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Sensitive and Efficient Detection of *RB1* Gene Mutations Enhances Care for Families with Retinoblastoma

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Timely molecular diagnosis of *RB1* mutations enables earlier treatment, lower risk, and better health outcomes for patients with retinoblastoma; empowers families to make informed family-planning decisions; and costs less than conventional surveillance. However, complexity has hindered clinical implementation of molecular diagnosis. The majority of *RB1* mutations are unique and distributed throughout the *RB1* gene, with no real hot spots. We devised a sensitive and efficient strategy to identify *RB1* mutations that combines quantitative multiplex polymerase chain reaction (QM-PCR), double-exon sequencing, and promoter-targeted methylation-sensitive PCR. Optimization of test order by stochastic dynamic programming and the development of allele-specific PCR for four recurrent point mutations decreased the estimated turnaround time to <3 wk and decreased direct costs by one-third. The multistep method reported here detected 89% (199/224) of mutations in bilaterally affected probands and both mutant alleles in 84% (112/134) of tumors from unilaterally affected probands. For 23 of 27 exons and the promoter region, QM-PCR was a highly accurate measure of deletions and insertions (accuracy 95%). By revealing those family members who did not carry the mutation found in the related proband, molecular analysis enabled 97 at-risk children from 20 representative families to avoid 313 surveillance examinations under anesthetic and 852 clinic visits. The average savings in direct costs from clinical examinations avoided by children in these families substantially exceeded the cost of molecular testing. Moreover, health care savings continue to accrue, as children in succeeding generations avoid unnecessary repeated anaesthetics and examinations.

Introduction

Mutation and subsequent biallelic inactivation of the human retinoblastoma susceptibility gene, *RB1* (GenBank accession number L11910), on chromosome 13q14 (Friend et al. 1986), initiates retinoblastoma (MIM 180200), a tumor of the eye in children. Bilateral retinoblastoma arises from predisposition imposed by a de novo or inherited germline *RB1* mutation, whereas in 85% of children with unilateral retinoblastoma and no known family history, both *RB1* alleles are mutated in a developing somatic retinal cell that becomes malignant.

Precise identification of the *RB1* mutations that account for retinoblastoma in each family has been predicted to enhance the quality of clinical management of the affected patient and relatives at risk (Gallie et al. 1995). Children at risk to develop retinoblastoma undergo a series of clinical examinations, including examination under anesthetic (EUA), to diagnose and treat tumors as early as possible. If the proband's *RB1* gene status is determined by molecular testing, only those relatives with the mutation require clinical surveillance, whereas those proven to be noncarriers require no further examinations. The direct costs of molecular testing are predicted to be significantly less than conventional clinical examinations for each family (Noorani et al. 1996). If a family's mutation is known, prenatal molecular testing allows careful planning of perinatal management for infants with *RB1* mutations, including premature delivery to facilitate early treatment and optimize visual outcome (Gallie et al. 1999).

Despite clear clinical benefits of molecular testing for retinoblastoma, *RB1* mutation detection has not been widely implemented. Highly heterogeneous inactivating mutations are distributed along the entire length of the gene, which suggests that no single technology will be fully sensitive and efficient and that testing for *RB1* mutations may be costly. Cost-effective health care would logically require that a molecular test methodology be shown to be ethically justified, sensitive, accurate, and economically feasible (Ganguly and Williams 1997; Rebbeck et al. 1997) before it becomes

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routine clinical care. Although there are many articles on ethical standards and the clinical relevance of genetic testing in general, there is little published analysis of the impact of molecular test sensitivity and efficiency on health care.

Here we present a sensitive and efficient strategy to screen probands with retinoblastoma for *RB1* mutations. The method combines quantitative multiplex PCR (QM-PCR), two-dye double-exon sequencing, and, for unilateral tumors, methylation-sensitive PCR (MS-PCR). The potential of allele-specific PCR (AS4-PCR) to increase efficiency was also evaluated. We used stochastic dynamic programming to model the search problem and to derive optimal orderings of 19 different tests for two proband groups: (1) probands with bilateral and familial unilateral retinoblastoma and (2) probands with sporadic unilateral retinoblastoma. Efficient use of molecular testing reduced the estimated surveillance cost for 20 randomly selected families with retinoblastoma.

Material and Methods

Patient Samples

We examined 134 probands with unilateral, nonfamilial retinoblastoma and 224 probands with bilateral or familial unilateral retinoblastoma, referred from Canada, the United States, and several other countries. All participating families provided informed consent for the research team to perform clinical tests and to use the samples for retinoblastoma research in an anonymous manner. No person selected the option to exclude their sample or information from research, in which case their information would have been excluded from this analysis.

Peripheral blood lymphocytes (PBL) of patients with bilateral and familial unilateral retinoblastoma were studied for *RB1* mutations. Since few (15%) of the persons with sporadic unilateral retinoblastoma are predicted to have a germline *RB1* mutation, failure to find a mutation in PBL is of marginal predictive value. Therefore, we searched unilateral tumor DNA for biallelic inactivating mutations and then examined PBL for those specific mutant alleles, to rule a germline mutation in or out.

Total genomic DNA from PBL and tumor samples was extracted using the Puregene kit, according to the manufacturer's directions (Gentra).

QM-PCR

We used QM-PCR to screen for changes in exon size and copy number. All 27 exons of the gene were amplified using intronic primers designed to include splice sites. The 3′ end of the promoter, containing binding sites for ATF, E2F, SP1, AP1, and HRE elements (T'Ang et al. 1989; Gill et al. 1994), was amplified as a single fragment. Amplification was performed in six multiplex sets containing 1–8 fluorescent-labeled primer pairs (Cy 5.5) that yielded products of different sizes to allow simultaneous visualization of fragments. Reactions were performed with the Ampli*Taq* DNA polymerase kit (Applied Biosystems). PCR conditions were optimized so that each exon was amplified quantitatively. Either a 282-bp or a 329-bp product from exon 4 of the human retinaldehyde-binding protein gene (chromosome 15) was used as an internal control for quantitation against external controls known to have the following *RB1* statuses: nullisomic (WERI-RB1 retinoblastoma cell line) (McFall et al. 1977), monosomic (EL cell line) (Benedict et al. 1983), and diploid (normal). Amplified products were heat denatured, separated on a 6% polyacrylamide gel, and analyzed using the OpenGene Automated DNA System (Visible Genetics). Fragment detection and subsequent calculations were performed by Gene Objects 3.1 software (Visible Genetics). Ratios of *RB1*-derived peaks to internal control peaks were used to calculate gene copy number. Size standards labeled with Cy5.5 were used to identify aberrantly migrating bands indicating suspect intraexonic insertion or deletion, which were verified and further characterized by bidirectional sequencing.

When QM-PCR indicated suspect insertion or deletion involving one or more exons, long PCR was used to distinguish between true genetic alteration and artifacts generated by inefficient amplification due to intronic primer site polymorphisms. Amplimers were generated from genomic DNA through use of primers flanking the span of exons suspected to be deleted, using the XL PCR kit according to the manufacturer's instructions (Perkin-Elmer). PCR products were analyzed by conventional agarose gel electrophoresis.

We collected 3,223 QM-PCR readings from samples known to have either two copies or only one copy of a particular exon. Then we estimated the statistical power of QM-PCR to distinguish between one-copy and two-copy genotypes. The number of data points for two-copy QM-PCR readings was 48–80 per exon, and, for one-copy readings, 4–17 data points. For each exon and the promoter region, statistical power was estimated by computing the two-copy confidence level associated with the lowest critical value that exceeds all observed one-copy QM-PCR readings for the exon in question.

