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Unilateral retinoblastoma, lack of familial history and older age does not exclude germline RB1 gene mutation

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ABSTRACT

Conclusive identification of *RB1* mutations in retinoblastoma is predicted to improve the clinical management of affected children and relatives. However, despite clear clinical benefits, *RB1* screening remains difficult, most of the alterations being unique and randomly distributed throughout the entire coding sequence. In this report, we present the results of a constitutional *RB1* analysis undertaken in our institution over the last four years. The detection of *RB1* gene deletion or mutation was performed by Southern blot and sequence analyses in 73 patients (including three families with 2, 3 and 3 probands, respectively). Complementary constitutional chromosome and fluorescent in situ hybridization (FISH) analyses of *RB1* gene were applied in cases where hereditary retinoblastoma was suspected despite negative detection. Altogether, germline abnormalities were found in 11% (4/36 patients) of sporadic unilateral retinoblastoma (median age, 21.5 months) and 86% (32/37 patients) of sporadic bilateral or positive familial history retinoblastoma (median age, 5 months). The spectrum of germline alterations found in 31 distinct families included 12 nonsense mutations (39%); 10 point insertions or deletions with frameshift (32%); 4 mutations and 1 deletion affecting splice sites (16%); 2 missense mutations (6%); and 2 large deletions (6%). A total of 15 mutations have not been previously reported. In this small series, splicing mutations were associated with bilateral disease whilst most of the frameshift mutations were identified in patients with an early age at diagnosis, bilateral disease or hereditary forms of the disease.

This study confirms that screening for constitutional *RB1* mutation should become an integral part of current management of any patient affected by retinoblastoma irrespective of the tumour laterality and familial background.

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1. Introduction

Retinoblastoma, an embryonic neoplasm of retinal origin, is the most common intraocular malignant tumour in childhood, with an average incidence of one case every 15,000–20,000 live births. It occurs as a result of mutations in the retinoblastoma gene (*RB1*) located at 13q14. Approximately, 45% of retinoblastoma patients are hereditary cases (15% unilateral and as many as 30% bilateral cases) whilst the others are sporadic and present as unilateral tumours [1]. Approximately, 12–15% of patients with retinoblastoma already have a family history, where the tumour phenotype segregates as an autosomal dominant trait with high (90%) penetrance. Individuals harbouring a germline *RB1* gene mutation are predisposed to the development of multiple other cancers throughout life including bone and soft tissue sarcomas, melanoma, brain tumours and have a 50% risk of transmitting their germline *RB1* gene mutation to an offspring [1,2].

While most patients with hereditary retinoblastoma can be clinically identified (bilateral or multifocal tumours, positive family history), 5–15% of patients have single-eye tumours and no familial history, making them indistinguishable from patients with non-hereditary retinoblastoma. Identification of germline alterations in this subset of unilateral retinoblastoma probands is crucial for appropriate treatment and long-term follow-up. Furthemore, precise identification of the *RB1* mutation in a child has been predicted to enhance the quality of clinical management of the affected patient and relatives [3,4]. Children at risk for retinoblastoma undergo intensive clinical surveillance with regular ophthalmologic examinations under anaesthesia. If a germline *RB1* gene mutation is detected, only relatives with the *RB1* gene anomaly require this follow-up whereas those proven not to be carriers require no further examinations [5,6]. Moreover, pre-implantation genetic diagnosis for retinoblastoma can be planned in pregnancies at risk [7].

Although enucleation and external beam radiotherapy have been the most common treatments for retinoblastoma in the last decade, standard therapeutic approach has evolved to systemic chemotherapy combined with local therapies to preserve eye and vision. Identification of patients with germline *RB1* mutation is crucial for the therapeutic choice. External irradiation will indeed be excluded in patients with constitutional *RB1* mutation to avoid development of secondary osteosarcoma. Conservative management will be attempted particularly in patients with bilateral retinoblastoma or at risk of developing bilateral tumours.

If *RB1* screening has demonstrated clear clinical benefits, *RB1* testing remains extremely challenging, as the majority of the mutations are unique and randomly distributed over the entire coding sequence. To date, more than 368 mutations have been reported in *RB1* germline mutation database [8–12]. All these difficulties explain why extensive *RB1* mutation detection has not been widely implemented [10].

