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Carlos Rodríguez-Martín, Cristina Robledo, Gema Gómez-Mariano, Sara Monzón, Ana Sastre, Jose Abelairas, Constantino Sábado, Nieves Martín-Begué , Joan Carles Ferreres, Ana Fernández-Teijeiro, Ricardo González-Campora, María José Rios-Moreno, Ángel Zaballos, Isabel Cuesta, Beatriz Martínez-Delgado, Manuel Posada, Javier Alonso.

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FREQUENCY OF LOW-LEVEL AND HIGH-LEVEL MOSAICISM IN SPORADIC RETINOBLASTOMA: GENOTYPE-PHENOTYPE RELATIONSHIPS

Carlos Rodríguez-Martín[1,$], Cristina Robledo[1,$], Gema Gómez-Mariano[2,$], Sara Monzón[3], Ana Sastre[4], Jose Abelairas[4], Constantino Sabado[5], Martín-Begué N[6], Joan Carles Ferreres[7], Ana Fernández-Teijeiro[8], Ricardo González-Campora[9], María José Rios-Moreno[9], Ángel Zaballos[10], Isabel Cuesta[3], Beatriz-Martínez-Delgado[2,11], Manuel Posada[2,11] and Javier Alonso[1,11,*]

[1] Unidad de Tumores Sólidos Infantiles. Instituto de Investigación de Enfermedades Raras. Instituto de Salud Carlos III. Majadahonda, Madrid, Spain.

[2] Instituto de Investigación de Enfermedades Raras. Instituto de Salud Carlos III. Majadahonda, Madrid, Spain.

[3] Bioinformatics Unit, Core Scientific and Technical Units, Instituto de Salud Carlos III. Majadahonda, Madrid, Spain.

[4] University Hospital La Paz. Madrid, Spain

[5] Pediatric Oncohematology Deparment. Vall d'Hebron Hospital. Barcelona, Spain.

[6] Pediatric Ophthalmology Department. Vall d'Hebron Hospital. Barcelona, Spain.

[7] Parc Taulí Hospital Universitari. Institut d'Investigació i Innovació Parc Taulí I3PT. Universitat Autònoma de Barcelona. Sabadell, Spain

[8] Pediatric Onco-Hematology Unit. Hospital Universitario Virgen Macarena, Sevilla, Spain

[9] Department of Anatomic Pathology. Hospital Universitario Virgen Macarena, Sevilla, Spain

[10] Genomics Unit, Core Scientific and Technical Units, Instituto de Salud Carlos III. Majadahonda, Madrid, Spain.

[11] Centro de Investigación Biomédica en Red de Enfermedades Raras, Instituto de Salud Carlos III (CB06/07/1009; CIBERER-ISCIII), Spain.

[$] These authors contributed equally to this work

*** Corresponding author:**

Javier Alonso

Unidad de Tumores Sólidos Infantiles.

Instituto de Investigación de Enfermedades Raras.

Instituto de Salud Carlos III.

Ctra. Majadahonda-Pozuelo Km 2,

Majadahonda, 28220 Madrid

Spain

e-mail: fjalonso@isciii.es

Conflict of interest statement

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ABSTRACT

Somatic mutational mosaicism is a common feature of monogenic genetic disorders, particularly in diseases such as retinoblastoma, with high rates of *de novo* mutations. The detection and quantification of mosaicism is particularly relevant in these diseases, since it has important implications for genetic counseling, patient management and probably also on disease onset and progression. In order to assess the rate of somatic mosaicism (high and low-level mosaicism) in sporadic retinoblastoma patients, we analyzed a cohort of 153 patients with sporadic retinoblastoma using ultra-deep next generation sequencing. High level mosaicism was detected in 14 out of 100 (14%) bilateral patients and in 11 out of 29 (38%) unilateral patients in whom conventional Sanger sequencing identified a pathogenic mutation in blood DNA. In addition, low level mosaicism was detected in 3 out of 16 (19%) unilateral patients in whom conventional screening was negative in blood DNA. Our results also reveal that mosaicism was associated to delayed retinoblastoma onset particularly in unilateral patients. Finally we compared the level of mosaicism in different tissues to identify the best DNA source to identify mosaicism in retinoblastoma patients. In light of these results we recommended analyzing the mosaic status in all retinoblastoma patients using accurate techniques such as next generation sequencing, even in those cases in which conventional Sanger sequencing identified a pathogenic mutation in blood DNA. Our results suggest that a significant proportion of those cases are truly mosaics that could have been overlooked. This information should be taking into consideration in the management and genetic counseling of retinoblastoma patients and families.

