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Enhanced Sensitivity for Detection of Low-Level Germline Mosaic *RB1* Mutations in Sporadic Retinoblastoma Cases Using Deep Semiconductor Sequencing

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Abstract

Sporadic retinoblastoma (RB) is caused by de novo mutations in the *RB1* gene. Often, these mutations are present as mosaic mutations that cannot be detected by Sanger sequencing. Next-generation deep sequencing allows unambiguous detection of the mosaic mutations in lymphocyte DNA. Deep sequencing of the *RB1* gene on lymphocyte DNA from 20 bilateral and 70 unilateral RB cases was performed, where Sanger sequencing excluded the presence of mutations. The individual exons of the *RB1* gene from each sample were amplified, pooled, ligated to barcoded adapters, and sequenced using semiconductor sequencing on an Ion Torrent Personal Genome Machine. Six low-level mosaic mutations were identified in bilateral RB and four in unilateral RB cases. The incidence of low-level mosaic mutation was estimated to be 30% and 6%, respectively, in sporadic bilateral and unilateral RB cases, previously classified as mutation negative. The frequency of point mutations detectable in lymphocyte DNA increased from 96% to 97% for bilateral RB and from 13% to 18% for unilateral RB. The use of deep sequencing technology increased the sensitivity of the detection of low-level germline mosaic mutations in the *RB1* gene. This finding has significant implications for improved clinical diagnosis, genetic counseling, surveillance, and management of RB.

Keywords

mosaic mutation; next-generation sequencing; sporadic retinoblastoma; *RB1*

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Additional Supporting Information may be found in the online version of this article.

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Introduction

Retinoblastoma (RB) is the most common intraocular cancer in young children (MIM #180200). It occurs in approximately one in 20,000 live births with about 300 new cases diagnosed each year in the United States and about 5,000 cases worldwide [Shields and Shields, 2004; Broaddus et al., 2009]. Clinically, RB can present as either bilateral (both eyes) or unilateral (one eye) disease. Germline mutations in the *RB1* gene are associated with bilateral RB, whereas somatic mutations on both alleles of the *RB1* gene in a retinal cell are associated with unilateral disease [Nichols et al., 2009]. An important aspect of RB is that it is characterized by a very high incidence of sporadic cases. Almost 80% of all newly diagnosed bilateral RB cases are sporadic without a family history, and are caused by de novo germline mutations in the *RB1* gene. In the case of unilateral RB, almost 87% of cases are sporadic and do not carry a germline mutation [Lohmann, 2013].

De novo mutations can occur prior to the conception or after the conception. Preconception mutation events occur mainly during spermatogenesis and result in germline mutations in the affected child—usually with bilateral RB [Dryja et al., 1997; Munier et al., 1998]. In contrast, postzygotic mutation events occurring at early stages of embryo development can lead to mosaicism that can extend to various organs and tissues, including the retina, lymphocytes, or even the gonads. The mosaic mutations can be detected in lymphocyte DNA using Sanger sequencing depending on the degree of mosaicism [Munier et al., 1998; Sippel et al., 1998; Barbosa et al., 2008].

The detection of mosaic mutations can be challenging. Sanger sequencing has been the gold standard for the screening of *RB1* gene for mutations in the promoter region and in the coding sequences. However, the threshold for the degree of mosaicism detectable by Sanger sequencing is quite high [Richter et al., 2003; Rushlow et al., 2009]. Previously, allele-specific PCR was used for the efficient detection of 11 recurrent nonsense mutations on CpG sites within the *RB1* gene that were present at a level less than 15% of the normal allele [Rushlow et al., 2009]. This method increased the sensitivity for the detection of mosaic mutations, and based on this technology, the frequency of germline mosaic mutations was estimated to be ~ 5.5% in bilateral and ~3.8% in unilateral RB patients [Rushlow et al., 2009]. However, the method was limited to the targeted search for 11 specific mutations and could not be extended to an unbiased screen of other mutations in the *RB1* gene.