Sequencing

Duplex PCRs that amplify pairs of exons were performed with the Ampli*Taq* DNA polymerase kit (Applied Biosystems). Each pair of exons was sequenced simultaneously through use of the Cy5/Cy5.5 Dye Primer Cycle Sequencing Kit (Amersham, Quebec/Visible Genetics). Each primer mixture contained two prim-

ers, labeled with either Cy5 or Cy5.5, that target the amplified exons. Primers were designed to include an average of 50 bp of intronic sequence to encompass recognized splice sites. Exons were duplexed on the basis of similar mutation yields (to load early tests with positive results) or compatible reaction conditions. To sequence all 27 exons and the promoter region of *RB1* required 14 duplex sequencing reactions. The sequences generated were compared with wild-type *RB1* (GenBank accession number L11910) for sequence alterations, using the OpenGene Automated DNA System and Gene Librarian software, version 3.1 (Visible Genetics).

Conventional nomenclature was used to name mutations in a way meant to clearly identify the predicted result on the expected protein product. Nucleotide position was numbered on the basis of the cDNA sequence, using the first base of the initiator codon as cDNA position number 1. *RB1* mutations are also commonly reported with reference to the genomic sequence (GenBank accession number L11910) (Antonarakis 1998; Lohmann et al. 2002). Splice mutations were named according to their position relative to the closest exonic base where the first base 5′ of the intronic-exonic boundary was denoted as −1 (and, similarly, the first base 3′ of the intronic-exonic boundary was denoted as +1). When relevant, mutations were also named by their effect on the amino acid at specific codons.

AS4-PCR

PCR primers were designed for specific multiplex amplification of mutant sequences of four recurrent mutations: R358X, R251X, R579X, and R455X, in fragments of 202, 253, 287, and 730 bp, respectively. The sense primers were specific for either the wild-type or mutant genomic DNA templates at the ultimate 3′ base position. Reactions were performed with the Ampli*Taq* DNA polymerase kit (Applied Biosystems). PCR conditions were optimized so that samples with these mutations would amplify strongly and only faint amplification would occur in the absence of the mutant alleles (for primer and template mismatches). Products were resolved by 2% agarose gel electrophoresis, and the intensity of the amplified band indicated a mutant allele.

Promoter Methylation Assay

For patients with unilateral retinoblastoma, no family history, and no more than one somatic mutation in tumor revealed by QM-PCR and sequence analysis, we tested for methylation of the *RB1* promoter. The sodium bisulfite conversion and methylation-specific PCR (MS-PCR) was performed as described by Zeschnigk et al. (1999). PCR products from hypermethylated (201 bp) and normal (154 bp) *RB1* promoter were separated on a 2% agarose gel.

Performance Measures

Sensitivity was calculated from the clinical samples by the mutation-detection strategy that combines QM-PCR, double-exon sequencing, and MS-PCR. The sensitivity of allele-specific PCR was estimated as the ability to correctly identify four different mutations in samples that were discovered, by sequencing, to contain those mutations. For each assay, the cost (supplies and labor) and time to perform the assay was measured. The optimal rank order for assays was determined by using stochastic dynamic programming to minimize forecast average cost (or turnaround time, if desired), under the assumption that the mutation frequency spectrum observed for our sample is representative of the population.

Optimal Test Orders

We used the following strategy to approximate the optimal sequence of 19 different molecular assays to discover unknown retinoblastoma mutations. Working backwards from diagnosed mutations, we listed the minimal set of assays required to completely diagnose each family's mutation(s). For each of 24 permutations of QM-PCR multiplexes, we counted the number of mutations first detected by each QM-PCR multiplex when performed in the permuted sequence, to identify the permutation(s) with the fastest discovery rate.

For each order of the 19 assays with QM-PCR assays in the optimal sequence, we calculated the total direct cost of performing assays in the chosen order until the first sign of a mutation was discovered, as well as the cost of confirming the diagnosis by certain other assays in the minimal set, family by family. The average cost per proband to diagnose unknown mutations, for families with germline and sporadic mutations, is the performance measure used to rank candidate assay permutations. The direct cost calculation for unilaterally affected probands included every test until the first mutation (M1) was discovered, the test required to determine whether M1 is a germline mutation, subsequent tests performed until the second mutation (M2) was discovered if the first mutation was not in the germline, and the test required to determine whether M2 was a germline mutation.

Clinical Impact of RB1 *Mutation Identification*

For each relative found to have the *RB1* mutation of the proband, we noted the ocular outcome. The impact of molecular analysis was compared with conventional clinical screening of 20 families with retinoblastoma whose complete pedigree information was available, including for second-degree child relatives. The number of clinical examinations counted for each person at risk followed standard surveillance protocols for relatives at

different ages and with differing levels of risk. Each child at high risk to have retinoblastoma would conventionally receive eight EUAs and five clinic examinations, depending on risk group, until age 4 years, and approximately eight clinic visits until age 10 years. Relatives who are older than 10 years are exempt from surveillance examinations, because the risk of developing retinoblastoma tumors becomes very small. Relatives at medium risk, such as first cousins of bilateral probands, would conventionally receive five EUAs and 14 clinic visits, and those at low risk (first cousins of unilateral, nonfamilial probands) would conventionally receive no EUAs and 17 clinic visits.

The direct costs of surveillance for retinoblastoma were estimated, for each family, with and without molecular testing. To estimate clinical surveillance costs, we adjusted the 1994 cost of EUA and clinic examination at the Hospital for Sick Children (Noorani et al. 1996) for 3% inflation (the average change in the Toronto Consumer Price Index). Costs for EUA and for clinical examination were estimated at Can$740 (US$468) and Can$108 (US$68), respectively. Cost of molecular analysis was estimated at Can$5,000 (US$3,200) to search for an unknown mutation, and Can$500 (US$320) to test a relative at risk for a known mutation. Under the molecular testing strategy, the carrier statuses of the probands and their first-degree relatives determined which, if any, additional relatives needed molecular testing. Molecular test results were assumed to be available 60 d after each proband's clinical diagnosis, except for those families in which the causal mutation could not be identified.

Results

Mutation Types and Recurrent Mutations

All functional classes of mutations were identified (table 1). The germline mutations in 199 probands with bilateral retinoblastoma were 93% null, 6% in-frame, and 1% in the promoter region. The germline mutations identified in 30 probands with unilateral retinoblastoma (both familial and nonfamilial) were 57% null, 40% in-frame, and 3% promoter, consistent with these mutations being associated with reduced penetrance. The mutant alleles that were identified in retinoblastoma tumors from 112 unilaterally affected probands but were not detectable in blood and therefore were presumed somatic were 89% null, 3% in-frame, and 8% methylation of the promoter.

Of 126 sporadic retinoblastoma tumors in which mutations were identified, the second allele was mutated by loss of heterozygosity (LOH) in 66 (52%). Interestingly, the likelihood that the M2 event would be LOH varied with the type of M1 event. Only 23% of tumors with M1 whole-gene deletions and only 27% of tumors with exonic deletions showed LOH, whereas 88% of tumors in which M1 was methylation of the promoter showed LOH.

The distribution of the 229 germline mutations is illustrated in figures 1 and 2 and table 1. The majority (93% for bilaterally affected probands and 57% for unilaterally affected probands) were null mutations that predict a truncated protein or mRNA with a premature stop codon that is anticipated to be degraded by nonsense-mediated decay (NMD) (Culbertson 1999; Frischmeyer and Dietz 1999; Hentze and Kulozik 1999). These null mutations, including whole allele deletions, deletions involving one or more exons and splice mutations (fig. 1), small frameshifting intraexonic insertions and deletions, and nonsense mutations (fig. 2), were distributed throughout the *RB1* gene. Most of the missense mutations were clustered in *RB1* domains A and B, which are known to be important in the regulation of transcription by the *RB1* protein product, pRB (DiCiommo et al. 2000).