INTRODUCTION

Retinoblastoma (RB, OMIM 180200, ORPHA 790) is the most frequent intraocular cancer in children with an incidence of approximately 1 in 20,000 live births. Retinoblastoma is the prototype of hereditary cancer caused by biallelic inactivation of a tumor suppressor gene. Thus, inactivation of the retinoblastoma tumor susceptibility gene *RB1* (HGNC 9884, LRG 517) at both loci is the cause of the disease[1-3]

In approximately 60% of retinoblastoma cases, inactivating mutations in both alleles occur in one retinoblast during retina development generating a sporadic unilateral retinoblastoma. Since *RB1* mutations are only present in the tumor cells (all of them derived from a unique progenitor with biallelic mutations), these patients are not at risk to have offspring with retinoblastoma. The other 40% of retinoblastoma patients are carrying one germline mutation in the *RB1* gene that predispose to retinoblastoma. The majority of these carriers will develop bilateral retinoblastoma usually in the first year of live, although some type of mutations have been associated with retinoblastoma of low penetrance (unilateral cases) and low expressivity (late retinoblastoma)[4-8]. Germline mutations, which are present in all cells of the body, can be inherited from one progenitor (familial retinoblastoma) or take place in a gamete during gametogenesis (sporadic retinoblastoma). Retinoblastoma is characterized by a very high incidence of sporadic cases. Almost 80% of all newly diagnosed bilateral RB cases are sporadic without a family history of the disease, and thus it is thought that they are caused by *de novo* germline mutations in the *RB1* gene. Most sporadic cases of retinoblastoma harboring germline mutations

occur as a consequence of *RB1* mutations in the male gametes during spermatogenesis[9].

Somatic mutational mosaicism is a common feature of monogenic genetic disorders, particularly in diseases like retinoblastoma, with high rates of de novo mutations[10,11]. Somatic mosaicism occurs when a mutation appears in a postzygotic, early embryonic cell, and thus the mutation is not distributed evenly among the different tissues of the body. Since the grade of mosaicism may be variable it can affect to various organs and tissues, including for example the retina, lymphocytes or even the gonads. The extent of mosaicism, particularly whether or not it affects the gonads, has important implications for genetic counselling[11,12].

The detection of somatic mosaicism is challenging. Usually, genetic testing has been focused on the analysis of constitutional DNA isolated from blood cells using Sanger sequencing which has a limited sensitivity to detect mutations below 15% of alternative allele[13]. Since this threshold is quite high, a significant number of cases with low-level mosaicism can remain undetectable. In addition, because Sanger sequencing is a semi-quantitative technique, exact quantification of the allele mutant frequency is inaccurate, making the distinction between high-level mosaicism and true-heterozygotes challenging.

Highly sensitive mutation detection methods such as allele specific-PCR (AS-PCR) have been used to identify low-level mosaicism disease-causing mutations not detectable by standard sequencing techniques in

retinoblastoma[14]. However, these methods are usually limited to study the most frequent mutations, and could not be extended to an unbiased screen of all mutations described in a gene, particularly in the cases, like retinoblastoma, where the number of unique mutations is particularly high[4].