In the current study, we present the results of the constitutional *RB1* analysis that was carried out over the last four years to improve the management of retinoblastoma patients and their families. Detection of *RB1* alteration was performed in 73 patients (including three families with 2, 2 and 3 probands) by the means of Southern blot and sequence of analyses of the 27 exons as well as part of the promoter and intronic regions. Constitutional chromosome and fluorescent in situ hybridization (FISH) analyses of gene were restricted to patients with a suspicion of hereditary retinoblastoma despite negative Southern blot and sequence analyses. We present the spectrum of *RB1* constitutional alterations, and discuss the benefits of this screening strategy in daily clinical practice with reference to existing literature [10–17].

2. Patients and methods

2.1. Patients

A total of 73 probands from 68 families with retinoblastoma, mostly of European countries including 18% of families from Spanish origin (12/68), were assessed for the presence of a constitutional *RB1* alteration. All the patients were followed at the Cliniques Universitaires Saint Luc, Brussels, Belgium. We studied 37 patients with bilateral retinoblastoma with or without familial history (including three families with 2, 3 and 3 probands, respectively) and 36 patients with unilateral retinoblastoma. Only one of the unilateral patients had a positive familial history at diagnosis. The sex ratio (M/F) was 0.7 for unilateral and 1.4 for bilateral groups and the median age was 19 and 5 months, respectively. All participating families provided an informed consent for the genetic analysis. Ophthalmologic examination was performed in each parent of the affected children. Southern blot and sequence analyses of the *RB1* gene were performed in all patients. Complementary constitutional chromosome and FISH analysis of *RB1* gene were carried out on blood DNA from bilateral retinoblastoma patients and patients under 1 year of age when Southern blot and sequence analyses were negative. They were also carried out initially on probands with abnormal phenotype such as developmental delay or facial dysmorphism.

2.2. Southern blot analysis

Detection of specific DNA fragments by gel transfer hybridization was performed from peripheral blood lymphocytes. DNA was digested with *Hin*dIII enzyme (Roche 656313) and separated by an agarose electrophoresis gel. The DNA was transferred to Zeta-Probe GT Genomic membrane (Biorad, ref:162-0194). Labelling and hybridization were performed according to the manufacturer's instructions by sequentially using two radioactive probes derived from cDNA *RB1* 3.8 and 0.6 kb (Dryja). After each experiment, labelled DNA probes hybridized to complementary DNA bands were visualized by autoradiography.

2.3. Sequence analysis of the retinoblastoma gene

Genomic DNA was isolated from peripheral blood lymphocytes. Amplification of each of the 27 individual exons, the promoter (520 bp) and intronic regions (5–80 bp, according to the location of the exons) was performed with primers and according to conditions listed in Table 1. Amplification products were purified from free nucleotides and primers using the QIAquick PCR Purification kit (Qiagen). Sequence analysis

Table 1 – Primer selection for amplification and sequencing the promotor and exons of Rb-1 gene