The 223 somatic null mutations (M2 for germline cases or M1/M2 for nongermline cases) were similarly distributed between mutation types, except that methylation of the promoter accounted for 8% of somatic mutations (table 1). We have tested blood from 7 of 28 probands with germline mutations in whom we have not found an *RB1* mutation, and none showed promoter methylation (data not shown).

Excluding the 66 mutant alleles due to LOH, of 386 mutant *RB1* alleles (235 germline and 151 somatic), 45% occurred only once (123 germline and 52 somatic = 175 overall). One-third of the "recurrent" mutations were large deletions or methylation of the promoter and were not defined at a nucleotide level (table 2). All of the 13 point mutations that occurred four or more times involved C→T transitions at CpG dinucleotides attributed to deamination of 5-methylcytosine (Rideout et al. 1990; Schmutte and Jones 1998). Twelve of these mutations affected arginine codons, and one created a splice mutation (1354+1G→A).

Molecular analysis of tumor tissue in one unilaterally affected patient identified three individual *RB1* mutations: heterozygous deletion of all exons and the promoter, and S795X and 713delAA, both presumed to be hemizygous (rather than homozygous). Since tumors are clonal, it is possible that the three mutations represent two different subclones with different M2 mutations but with the same M1 deletion. None of the three mutations were present in the patient's blood. This is the only retinoblastoma tumor in which more than two *RB1* mutations were observed, but we have not exhaustively looked for more than two mutations. Mutation analysis of tumor DNA is discontinued once two genetic changes predicted to be causative have been identified.

Table 1

Characterization of 452 Mutant *RB1* Alleles from 378 Probands with Retinoblastoma

Mutation Type	Bilateral and Familial Unilateral Germline (Studied Blood)			Unilateral Germline (Studied Tumor)		Mutant Alleles in Tumors (Unilateral)	Unilateral Sporadic (Studied Tumor)				Total Count[a]
	Bilateral	% M1 Found for Probands with Bilateral Disease	Familial Unilateral	Sporadic Germline Unilateral	% M1 Found for Probands with Unilateral Disease		No. Somatic M1/M2 Including LOH	%	No. of Tumors with LOH/Total Tumors	%[b]	
Null:											
Whole-gene deletions (P→27)	11	6	0	4	13	33	29	13	6/26	23	
Exonic deletions	14	7	0	0	0	18	18	8	3/11	27	
Intraexon deletions or insertions	47	24	4	2	20	41	39	17	13/21	62	
Splice mutation within two bases of exon	25	13	1	2	10	21	19	9	5/10	50	
Splice mutation more than two bases from exon	5	3	0	0	0	1	1	0	0/0	NA	
Nonsense mutations	84	42	2	2	13	95	93	42	29/45	64	
Total	***186***	93	***7***	***10***	57	209	***199***	89	56/113	50	***402***
In-frame (not truncating):											
Deletion 3 bp, exon 4, 13, 24–25	2	1	2	0	7	2	2	1	1/1	100	
In-frame splice deletions	0	0	0	0	0	0	0	0	0/0	NA	
Missense mutations	10	5	7	3	33	8	5	2	2/4	50	
Total	***12***	6	***9***	***3***	40	10	***7***	3	3/5	60	***31***
Promoter:											
Promoter sequence mutations	1	1	1	0	3	0	0	0	0/0	NA	
Methylation	0	0	0	0	0	17	17	8	7/8	88	
Total	***1***	1	***1***	***0***	3	17	***17***	8	7/8	88	***19***
Total alleles identified	***199***		***17***	***13***		236	***223***				***452***
Total Tumors with LOH									66/126	52	

Note.—Apparent discrepencies in totals are due to rounding.

[a] The values summed to make up each total are shown in boldface italic type.

[b] NA = not applicable.

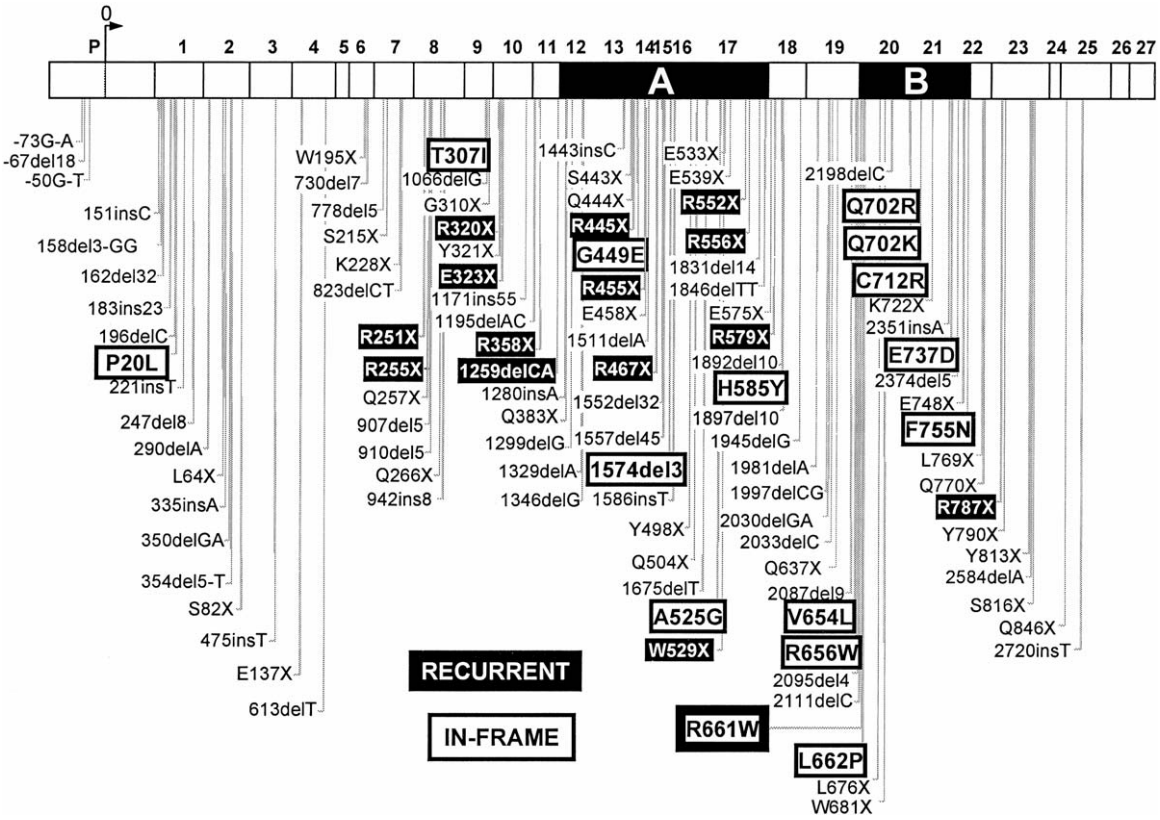

Figure 1 The distribution, within the *RB1* cDNA and promoter, of 111 different missense mutations and small insertions and deletions in the germline of 165 persons. Ninety-five families had unique mutations. Fourteen recurrent mutations that affected 70 independent families are shown in black; 14 in-frame mutations are outlined in black; R661W is both recurrent (all seven occurrences in germline) and in-frame, indicated by a wide black border.

Genotype-Phenotype Correlations

For 93% (186/199) of probands with bilateral retinoblastoma, the germline *RB1* mutant alleles were "null" (table 1) and were expected to result in premature truncation of translation and unstable mRNA (Dunn et al. 1989*b*) due to nonsense-mediated decay (Culbertson 1999; Frischmeyer and Dietz 1999; Hentze and Kulozik 1999), resulting in no detectable protein. Of the 203 probands with null germline mutations, 92% had bilateral tumors, whereas only 50% (12/24) of probands with in-frame mutations developed bilateral tumors (table 1). The diseased eye ratio (*der;* the ratio of the sum of affected eyes to the number of mutation carriers in a family) (Lohmann et al. 1994) is 1.9 for null *RB1* alleles and <1.5 for in-frame and promoter mutant alleles.