This manuscript describes targeted resequencing of the coding exons of *RB1* gene using deep sequencing on the Ion Torrent Personal Genome Machine (PGM) platform for the detection of low-level mosaic mutations in germline DNA from lymphocytes of individuals diagnosed with sporadic RB and deemed mutation negative by standard Sanger sequencing.

Materials and Methods

Patient Samples

Patient samples were submitted to Genetic Diagnostic Laboratory (GDL), University of Pennsylvania, for clinical testing of *RB1* gene. Physicians from across the country and worldwide submitted the samples. Blood samples were collected in EDTA-containing tubes

and shipped at room temperature. For tumors, either flash-frozen or paraffin-embedded tissue was submitted. A total of 20 bilateral and 70 unilateral RB cases were analyzed. Informed consents signed by the parents were obtained for all affected children being tested. The protocol for retrospective reanalysis was approved by the IRB of the University of Pennsylvania.

DNA Isolation

Genomic DNA was isolated from 3 ml blood using Qiagen Gentra Puregene blood DNA isolation kit (Valencia, CA) following the manufacturer's instructions. For tumor tissue, the QIAGEN® DNeasy Blood and Tissue Kit (Germantown, MD) was used.

Detection of mutations in RB1 gene using Sanger sequencing—The protocol for the detection of germline mutations in *RB1* gene is different for bilateral and unilateral RB cases. The optimal work-flow is described in Figure 1. PCR amplification of the 27 coding exons and the promoter region of the *RB1* gene was performed using AmpliTaq Gold 360 Master Mix (Life Technologies, Carlsbad, CA). Each 25 μ l reaction contained 40 ng of genomic DNA (isolated from lymphocytes or tumor), 10 pmol each of forward and reverse primer, 12.5 μ l master mix, and 1 μ l GC enhancer, which is included in the kit. Thermal cycling conditions and PCR primers have been described previously [Nichols et al., 2005]. Sanger sequencing was performed using the BigDye Terminator kit version 3.1 (Life Technologies) according to the manufacturer's instructions. Sequencing products were analyzed on a 3130 Genetic Analyzer (Life Technologies). Sanger sequencing data alignment and analysis were performed with Sequencher 5.0 software (Gene Codes Corporation, Ann Arbor, MI). The list of somatic mutations identified by Sanger sequencing in the 70 unilateral RB tumors is included in Supp. Table S1. These mutations have been submitted to Clin-Var (<http://www.ncbi.nlm.nih.gov/clinvar/>) and the submission ID is MDI-2436.

Sequencing on Ion Torrent PGM platform—Deep sequencing was used to investigate the presence of low-level mosaic mutations in the lymphocyte DNA of sporadic RB cases.

A group of 20 bilateral cases without detectable mutations was selected. The 27 PCR amplicons from each lymphocyte DNA were pooled in equimolar ratio and fragmented using Ion Shear Plus Reagents (Life Technologies). The quantity and size distribution of sheared fragments were assessed using an Agilent 2100 Bioanalyzer following manufacturer's instructions (Agilent Technologies, Clara, CA) and ensured that the mean fragment size was between 100 and 200 bp. The pooled fragments for each sample were ligated to bar-coded adapters using Ion Express Plus Fragment Library kit (Life Technologies). Ten barcoded libraries were combined at a time in equal molar proportion, and amplified using the Ion OneTouch system (Life Technologies). After enrichment, the Ion Sphere Particles carrying the amplified fragments were loaded and sequenced on a single 318 chip using the Ion PGM Sequencing Kit (Life Technologies).

In addition, three barcoded libraries, each one containing all 27 exons of *RB1* gene from a single healthy control individual, were sequenced on a 316 chip following the same procedure. The choice of the chip, 318 versus 316, was determined by the predicted

throughput and the required coverage as described below. The total number of mapped sequence reads, on-target reads, and average coverage for the 20-barcoded libraries from bilateral RB cases, are summarized in Supp. Table S2. The sequencing data from the healthy control individuals allowed the assessment of background noise and filtering of false positive variant calls due to the presence of homopolymer regions (Supp. Table S3). Each sequencing experiment was performed in duplicate.