Promotor and exons	nom	PCR Primer 5′ → 3′	Annealing temperature	Size	Number of intronic bases upstream	Exon size	Number of intronic bases downstream
Promotor	Prom. S	TCCCAAAAGGCCAGCAAGTGTCT	60	589			
	Prom. A	CGTCCCCTGAGAAAAACCGGAC					
1	EX. 1 S	TCCCGCGGTTGGAC	60	348	171	137	10
	EX. 1 A	GACGGCGGCTCT					
2	EX. 2 S	CAAGTATGTACTGAATCAA	51	230	48	127	11
	EX. 2 A	AAAATTTCAAAACGTTTTAAG					
3	EX. 3 S	ATGAAATATTTGATCTTTATTT	51	214	11	116	42
	EX. 3 A	GGAAAATCCAGAATTCATT					
4	EX. 4 S	TTACTGATTTACTTTTTTC	51	195	17	120	17
	EX. 4 A	ATCAGAGTGTAACCCTAA					
5	EX. 5 S	AGATGAATAAAGCATGAG	51	201	63	39	58
	EX. 5 A	AACTATCAAGATGTTTGAG					
6	EX. 6 S	CTGTTTTTTTTCTGCTTTC	51	211	14	68	89
	EX. 6 A	GGAGGAGTACATTACTC					
7	EX. 7 S	AAGATCTGAATCTCTAAC	51	221	32	111	39
	EX. 7 A	CAACTGCTGAATGAGAA					
8	EX. 8 S	GTTATAGTTAGAATACTTCA	51	287	54	143	48
	EX. 8 A	TTGTTAGGGAGAACTTAC					
9	EX. 9 S	TGTTCAAGAGTCAAGAGA	51	230	52	78	59
	EX. 9 A	ATCTAAGAAAGTTAGACAA					
10	EX. 10 S	TAATGAAATCTGTGCCTC	51	252	61	110	41
	EX. 10 A	CCTATATCAGTATCAACC					
11	EX. 11 S	CATAAAGCACAAATTGTAA	51	249	39	78	89
	EX. 11 A	ATTTCTTACATGTATGTATG					
12	EX. 12 S	TTTCTCCCTTCATTGCTT	51	247	30	88	88
	EX. 12 A	TGTTAGATAGGAGATTAGT					
13	EX. 13 S	TCATATATTATGGAGCAG	50	264	61	117	47
	EX. 13 A	GGAAAGATGCTGCTTTT					
14	EX. 14 S	ATTGTGATTTTCTAAAATAGCAGG	60	223	29	57	89
	EX. 14 A	TTGATGCCTTGACCTCCTGA					
15–16	EX.15–16 S	TTTTTTTTTTTTTAAATTATCT	51	277	7	32	80
	EX.15–16 A	CCACATTTTAACTTTAAATTGAAC			80	77	31
17	EX. 17 S	AAAAAATACCTAGCTCAAGGGT	60	342	40	197	57
	EX. 17 A	TTAAGAAACACCTCTCACTAAC					
18	EX. 18 S	TACCTGGGAAAATTATGCTTAC	60	221	34	119	20
	EX. 18 A	ATTTGCAGTTTGAATGGTCAAC					
19	EX. 19 S	AAGTGTATGTATAATCTGT	56	276	48	146	43
	EX. 19 A	TTTGAACCCAGTCAGC					
20	EX. 20 S	TTTGAAAAAAATCTACTTGTAATTC	60	266	42	146	28
	EX. 20 A	TTAACAAGTAAGTAGGGAGGA					
21	EX. 21 S	CCATGTAATAAAATTCTGAC	56	205	27	105	27
	EX. 21 A	GTTATGGATATGGATTTATCAA					
22	EX. 22 S	TTTTTTTTACTGTTCTTCC	51	194	4	114	31
	EX. 22 A	TTGGTGGACCCATTACATTAGA					
23	EX. 23 S	TGTAATGGGTCCACCAA	51	262	40	164	19
	EX. 23 A	TCATTCTTTACTACTTCC					
24	EX. 24 S	AATGATGTATTTATGCTCA	51	212	60	31	80
	EX. 24 A	TTGAATAACTGCATTTGG					
25	EX. 25 S	AACTTGAGGTTGCTAACT	51	257	51	143	25
	EX. 25 A	ACCATCTCAGCTACTG					
26	EX. 26 S	TGGAAATTTGAGTTTTCC	51	179	45	50	45
	EX. 26 A	AAGACTTCTTGCAGTGT					
27	EX. 27 S	TGCCAACTTACCCAGT	51	202	39	71	55
	EX. 27 A	ATACAGTCACATCTGTG					

Uniform PCR conditions were used for amplification of the promoter and exons from genomic DNA, and consisted of an initial denaturation of 7 minutes at 94 °C, followed by 30 cycles (40 seconds at 94 °C, 40 seconds at 51 to 60 °C according to the primer pair as defined supra, and 1 minute 30 seconds at 72 °C), and a final elongation of 7 minutes at 72 °C. PCR conditions for the amplification of exon 1 and the promoter region included the addition of 5% and 7% dimethyl sulfoxide [volume/volume], respectively. $MgCl_2$ final concentration was 3.25 mM for exon 13 and 2,5 mM for all the other exons.

was carried out in both orientations on an automated ABI 3100 Apparatus (Applied Biosystems, Foster city, USA), using the Taq Dye Deoxy Terminator Cycle Sequencing kit from the same manufacturer and according to its instructions. The *RB1* reference sequence corresponds to GenBank Accession No. L11910.