The recurrent missense mutation R661W occurred in 7 (3%) of 235 unrelated families with germline mutations (table 2). Four of the seven R661W probands were unilaterally affected (*der* 1.6). Of 30 unilateral germline probands, four (13%) had R661W. This mutation has been associated with reduced penetrance (Lohmann et al. 1992; Onadim et al. 1992), and the protein product of the R661W allele has been shown to have partial activity (Otterson et al. 1997; Whitaker et al. 1998). Therefore, the patients with sporadic unilateral retinoblastoma and with R661W germline mutations can be considered the founders of families with reduced penetrance. Of the 16 missense mutations, 13 involved the A/B "pocket" domain of pRB (15 germline missense mutations are shown in fig. 1), which is critical in the interaction of pRB with the transcription factor E2F (DiCiommo et al. 2000). For example, in vitro studies of R661W showed decreased binding to the transcription factor E2F1 but a retained ability to undergo cyclin-mediated phosphorylation (Otterson et al. 1997).

Lysine substitution for valine (V654L) resulted in reduced penetrance, with a *der* of 0.5, but substitution of glutamic acid for valine at the same position has been shown to yield a more highly penetrant phenotype (Lohmann, personal communication). A V654L mutant allele was also heterozygous with the null allele R556X in a unilateral nonhereditary tumor, consistent with the concept that LOH for the reduced-penetrance V654L allele might be insufficient for tumorigenesis (DiCiommo et al.

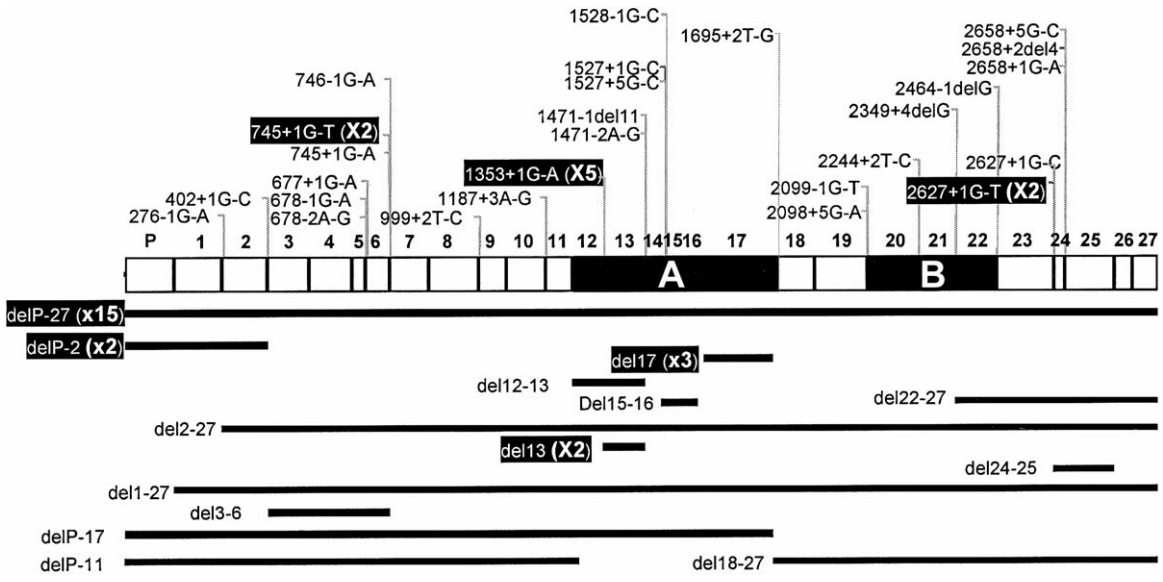

Figure 2 The distribution within the *RB1* cDNA and promoter of 41 splicing mutations and large deletions identified in the germline of 65 probands with retinoblastoma. The mutations in black occurred in more than one independent family; the number of occurrences is in brackets.

2000). Functional studies may provide greater insight into the importance of this valine residue, which lies 90%–100% buried (Lee et al. 1998) within the pocket domain.

Of 15 probands with complete deletion of *RB1* (delP→27), only 11 (73%) had bilateral retinoblastoma. The delP→27 mutation accounted for 13% (4/30) of total unilateral germline mutations but only 6% of all bilateral germline mutations (table 1). The tendency for total deletions to cause fewer tumors has been hypothesized to be due to contiguous deletion of an adjacent unknown gene that is essential for cell survival (DiCiommo et al. 2000). Although LOH is the M2 event in 52% of all retinoblastoma tumors studied, it occurred in only 22% of whole-gene deletions. Presumably, LOH for a deletion that encompassed both *RB1* and the adjacent "lethal" gene would result in total loss of the essential gene, causing cell death rather than retinoblastoma. Therefore, fewer tumors would form, resulting in a higher frequency of unilateral disease. Tumors would result only if M2 were a different mutation within *RB1,* leaving the unidentified lethal gene intact.

Sensitivity to Identify RB1 *Mutation(s) in Probands*

In total, the combination of molecular techniques described above characterized 452 mutant *RB1* alleles (table 3). We identified the germline mutation in 199 of 224 probands with bilateral retinoblastoma (89% sensitivity) and in 17 of 20 (85%) probands with unilateral familial retinoblastoma. In the tumors from 134 patients with unilateral sporadic disease, we characterized 236 mutated alleles (89% sensitivity to discover mutant alleles). The germline status in 112 (84%) of these 134 patients was determined. No mutations were detected in the tumors of eight probands with unilateral sporadic disease. In tumors of 14 probands with unilateral sporadic disease, we characterized only one mutant allele that was not detected in blood. These 22 cases are considered incomplete. Thirteen (12%) of the 112 probands with unilateral, sporadic disease had germline mutations despite the absence of family history.

QM-PCR Analysis

QM-PCR is sensitive to insertions and deletions ranging in size from 2 bp (fig. 3) to the whole *RB1* gene. QM-PCR covering the whole gene in six multiplex sets of 1–8 exons (2, 1, 8, 6, 6, and 5 exons in six multiplex groups) detected 31% of the mutant alleles in our sample. However, QM-PCR is a more powerful assay of allelic copy number for some exons than for others (table 4). For 19 exons, QM-PCR distinguished correctly between samples that have one allele and samples that have two alleles, at a 97% confidence level. For 22 exons, QM-PCR distinguished correctly at a ⩾95% confidence level. For four exons, QM-PCR showed moderate power (confidence level 60%–80%), and, for exon 1, QM-PCR did not discriminate between one-copy samples and two-copy samples. We have developed a replacement set of four QM-PCR multiplex sets (Q1–Q4) with 1–13 exons