Sporadic unilateral RB is predominantly associated with somatic mutations in *RB1* gene. Thus, the tumor DNA was first analyzed using Sanger sequencing chemistry. After the tumor mutations(s) were identified, the DNA isolated from matched lymphocytes was tested to look for the presence of the same mutation. No mutation was detected in the lymphocyte DNA in these samples. Therefore, for each lymphocyte DNA, amplicons of the specific exons were prepared that harbor mutations in the corresponding tumors. Next, the individual amplicons were pooled in equimolar ratios as detailed in Supp. Table S1. Five individually barcoded libraries were prepared and only unique mutation-bearing amplicons were included in each library. These libraries were pooled and sequenced on a 316 chip. The sequencing experiments were performed in duplicate. The total number of mapped sequence reads and average coverage are listed in Supp. Table S4.

Taqman Single-Nucleotide Polymorphism genotyping assay—Taqman single-nucleotide polymorphism (SNP) genotyping assays for seven recurrent nonsense mutations (CGA>TGA) within the *RB1* gene (p.Arg251X, p.Arg320X, p.Arg455X, p.Arg552X, p.Arg556X, p.Arg579X, and p.Arg787X) were designed (Life Technologies). These seven mutations account for ~18% of all known mutations in the *RB1* gene. The primer and probe sequences are listed in Supp. Table S5. Each 25 μ l reaction contained 2 ng lymphocyte DNA, 1.25 μ l SNP genotyping assay (20X stock) and 12.5 μ l TaqMan universal PCR master mix without AmpErase UNG. SNP genotyping assays were performed in the StepOnePlus Real-Time PCR System (Life Technologies). Each genotyping assay was performed in triplicate as required by the manufacturer.

Calibration curve—Experiments to draw the calibration curves for defining the limit of detection (LOD) and limit of linearity for the deep sequencing experiments were performed by using serial dilutions of a DNA, which is heterozygote for the three mutations being assayed, with wild-type DNA to mimic the presence of mutations at levels of 50%, 33%, 16.5%, 8.25%, 4.12%, and 0% (wild type).

Pooled samples of individually barcoded serial dilutions of each mutation tested were sequenced twice on the Ion PGM platform. The number of reads harboring the mutant allele was tabulated. To estimate the background sequencing error, the number of nonreference alleles present at the same location as the mutation in the wild-type DNA sequence read was also tabulated.

The data were analyzed using the calibration curve drawing protocol included in EXCEL 2007.

Data Analysis

The sequence alignment against the human *RB1* genomic sequence (GenBank Accession L11910.1) and variant calling of the sequence reads were made using Ion Torrent Suite (Life Technologies). The reads were automatically barcode-sorted followed by the removal of the reads with low quality. All variant calls were confirmed using a second software package (NextGENe, SoftGenetics, State College, PA). For the purpose of this analysis, variants were selected when there was at least 400× coverage for the reference sequence, and 20× coverage of the variant reads (5%). When the variant frequency was as low as 2% of the reference allele, the coverage of the nonreference base had to be at least 20 with the number of forward (F) and reverse (R) reads balanced (ratio $\times > 0.25$). Variants with F/R read balance < 0.25 and small homopolymer insertion/deletions with F/R balance < 0.25 were filtered out. The BAM and BAI files of Ion PGM runs were loaded into Integrated Genomics Viewer (IGV) software V2.1 (Broad Institute, Cambridge, MA) to visualize alignment with respect to the reference sequence. To reduce the number of false positive variant calls due to sequencing errors associated with homopolymer regions, the locations of all variants were checked visually on the IGV browser for the proximity of mononucleotide repeat sequences that could be as small as four bases. The variant calls were also compared with the sequences obtained from three healthy individuals and eliminated when present in the latter group.