2.4. Constitutional chromosome study and FISH of RB1 gene

Standard karyotypic examinations were performed on standard G-banded metaphase of synchronized 24 h peripheral lymphocyte culture. Rearrangement of the *RB1* gene was investigated by FISH using commercial LSI 13 (*RB1*) 13q14 Spectrum Orange probe (Vysis nr 32-190001, Downer's Grove, IL, USA) according to the manufacturer's instructions. The probe was used to hybridize 20 blood metaphases from each patient and centromeric probe specific of chromosome 16 hybridized the same metaphases as internal control. These techniques were performed to exclude large deletions involving 13q14 undetected by sequencing, e.g., whole-gene deletions.

3. Results

Germline abnormalities were found in 11% (4/36) sporadic unilateral retinoblastoma and in 86% (32/37) bilateral and/or familial history retinoblastoma patients. Expressed on a family rather than on a proband basis, the rate of RB1 germline alteration reached 87% (27/31) in this latter group. The global spectrum of these genetic abnormalities found in 31 distinct families consisted of 12 nonsense (39%), 10 point insertion or deletions with frameshift (32%), 4 mutations and 1 deletion affecting splice sites (16%), 2 missense mutations (6%) and 2 large deletions (6%) (Tables 2–4).

A total of 26 different germline alterations were identified by sequence analysis in 48% (34/71) of the patients (Fig. 1) amongst which, 15 mutations had not been previously reported and consisted of 10 frameshifts, 3 nonsense and 2 splice mutations (Tables 2 and 3).

Median age in unilateral and bilateral retinoblastoma patients with germline mutations was 21.5 and 5 months, respectively. If we assessed separately the 31 bilateral (26 families) and the 15 familial cases (10 families), median age was similar in both groups (5 and 6 months). Whereas age in both groups was unusual, it must be pointed out that 9 familial cases did not benefit from prospective screening (Tables 2 and 3) due to: one of the parents being asymptomatic carrier ($n = 4$; probands 3b, 23b, 2u, 3u); lack of genetic counselling despite a history of bilateral retinoblastoma in a parent ($n = 2$; probands 22b, 1u); or mosaicism with several siblings affected in a same family without apparent germline mutation in the parents ($n = 3$; family 20b).

In unilateral retinoblastoma, there was a single case with familial history at diagnosis (a 2 bp deletion with frameshift) whereas four cases were considered as non-hereditary at diagnosis (2 missenses and 2 nonsense mutations) (Table 2). The genetic analysis revealed however that two of them were hereditary forms (probands 2u, 3u).

In bilateral retinoblastoma (26 families), the spectrum of mutations included 10 nonsense (38%), 9 point insertion or deletions with frameshift (35%), 4 mutations and 1 deletion affecting splice sites (19%) (Table 3); whereas large deletions on q13 were identified by means of constitutional chromosome study and FISH in another two cases (8%) presenting with a dysmorphic phenotype (Table 4). In one of those cases, del (13)(q32-ter) did not directly affect the 13q14 locus but direct or indirect alteration of this locus during the chromosome end rearrangement cannot be ruled out. Despite the lack of clinical symptoms, the abnormal karyotype prompted an ophthalmologic examination which led to the diagnosis of bilateral retinoblastoma. In the second patient, a bilateral retinoblastoma was serendipitously discovered on a brain CT scan performed for assessing an asymptomatic macrocephalia. Besides these two cases in which karyotyping result preceded the diagnosis of retinoblastoma, constitutional chromosome analysis completed by FISH failed to identify alterations that would have potentially been missed by Southern blot and sequence analyses.

In the current series of patients, it is of note that no mutation was found in the *RB1* promoter region. On the basis of an abnormal Southern blot result, a large deletion was suspected but could not be confirmed by karotype, FISH or sequence analysis in another two patients with unilateral retinoblastoma.