Table 2

Recurrent Mutations Identified by QM-PCR and Bidirectional Sequencing

Type of Mutation and Test	Exon/ Promoter	Mutation	Total	Somatic	Germline		
					All	Unilateral	Bilateral
Small deletions:							
QM-PCR	9	1066delG	2	0	2	1	1
QM-PCR	11	1259delCA	2	0	2	0	2
Nonsense mutations:							
Sequence	10	R320X	18	6	12	0	12
Sequence	15	R467X	15	8	7	2	5
AS4-PCR	18	R579X	13	6	7	1	6
AS4-PCR	14	R455X	11	6	5	1	4
Sequence	14	R445X	10	3	7	0	7
AS4-PCR	11	R358X	8	3	5	0	5
Sequence	23	R787X	7	3	4	0	4
AS4-PCR	8	R251X	8	4	4	1	3
Sequence	17	R552X	6	4	2	0	2
Sequence	17	R556X	4	2	2	0	2
Sequence	23	Y790X	3	2	1	0	1
Sequence	18	Q575X	2	2	0	0	0
Sequence	8	R255X	4	1	3	0	3
Sequence	17	W529X	2	1	1	1	0
Sequence	25	Q846X	2	1	1	0	1
Sequence	13	S443X	2	1	1	0	1
Sequence	10	E323X	2	0	2	0	2
Sequence	19	Q637X	2	1	1	0	1
Splice mutations:							
Sequence	12	1353+1G→A	7	2	5	1	4
Sequence	23	2627+1G→T	2	0	2	0	2
Sequence	6	745+1G→T	2	0	2	1	1
Sequence	6	678-1G→A	2	1	1	0	1
Missense mutations:							
Sequence	20	R661W	7	0	7	4	3
Sequence	19	V654L	2	1	1	1	0
Promoter mutations:							
Methylation	Promoter	Pmeth	10	10	0	0	0
Large deletions:							
QM-PCR		delP→27	38	22	16	5	11
QM-PCR		del3→27	5	5	0	0	0
QM-PCR		del18→27	3	2	1	0	1
QM-PCR		delP→2	3	1	2	0	2
QM-PCR	17	del17	3	0	3	0	3
QM-PCR		delP→17	2	1	1	0	1
QM-PCR	13	del13	2	0	2	0	2
No. of mutations that occurred more than once			211	99	112	19	93
% of mutant alleles				66	48	53	47
No. of mutations that occurred only once				52	123	17	106
% of mutant alleles				34	52	47	53
Total no. of mutant alleles excluding LOH				151	235	36	199
Tumors with LOH				61		5	
Total no. of mutant alleles including LOH				212		41	

each, to increase efficiency in mutation identification, which are now being validated.

Allele-Specific PCR

Analysis of mutation type and distribution along the *RB1* gene revealed that, although the gene lacks hot spots that would predict important functional domains, 13 point mutations recurred with significant frequency, particularly CpG transitions (Cowell et al. 1994) (table 2). We developed AS4-PCR for four of these recurrent mutations in one multiplex, which clearly distinguished the specific alleles from the background level of wild-type allele amplification (fig. 4). This multiplex is fast and inexpensive, since only one PCR and one agarose gel are required to detect 10% (40/401) of all mutant

Table 3

Sensitivity of Combined Molecular Techniques to Identify *RB1* Mutations

	Bilateral (Study Blood)	Familial Unilateral (Study Blood)	Sporadic Unilateral (Study Tumor)	Total
No. of probands analyzed	224	20	134	378
No. of probands diagnosed	199	17	112	328
Sensitivity to diagnose proband	89%	85%	84%	87%
No. of alleles examined	224	20	266[a]	510
No. of mutant alleles identified (including LOH for tumors)	199	17	236	452
Sensitivity to identify allele	89%	85%	89%	89%

[a] Tumors from 134 patients with sporadic unilateral retinoblastoma contained 268 mutant alleles; however, for two tumors, the first allele discovered was shown to be in the germline, so mutations were not identified in the second alleles.

alleles (12% of somatic and 8% of germline mutant alleles).

Assay Order Analysis and Optimization

Our search for optimal orderings revealed statistical dependence between assays in at least two ways. First, characterization of some multiexon deletions requires more than one multiplex, so the sensitivity of the next multiplex depends on the subset of multiplexes already performed. For example, multiplex Q2 scores 10, 13, or 17 mutations, depending on which multiplexes precede it in the test order. Consequently, QM-PCR multiplexes cannot be optimally ordered by simply comparing test sensitivity.

We solved this problem by isolating the set of QM-PCR multiplex permutations that achieves minimum time to discovery. We ignored materials costs and time differences, because each multiplex takes almost the same time and incurs almost the same materials cost. We found the unique best multiplex order to achieve complete diagnosis for bilaterally affected families and a different optimal order for unilaterally affected probands. After the QM-PCR multiplexes were ordered, ranking proceeded for the entire list of tests by iteration, comparing all test permutations with the order of QM-PCR multiplexes constrained to the optimal order. We found different optimal multiplex orders for the 199 proband germline *RB1* mutations and the 112 patients whose testing was completed by identification of somatic mutations.

Second, statistical dependence appears when analyzing sporadic unilateral mutations. In the 48% of tumors that do not show LOH, we must search for two different mutations. Any pair of mutations that occurs together with more than random probability introduces history-dependent sensitivity ranking, so the likelihood that the next assay will complete the molecular characterization of disease depends on which tests have already been performed. We solved this problem by using an iterative sort procedure that isolates minimum-cost orderings (multiple answers are possible) through pairwise comparisons, widely known as a "bubble" sort. Though several methods for sorting a static list of values are known to be quicker than a series of iterative pairwise comparisons, in this case the ranking measure is not static and changes with the sort order. Therefore, iterative pairwise comparisons are essential to ensure correct rank.

Our search for optimal orderings showed progressive improvement. Initially, we naïvely performed the six original QM-PCR multiplexes (M1–M6), then sequenced exons and the promoter in duplexes ordered by casual observation of the exons most likely to contain mutations. Because both *RB1* mutant alleles must be identified for tumors, we analyzed germline and somatic mutations separately. Using the original naïve method, projected turnaround time was 6.4 wk for probands with heritable (bilateral or familial unilateral) disease. If sequence duplexes are interleaved between QM-PCR multiplexes, projected turnaround time drops to 5.13 wk. Finally, with redesigned QM-PCR multiplexes (Q1–Q4) and AS4-PCR, projected turnaround time is 2.7 wk.

Impact of RB1 *Mutation Identification on Quality of Health*

Molecular analysis was useful to families with retinoblastoma in several ways. Thirteen of 29 offspring of adult retinoblastoma survivors, who were tested at various stages of pregnancy or postnatally, were shown to carry their family's mutation. The offspring who did not carry the *RB1* mutation did not develop retinoblastoma. Four infants had the molecular test and were clinically examined shortly after birth. All had tumors at birth and, in the next few months, all developed bilateral tumors that were treated with focal therapy and chemotherapy. One of the eight eyes was eventually enucleated.

Prenatal testing identified nine fetuses that carried the family's *RB1* mutation. Five of these families, all of whom had experienced children dying of retinoblastoma or second primary tumors, chose termination of the pregnancy. One family that had undergone a very neg-

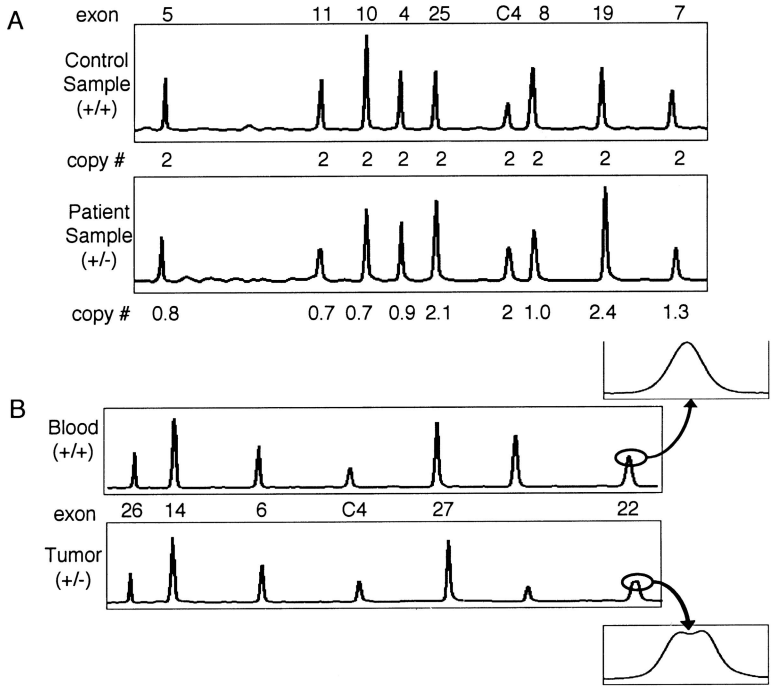

Figure 3 Mutation detection using QM-PCR. *A,* Detection of a multiexonic deletion spanning at least exons 4–11, while exons 19 and 25 remain two-copy. *B,* Detection of a 2-bp deletion in exon 22 by QM-PCR.

ative experience with retinoblastoma terminated a pregnancy that carried the *RB1* mutation and subsequently delivered a baby without the mutation, who did not develop tumors.