Results

A comprehensive screening protocol for the detection of *RB1* germline alterations including sequence analysis of all coding exons plus the promoter region, as well as deletion/duplication assays for the estimation of copy-number changes of intragenic exons has been previously described as in Figure 1 [Nichols et al., 2005]. For tumor DNA, somatic mutation analysis by standard Sanger sequencing is followed by studies to determine loss of heterozygosity (LOH) and promoter methylation status as described [Nichols et al., 2005].

Using standard Sanger sequencing, five mosaic mutations in bilateral RB cases and five in unilateral RB cases were identified by analysis of lymphocyte DNA. The results are summarized in Table 1. These mosaic mutations were present at a level that is less than what is observed in a conventional heterozygote, where the peak heights of each allele—reference and variant—are almost equal (Supp. Fig. S1). Three frameshift mutations and seven point mutations were detected. The latter included four mutations involving the CpG dinucleotides, which are hotspots for spontaneous mutation.

Based on the ratio of the peak heights of the reference and variant alleles as obtained by the sequencing of serial dilution of a DNA, heterozygote for a mutation, with a normal DNA that is homozygous for the wild-type allele (Fig. 2), it is estimated that the lowest proportion of the mutant alleles that can be detected unambiguously by Sanger sequencing is between 15% and 30% and depends on the specific mutation and its sequence context. The mutation in the lymphocyte DNA when present at a very low proportion can often be detected when the location of the mutation is previously defined by sequencing the tumor DNA (Supp. Fig. S2). A subset of these samples was investigated by deep sequencing on Ion Torrent platform

and the results are summarized in the next section (Fig. 3). The results concurred with Sanger sequencing (Fig. 2).

Calibration Curve

To determine the threshold of the detection of mosaic mutations in the lymphocyte DNA, three separate DNAs harboring three separate heterozygous mutations, were serially diluted with wild-type DNA and used for Sanger sequencing (Fig. 2) and deep sequencing on Ion PGM. Figure 3 shows the calibration curve for one point mutation, g.70330G>A, in exon 12 and defines the LOD value as ~4% by deep sequencing, which is lower than ~15% by Sanger Sequencing, as indicated by the arrows (Fig. 2).

To investigate the presence of mosaic germline mutations that may be present at <20% level, we selected 20 bilateral and 70 unilateral RB cases where Sanger sequencing had ruled out the presence of mutations in the lymphocyte DNA. For the unilateral cases, a total of 95 point mutations were identified in tumor DNA, and were not detected in the corresponding lymphocyte DNA (Supp. Table S1).

Germline Mosaic Mutations Identified in Bilateral RB cases

A total of six mosaic mutations were detected in 20 bilateral RB samples and the results are summarized in Table 2. The frequencies of the mutant alleles varied from 2.1% to 15.3%. There were three samples with the g.150037C>T mutation in exon 18, leading to a premature stop codon p.Arg579X in the translated protein. This mutation, along with g.65386C>T and leading to p.Arg358X found in one sample, are known as hotspots for mutations in the *RB1* gene and occur in the context of CpG di-nucleotides [Rushlow et al., 2009]. In contrast, the mutations g.70330G>A found in RBM4 and g.162203G>A found in RBM5 do not arise in the context of CpG di-nucleotides; however, these mutations have been previously reported multiple times as germline mutations [Lohmann, 2010] that affect the consensus sequences of splice sites.

The coverage of the target region, which is 27 exons of *RB1* gene, was 100% for the entire sequenced region (Supp. Fig. S3). The average depth of read for individual *RB1* exons across the 20 samples was not uniform; different exons have differences in length and G/C content that can lead to different sequencing efficiency. Exon 1 was one of the largest exons assessed and also had a high GC content. Thus, the mean coverage on exon 1 was the lowest among all exons, but was still as high as 1,392. The mean coverage of all 27 exons was 5,275× and ranged from 1,392× to 7,838×. Hence, the coverage for all samples assessed was above the set threshold of a minimum read depth of 400 as described before and was sufficient for effective variant calling.

