients from the Netherlands.

Methods

From the 1173 Rb patients registered in the Dutch National Retinoblastoma Register until January 2013, 529 patients from 433 unrelated families could be included. *RB1* mutation scanning was performed with different detection methods, depending on the time period.

Results

Our mutation detection methods revealed *RB1* mutations in 92% of bilateral and/or familial Rb patients and in 10% of non-familial unilateral cases. Overall an *RB1* germline mutation was detected in 187 (43%) of 433 Rb families, including 33 novel mutations. The distribution of the type of mutation was: 37% nonsense, 20% frameshift, 21% splice, 9% large indel, 5% missense, 7% chromosomal deletions and 1% promoter. Ten per cent of patients were mosaic for the *RB1* mutation. Six three-generation families with low-penetrance *RB1* mutations were found. We found evidence that two variants, previously described as pathogenic *RB1* mutations, are likely to be neutral variants.

Conclusion

The frequency of the type of mutations in the *RB1* gene in our unbiased national cohort is the same as the mutation spectrum described worldwide. Furthermore, our *RB1* mutation detection regimen achieves a high scanning sensitivity.

INTRODUCTION:

Retinoblastoma (Rb) is the most common intraocular malignancy in children and is almost always initiated by biallelic inactivation of the *RB1* gene.[1-3] Some Rb patients (40%) have a heritable predisposition, caused by either an inherited familial *RB1* mutation (familial heritable Rb) or a de novo germline *RB1* mutation (sporadic or non-familial heritable Rb). Inactivation of the second *RB1* allele in *RB1* mutation carriers occurs in developing retinal precursor cells, either by mutation, loss of the wild type allele (with loss of heterozygosity (LOH) as a consequence), or promoter hypermethylation. *RB1* mutation carriers can transmit the *RB1* mutation in an autosomal dominant manner. The majority of *RB1* mutation carriers develop bilateral Rb at a relatively early age, with an average age at diagnosis of 12 months. In addition to a predisposition for the development of Rb, germline *RB1* mutation carriers have an increased risk for the development of second primary tumours.[4-6] Non-familial non-heritable Rb is usually caused by two somatic *RB1* inactivating mutations in developing retinal cells, these patients are unilaterally affected (60% of all cases).[7]

Children at increased risk for Rb are subjected to regular ophthalmological examination from birth until the age of four years, performed under a general anaesthetic from the age of three months. These eye exams would no longer be necessary if an increased risk could be excluded through DNA testing. Furthermore, knowledge of the presence of an *RB1* mutation in the germline, or two *RB1* mutations in the Rb tumour and none in the germline, can aid families in reproductive decision making[8 9], emphasising the clinical importance for *RB1* mutation scanning.

The *RB1* gene contains 27 exons, encoding the 928 amino acid long Rb protein (pRb). The Rb protein belongs to the family of pocket proteins, together with p107 (*RBL1*) and p130 (*RBL2*), which regulate the cell cycle by binding to E2F transcription factors.[10-12] Since the discovery of the *RB1* gene, a broad spectrum of germline and somatic *RB1* mutations have been described, and can be accessed through many articles[13-17], and the online Leiden Open Variation Database (LOVD), which includes over 1000 different *RB1* mutations reported worldwide.[18] The majority of *RB1* mutations are scattered throughout the gene, although several recurrent mutations and mutation hotspots exist.[15 17 19] We describe the distribution of *RB1* mutations in a comprehensive national cohort, including more than 500 Dutch Rb patients from the Dutch Retinoblastoma Register. More than 180 *RB1* germline mutations were detected including 33 novel mutations.

METHODS
Patient selection

The Dutch National Retinoblastoma Register collects information on all Rb patients diagnosed in the Netherlands from 1862 and is complete from 1945 onwards. Throughout the years, the information has been updated regularly with clinical and genetic data.[4][20] Since 1992, all newly diagnosed Rb patients are treated in the National Retinoblastoma Treatment Center at the VU University Medical Center. DNA testing for all patients with Rb in the Netherlands is performed in one single laboratory: the DNA diagnostic laboratory of the Department of Genetics at the University Medical Center Groningen. For this study, all Dutch Rb patients were included who were tested by *RB1* mutation scanning and/or chromosome analysis until January 2013. In 33 included familial cases, the *RB1* gene was not actually tested, but these cases were either obligate carrier of the familial *RB1* mutation or had been diagnosed with Rb and had died before confirmation of the familial *RB1* mutation could be performed. Eleven non-familial cases and three affected parent-child pairs were excluded because DNA testing methods prior to 1998 were not able to detect a mutation. Testing with newer methods was not possible because DNA was no longer available and the patients could not be contacted again or had died.

All parents of newly diagnosed non-familial patients were given an eye examination, as part of the diagnostic work-up.

This study was conducted in accordance with the principles of the Helsinki declaration.

Scanning of *RB1* mutations in DNA from peripheral blood lymphocytes

Between 1990 and 1998 mutation detection in blood was first performed by means of Southern blotting, after which it was replaced by Single Strand Conformation Polymorphism analysis (SSCP). From 1998 until 2011, DNA analysis included direct sequencing of exons 1 and 15, as well as the *RB1* promoter and Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the other exons and flanking intronic sequences. To detect large deletions and duplications, Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was performed using SALSA MLPA kit p047 *RB1* (MRC-Holland, Amsterdam, the Netherlands). As of 2011, direct sequencing of all exons and the promoter, using in-house derived primers, was used instead of DGGE. In cases where no mutation was detected by Southern blotting and/or SSCP, mutation analysis was later repeated by DGGE, MLPA and/or sequencing, in so far as DNA was available. When novel putative splice mutations were detected (>2 nucleotides from intron exon boundary) and RNA was available, cDNA was studied to detect aberrant splicing.

If warranted, e.g. when dysmorphic features or mental retardation was noted, karyotyping was performed in blood lymphocytes to detect chromosomal rearrangements.

When an *RB1* mutation was found, DNA of the parents (if still alive) was tested for the mutation.

Scanning of *RB1* mutations in tumour DNA

Since 2007 all non-familial unilateral cases in whom the eye was enucleated (n=34) were first tested for the *RB1* mutations in tumour material and then DNA from blood was tested for the tumour mutations (n=34). For 19 non-familial unilateral cases diagnosed prior to 2007, tumour DNA was available and therefore these tumours were also tested. In addition, tumour DNA was tested of two out of 14 non-familial bilaterally affected patients in whom regular scanning of blood DNA did not detect a mutation. In addition to DGGE and/or sequencing analysis, as in analysis of DNA from blood, hypermethylation of the promoter was analysed by MLPA using MRC-Holland kit ME002-C1. LOH was investigated by two intragenic microsatellite markers (Rbi.2 and *RB1*.20). If these methods did not detect two mutations, MLPA of the tumour DNA was performed with the SALSA MLPA kit p047 *RB1* (MRC-Holland, Amsterdam, the Netherlands) to detect large deletions and duplications.

Additional mutation scanning

Additional mutation scanning was performed for several cases with non-familial bilateral or familial Rb without a detectable *RB1* mutation using regular scanning techniques. Lymphoblast cell lines were made of peripheral blood lymphocytes from two patients with non-familial bilateral Rb and three patients with familial Rb, RNA was extracted from cell lines and cDNA was analysed to detect possible deep intronic mutations that can lead to mis-splicing. The coding regions of *RB1*, *RBL1* and *RBL2* were covered by RT-PCR and analysed by electrophoresis (*RB1*, *RBL1* and *RBL2*) and sequencing (*RBL2*). In addition an extended region of the promoter (-2500 to +700) was sequenced in blood DNA of four patients with familial Rb and nine patients with non-familial bilateral Rb to reveal possible promoter mutations that might alter the expression of *RB1*. See supplementary information for detailed methods.