Four infants known to have *RB1* mutant alleles were delivered prematurely at ~35 wk gestation, in order to treat potential macular tumors early. One of the four infants had a unilateral macular tumor at 36 wk gestation. The other three children developed bilateral tumors 1 mo to 1 year later. All eight eyes were treated with laser and cryotherapy only, and all have 6/6 vision.

Only 13 of 112 (12%) unilaterally affected probands with no family history of retinoblastoma were germline carriers of one of the two *RB1* mutations detected in their tumor. None of the 99 unilateral probands whose identified tumor mutations were not present in blood developed retinoblastoma in the unaffected eye. Of all germline *RB1* mutation carriers, ~10% (Sippel et al. 1998; Smith et al. 2000) are mosaic and might not be detected by our screens. A 1.2% (10% mosaicism × 12% germline mutation in sporadic unilateral disease) risk remains that a unilaterally affected patient in whom the tumor mutations are not detectable in blood has germline mosaicism, with 0.6% residual risk for the next generation. Precise knowledge of *RB1* mutations in the tumor of a potentially mosaic unilaterally affected proband would permit accurate testing of future offspring for the same mutations.

Within our cohort of 212 nonfamilial probands with germline mutations, >95% of parents tested normal for their child's mutation. One percent (10% mosaicism × 10% germline) of such parents could be mosaic, thereby putting siblings at a 0.5% calculated risk (50% chance of inheriting the mutant allele × 1.0% chance that the parent who tests normal is mosaic) of inheriting the same mutant *RB1* allele as the proband. Since mosaicism cannot be inherited, siblings and other relatives are at the population risk to develop retinoblastoma if a unilaterally affected proband's tumor mutation does not show in blood.

Impact of RB1 *Mutation Identification on Cost of Health Care*

To measure the impact of *RB1* molecular testing on health outcomes and health care costs for whole families, we counted the number of surveillance examinations performed on first- and second-degree child relatives for a representative sample of 20 Ontario families (fig. 5). For each family, we counted the number of examinations saved by the molecular strategy and the net savings in direct surveillance cost.

With molecular testing, individual relatives required

Table 4

Statistical Power of Test for Allelic Quantity by QM-PCR

Exon	Two-Copy Sample Size (n)	Smallest α for Zero β[a] (%)	Critical Value (Measured as Copy Number)	Implied % Confidence Level	Implied Statistical Power[b]		
					Strong ($p > .95$)	Moderate ($.60 < p < .95$)	Weak ($0 < p < .60$)
7	78	0	1.2	100	X		
19	78	0	1.2	100	X		
27	72	0	1.2	100	X		
3	72	0	1.3	100	X		
12	80	0	1.4	100	X		
15/16	80	0	1.4	100	X		
25	78	0	1.4	100	X		
9	72	0	1.5	100	X		
5	78	2	1.2	98	X		
8	78	2	1.2	98	X		
10	78	2	1.2	98	X		
24	72	2	1.2	98	X		
26	72	2	1.2	98	X		
23	67	2	1.3	98	X		
17	80	2	1.5	98	X		
4	78	3	1.1	97	X		
22	72	3	1.3	97	X		
6	72	3	1.4	97	X		
20	70	3	1.5	97	X		
11	78	4	1.3	96	X		
13	70	5	1.2	95	X		
14	72	5	1.3	95	X		
2	48	23	1.7	77		X	
21	79	23	1.7	77		X	
18	80	34	1.8	66		X	
P	63	38	1.9	62		X	
1	65	94	2.3	6			X
Count total					22	4	1

[a] α = the probability of falsely categorizing two-copy DNA as one-copy DNA; β = the probability of falsely categorizing one-copy DNA as two-copy DNA.

[b] p = frequency not two-copy.

fewer or no examinations for clinical surveillance. In the 20 families, 88 children avoided a total of 313 EUA, and 107 children avoided 852 clinic examinations (fig. 5*A*). The mean savings in health care spending for all 20 families was Can$6,591 (US$4,200) per family (fig. 5*B*). In 12 of 20 families surveyed, molecular analysis saved Can$1,000–$38,000. In the remaining eight families, surveillance with molecular analysis cost Can$1,000–$6,300 more than conventional surveillance. Even when the molecular strategy cost more, the quality of care was better for families in which molecular diagnosis allowed children to avoid clinical examinations. In five families, children at risk were relatively few or relatively old, so the usual savings were diminished. In one family that carries a low-penetrance mutation, a large proportion of people in the pedigree needed to be tested. In two families whose mutant *RB1* allele was not identified by all the assays performed, the molecular route cost more because no clinical advantage was obtained despite the work.

Discussion

Using a combination of molecular techniques, we detected 452 *RB1* mutations in clinical samples from 378 probands with retinoblastoma (tables 1 and 3). Twenty-seven of these mutations that we previously published are included in a review of 368 reported *RB1* mutations posted in the *RB1* Gene Mutation Database (Lohmann 1999). A meta-analysis of germline mutations of 192 patients (Harbour 1998) reported that 90% of mutations were likely to result in the absence of RB protein. We confirm that mutations likely to result in residual protein (missense mutations, in-frame deletions and splice abnormalities, and promoter mutations) are rare (7%) in bilaterally affected probands but represent 43%

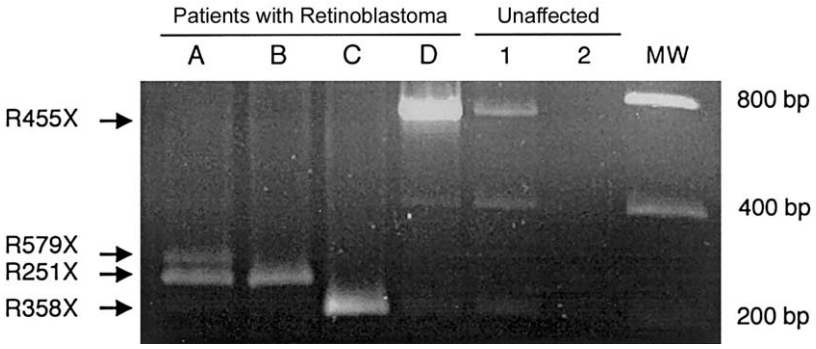

Figure 4 Multiplex AS4-PCR for rapid detection of recurrent mutations in *RB1*. Four samples from patients with retinoblastoma (patients A–D) and two control normal samples were tested by AS4-PCR containing primers specific for the recurrent mutations R455X (730 bp), R579X (287 bp), R358X (251 bp), and R251X (202 bp). Strong amplification occurred when the mutation identified by the specific PCR primer was present. Unaffected individual 1 and patient D also show low levels of cross-hybridization to the wild-type alleles with the primers for R455X and R358X.

of germline mutations in unilaterally affected probands. Neither of the previous studies reports whole-exon or multiexon deletions, which accounted for 14% of the bilaterally affected probands in our study. It is likely that the techniques used in the previous studies were insensitive to exon copy number. Karyotype analysis will detect 5%–7% of *RB1* mutations that are large rearrangements but has insufficient resolution for small exonic deletions.