The automated variant call output includes SNPs previously reported in the RB1-LOVD database (RB1-lsdb, Leiden Open Variation Database) or the in-house database of GDL. All these known SNPs were confirmed by comparing to Sanger sequencing results and filtered out (Supp. Table S3).

To validate the results of next-generation sequencing, we used an alternate highly sensitive platform. We designed Taqman SNP assays targeting seven recurrent nonsense mutations

within the *RB1* gene (Supp. Table S5) and investigated the same 20 bilateral RB cases without germline mutations. The mosaic mutation (g.150037C>T; p.Arg579X; nonsense mutation) was confirmed on samples RBM1, RBM2, and RBM3 (data not shown). Among them, one nonsense mutation, detected in RBM2, was not called by the automated variant caller output of Ion Torrent suite. In this case, the variant frequency of 2.1% was below the predefined detection threshold of Torrent suite [20×/400×; 5%] as described in the *Materials and* section. But, reanalysis of the reads using NextGENe software and visualization of the reads using IGV confirmed the presence of this mutation. As can be seen in Figure 4, this mutation was barely detectable on the Sanger sequencing chromatogram; however, both Taqman assay and deep sequencing on Ion PGM detected the presence of the mutant allele without any ambiguity.

Therefore, the observed frequency of low-level mosaicism detectable in lymphocyte DNA in the bilateral RB cases detected by deep sequencing is approximately 30% (six out of 20 cases), which were previously mutation negative by standard Sanger sequencing.

Germline Mosaic Mutations Identified in unilateral RB Cases

A total of 70 unilateral RB cases were selected for mutation analysis on lymphocyte DNA where each tumor had biallelic inactivation of the *RB1* gene that was detected by standard Sanger sequencing. Each tumor carried at least one coding sequence mutation, while the second allele was inactivated by promoter methylation or LOH. In multiple tumors, two separate mutations were identified on two different alleles of the *RB1* gene. A total of 95 somatic mutations were identified in these 70 tumor samples (Supp. Table S1). Sanger sequencing of the DNA isolated from the matched lymphocytes of each individual with the tumor ruled out the presence of that specific mutation in the corresponding exon. To investigate the presence of a low-level mosaic mutation using deep sequencing, individual amplicons from different samples, each contributing a unique exon bearing a unique mutation, as described in the *Material and Methods* section, were pooled after bar-coding and sequenced (Supp. Table S1). The read depth for each exon across the different libraries ranged between 4019X and 6342X (Supp. Table S4).

A total of four unique mutations, previously detected in tumors, were detected in the germline DNA of the 70 unilateral RB cases (RBU12, RBU26, RBU37, and RBU52) (Table 3). The depths of read with variant calls ranged between 752X and 1091X translating to variant frequencies of 11.7%–27.2%. Supp. Figure S4 illustrates the PGM reads and Sanger sequencing chromatograms of the same exons using germline DNA and the corresponding tumor DNA from RBU52, respectively. This mutation was present in a heterozygous state in the tumor DNA. There was a hint of the mutant allele upon re-examination of the Sanger sequencing chromatograms of the lymphocyte DNA. But, the presence of the mutation call was challenging and would have been unreliable without the supporting data from the Ion PGM sequencing reads. To exclude the possibility that the variant calls are due to sequencing errors of Ion PGM, the frequencies of any variant calls at the corresponding locations were extracted by visual inspection of sequencing reads on lymphocyte DNA from three healthy individuals on the IGV browser (Table 4). Statistical analysis using the *t*-test indicated that the variants in the four cases were called with high confidence ($P < 0.0001$).

In summary, the Ion PGM sequencing data indicated that the frequency of mosaic mutations in lymphocyte DNA in unilateral RB samples, with known somatic mutations identified in the tumor, was 5.7% (four out of 70 cases).