Correlation between the type of molecular alterations and the phenotypic expression of the disease was assessed according to the laterality (uni or bilateral tumour), the presence of a familial history, and age at diagnosis with a cut off at 1 year (Table 5). In this small series, frameshift was not identified in patients older than 1 year, nor in patients with unilateral sporadic retinoblastoma. Missense mutations were found twice in the latter group. In contrast, frameshifts were more often found in hereditary cases (4/10 families; 40%), in bilateral disease (9/26 families; 35%) and in patients with an

Table 2 – Distribution of RB1 mutations in unilateral (u) cases

Country of origin	Site	Age (months)	Genetic alteration and description		Germline carrier other than proband	Comments
Family history at diagnosis						
1u	Exon 21	6	**g.160815-60816del2T**	L731fsX	Yes	Father affected
No family history at diagnosis						
2u Spain	Exon 1	19	g.2118C > T	P20L	Yes	Mother and sister: asymptomatic carriers
3u	Exon 9	10	g.61788C > T	T307I	Yes	Father: asymptomatic carrier
4u	Exon 17	35	g.78238C > T	R552X		
5u	Exon 23	24	g.162237C > T	R787X		

Unpublished mutation is noted in boldface type. All the probands are of Belgian origin except when otherwise stated.

Table 3 – Distribution of RB1 mutations in bilateral (b) cases (24 families)

	Country of origin	Site	Age (months)	Genetic alteration and description		Family history	Comments
1b		Exon 2	5	**g.5442insCA**	T52fsX		
2b*		Intron 6	0.2–1-6	g.45867G > A	Splice	Yes	One proband, two affected brothers, mother affected
3b		Exon 7	5	**g.56858delG**	V205fsX	Yes	Father asymptomatic carrier
4b	Spain	Exon 10	6	g.64348C > T	R320X		
5b		Exon 10	4	**g.64415-64416delCT**	T342fsX	Yes	Mother affected
6b	Spain	Exon 12	5	**g.70307delA**	E398fsX		Southern blot positive in proband and father
7b	Spain	Intron 12	1	g.70330G > A	Splice		
8b	Republic Democratic of Congo	Intron 12	15	g.73724A > G	Splice		
9b	Yugoslavia	Exon 14	2	g.76430C > T	R445X		
10b	Yugoslavia	Exon 14	4	**g.76460C > T**	R455X		
11b–12b		Exon 15	6–12	g.76898C > T	R467X	Yes (11b)	In two unrelated patients (among which one familial case: father affected)
13b		Exon 15	1	**g.76889G > T**	E464X		
14b	Yugoslavia	Exon 17	11	**g.78119-78120delTG**	L512fsX		
15–16b	Spain (16b)	Exon 17	0.1–9	g.78238C > T	R552X		In two unrelated probands
17b	Yugoslavia	Exon 18	5	g.150037C > T	R579X		
18b	Spain	Exon 18	3	**g.150104delC**	T601fsX		
19b		Exon 19	12	**g.153218delC**	P609fsX		
20b*	Marocco	Exon 20	6–11(2)	**g.156819-156822 delGAGAA**	R696fsX	Yes	One proband, two twin brothers affected
21b		Exon 20	2	**g.156804delA**	E691fsX		
22b		Intron 22	7	**g.162112delT**	Splice	Yes	Mother affected
23b*		Exon 23	3–4	**g.162327G > T**	E817X	Yes	One proband, one sister affected; father was asymptomatic carrier
24b	Spain	Intron 23	38	**g.170364C > G**	Splice		

Unpublished mutations are noted in boldface type. All the probands are of Belgian origin except when otherwise stated. The three families with multiple probands (3, 3 and 2 children) are marked with an asterisk (*). In those families, only the first proband was considered when the frequency of genetic alterations was examined.

early age at diagnosis (10/26 families; 38%) whilst splicing mutations were associated with bilateral disease. The two large deletions detected by constitutional chromosome study and FISH of *RB1* gene were associated with a dysmorphic phenotype and bilateral retinoblastoma (Table 4).

4. Discussion

In our series of patients, unilateral sporadic cases represented nearly half of the cohort of probands, in line with the 60% rate expected in the general population [18,11], as opposed to previous studies focusing only on bilateral/familial [12,19]. Nonsense mutations (12/31 families, 39%) and insertions/deletions with frameshift (10/31 families, 32%) were frequently found, followed by mutations or deletions affecting splice junctions (5/31 families, 16%). All but one intronic alterations (g.73724) occurred in the vicinity of splicing consensus sites (either donor or acceptor sites). Suppression of the normal splice site was confirmed by splice site prediction program (available at www.fruitfly.org/seq_tools/slice.htlm), except for the g.73724 that is reported as a single nucleotide polymorphism in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). Functional studies should be carried out to confirm whether or not g.73724 and g.170364 intronic mutations are deleterious but these data were lacking: patient 8b (Table 3) with g.73724, who originated from Congo, died from tumour progression, whereas patient 24b (Table 3) with g.170364, from Spain was lost to follow-up. Surprisingly, the distribution of mutations and their relative proportions in our cohort were similar to those of southern European patients with a high proportion of small length mutations. In contrast, northern European countries (Germany, United Kingdom) reported a higher rate of nonsense nucleotide substitutions of the *RB1* gene [11,12,20]. It is unlikely that the differences arise from sensitivity of our methods of detection, since our detection rate (86% in sporadic bilateral