The introduction of next generation sequencing technology, which allows to reach high levels of sensitivity and specificity and to quantify the exact number of molecules and the exact percentage of variants, has offered an excellent opportunity to detect mosaicism in different diseases[15-17], including retinoblastoma[18-22]. In this work we applied ultra-deep next generation sequencing to detect high-level and low-level mosaicism in a cohort of 153 sporadic retinoblastoma patients. Our study provides valuable information about the incidence of somatic mosaicism in sporadic retinoblastoma and their correlation with clinical parameters.

MATERIAL AND METHODS

Editorial Policies and Ethical Considerations

This project was approved by the institutional ethics committee of the Instituto de Salud Carlos III (PI 17_2012-v2) and written consent were obtained from all patients.

Patients and samples

153 patients with sporadic retinoblastoma diagnosis were included in this study: 46 unilateral and 107 bilateral. In addition, we analyzed 36 retinoblastoma patients carrying a mutation inherited from a progenitor (second generation retinoblastoma) to calculate the experimental range of true-heterozygosity ratio. Patients were remitted to our genetic laboratory from January 1998 to December 2015. To identify mutations in the *RB1* gene, all patients were studied according the scheme implemented in our laboratory that includes analysis of blood DNA by Sanger sequencing of all exons and exon/intron boundaries to detect nucleotide substitutions and small insertion/deletions and MLPA (MLPA kit #P047, MRC-Holland, Amsterdam, The Netherland) to detect gross deletions involving part or all *RB1* gene[5,23]. When tumors were available after therapeutic enucleation, similar analysis were carried out to characterize the mutations present in the tumor. In these cases, STR analysis was also used to confirm gross deletions and loss of heterozygosity (LOH) in tumors as previously reported[23]. Figure S1 (supplementary information) shows the distribution of all patients studied in our laboratory from January 1998 to December 2015, indicating form of presentation, molecular screening carried

out, results obtained in each step and patients studied by ultradeep NGS in this work with indication of the result table associated.

Next generation sequencing and bioinformatics analysis

RB1 exons were amplified separately using the same PCR conditions used for standard genetic analyses except that Phusion High-Fidelity Polymerase was used (ThermoFisher Scientific, Waltham, MA, USA). In all cases, two independent amplicons for each mutation analyzed were generated and sequenced. Amplicon size ranged from 167 bp to 472 bp (mean size 324 bp) in order to satisfy sequencing requirements. Amplicons from different *RB1* exons were indexed according to manufacturer instructions (New England Biolabs, Ipswich, MA, USA) and pooled. Sequencing was performed in a MiSeq sequencer (Illumina, San Diego, CA, USA). Amplicons with less than 1,000 reads were discarded, which corresponded to 7 bilateral and 1 unilateral patients. Thus, a total of 145 sporadic patients were available for analysis (Supplementary Figure S1). Depth of coverage per amplicon ranged from 1,118x to 200,061x.

Quality control of fastq files was performed with FastQC software v. 0.11.3 and filtered with NGSQCtookit v2.2.3 keeping reads in which 70% of nucleotides had PhredQ values greater than Q20. Reads were aligned to the genomic *RB1* reference sequence L11910.1 using BWA-MEM aligner v.0.7.12. Aligned reads were processed with Samtools mpileup v1.2. SNP and indels counts per position were extracted parsing pileup format to a tab delimited file using in-

house scripts. Alternative allele frequencies, as well as forward and reverse percentages, were calculated with an in-house R package.

Statistical analysis

For a single comparison of two groups, two-tailed Student's T-test was used. For proportions, Fisher's exact test was used. For all analyses, the level of significance was set at P=0.05. All statistical calculations were performed using the GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

The aim of the present study was to determine the frequency of high- and low-level mosaicism in a large cohort of 153 patients with sporadic retinoblastoma. In all these patients, previous analysis was able to identify pathogenic mutations in blood or tumor DNA using conventional screening strategies such as Sanger sequencing (Supplementary Table S1). However, standard Sanger sequencing could overlook several cases of mosaicism. Thus, our objective was the identification of mosaic patients using targeted ultra-deep next generation sequencing to determine accurately the proportion of reference and alternative allele in the DNA samples analyzed.