Incomplete penetrance mutations

Mutations were regarded as incomplete penetrance mutations, when the diseased eye ratio (DER) in the family was ≤1.5, defined as the total number of affected eyes per family divided by the number of mutation carriers in the family.[21] To exclude possible mosaicism as a cause of milder expression, the oldest mutation carrier in these families was excluded from the analysis.[14]

Statistics

To determine differences in the frequencies of observed mutation types (nonsense, missense, splice and promoter) in our cohort and worldwide reported mutation frequencies from a meta-analysis by Valverde et al.[15], the Chi-square goodness-of-fit test was performed. To determine differences in the distribution of *RB1* mutations over the different exons and adjacent introns between familial, non-familial bilateral and non-familial unilateral patients, cross tables were analysed with the Fisher's exact test. P-values of <0.05 were considered significant.

RESULTS

As of January 2013, 1173 patients were included in the Dutch Retinoblastoma Register. In **Figure 1A** the number of patients according to year of birth and the number of those patients scanned for *RB1* mutations is depicted. From 1992, almost all newly diagnosed patients were tested at diagnosis, many patients diagnosed before 1992 were tested in retrospect. **Figure 1B** shows the inclusion of patients and the distribution of the mutations according to non-familial or familial cases and laterality. A total of 529 patients treated for Rb, 20 non-penetrant *RB1* mutation carriers, and two *RB1* mutation carriers with retinoma, were included in our study. Fifty-two per cent of the Rb patients were male. Of these 529 Rb cases, 307 (58%) patients were unilaterally affected and 222 (42%) were bilaterally affected. The total of 529 Rb patients concerned 382 isolated non-familial cases and 147 familial cases belonging to 51 unrelated families, making the total of included unrelated families 433. An *RB1* mutation in blood was detected in 187 (43%) out of these 433 unrelated families. The mutation detection rate for all bilaterally affected and/or familial cases was 92%, while of all non-familial unilaterally affected patients 10% turned out to have a germline *RB1* mutation.

Mutations were distributed across the entire gene and the promoter, excluding exons 5, 26 and 27 (**Figure 2**). Distribution per exon/intron was in accordance with previous studies.[15 17] We did detect a difference in the frequency of mutations over the different exons when we compared *RB1* mutations in familial, non-familial bilateral and non-familial unilateral patients (Fisher's exact test, p-value 0.02). **Figure 3** and **Table 1** show the frequency and percentages of the different types of mutations found, for all cases (**Figure 3A**) and separately for familial and non-familial cases (**Figure 3B**). We compared the frequency of the type of mutation (nonsense, missense, splice and frameshift, excluding promoter mutations, large indels, and chromosomal deletions) with worldwide frequencies from a meta-analysis by Valverde et al.[15] describing 925 published *RB1* mutations, belonging to Rb patients from 21 countries. No significant differences in the frequency of the type of mutation between our Dutch cohort and the worldwide frequencies were detected (p = 0.29) (**Figure 3C**).
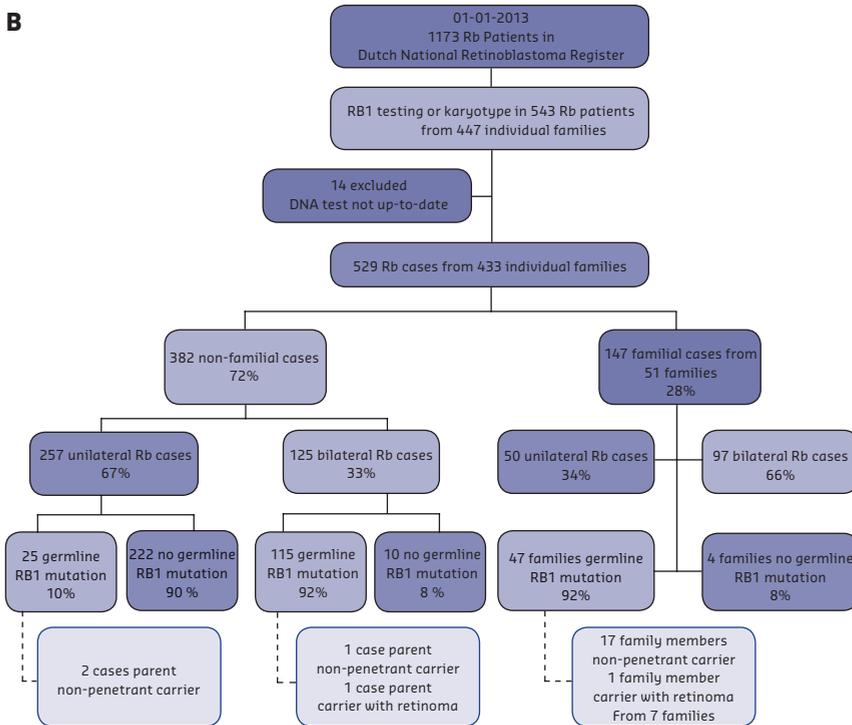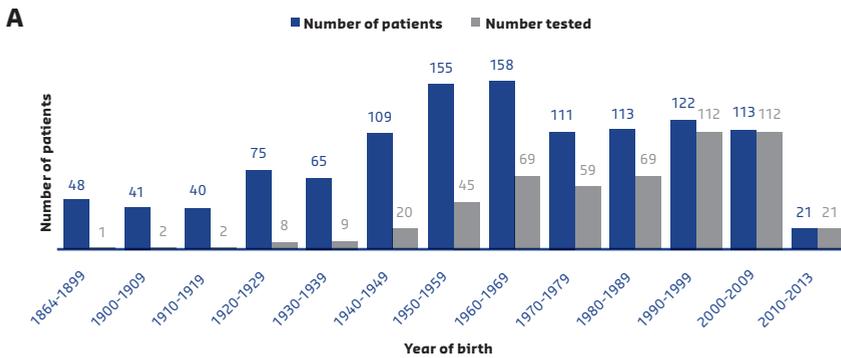
Figure 1. Rb patients from the Dutch National Retinoblastoma Register included in this study. (A) Number of patients by year of birth from the registry (blue bars) and the number of patients tested from that birth year (grey bars), shown from 1900 until 2010 by decade. Before 1900 and from 2010 until 2013 the numbers are taken together (B) Flow chart showing inclusion of retinoblastoma patients from the Dutch National Retinoblastoma Register for whom *RB1* mutation scanning has been performed.
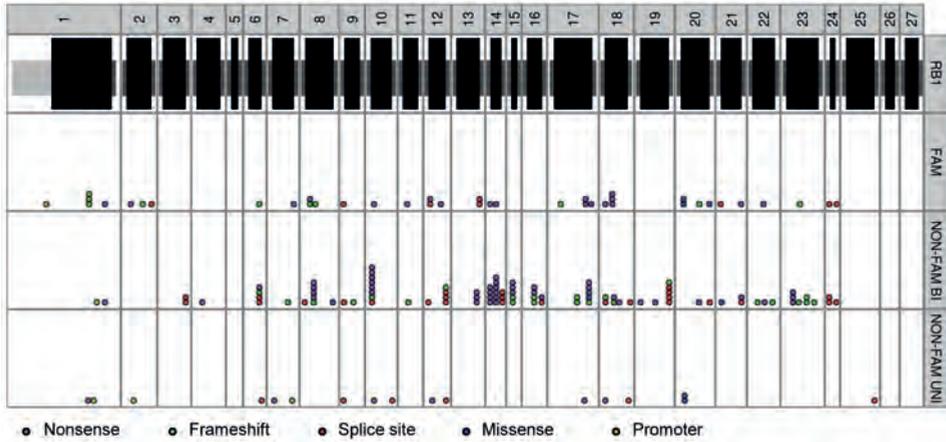
Figure 2. Distribution of germline mutations across the RB1 gene. All nonsense, frameshift, splice site, missense and promoter mutations from this study across the RB1 gene are shown. The top panel indicates 200 bp of the RB1 promoter (in light grey), exons (in black) and 20 bp of flanking intronic sequences (in dark grey). Familial *RB1* mutations are shown in the second panel from above (one mutation per family is shown), non-familial *RB1* mutations are shown in the bottom 2 panels, with non-familial bilateral and unilateral mutations respectively. Dots indicate mutations, dot colour indicates type of mutation (nonsense in pink, frameshift in green, splice site in red, missense in blue and promoter in orange).
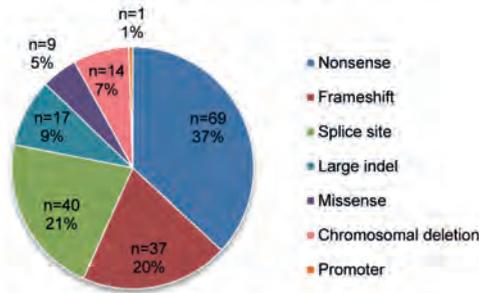
Novel and recurrent mutations