Discussion

Germline mosaicism for a *RB1* mutation that can be detected in lymphocyte DNA has important clinical implications and can alter genetic diagnosis, counseling, and disease management. However, the detection of mosaic mutations can be difficult. The use of deep sequencing technologies has changed the realm of detecting mosaic mutations present at low level in the background of normal sequence. In this manuscript, we have used semiconductor-based deep sequencing on the Ion Torrent PGM platform to identify the underlying molecular defect in *RB1* gene in families with sporadic RB.

Bilateral RB is a monogenic disease caused by germline mutations in the *RB1* gene. Therefore, it is expected that the sensitivity of mutation detection in *RB1* gene should be approaching 100% when analyzing lymphocyte DNA in bilateral cases. Accordingly, we chose to investigate 20 bilateral RB cases where we did not detect any coding sequence mutation by standard sequencing, or, evidence for any intragenic deletion/duplication or rearrangement. Six individuals were identified to be carriers of mosaic mutations by deep sequencing (Table 2). This number is almost the same as the mosaic mutations detected by conventional Sanger sequencing where the degree of mosaicism was at least 20% of the normal allele (Table 1). The degree of mosaicism detected by next-generation sequencing is even lower and ranged between 2% and 15%. This contributed to an overall increase of 1% (96%–97%) to the overall detection efficiency of *RB1* mutation in bilateral cases.

Similarly, unilateral RB has been known to be caused by germline mutations in ~13% of cases. Here, we show that the detected mosaics increase the proportion of putative germline mutation-positive unilateral cases from 13% to 18%.

The significance of these results has to be evaluated in the context of the original reason for molecular testing of individuals with RB disease [Gallie, 1997; Lohmann, 2010]. The motivation behind genetic testing is first to establish a molecular diagnosis in the affected child such that the clinical management can be decided. Second, the presence of a germline mutation in the child warrants testing of the parents and the siblings as well. For a mutation-positive parent, the option of testing future offspring becomes possible and early detection of tumors is also a reality. When a child carries a mosaic mutation, which is clearly a postzygotic event, the risk of RB in all other members of the immediate family becomes minimal. This leads to significant emotional and economic benefits for the child and the respective family as well as for the society, and the consequent financial benefits have been documented [Richter et al., 2003].

Among the 10 individuals, who were previously identified with mosaic mutations by Sanger sequencing (Table 1), six individuals were adults and had RB as a child. The results of testing additional family members, where available, are included in the last column of the table. There was no apparent correlation of the degree of mosaicism in a parent and the

detection of the same mutation in a child. This implies somatic mosaicism in the parent with reduced risk of having an affected child. However, for every child without the familial mutation, the risk of RB was reduced to practically zero and eliminated multiple examinations under anesthesia for early detection of a tumor, which is associated with significant financial burden.

The demographics of the individuals identified with low-level mosaic mutations in the *RB1* gene are shown in Table 5; it is obvious that there is no correlation between the degree of mosaicism in lymphocyte DNA and the age at diagnosis of RB. None of the family members of a child with a mosaic mutation were positive for the same mutation as was expected.

Therefore, coding sequence mutations in *RB1* gene, including mosaic mutations, explain 97% of bilateral RB cases and 18% of unilateral RB cases. The increase in the fraction of unilateral cases is large and this changes how these patients and their parents are counseled with respect to their genetic risks and treatment options.

Conclusions

The current study indicates that amplicon-based, multiplexed deep sequencing approach can increase the detection rate of mosaic germline mutations in RB patients. The sensitivity of germline mutation increased from ~96% to 97% for bilateral cases, and for sporadic unilateral RB cases, the rate increased from ~13% to ~18%. Given the fact that this current study was limited to the coding exons of *RB1* gene, and the intronic and promoter regions were not tested, the frequency of germline mosaicism in RB can be even higher than what has been observed here. This finding highlights the potential utility of the deep sequencing technology for clinical genetic testing of RB. An important implication is that it changes the individualized genetic counseling of the RB cases for the risk of secondary cancers and the risk of disease in future offspring for a subset of individuals previously defined as germline mutation negative.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Workflow for the detection of mutations in *RB1* gene in RB cases. This workflow illustrates the different approaches used for sequencing bilateral and unilateral RB cases.