Determining the upper threshold defining true-heterozygosity

First of all, we determined the range of heterozygosity values that we could consider true heterozygosity. Genetic variants or mutations that are expected to be heterozygous should show a reference:alternative allele ratio very closed to 0.5 (i.e. a theoretical 50:50 proportion). However, it is possible to find small variations in this perfect ratio due to technical variability at different levels (PCR, sequencing, read mapping, variant caller, etc.). In order to define the range of heterozygosity ratios that can be considered true heterozygosity, we analyzed a series of second generation, hereditary retinoblastoma patients, which were thus expected to be carriers of the pathogenic mutation in true heterozygosity in blood DNA. A total of 36 patients from 36 independent families (36 different mutations, 12 indels, 24 point mutations) were analyzed by ultra-deep sequencing (mean depth 109,194x, range 4,049x-200,061x). As shown in Figure 1A and supplementary Table S1, the mean heterozygosity ratio in this

group of true-heterozygous patients was 0.496±0.019 (mean±standard deviation, range 0.458-0.528), indicating that although some degree of variability was observed, the mean heterozygosity ratio observed was not statistical significant in comparison with the theoretical 0.5 expected value (*P*=0.1929, one sample T Test). In order to stablish a conservative upper threshold for high level mosaicism we stablished that mutations below 0.439 heterozygosity ratio (that is, mean minus three standard deviations) represent mosaic patients.

Mosaicism in patients in which a mutation was detected in blood DNA using Sanger sequencing (High level mosaicism)

Next, we used ultra-deep NGS to quantify the heterozygosity ratio present in the constitutional DNA extracted from patients with sporadic bilateral and unilateral retinoblastoma in which a mutation was previously detected in blood DNA using standard Sanger sequencing. We analyzed by ultra-deep sequencing a total of 107 patients with sporadic bilateral retinoblastoma and 30 patients with sporadic unilateral retinoblastoma. After ultra-deep NGS, 7 bilateral and 1 unilateral patients were discarded by present low quality/coverage data. Consequently, 100 bilateral and 29 unilateral patients were available for analysis (Figure S1, Tables S2 and S3).

In the sporadic bilateral retinoblastoma group (Figure 1B and supplementary Table S2), 14 out of 100 patients (14.0%) presented a heterozygosity ratio below 0.439, thus representing mosaic patients according our previously stablished threshold. The heterozygosity ratio in these mosaic patients ranged from 0.241 to 0.431.

In the sporadic unilateral retinoblastoma group (Figure 1C and supplementary Table S3), 11 out of 29 patients (37.9%) presented a heterozygosity ratio below 0.439 and thus these patients were considered mosaics. The heterozygosity ratio in this group of mosaic patients ranged from 0.134 to 0.433.

Mosaicism in patients in which two mutations were identified in tumor DNA but neither of them was detected in blood DNA using Sanger sequencing (Low level mosaicism)

Genetic analysis of the tumor can be helpful when screening for mutations in the RB1 gene in the blood DNA resulted negative. When the two inactivating mutations in both alleles are identified in the tumor, it is possible to rule out or confirm the existence of these *RB1* mutations at the constitutional level. However, conventional sequencing is not sensitive enough to detect mutations in low proportion below heterozygosity ratios of 0.10-0.15 (i.e. low-level mosaicism), which however can be achieved accurately with ultra-deep next generation sequencing. To avoid any bias associated with the non-identification of one of the two *RB1* biallelic mutations, only patients in whom both mutations were identified in the tumor and in which at least one of them was a point mutation or indels were included in this part of the study.