Table 4 – Abnormalities in constitutional chromosome study and FISH of RB1 gene in two bilateral retinoblastoma patients without family history[a]

Description	Age (months)	Comments
46,XX,t(8;13)(q24;q14)	4	Asymptomatic macrocephalia
46XY,del(13)(q32-ter)	11	Moderate mental retardation, mild facial dysmorphism

a It is of note that no germline mutation was found in parents and sibling of both probands.

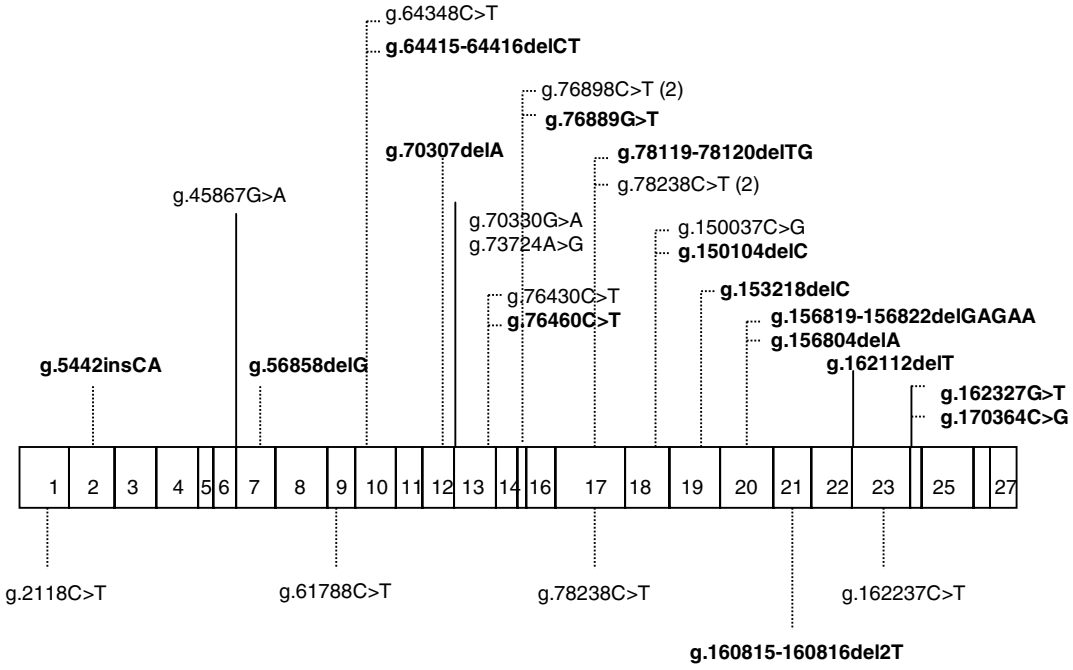

Fig. 1 – Distribution of the 26 different germline *RB1* mutations identified by sequence analysis in 36 patients. *RB1* sequence is drawn to scale. Mutations were identified in bilateral (above the coding sequence) and unilateral cases (below). Unpublished mutations are noted in boldface type. Recurrent mutations are noted in brackets.

or familial retinoblastoma) is largely comparable to other reports [10–12,17]. In this respect, it is of note that 18% (12/68 families) of all our patients were of Spanish origin with an unbalanced selection. Those patients were indeed affected by bilateral retinoblastoma and referred for therapeutic management. Of the mutated families, 13/31 (42%) came from other countries: 7 from Spain, 4 from Yugoslavia and 2 from Africa.