A total of 144 different germline *RB1* mutations were detected in 140 non-familial Rb cases and 47 Rb families, with 125 unique mutations occurring in only one Rb patient or Rb family of our cohort and 19 mutations occurring in two or more patients/ Rb families, which we defined as recurrent (**Supplementary Table 8**). Seventeen of the recurrent mutations were reported in the LOVD database[18], including nine of the 11 well-known recurrent RB1 CGA>TGA nonsense mutations[13] [22,23], two of the recurrent mutations were not previously described (c.862-5T>G and c.1398del/ p.Glu466Aspfs*12).

The 144 *RB1* mutations detected in our cohort included 33 novel mutations, determined as mutations not previously detected in Rb according to the LOVD database.[18] These mutations are summarized in **Supplementary Table 9**.
In **Supplementary Tables 10 and 11** the germline mutations detected in our cohort in the *RB1* gene that were not summarized in **Supplementary Tables 8 and 9** are listed (not published).

Figure 3. Spectrum of RB1 mutation types. (A) All detected germline RB1 mutations by mutation type. (B) Type of germline RB1 mutations in familial (one mutation per family is shown) and non-familial Rb patients. (C) Frequencies of RB1 mutation type in the Netherlands compared to worldwide distributions reported by Valverde et al.[15], only nonsense, frameshift, splice site and missense mutations are included.

Chromosomal deletions

In fourteen patients a de novo large microscopic deletion involving chromosome 13q band 14.2 was detected by karyotyping of peripheral blood lymphocytes (4% of non-familial patients). **Figure 4** shows the different cytogenetically visible chromosomal anomalies. In two cases the 13q-deletion was only seen in respectively 9% and 10% of peripheral blood lymphocytes. Seven patients with a microscopic deletion were unilaterally affected and seven were bilaterally affected. All patients had developmental delay, ranging from mild to severe mental retardation.

Non-pathogenic mutations

We detected two variants in *RB1* that were previously described as disease-causing in Rb, but additional evidence showed that these variants are more likely to be neutral variants.

Nichols at al.[24] described the synonymous mutation c.42C>T, p.= in blood of a bilateral non-familial Rb patient, Hogg et al.[25] detected the same variant in a unilateral Rb tumour. We detected the variant in blood of two (unrelated) non-familial bilateral patients along with established pathogenic nonsense mutations in *RB1*, while both patients had healthy family members carrying the c.42C>T, p.= variant, including the father of one of the patients who had the variant in homozygous state.

The variant c.1966C>T, p.Arg656Trp has been described as missense germline *RB1* mutation by Nichols et al.[24] and Richter et al.[26]. The variant was detected by our laboratory in the germline of two non-familial unilateral patients and seven of their unaffected family members. The c.1966C>T, p.Arg656Trp variant was detected in the germline of a non-familial bilaterally affected patient in Germany as well. The patient also carried a deletion of exon 25-26 in the germline (personal communication D. Lohmann, Institut für Humangenetik, Essen, Germany). Further testing in Canada of tumour of the non-familial unilaterally affected patient with the c.1966C>T, p.Arg656Trp variant in the germline described by Richter at al.[26] showed two pathogenic mutations in the tumour (a nonsense mutation and promoter hypermethylation) along with c.1966C>T, p.Arg656Trp (personal communication D. Rushlow, Impact Genetics Inc., Canada). Therefore c.42C>T, p.= and c.1966C>T, p.Arg656Trp are likely to be rare non-pathogenic variants.
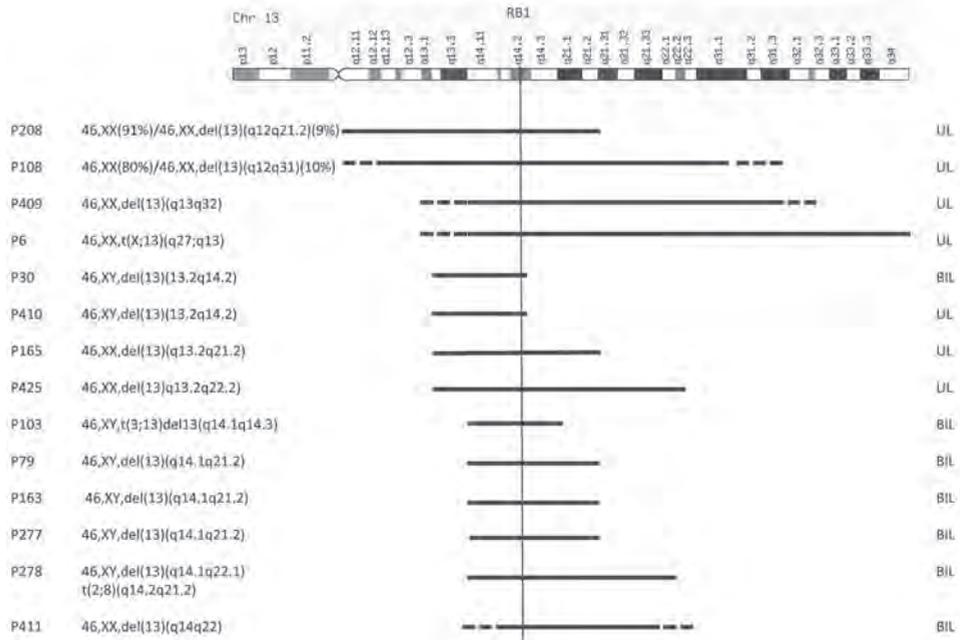
Figure 4. Chromosomal deletions including chromosome 13q14.2 in non-familial Rb patients. Fourteen deletions of part of chromosome 13 including the RB1 gene are shown. Lines depict the approximate length of the deletions. When the breakpoints were determined on karyotypes with a resolution of less than 500 bands, a dotted end of the line is shown. Percentages indicate mosaicism of different karyotypes. UL = unilateral Rb; BIL = bilateral Rb

Familial Rb

Our cohort included 147 familial Rb patients belonging to 51 families. In 47 families an *RB1* mutation was detected. In four families consisting of an affected parent with affected children, *RB1* mutation analysis did not detect a germline mutation. Of all familial cases 50 (34%) were diagnosed with unilateral Rb and 97 (66%) with bilateral Rb.