Conventionally, bilateral RB is associated with germline mutations and the analysis begins with lymphocyte DNA; in contrast, unilateral RB is mainly sporadic and associated with somatic mutations; thus, the analysis starts with tumor DNA when available. After standard Sanger sequencing and other assays, mutation-negative samples are reanalyzed using deep sequencing on the Ion PGM platform.

Figure 2.

Sanger sequencing chromatograms of serial dilutions of mutant DNA with wild-type DNA in three separate mutation contexts. As is evident, the presence of the mutant allele becomes less pronounced as the proportion of the wild-type allele increases. The arrows indicate dilution levels beyond which the mutant allele is not detectable with confidence. The value of the mutant allele frequency is around 15% for the point mutations, and around 10% for the frameshift mutation.

Figure 3.

Calibration curve for the detection of the g.70330G>A mutation by deep sequencing. The curve indicates the LOD as 4% of mutant allele frequency. The results for the other two mutations were similar (data not shown).

Figure 4. Sanger sequencing chromatogram, Taqman SNP genotyping assay, and the screen shot of the alignment of Ion PGM reads against the reference sequence on IGV are displayed for a mosaic mutation p. Arg579X (g.150037C>T) identified in a bilateral RB sample (RBM2). The degree of mosaicism is estimated to be 2.1% by tabulating the reads from deep sequencing and 1.8% by Taqman analysis.

Table 1

Summary of Mosaic Mutations Detected by Sanger Sequencing and Results of Testing Additional Family Members

Sample ID	BL/UL	Age at testing (years)	Age of diagnosis	Ethnicity	Mosaic mutation	Variant frequency	Additional family members tested	Result of mutation screening of family member
RBM11	BL	3	3 years	Caucasian	g.160832–160838dup7, exon 21	~20% ^a	None	Not tested
RBM12	BL	23	Unknown	Caucasian	g.156803G>T, exon20	~25% ^a	Fetus	Positive
RBM13	BL	23	8 months	Caucasian	g.70330G>A, intron 12 (+1)	~20% ^a	Son	Negative
RBM14	BL	37	Unknown	Caucasian	g.77011delA, exon 16	~20% ^a	Fetus	Negative
RBM15	BL	38	5 months	Caucasian	g.76430C>T, exon 14	~20% ^a	Fetus	Negative
RBM16	UL	39	20 months	Caucasian	g.150337C>T, exon 18	~20% ^a	Son	Positive
							Daughters	Negative
RBM17	UL	1	7 months	Unknown	g.162346 delC, exon 23	~20% ^a	No additional family members	Not tested
RBM18	UL	3	12 months	Hispanic	g.78238C>T, exon 17	~20% ^a	No additional family members	Not tested
RBM19	UL	6	4 years	Caucasian	g.76430C>T, exon 14	~20% ^a	Sibling	Negative
RBM20	UL	35	13 months	Unknown	g.70330G>A, intron 12 (+1)	~30% ^a	No additional family members	Not tested

^aThe variant frequency is based on the ratio of the heights of the normal and mutant alleles and compared that with a known heterozygote for a mutation at the same position.

Table 2

Germline Mutations Identified in Bilateral RB Samples by Deep Sequencing

Sample ID	Mutation	Total coverage	Variant coverage	Variant frequency (%)	<i>P</i> value	Mosaic (95% CI)
RBM1	g.150037C>T, exon 18	4,267	352	8.30	1.00E-10	8.3% (7.5%, 9.1%)
RBM2	g.150037C>T, exon 18	6,781	146	2.10	1.00E-10	2.1% (1.8%, 2.4%)
RBM3	g.150037C>T, exon 18	5,984	650	10.90	1.00E-10	10.9% (10.1%, 11.7%)
RBM4	g.70330G>A, intron 12(+1)	3,414	449	13.20	1.00E-10	13.2% (12.1%, 14.3%)
RBM5	g.162203G>A, intron 22	1,460	223	15.30	1.00E-10	15.3% (13.5%, 17.2%)
RBM6	g.65386C>T, exon 11	6,429	483	7.50	1.00E-10	7.5% (6.9%, 8.1%)