Of 15 missense mutations, 13 are clustered in the A/B pocket and the intervening spacer region, confirming that in-frame mutations favor critical regions of *RB1*. At least three of the missense mutations (R661W, V654L, and C712R) have been associated with reduced penetrance (DiCiommo et al. 2000). Only one of the missense mutations was recurrent, R661W. In addition, the mutations identified in the germline of unilaterally affected children, 43% of which are in-frame, can also be considered to have reduced penetrance. The recurrent mutations, including R661W, all involve C→T transitions (fig. 1), usually resulting in CGA (arginine) mutating to TGA (stop codon) (Cowell et al. 1994). Of 46 arginine codons in *RB1,* 14 are encoded by CGA/G, and 13 of 14 are targets for recurrent mutations. The 14th CGA arginine is in exon 27. Since no mutations are observed yet in exons 26 and 27, change in the C-terminus of the RB protein may be insufficient to induce retinoblastoma.

We show that QM-PCR is a very efficient method to identify copy number and exon size changes and is amenable to tight statistical control for accuracy and sensitivity in clinical application. Mutation identification strategies for other genes, such as *BRCA1* and *MLH1* (Nakagawa et al. 2002), have not efficiently identified this class of mutation. Generation of stable murine-human hybrid cell lines carrying selected human chromosomes in only a single copy (Yan et al. 2000) or by limiting dilution of DNA to a single template (Zhou et al. 2002) has been used to obtain copy-number mutations in the *hMSH1* and *hMSH2* genes in patients (Yan et al. 2000; Nakagawa et al. 2002). However, these techniques are extremely labor- and technology-intensive and are unlikely to achieve cost effectiveness in health care applications. QM-PCR would have identified these mutations efficiently without the complexity of producing hybrids. We have used QM-PCR, in addition, to efficiently identify narrow regions of common genomic gain (Chen et al. 2002) and loss (D. Chen, M. Harmandayan, C. Lee, M. Marchong, and B. Gallie, unpublished data), other than the *RB1* changes, that occur commonly in retinoblastoma. Thus, QM-PCR presents clear opportunity for multiple uses in mutation identification and in studies of cancer prognosis.

Of 126 sporadic retinoblastoma tumors in which mutations were identified, the second allele was mutated by LOH in 66 (52%). Interestingly, the likelihood that the M2 event would be LOH varied with the type of M1 event. Only 23% of tumors with M1 whole-gene deletions and only 27% of tumors with exonic deletions showed LOH, whereas 88% of tumors in which M1 was methylation of the promoter showed LOH.

Exons have been screened for mutations through use of a variety of techniques, including SSCP analysis (Hogg et al. 1992; Shimizu et al. 1994; Blanquet et al. 1995; Asher et al. 1996), pulsed field gel electrophoresis (Janson and Nordenskjold 1994), denaturing gradient gel electrophoresis (Blanquet et al. 1995), heteroduplex analysis (Szijan et al. 1995; Zhang and Minoda 1995), and Southern blotting (Blanquet et al. 1991; Kloss et al. 1991; Zajaczek et al. 1999). Aside from direct sequencing of exons (Yandell et al. 1989; Zajaczek et al. 1999), the majority of these methods rely on an initial

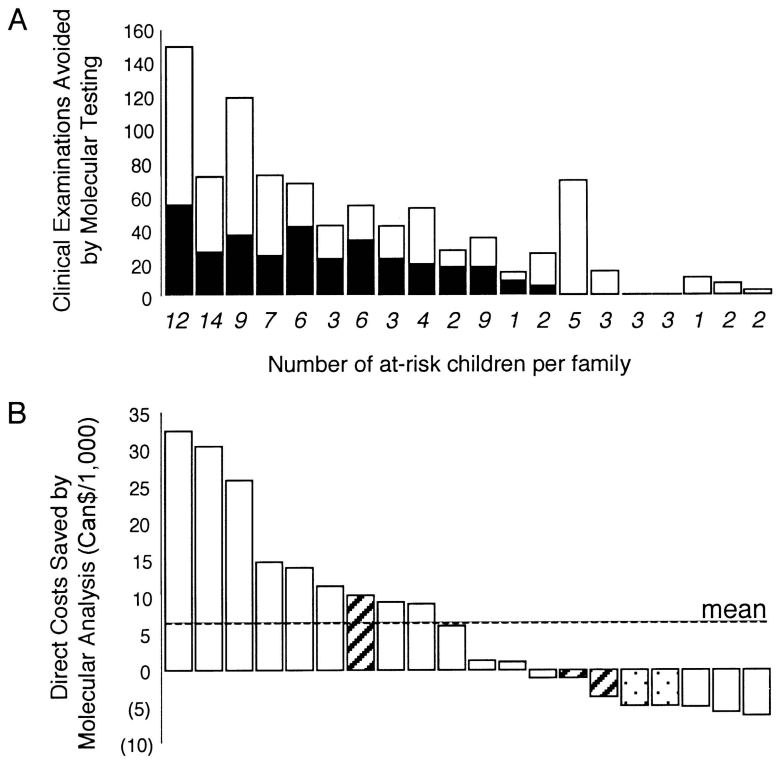

Figure 5 Impact of molecular *RB1* testing. *A*, Histograms of the number of examinations avoided because of molecular results. Clinic examinations are shown in white bars, EUAs in black bars. *B*, Histogram of direct surveillance costs saved for 20 families because of molecular *RB1* testing. Mean savings was Can$6,591 per family (*horizontal line*). Families with bilateral and familial retinoblastoma are shown in white bars, and families with unilateral nonfamilial disease are shown in bars with oblique hatches. The molecular strategy cost more than conventional surveillance for two families in which all assays failed to show the proband's mutation (*dotted bars*) and for six families in which at-risk family members were few in number or significantly older than the proband.

exon screen followed by sequencing of suspect amplified fragments. Denaturing high performance liquid chromatography, which appears to be efficient at finding heterozygous mutations once optimized for the gene being studied (Bennett et al. 2001; Han et al. 2001), has been effectively applied to *RB1* mutation screening (D. Lohmann, personal communication).

We have used double-exon sequencing without a prescreen for exonic changes and found that this approach could be efficient if test order is optimized. By analysis of the *RB1* mutational spectrum and calculation of the frequency of occurrence for mutation types that would be refractory to characterization by exon sequencing, we were able to use a statistical approach for identifying a combination of assays to find mutations with the smallest number of required assays. This requires that QM-PCR be performed at predictable stages between the sequencing of particular exons. Statistical analysis also showed that time to the discovery of a mutation was hindered when this approach was not used.

Detection of *RB1* mutant alleles has required the analysis of genomic DNA, because, in blood, mRNA from null alleles was undetectable (Gallie et al. 1988; Dunn et al. 1989*a*; Kato et al. 1994). This has been attributed to message instability due to NMD (Culbertson 1999; Frischmeyer and Dietz 1999; Hentze and Kulozik 1999). However, null alleles are easily detected in mRNA in retinoblastoma tumors. This may occur either because NMD is less active in tumors or because the mRNA from tumor cell lines was prepared without the delay usual for blood samples, allowing no time for NMD, or because the absence of pRB leads to upregulation of transcription to levels where even degrading mRNA is detectable by PCR (Dunn et al. 1989*b*). Stabilization of mRNA showing premature truncation of translation by puromycin or cycloheximide (Carter et al. 1995) prior to RT-PCR might allow detection of mRNA from null alleles in blood, adding efficiency and a potential increase in sensitivity of *RB1* mutation identification.