In eleven families with Rb, one or more family members carried an *RB1* mutation but were either unaffected or had a benign retinoma. Incomplete penetrance mutations were found in six three generation families with non-penetrant *RB1* mutation carriers, as shown in **Figure 5**. The mutations were three splice site mutations, two missense mutations and small deletion leading to a frameshift in exon 1. Pathogenicity of the splice site mutation c.862-5T>G mutation was confirmed by cDNA studies, showing a transcript missing exon 9. The c.862-5T>G mutation was confirmed in an Rb tumour from one of the c.862-5T>G carriers with the second hit being a nonsense mutation: c.1918A>T/ p.Lys640*. Pathogenicity of the splice site mutation c.2520+6T>C was confirmed by cDNA studies, showing a transcript missing exon 24. The mutation of family 5 (c.1970T>C/p.Leu657Pro) was not described previously as incomplete penetrance mutation. The other three mutations were previously described as *RB1* mutations with incomplete penetrance.[27-29] Family 6 was previously described by Scheffer et al[30]. As depicted in the family trees, three family members without Rb who carried an *RB1* mutation developed a potentially *RB1*-related tumour.

Three non-familial Rb patients with an *RB1* mutation (one with bilateral Rb and two with unilateral Rb) had an unaffected parent who turned out to be a carrier. Two of these mutations are known to be mutations with incomplete penetrance: c.862-5T>G (this paper); c.1981C>T (p.Arg661Trp).[28] The third mutation was a deletion of the complete *RB1* allele.[29] Karyotyping and FISH analysis (LSI *RB1*) of skin fibroblasts showed the carrier parent to be mosaic for the deletion in 94% of metaphase cells.

A remarkable observation was that an unaffected carrier of the c.2268T>A / p.Tyr756* nonsense mutation had three children and four grandchildren, all affected with bilateral Rb. Regular testing of blood lymphocytes did not show the (grand)mother to be mosaic. Lastly, a carrier of the nonsense mutation c.1333C>T / p.Arg445*, detected in her bilaterally affected child, did not develop Rb, but had a retinoma on fundus examination.

Seven parents with Rb -six unilaterally affected and one with bilateral Rb- were unaware of the hereditary nature of their disease until the moment their child was diagnosed with Rb. Five of these children were born after 1992 and DNA testing would have been possible, had the parent been referred for *RB1* testing.
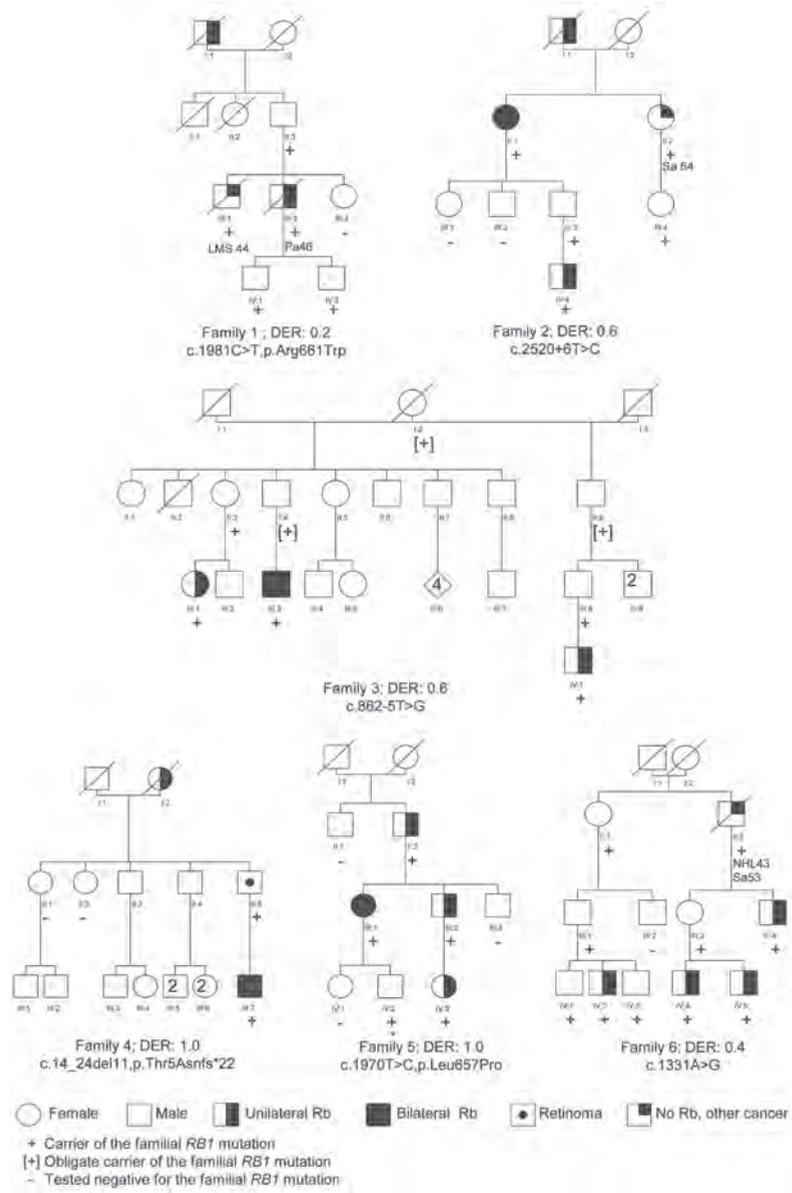
Figure 5. Incomplete penetrance mutations. Family trees of three-generation families with non-penetrant mutation carriers. All family members of the youngest generation are above 4 years of age, except one *RB1* mutation carrier in family 5, indicated with *. Family 6 was also described in a paper by Scheffer et al[30]. DER = diseased eye ratio, Rb = retinoblastoma, LMS = leiomyosarcoma, Pa = pancreas carcinoma, Sa = sarcoma, NHL = Non-Hodgkin lymphoma

Bilateral non-familial and familial Rb without a mutation in *RB1*

In ten patients with non-familial bilateral Rb and four families with familial Rb no *RB1* mutation could be detected by standard *RB1* DNA diagnostic analysis. Several of these patients were examined using additional methods to reveal possible deep intronic *RB1* mutations, *RB1* promoter mutations not investigated with standard mutation diagnostics or mutations in the *RB1* family members *RBL1* (p107) and *RBL2* (p130). Although several (known) SNPs were detected, these additional methods did not reveal evidence for pathogenic mutations in *RB1*, *RBL1* and *RBL2* (See supplementary results).

Non-familial Rb

A germline *RB1* mutation was detected in 140 out of 382 non-familial Rb patients. In 25 out of 257 non-familial unilaterally affected cases a mutation was detected (mutation detection rate: 10%) and in 115 of 125 bilateral non-familial cases a mutation was identified (mutation detection rate: 92%). Fourteen of the 140 mutations (10%) were shown to be somatic mosaics by regular scanning techniques. Three patients with an *RB1* mutation in mosaic state were unilaterally affected (1.2% of non-familial unilateral cases), while eleven had bilateral Rb (8.8% of non-familial bilateral cases). In two bilateral mosaic cases, the mutation was not detected in DNA from blood at first, but after detection of two mutations in tumour DNA it was possible to detect the mosaic mutation in blood DNA.