Considering that the rate of *RB1* germline alteration reached 87% (27/31 families) in bilateral and/or familial history retinoblastoma patients, analyses failed to detect germline mutations in 13% of suspected hereditary cases. As illustrated in the current study, this may be attributed to limited sensitivity of Southern blot and karyotype analyses while sequence analysis may overlook large deletion. Moreover, promoter-targeted methylation was not investigated. Despite accounting for 8% of the somatic alterations in retinoblastoma, *RB1* promoter methylation has not yet been proven to be involved in the ontogeny of hereditary retinoblastoma [1], and was not performed in our study. However, routine screening for hypermethylation should be part of future investigations as this was found as a constitutional abnormality in a recently reported case of hereditary nonpolyposis colorectal carcinoma [21]. This is also justified by the availability of new reliable and easy to use methods such as quantitative analysis of methylated alleles [22].

Among the spectrum of *RB1* genetic alterations, 15 mutations have not yet been reported [8,9,20,23–26]. Ongoing identification of novel genetic alterations, as reported in recent studies from various countries, suggests that the spectrum of *RB1* mutations is not yet entirely deciphered, the achievement of which could even be an endless task.

In line with published literature, we noted that nonsense mutations or mutations affecting splice junctions were usually associated with bilateral disease and early onset [20,27,28], as also were large deletions in children with a dysmorphic phenotype. Moreover, a majority of mutations were distributed between exon 12 and 22, a coding region corresponding to the *RB1* pocket domain, which is critical for transcriptional repression [29]. In contrast, germline missense mutations found in sporadic unilateral retinoblastoma have

Table 5 – Correlation between clinical features and type of mutations

Clinical features	Number of probands (number of families affected)	Germline mutations (number of families affected is given when its differs from the number of probands)
Age >1 year at diagnosis	5 (5)	2 nonsense, 2 splicing defect, 1 missense mutations
Age ⩽1 year at diagnosis	31 (26)	12 (10) frameshift, 13 (10) nonsense and 1 missense mutations, 3 splicing defect, 2 large deletions
Bilateral disease	31 (26)	13 (10) nonsense mutations, 11 (9) frameshift, 5 splicing defect, 2 large deletion
Hereditary forms (germline carrier other than proband)	15 (10)	6 (4) frameshift, 2 splicing defect, 5 (2) nonsense and 2 missense mutations

usually lower penetrance. However, the number of patients in our series is too limited to reliably correlate phenotypic expression of retinoblastoma and molecular alterations of *RB1* while non-genetic factors like ethnic or environmental background can also interfere with the gene expression [12].

In line with previous data [30], median age in unilateral (19 months) and bilateral (5 months) retinoblastoma patients with germline mutations was significantly different. Median age at diagnosis for sporadic unilateral retinoblastoma with (21.5 months) or without (16.5 months) germline mutation were comparable. This observation contradicts previous recommendation based on the age at diagnosis in the group of patients with sporadic unilateral tumour [6,31]. It is however, in agreement with the recent report of Schüler that age at diagnosis of isolated unilateral retinoblastoma does not distinguish patients with or without a constitutional *RB1* gene mutation [32].

In sporadic unilateral retinoblastoma, germline abnormalities were found in 4/36 (11%) probands which is twice as high as a recent report [11], but in accordance with Vogel's postulate [33] and other reports [10] stating that 10–12% of sporadic unilateral cases carry germline mutations when penetrance of 90% is assumed. The finding of a familial unilateral retinoblastoma in our series is also in accordance with the observation that up to 2% of sporadic unilateral cases may have a family history [6,30]. In unilateral retinoblastoma, correlation between intraocular multifocal tumours and germline mutations could not be assessed because in these patients, the tumour was usually bulky at diagnosis.

Constitutional *RB1* gene mutations were found at diagnosis in three children over 19 months age (probants 2u, 4u, 5u) who were first classified as sporadic unilateral retinoblastoma. Accordingly, and even if the performance of mutation screening remains low in unilateral retinoblastoma, this observation confirms that the diagnosis of a unilateral sporadic case should prompt genetic screening and counselling.