The optimization of multiple steps for mutation detection in large genes may be generalized to other genes that cause disease with a high frequency of private null alleles, such as *BRCA1* and *BRCA2*. Mutation identi-

fication for *BRCA* genes is similar to *RB1,* in that knowledge of carrier status can provide very real advantages to the family (Kutner 1999) and there are many ways that the genes can be mutated, including copy number changes. However, the advantages we have in testing of families with retinoblastoma (i.e., only one gene [*RB1*], >95% penetrance to get tumors for null alleles, all tumors showing loss of both alleles, clear benefit in health outcome, effective treatment in the first years of life) are not so obvious for familial breast cancer (multiple genes, including *BRCA1, BRCA2,* and *CHEK2* [Liede et al. 2002; Meijers-Heijboer et al. 2002]; penetrance variable and much less than 90%; somatic tumor mutation heterogeneity; health benefit and treatment less obvious; and inhibition of optimization of molecular testing due to patenting of the *BRCA1* and *BRCA2* genes). The exonic copy number changes that we detect readily with QM-PCR in *RB1* are common in the *BRCA* genes (Gad et al. 2001) but are not commonly looked for in screening strategies (Hegde et al. 2000).

Genotype-phenotype correlations can be used to further optimize time to diagnosis of the mutation. For example, for families with a reduced number of affected eyes and a high number of unilaterally affected children, the order of assays can be further optimized to reflect certain genotype-phenotype correlations. Thirteen percent of families with reduced penetrance carry the R661W mutation, in comparison with 1% of bilateral families with full penetrance and expressivity. In addition, two families in our study showed the 745+1G→T mutation (IVS6+1G→T) leading to a stop codon in exon 7 and the reported characteristic highly unusual inheritance pattern (Klutz et al. 2002). Whenever this splice mutation is paternally inherited, it shows high expressivity and nonsense-mediated decay of one copy of the mRNA, as observed for other null *RB1* alleles. Conversely, maternal inheritance of the mutation is associated with extremely low expressivity, and the misspliced message (missing exon 6) is easily detectable. We already base the assays on phenotype, in that bilateral familial and unilateral nonfamilial patients are approached very differently. However, we can further optimize on the basis of genotype-phenotype knowledge. For example, we can now test unilaterally affected familial probands first for total deletion and R661W, since these two assays would find 26% of germline alleles. Similar test order would also be applicable to the study of unilateral, nonfamilial tumors, to optimize time to discover the rare germline mutations in the first member of a family with reduced penetrance.

Intronic translocations, which remain undetectable by our current techniques, may account for some of the 10% of families for which no mutation was identified. FISH, a method well suited to the detection of translocations, has not yet revealed any translocations in samples in which all our assays were negative. Some of the unidentified 11% of germline alleles are likely to be accounted for by mosaicism. Given the suggestion that mosaicism for *RB1* mutations occurs in ~10% of families (Sippel et al. 1998), we may be close to the maximally achievable sensitivity for detection of germline mutations, but mosaicism can not account for the missing tumor mutations or familial mutations. Our sequencing is sensitive to 20%–30% heterogeneity, and therefore we can sometimes identify mosaicism (Sippel et al. 1998), so when no obvious mutation was found, we reviewed the primary data for possible mosaicism. The 18% of unidentified somatic mutant alleles in tumors may be accounted for by regulatory mutations in regions not yet known to be important in *RB1* function.

Human papilloma virus (HPV) E7 protein binds and inactivates pRB in the process of deregulation of the cell cycle (Dyson et al. 1989). In a recent study, an apparent correlation of PCR-detectable HPV sequence, as well as both the lower proliferation indices and the lower risk of extraocular disease in 14 of 39 retinoblastoma tumors, led to the hypothesis that the virus may play a role in sporadic disease (Orjuela et al. 2000). We are currently investigating the DNA of unilaterally affected patients for whom no oncogenic mutations were found, to look for evidence of HPV.

High assay sensitivity to discover mutant allele(s) is crucial to clinical implementation of *RB1* mutation identification. The costs of failing to identify a mutation include substantial wasted lab costs, the costs of counseling the family, and a significant opportunity cost, since irreplaceable tumor samples must be used for patients with unilateral, sporadic disease. We report 89% sensitivity to identify *RB1* mutant alleles, the highest yet reported. Other investigators indicate sensitivity to identify mutant *RB1* alleles between 40% (Sippel et al. 1998) and 80% (Mateu et al. 1997; Harbour 1998; Lohmann 1999; Zajaczek et al. 1999; Alonso et al. 2000; Najera et al. 2001). Linkage studies indicate that the unidentified mutant alleles in probands with hereditary retinoblastoma still involve the *RB1* gene, even when mutations are not found. When all assays were negative, failure to identify a mutant allele was reported to the referring professionals, and work continued to identify the *RB1* mutation on a research basis.

The optimization algorithms provide a practical tool to evaluate prospective new tests. For example, the impact of developing multiplex AS4-PCR on test sensitivity, costs and turnaround time was assessed prospectively prior to use of the assay on clinical samples. The analysis showed that AS-PCR would be more effective than direct sequencing only if multiple point mutations with a relatively high frequency of occurrence were assayed in a single reaction. Thus, we implemented AS4-

PCR only after finding a combination of experimental conditions that allowed four recurrent nonsense mutations to be multiplexed and detected in a single agarose gel (fig. 4). If the 13 mutations that have occurred four or more times were tested together in one or two multiplex reactions, 112 of 402 (28%) *RB1* mutations could be detected. At present, we still confirm the AS4-PCR results by sequencing the suspect mutant allele, but no false positives have yet been found.

Our analysis demonstrates a significant positive impact on health care costs of *RB1* mutation identification. In 1994, we calculated that the cost of molecular testing for a family was equal to the cost of conventional surveillance for all children of the same generation as the proband. With the benefit of the proband's analysis, the cost of molecular surveillance for future generations was significantly lower than the cost of conventional screening (Noorani et al. 1996). We now show that, on average, molecular testing resulted in direct health savings equivalent to Can$6,591 (US$4,200) per family, before counting the benefit to children not yet born. These savings estimates clearly underestimate the true savings achieved by molecular testing if morbidity and indirect costs to families were measured. For 12/20 families, there was a net monetary benefit due to excluding children from EUAs and clinical exams, and for eight families there was a net increase in costs. However, 18 of the 20 families benefited from molecular testing by avoiding clinic examinations with inherent risk and by saving time away from work and travel, two of the many indirect costs not measured. We were unable to detect the proband's mutation for 2 of 20 families, consistent with our observed sensitivity. These two families incurred testing costs but attained no benefit.

Since the initial analysis of these data in 1994, the cost of conventional care has continued to rise, while the cost of molecular testing has decreased. We anticipate that the disparity in cost between conventional and molecular screening will continue to grow as new genetic methods further optimize testing. Our work shows the feasibility of using cost-effective improvements in the quality of care to justify changes to the standard of care and shows that new technology is not always more expensive.

Optimization of *RB1* mutation detection decreases test costs, decreases turnaround time, and increases the likelihood of clinical implementation. We have found that the spectrum of mutations, cost of methodology, sensitivity, efficiency, and phenotype-genotype correlations must all be considered before a truly comprehensive approach to mutation detection can be applied effectively and reliably in a clinical context. These factors override single, superficial factors like technical sophistication or cost efficiency.

Responsible public health care policy requires criteria such as the following, to ensure effective use of molecular testing (Secretary's Advisory Committee on Genetic Testing 2000; Foreman et al. 2001): (i) effectiveness to predict illness in a specific patient group, (ii) demonstrated technical competency, (iii) improved health outcomes as a result of testing, (iv) beneficial psychological impact, (v) appropriate pre- and post-test counseling, (vi) ongoing development to continually improve technology, (vii) interlaboratory validation of sensitivity and proficiency, and (viii) beneficial impact on health spending. The data presented in the present article support implementation of *RB1* mutation testing in routine health care that meets such criteria.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *RB1* [accession number L11910])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for retinoblastoma [MIM 180200])

RB1 Gene Mutation Database, http://www.d-lohmann.de/Rb/mutations.html

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