Of 53 unilaterally affected, non-familial Rb patients (21%), tumour DNA was tested for somatic *RB1* mutations. The results are listed in **Table 1**. A total of 96 *RB1* tumour mutations were detected including 45 small mutations (nonsense (n=29), frameshift (n=11), splice site (n=4), and missense (n=1)) of which 9 were novel mutations (**Supplementary Table 9**). The remaining mutations were large indels (n=9), promoter hypermethylation (n=8) and LOH (n=34). In three (6%) patients one of the tumour mutations was present in DNA from blood, two of which showed mosaicism. In 46 tumours (87%), two *RB1* mutations were found, while in four tumours (7.5%), mutation scanning could detect only one mutation; in one tumour only LOH was detected, another tumour showed only a missense mutation in exon 14 (c.1345G>A /p.Gly449Arg), another only a deletion of *RB1* and in the fourth tumour just one nonsense mutation (c.1072C>T /p.Arg358*) in exon 11 was found. In another three tumours (6%), no *RB1* mutations could be identified at all. In all these three tumours a high-level amplification of MYCN was detected, as described in our previous study.[31]

Table 1. Germline and somatic *RB1* mutations detected in the Dutch retinoblastoma cohort

	Germline Familial		Non-Familial Unilateral		Bilateral		Total		Somatic		Total
Mutation Type	n	%	n	%	n	%	n	%	n	%	n
Nonsense	18	38	6	24	46	40	70	37	29	30	99
Frameshift	10	21	3	12	23	20	36	19	11	11	47
Missense	4	9	2	8	3	3	9	5	1	1	10
Splice Site	8	17	6	24	26	23	40	21	4	4	44
Large indel	6	13	1	4	10	9	17	9	9	9	26
Chromosomal deletion	0	0	7	28	7	6	14	7			14
Promoter	1	2	0	0	0	0	1	1			1
Promoter methylation	-	-	-		-		-		8	8	8
LOH	-	-	-		-		-		34	35	34
Total	47		25		115		187		96		283
No mutations	4		10						10*		
Detection rate		92				92				96†	

*Including 3 *RB1*+/+MYCN^AMP tumours; †To calculate the detection rate in tumours, the 3 *RB1*+/+MYCN^AMP tumors were excluded, since no *RB1* mutations are expected in these tumors.

DISCUSSION

In this study we describe all *RB1* mutations detected in a comprehensive long-term national cohort of 529 Rb patients, 20 non-penetrant mutation carriers and two mutation carriers with retinoma from 433 unrelated families, tested in the Netherlands since 1990.

The national health insurance in the Netherlands covers genetic counselling and testing of all relevant genes. Therefore all newly diagnosed Rb patients have been offered DNA testing since the beginning of the 1990s and extensive follow-up procedures have led to the testing of virtually all Rb patients still living from the Dutch National Retinoblastoma Register.[20] Our study cohort thus forms a unique unbiased nationwide group of Rb patients.

In our cohort we detected an *RB1* mutation in 10% of non-familial unilateral cases. When an *RB1* mutation is detected in an Rb patient, the sibs and future children of the patient can be tested for the mutation and appropriate ophthalmological screening from birth can be provided when the mutation is present. In addition, knowledge of the absence of an *RB1* mutation in sibs and children can avoid unnecessary and costly eye examinations under anaesthesia. Furthermore,

appropriate counselling about reproductive options can be provided to *RB1* carriers and parents of children with Rb. As we have shown in previous studies, reproductive behaviour is influenced by Rb for many parents at increased risk for a child with Rb.[8 9] Several patients with Rb in our cohort were unaware of the hereditary nature of their disease and conceived children who developed Rb and were not screened from birth, nor had the parents had the opportunity to consider other reproductive options, e.g. prenatal diagnosis or pre-implantation genetic diagnosis. This stresses the importance to perform *RB1* mutation scanning and offer genetic counselling, irrespective of laterality or familial occurrence. The distribution of the different types of mutations is in agreement with most previous studies (**Figure 3C**).[15 17 19 24 32 33] In total, 33 out of 187 detected mutations were *RB1* mutations not previously described in literature or the LOVD database.[18] Two of the novel mutations were recurrent, the c.862-5T>G mutation was detected as incomplete penetrance mutation in a large family and a non-familial patient who had an unaffected parent that also carried the mutation. Although we did not have evidence that these two families were related we cannot rule out the possibility of a common founder. The other novel recurrent mutation (c.1398del) was detected in two unrelated non-familial patients. Furthermore, we have evidence that two *RB1* variants, c.42C>T, p.= and c.1966C>T, p.Arg656Trp, previously described as pathogenic[24-26] are more likely to be non-pathogenic variants.

We detected two mutations in 46 out of 53 Rb tumours, in four tumours only one mutation was identified, and in three other tumours no mutations could be detected. The latter three tumours were shown to be caused by high-level amplification of MYCN in the absence of mutations in *RB1*.[31] With the MYCN amplified tumours excluded, we detected 96% (96/100) of expected *RB1* mutations in tumours. In the majority of tumours the second hit was LOH (34 / 64%), in line with previous reports.[17 26 34 35]

We have shown several three-generation families with incomplete penetrance for Rb. Most of the families with incomplete penetrance, had splice mutations or missense mutations, confirming other observations described in literature.[14 15 21 36 37] In three of these families one of the *RB1* carriers without Rb had developed a sarcoma that may be *RB1*-related. In a previous study we have shown that the risk of a second cancer for carriers of a low penetrance *RB1* mutation is much lower than for carriers of a mutation with high penetrance.[20] Nevertheless awareness of a possible increased risk is important, and therefore *RB1* mutation testing of family members can be useful, even when they will not develop Rb anymore. The detection rate of 92% in familial and/or bilateral cases with the testing scheme we used is similar to detection rates reported by other studies.[13 15 17 24] Germline mosaicism of the *RB1* mutation was found with our current screening protocol in 14 non-familial cases (10% of all non-familial patients with a germline

mutation) and in one unaffected parent. Another unaffected parent with multiple bilaterally affected (grand)children may be mosaic, but regular testing did not show mosaicism. In 9.6% of bilaterally affected patients the *RB1* mutation was present in a mosaic state, whereas 12% of unilaterally affected patients with a germline *RB1* mutation were shown to be mosaic. In the near future we will probably use next generation sequencing as the main diagnostic tool for Rb mutation scanning. Next generation sequencing is expected to increase our mutation detection rate, especially by increasing the detection rate for germline mosaicism. Rushlow et al. increased their mutation detection rate from 92.6% to 94.5% by using allele specific-PCR (AS-PCR) for mosaicism of 11 recurrent *RB1* CGA>TGA mutations.[13] A recent study described next generation deep sequencing on blood samples from 20 bilateral and 70 unilateral non-familial Rb patients in whom Sanger sequencing did not detect an *RB1* mutation.[38] They detected six low-level mosaic mutations in bilateral Rb and four in unilateral Rb, increasing their detection rate from 96% to 97% for bilateral Rb and from 13% to 18% for unilateral Rb. Since the detection of germline mosaicism has important clinical consequences for the patient and family members as has been pointed out in many papers[26 39], the ultimate goal remains to detect all *RB1* mutations, both in the tumour and the germline.