List of total coverage, variant read coverage, and variant frequencies of six mosaic mutations identified in DNA isolated from lymphocytes of bilateral RB cases by Ion Torrent Suite are listed. The *P* value of variant calls and degree of mosaicism along with 95% confidence interval are indicated.

CI, confidence interval.

Table 3**Germline Mutations Identified in Unilateral RB Samples by Deep Sequencing**

Sample ID	Mutation	Total coverage	Variant coverage	Variant frequency (%)	<i>P</i> value	Mosaic% (95% CI)
RBU12	g.76430C>T, exon 14	2,767	752	27.2	1.00E-10	27.2% (25.5%, 28.8%)
RBU26	g.77051T>C, exon 16	9,344	1,091	11.7	1.00E-10	11.7% (11.0%, 12.3%)
RBU37	g.64411A>T, exon 10	4,000	905	18.4	1.00E-10	18.4% (17.2%, 19.6%)
RBU52	g.70330G>A, intron 12	5,591	873	13.5	1.00E-10	13.5% (12.6%, 14.4%)

List of total coverage, variant read coverage, and variant frequencies of four mosaic mutations identified in DNA isolated from lymphocytes of probands with unilateral RB. The *P* value of variant calls and degree of mosaicism along with 95% confidence interval are indicated.

CI, confidence interval.

Table 4

t-Test of Variant Frequencies for Four Mosaic Mutations Identified in Unilateral RB Samples Against Background Noises from Control Runs of Three Healthy Individuals

Mutations	Unilateral RB cases		Control cases			<i>t</i> test
	1	2	1	2	3	
g.76430C>T	27.18%	25.53%	1.52%	1.63%	1.57%	$P < 0.0001$
g.77051T>C	11.68%	12.0%	0.17%	0.11%	0.08%	$P < 0.0001$
g.64411A>T	18.44%	18.44%	1.59%	1.57%	2.01%	$P < 0.0001$
g.70330G>A	13.50%	14.87%	0.16%	0.12%	0.21%	$P < 0.0001$

The frequencies of sequencing errors as determined by the presence of variant bases at the same locations as the mosaic mutation in the sequence reads derived from three healthy individuals were calculated. A “*t*-test” was performed to determine the significance of the variant frequency in the unilateral RB cases when compared with that of the background from sequencing errors in the healthy individuals where all 27 coding exons of *RB1* gene were sequenced in parallel with RB cases.

Table 5

Summary of Mosaic Mutations Detected by Deep Sequencing and Additional Familial Testing Results

Sample ID	UL/BL	Current age (year)	Age of diagnosis	Ethnicity	Variant frequency (%)	Additional family members tested	Result of mutation screening of family member
RBM1	BL	40	4 months	German	8.3	Two sons	Negative
RBM2	BL	5	Not specified	Caucasian/Hispanic	2.1	No additional family	
RBM3	BL	3	14 weeks	Caucasian	10.9	Mother and father	Negative
RBM4	BL	22	8 months	Caucasian	13.2	Son	Negative
RBM5	BL	31	Not specified	Caucasian	15.3	No additional family	
RBM6	BL	6	1 year	No ethnicity listed	7.5	Mother and father	Negative
RBU10	UL	2	14 weeks	No ethnicity listed	27.2	No additional family	
RBU26	UL	6	Not specified	Hispanic	11.7	Mother	Negative
RBU37	UL	7	2 years	Black/Hispanic	18.4	Mother	Negative
RBU52	UL	6	Not specified	No ethnicity listed	13.5	No additional family	