Parents of each *RB1* mutation carrier were tested for the mutation. Such screening strategy allowed to identify four asymptomatic carriers without familial retinoblastoma or cancer but with offspring presenting unilateral (patients 2u and 3u) or bilateral retinoblastoma (patients 3b and 23b). In unilateral cases, and unlike recent observation [32], the patient with the maternally inherited RB1 allele (patient 2u) did not develop retinoblastoma at an earlier age than the patient with the paternally inherited RB1 allele (patient 3u). Recent advances in the understanding of the structure and function of the retinoblastoma protein (pRB) now provide new insights into the molecular basis of this low-penetrance form of retinoblastoma. Low-penetrance retinoblastoma mutations either cause a reduction in the amount of normal pRB that is produced or result in a partial functional mutant pRB [34]. Leucine substitution for proline (P20L) and isoleucine substitution for threonine (T307I) are both in-frame mutations that have been associated with reduced penetrance [10,11,27]. This was confirmed here as P20L and T307I were found in unilateral retinoblastoma probands (probands 2u and 3u) with asymptomatic mother and sister carriers, or asymptomatic father carrier, respectively. In contrast, another asymptomatic father carrying the E817X mutation had his two children (probands 23b) affected by bilateral retinoblastoma, pinpointing the higher penetrance of this nonsense mutation. Interestingly, the intensity of the paternal signal on the electropherogram is intermediate between a normal and heterozygous mutated sequence which suggests paternal mosaicism. Another case of mutational mosaicism was illustrated with R696fsX mutation on exon 20 (family 20b) where bilateral retinoblastomas affected two twin brothers and a sister among 11 siblings, but were not found in the asymptomatic parents.

Altogether, the current data confirm that Southern blot is a totally inappropriate method to screen for gene rearrangements. There were only two abnormal Southern blots and none could be confirmed by other methods. This very low performance is also striking when our data are compared with those of other groups using the quantitative multiplex PCR of short fluorescent fragments (QMPSF), a method recently validated and recommended in this setting [10,11]. It confirms the need to replace the Southern blot by a robust and reliable methodology designed to detect exon deletions in *RB1* analysis. This improvement would alone be expected to further improve the detection rate of *RB1* alteration in our bilateral and sporadic unilateral retinoblastoma patients.

Likewise, cytogenetic examinations complemented by FISH analysis were only contributory in two cases presenting with dysmorphic phenotype. This analysis should therefore be restricted as a second-line screening or as first-line when gross rearrangement is suspected on the basis of the patient's phenotype [11].

Since 1999, systematic *RB1* molecular screening has been performed in all retinoblastoma patients followed in our institution. Richter et al. [10] demonstrated the significant positive impact of *RB1* mutation identification on health care costs. They calculated that the cost of molecular testing for a family becomes inferior to the cost of conventional surveillance for all children of the same generation as the proband. The direct cost savings consisted in avoidance of multiple ophthalmologic examinations under anaesthesia and clinic visits. Furthermore, the psychological positive impact of exact genetic counselling on the families is great and could not be estimated [10]. Available molecular techniques such a Southern blotting, single-strand conformation polymorphism analysis, heteroduplex analysis, restriction fragment length polymorphism analysis, direct sequencing or combination of multiple techniques are yet expensive and time-consuming even if some methods are slightly more rapid then others [13]. But no single procedure will be fully sensitive, efficient and cheap as highly heterogeneous inactivating mutations are distributed along the entire length of the gene. Our study confirms that genetic screening is efficient in retinoblastoma patients and the data confirm that routine *RB1* mutation testing meet the criteria of public health care policy [10,11]. This point is crucial as improvements in ophthalmologic technologies, medical therapies and efficacy of genetic *RB1* testing will increase the number of cases sent for genetic screening or counselling. However, because of inconsistent detection of the *RB1* gene abnormality, even in cases with clear hereditary form of retinoblastoma, clinical screening should still be proposed in relatives at risk when no *RB1* gene abnormality has been discovered in a proband.

Mutation analysis should become an integral part of current management of all patient with retinoblastoma and their families. This screening should not be restricted to high-risk

patients. In patients with sporadic unilateral retinoblastoma, we confirm other recent data that age at diagnosis is not a factor allowing to discriminate those who are expected or not to carry germline mutations [32]. Further studies are yet mandatory to better understand the correlation between genotype/phenotype, the mechanism of low-penetrance mutations, the implication of constitutional *RB1* methylation, the putative role of epigenetic factors or modifier gene in retinoblastoma oncogenesis.

Conflict of interest statement

None declared.

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