Besides mosaicism, possibilities for a missed mutation in *RB1* are deep intronic mutations that create cryptic splice sites[16] or mutations in regulatory regions other than the promoter region we routinely analyse. We therefore analysed *RB1* cDNA and performed additional testing of an extended region of the *RB1* promoter in several bilateral non-familial and familial Rb patients without detectable *RB1* mutations. In mice loss of one of the other Rb pocket proteins, p107 (*RBL1*) and p130 (*RBL2*), along with loss of *RB1* is required for the development of Rb.[40-43] Although there is currently no evidence for genetic heterogeneity in heritable Rb in humans, we hypothesized that mutations in these other Rb pocket genes might play a role in the development of Rb. However, analysing cDNA of *RBL1* and *RBL2* in patients without a detectable *RB1* mutation did not reveal evidence for mutations in these genes in our Rb patients.

Another possibility for a missed mutation is that genes other than *RB1*, and the other pocket protein genes, might cause Rb in patients where no *RB1* mutation can be detected. Several studies describe that polymorphisms in genes in the p53 pathway can modify susceptibility to Rb development.[44-47] In the future, whole genome sequencing could be used to study Rb families or non-familial bilateral Rb patients without detectable *RB1* mutations to identify possible mutations or modifying polymorphisms in genes besides *RB1*.

In conclusion, we have shown that sequencing and MLPA are accurate and fast testing methods to detect 92% of *RB1* mutations in bilateral and/or familial Rb patients. Distribution of mutation types in our comprehensive national cohort

does not differ substantially from the distribution described in most other papers. Further testing for mutations in mosaic state and deep intronic mutations will eventually lead to an even higher mutation detection rate, so more Rb patients and their families can receive accurate risk estimation and use this knowledge for risk management and reproductive decisions.

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SUPPLEMENTARY METHODS

Patients selected for additional mutation scanning

Several bilateral and/or familial Rb patients in whom no *RB1* mutation could be detected with standard RB1 DNA diagnostics were selected for additional mutation scanning. **Supplementary Table 1** summarizes included patients and the additional analyses that have been performed.

Supplementary Table 1. Included Rb patients for additional mutation scanning

Patient ID	Type of Rb	cDNA analysis performed (*RB1, RBL1, RBL2*)	Sequence analysis *RBL2* performed	Promoter analysis performed (extensive region *RB1* promoter)
22	Familial	+	+	+
40	Familial	+	+	+
185	Familial	+	+	+
224	Familial	-	-	+
2	Non-Familial bilateral	-	-	+
130	Non-Familial bilateral	-	-	+
140	Non-Familial bilateral	+	+	+
210	Non-Familial bilateral	-	-	+
242	Non-Familial bilateral	-	-	+
244	Non-Familial bilateral	+	+	+
288	Non-Familial bilateral	-	-	+
306	Non-Familial bilateral	-	-	+
321	Non-Familial bilateral	-	-	+

Cell culture

Epstein barr virus-immortalized Lymphoblastoid cell lines were established from blood samples. The patient cell lines (from familial Rb patients 22, 40 and 185 and non-familial bilateral Rb patients 140 and 244) and control Lymphoblastoid cell lines HSC93 and MAN-EBV were cultured in RPMI supplemented with 10% FBS and sodium pyruvate (Gibco). To prevent that possible alternative transcripts with premature termination codons were recognized and digested by nonsense mediated decay the cells were treated with cycloheximide.[1 2]

RNA and *RB1* promoter analysis

Total RNA was extracted from cycloheximide-treated lymphoblastoid cell lines with the High Pure RNA Isolation Kit (Roche Applied Science) followed by cDNA synthesis with the iScript™ cDNA Synthesis kit (Bio-Rad) or 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Applied Science) with Oligo-p(dT)15 primer.

RT-PCR was performed with cDNA and 9 primer sets for *RB1* (**Supplementary Table 2**) and 8 primersets for *RBL1* and *RBL2* (**Supplementary tables 3-4**) with platinum Taq polymerase (Bioke). Primersets were designed to cover all exons and the corresponding products had a length of approximately 500 bp. PCR products were analyzed on 2% agarose gels.

Genomic DNA was extracted from lymphoblastoid cell lines (patients 22, 40, and 244) with the QIAamp DNA blood mini kit (Qiagen) or DNA available from DNA diagnostics was used (patients 185, 224, 2, 130, 140, 210, 242, 288, 306, and 321). The 3226 bp *RB1* region containing the promoter, exon 1 and partially intron 1 were PCR amplified sequentially in 7 overlapping fragments of approximately 500 bp (**Table 5**), with platinum Taq DNA polymerase (Invitrogen) (primer 1-5), because of the high GC content of the regions targeted by primersets 6 and 7, the Accuprime kit (Invitrogen) was used to amplify the latter regions. The PCR was performed with 35 cycli, PCR products were analyzed on 2 % agarose gels.

Sequence analysis *RB1* promoter and *RBL2* trancripts

The RT-PCR products of *RBL2* and PCR products of the *RB1* promoter were used for sequence analysis. PCR fragments were incubated for 30 minutes at 37ºC and 15 minutes at 80ºC with Shrimp Alkaline Phosphatase (SAP) and Exonuclease I. The cycle-sequencing reaction was performed with BigDye® Terminator v3.1. Cycle Sequencing kit (Applied Biosystems, Bleiswijk, Netherlands). The generated DNAs were subsequently purified with Sephadex G50 Superfine and analyzed with an ABI 3730 DNA analyzer (Applied Biosystems, Bleiswijk, Netherlands).

Supplementary Table 2. Primers cDNA *RB1*

Sequence cDNA (5' → 3')	Coverage	Expected product size
F : AGGACCTGCCTCTCGTCAG R : GAACTGCTGGGTTGTGTCAA	exon 1 - exon 5	466 bp
F : AGTTTCATCTGTGGATGGA R : GAGCAACATGGGAGGTGAG	exon 2 - exon 7	421 bp
F : TGGATGGAGTATTGGGAGGT R: CTTTGAGCAACATGGGAGGT	exon 3 - exon 7	525 bp
F : AAATGGAAGATGATCTGGTGA R: CGTGGTGTTCTCTGTGTTTCA	exon 7 - exon 11	525 bp
F : TGGACTTCCAGAGGTTGAAAA R: CGCACGCCAATAAAGACATA	exon 10 - exon 16	600 bp
F : GGACAGGGTTGTGTCGAAAT R: TGATCAGTTGGTCCTTCTCG	exon 13 - exon 18	525 bp
F : TGCATGGCTCTCAGATTCAC R : TCTCCTGAACAGCATGAGGA	exon 17 - exon 22	675 bp

Table 2. Continued

Sequence cDNA (5' → 3')	Coverage	Expected product size
F : TGGCATATGCAAAGTGAAGA R: TCCATCTGCTTCATCTGATCC	exon 21 - exon 25	600 bp
F : TGGTGAATCATTCGGGACTT R: TGGCTTAGGAATCACCCAAA	exon 24 - exon 27	600 bp

Supplementary Table 3. Primers cDNA *RBL1*

cDNA sequence (5' → 3')	Coverage	Expected product size
F : CCTGGGAGGGAGAAAGAAGT R: CACTGCAAGGAATCCTCCTC	exon 1 – exon 4	565 bp
F : GGCTAGAGAGAAATTTTGAGGTG R: CCTCTCATCAAAATCACCAACA	exon 3 – exon 8	587 bp
F : AGAATGCCTCCTGGACCTTT R: CTTCGTGTTTCCTGAACCATT	exon 7 – exon 11	588 bp
F : GAACAGCCAGGATCTCACAT R : TTCGAAGACTCCCACTGTCA	exon 10 – exon 14	599 bp
F : AGGAAATGGAGGAAATGTGC R : CCAGTTCTCTTTGGCCTGTT	exon 14 – exon 16	561 bp
F : CACACTGATACCTCTTTCCATGA R : TCCACTGGAACAGTCAGGTG	exon 16 – exon 20	568 bp
F : TTCCAAGAGAAGTTGTGGCATA R : TGCAGAACAATCTGAAAGTGC	exon 19 – exon 22	594 bp
F : AGAATCCCCTGCCAAACG R : CAACCTTGGCTCACCACAAC	exon 22	560 bp

Supplementary Table 4. Primers cDNA *RBL2*

Sequence cDNA (5' → 3')	Coverage	Expected product size
F : CTATGCCGTCGGGAGGTG R : TGTCCTGAAAAATGGGTTCA	exon 1 – exon 4	410 bp
F : CCCCACATTTCAGAGAACGTA R : TCTCCAAAGTTCCCAGGTTC	exon 3 – exon 7	562 bp
F : GGAAGCAAAGGGGATAAAGG R : TTAGCAATAGCCTGGGTTGG	exon 6 – exon 10	519 bp
F : TCAGGAATGCACCAAGTGAG R : CTCTGGTTTCCATGCCAAAT	exon 9 – exon 13	500 bp
F : AGAGCAGAAGATGGCCTTTG R : GTTACCGTTTGCCCATTGTT	exon 13 – exon 15	515 bp

Supplementary Table 4. Continued

Sequence cDNA (5' → 3')	Coverage	Expected product size
F : CACACCAGTTCCTGGACAGA	exon 15 – exon 18	553 bp
R : TGTGACCTTTGCCATCACAT		
F : GGACCTGCTTTGAATTCTCC	exon 17 – exon 21	531 bp
R : GAGGGGAGCCTGTTCTTACA		
F : GGAGAGGGGAGACCTCATTC	exon 20 – exon 22	557 bp
R : TCACCAAAATGTCCCCTCAT		

Supplementary Table 5. Primers promoter *RB1*

DNA sequence (5' → 3')	Coverage	Expected product size
F : TTGTTCCCCTCTTCATGTCC	promoter	586 bp
R : AGCCCTGGAGCTGACCTAAG		
F : AGGCAATGATGATACCCAAGA	promoter	510 bp
R : TTTCTTGTGGGCAAATGTCA		
F : AATCTGGGGTGAGGTGAATG	promoter	511 bp
R : GAGCTTGCTGGGACTACCAC		
F : GACGAGTTCAGATGGGCAAT	promoter	547 bp
R : AATCCCCAGAAACAGCCTTT		
F : AGCAAACCTCCATGGCATAC	promoter	590 bp
R : CTATCCCGGGGTCTGATAGG		
F : ACTTTGCAAAACGGATTGG	Promoter - Exon 1	501 bp
R : CTCCCGACTCCCGTTACAA		
F : GCGGAAGTGACGTTTTCC	Exon 1 – Intron 1	551 bp
R : GTCAAGTTGAAGCCGAGACC		

SUPPLEMENTARY RESULTS

No evidence for alternative splicing of *RB1*, *RBL1* and *RBL2* transcript was detected with cDNA analysis. Sequence analysis of the *RBL2* transcript and an extended region of the *RB1* promoter did not show evidence for the presence of pathogenic mutations, although several annotated SNPs were detected (**Supplementary Table 6 and 7**).

Supplementary Table 6. SNPs in gDNA *RB1*

SNP	Location cDNA *RB1**	Alleles	MAF/ MinorAlleleCount	Ancestral allele	Patient ID	Homo-/ hetero-zygous
rs1573601	c.-1692C>A	A/C	A = 0.191/417	C	224	Homozygous
					242, 306, 321	Heterozygous
rs3092875	c.-733A>C	A/C	C = 0.016/35	A	224, 321	Heterozygous
rs2252544	c.137+107G>A	C/T (- strand)	G = 0.447/976	C	130, 2, 288, 140, 185, 244, 22	Homozygous
					242, 306, 210, 321, 40	Heterozygous

*Transcript NM_000321.2; MAF: Minor allele frequency

Supplementary Table 7. SNPs in cDNA *RBL2*

SNP	Location cDNA *RBL2* (NM_005611.3)	Exon	Alleles	Ancestral allele	MAF/ Minor-AlleleCount	Patient ID (all hetero-zygous)	Coding effect
rs147880565	c.423A>G	3	A/G	A	G = 0.012/26	140	Synonymous
rs17800727	c.629A>G	4	A/G	A	G = 0.141/308	185	p.Tyr210Cys
rs140896175	c.1646A>T	12	A/T	A	T = 0.012/26	140	p.Asn549Ile
rs1131220	c.2082G>A	15	A/G	A,G	A = 0.456/995	185, 22	Synonymous
rs11540358	c.2091C>G	15	C/G	C	G = 0.229/499	185	Synonymous
rs10748	c.2590T>C	17	C/T	C	C = 0.456/995	185, 22	Synonymous
rs3929	c.*96G>C	22	C/G	C	C = 0.451/985	185	Synonymous

*:3'UTR Alternative transcript; MAF: Minor allele frequency

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Supplementary Table 8. Recurrent *RB1* mutations

Type of *RB1* mutation	Consequence	Exon	Recurrence	# F	# N	Known recurrent
Nonsense (n =13)						
c.103C>T	p.Gln35*	1	2	1	1	no
c.751C>T	p.Arg251*	8	4	1	3	yes
c.958C>T	p.Arg320*	10	8	1	7	yes
c.1147C>T	p.Gln383*	12	2	1	1	no
c.1333C>T	p.Arg445*	14	5	1	4	yes
c.1363C>T	p.Arg455*	14	7	1	6	yes
c.1399C>T	p.Arg467*	15	3	0	3	yes
c.1467C>A	p.Cys489*	16	2	0	2	no
c.1654C>T	p.Arg552*	17	2	1	1	yes
c.1666C>T	p.Arg556*	17	4	1	3	yes
c.1700C>A	p.Ser567*	18	2	1	1	no
c.1735C>T	p.Arg579*	18	4	3	1	yes
c.2359C>T	p.Arg787*	23	3	0	3	yes
Frameshift (n=2)						
c.596del	p.Leu199Tyrfs*2	6	2	1	1	no
c.1398del†	p.Glu466Aspfs*12	15	2	0	2	no
Missense (n=1)						
c.1981C>T	p.Arg661Trp	20	3	1	2	no
Splice site (n=3)		**Intron**				
c.607+1G>A	Predicted: skip of exon 6	6	2	1	1	no
c.862-5T>G†	In frame deletion exon 9	9	2	1	1	no
c.1215+1G>A	Predicted: skip of exon 12	12	3	0	3	no

F = number of Rb families with the mutation; # N = number of non-familial Rb patients with the mutation; Known recurrent = one of the 11 recurrent CGA>TGA nonsense mutations; †Novel mutation

Supplementary Table 9. Novel *RB1* mutations

Type of Germline *RB1* mutation	Consequence	Phenotype	Exon
Nonsense (n =3)			
c.1700C>A	p.Ser567*	F	18
c.1770T>A	p.Cys590*	N	18
c.2268T>A	p.Tyr756*	F	22
Frameshift (n=17)			
c.14_24del†	p.Thr5Asnfs*22	F	1
c.46_65dup	p.Pro25Argfs*47	N	1
c.174_175del	p.Ala59Ilefs*50	N	2
c.709del	p.Glu237Asnfs*27	N	7

Type of Germline *RB1* mutation	Consequence	Phenotype	Exon
c.735_744delinsTGTCGACCC	p.Ile246Valfs*18	F	8
c.907_908del	p.Leu303Trpfs*6	N	9
c.1078_1082dup	p.Asn361Lysfs*8	N	11
c.1398del§	p.Glu466Aspfs*12	N	15
c.1453_1456del	p.Ser485Tyrfs*9	N	16
c.1683dup	p.Ala562Cysfs*10	N	17
c.1707_1710del	p.Phe570Ilefs*40	N	18
c.1752_1753insGGTTTCA	p.His585Glyfs*5	N	18
c.2239dup	p.Glu747Glyfs*4	N	22
c.2313del	p.Tyr771*	N	22
c.2416dup	p.Ile806Asnfs*9	N	23
c.2439dup	p.Lys814X	N	23
c.2465dup	p.Thr823Asnfs*15	N	23
Splice site (n=13)			**Intron**
c.719-3C>G	Predicted: frameshift	N	7
c.862-5T>G†‡	In frame deletion exon 9	F	8
c.862-2del	Predicted: skip of exon 9	N	8
c.940-2A>C	Predicted: skip of exon 10.	N	9
c.1128-2A>G	Predicted: skip of exon 12.	F	11
c.1215+4del	Predicted: skip of exon 12	N	12
c.1389+5G>T	Predicted: not predictable	N	14
c.1960+2T>G	Predicted: skip of exon 19	N	19
c.2106+1del	Predicted: skip of exon 20	N	20
c.2211+5G>C	Predicted: not predictable	N	21
c.2490-2_2490-1del	Predicted: skip of exon 24	N	23
c.2490-2A>G	Predicted: skip of exon 24	N	23
c.2520+6T>C†	Skip of exon 24	F	24
Type of Somatic *RB1* mutation	**Consequence**		**Exon**
Nonsense (n = 1)			
c.2527G>T	Glu843*		25
Frameshift (n = 6)			
c.171dup	p.Thr58Tyrfs*52		2
c.1149dup	p.Gln384Thrfs*11		12
c.1420_1421+30del	p.Ser474Phefs*8		15
c.1746_1749dup	p.Asp584Asnfs*2		18
c.2240_2241del	p.Glu747Glyfs*3		22
c.2496_2497del	p.Leu832Phefs*5		24
Splice site (n = 2)			**Intron**
c.862-5T>G‡	In frame deletion exon 9		8
c.2520+4A>G	Predicted: Not predictable		24

F: Familial; N: Non-familial; †: Incomplete penetrance mutation; ‡:Detected in a Rb family and a non-familial Rb patient with an unaffected parent that carried the mutation and a Rb tumor from another non-familial Rb patient; §: Detected in two unrelated non-familial Rb patients.

Supplementary Table 10. *RB1* mutations described in LOVD detected once in the Dutch cohort

Type of Germline *RB1* mutation	Consequence	Exon	Familial (F)/ Non-familial (N)	Unilateral (UL)/ Bilateral (BIL)
Nonsense (n=19)				
c.19C>T	p.Arg7*	1	N	UL
c.160G>T	p.Glu54*	2	F	
c.409G>T	p.Glu137*	4	N	BIL
c.584G>A	p.Trp195*	6	N	BIL
c.619C>T	p.Gln207*	7	N	BIL
c.717T>G	p.Tyr239*	7	F	
c.763C>T	p.Arg255*	8	N	BIL
c.1072C>T	p.Arg358*	11	F	
c.1190C>A	p.Ser397*	12	F	
c.1306C>T	p.Gln436*	13	N	BIL
c.1318G>T	p.Glu440*	13	N	BIL
c.1328C>A	p.Ser443*	13	N	BIL
c.1494T>A	p.Tyr498*	16	N	BIL
c.1818T>G	p.Tyr606*	19	N	BIL
c.1891C>T	p.Gln631*	19	N	BIL
c.2053C>T	p.Gln685*	20	N	BIL
c.2206C>T	p.Gln736*	21	N	BIL
c.2209G>T	p.Glu737*	21	F	
c.2273C>A	p.Ser758*	22	N	BIL
Frameshift (n=17)				
c.19dup	p.Arg7Profs*24	1	F	
c.32_63del	p.Ala11Glyfs*9	1	F	
c.62dup	p.Ala22Glyfs*9	1	N	BIL
c.219_220dup	p.Ala74Glufs*4	2	F	
c.687del	p.Ser230Hisfs*34	7	N	BIL
c.770_771dup	p.Asn258Argfs*7	8	F	
c.772_776del	p.Asn258Glufs*11	8	N	BIL
c.951_954del	p.Ser318Asnfs*13	10	N	BIL
c.1212del	p.Phe404Leufs*6	12	N	BIL
c.1450_1451del	p.Met484Valfs*8	16	N	BIL
c.1535del	p.Leu512Cysfs*7	17	F	
c.1605del	p.Phe535Leufs*8	17	N	BIL
c.1629_1630del	p.Glu545Asnfs*9	17	N	BIL
c.1959dup	p.Val654Serfs*14	19	N	BIL
c.2055_2056del	p.Thr687Profs*4	20	F	
c.2388del	p.Leu797Tyrfs*13	23	N	BIL
c.2394dup	p.Ile799Aspfs*16	23	F	

Type of Germline *RB1* mutation	Consequence	Exon	Familial (F)/ Non-familial (N)	Unilateral (UL)/ Bilateral (BIL)
Splice site (n=19)				
c.264G>A		2	F	
c.380G>A		3	N	BIL
c.380G>C		3	N	BIL
c.1331A>G		13	F	
c.1332G>A		13	F	
c.1960G>C		19		
Splice site		**Intron**		
c.607+1G>C		6	N	BIL
c.1049+1G>T		10	N	UL
c.1128-1G>A		11	N	BIL
c.1389+5G>A		14	N	BIL
c.1498+1G>A		16	N	BIL
c.1696-1G>A		17	N	BIL
c.1814+3A>C		18	N	UL
c.1814+11T>C		18	N	BIL
c.1960+1G>T		19	N	BIL
c.2107-1G>C		21	F	
c.2490-1_2490del		23	F	
c.2520+3_2520+6del		24	N	BIL
c.2663+1G>A		25	N	UL
Missense (n=6)		**Exon**		
c.857A>G	p.Asp286Gly	8	N	BIL
c.1688G>T	p.Trp563Leu	17	F	
c.1688G>C	p.Trp563Ser	17	F	
c.1970T>C	p.Leu657Pro	20	F	
c.2105A>G	p.Gln702Arg	20	F	
c.2111T>G	p.Met704Arg	21	N	BIL
Promoter				
c.-193T>G			F	

Supplementary Table 11. 13 large deletions and duplications of the *RB1* gene detected in the Dutch cohort

Exon deletion	Familial (F)/ Non-familial (N)	Unilateral (UL)/ Bilateral (BIL)
Del *RB1*	F/N§	
Del ITM2B-exon 17	N	BIL
Del promoter-exon 17	N	BIL
Del exon 3-17	N	BIL
Del exon 6-17	N	BIL
Del exon 9	N	BIL
Del exon 9-27	F	
Del exon 10 or 10+11	F	
Del exon 17	N	BIL
Del exon 18	F	
Dup exon 3	F	
Dup exon 3-6	N	BIL
Dup exon 10/11-17	N	UL

§ Detected in 2 families and 3 non-familial cases

REFERENCES

1   Lohmann D, Degen S: LOVD *RB1*. http://*RB1*-lovd.d-lohmann.de/home.php?select_db=*RB1*. Date accessed: 15 Nov 2013