A total of 16 patients met the criteria described above. All of these patients developed a sporadic unilateral retinoblastoma. In 12 of them, the first oncogenic event (M1) was a deleterious point mutation or indels in the *RB1* gene while the second hit (M2) consisted in the loss of the normal allele and duplication of the allele mutated (i.e. Loss Of Heterozygosity, LOH). In the

remaining 4 patients, both M1 and M2 hits consisted of point mutations. In the Figure 1D (and supplementary Table S4), it is shown the results obtained after ultra-deep sequencing of all exons harboring an oncogenic *RB1* mutation (mean depth 69,046x, range 28,187x-122,369). In agreement with the fact that no mutation could be detected by Sanger sequencing in blood DNA, no patients showed a heterozygosity ratio above 0.1-0.15, which can be considered an acceptable threshold for the detection of variants by experienced researchers using conventional Sanger sequencing. We considered low-level mosaicism those samples in which heterozygosity ratios ranged from 0.01 to 0.10. The upper limit is based on the 0.10 limit for the detection of mutations by Sanger sequencing while the lower limit is based in the unspecific background that we consistently observed across samples. Three patients had heterozygosity ratios above 0.01 for one of the mutations identified in the tumor. In the remaining 13 patients all mutations analyzed showed heterozygosity ratios below 0.001, indicating that in these blood samples no mutant allele could be detected. Consequently, 3 out of 16 patients (18.75 %) in this group were considered to harbor low-level mosaicism mutations according the specified criteria.

Distribution of mosaicism in body tissues

It is important to determine if the percentage of mosaicism is similar in different tissues or by contrast there are significant differences among them. This information can be useful to determine what tissues can be more relevant to identify mutations in low proportion. Thus, we analyzed by ultra-deep sequencing, DNA isolated from representative tissues of the three embryonic layers that can be obtained safely and painlessly from patients: hair (ectoderm),

urine (endoderm/mesoderm), oral mucosa (ectoderm), saliva (mesoderm), and blood (mesoderm).

We analyzed representative tissues from 7 patients with high level mosaicism (range heterozygosity ratio 0.139-0.365) and 2 patients with low-level mosaicism (heterozygosity ratios 0.054 and 0.086). Moreover, we analyzed 2 patients harboring a true-heterozygous *RB1* mutation in blood DNA that were here used as controls to assess the quality of the DNA isolated from the different tissues. The mutations present in these control patients were also present in two of the patients from the high-level mosaic group. As shown in Figure 2, the heterozygosity ratio of the mutant allele in the various tissues analyzed showed some differences that were particularly relevant in some specific cases. Generally, DNA isolated from blood samples showed the highest heterozygosity ratio. In only two patients out of 9, DNA isolated from other tissue, in particular hair, showed the highest values. By the other side, DNA isolated from oral mucosa showed the lowest heterozygosity ratio in most patients. As expected, the largest differences among tissues were observed in the group of high-level mosaicism. In one case these differences were especially remarkable, ranging from 0.053 in DNA isolated from oral mucosa to 0.365 in DNA isolated from blood. To rule out possible technical artefact related with the source of DNA, we analyzed two independent, true-heterozygous mutations, in two second generation retinoblastoma patients. As expected, heterozygosity ratios were closed to 0.5 and very similar among the different tissues analyzed.

Mosaicism, retinoblastoma phenotype and age at diagnosis

In summary, we detected high level mosaicism in 14% of patients with sporadic bilateral RB (14 out of 100) and in 37.9% of patients with sporadic unilateral RB (11 out of 29) in whch a mutation was previously identified by Sanger sequencing in blood DNA. In addition, low-level mosaicism was observed in 18.7% of patients with sporadic unilateral RB (3 out of 16) in which no mutation could be detected by Sanger sequencing in blood DNA. Taken together, mosaicism was detected in 19.3% of all RB cases analyzed (28 out of 145). As showed in Figure 3A, there was differences statistically significant between the percentage of mosaicism observed in sporadic bilateral and sporadic unilateral patients with high-level mosaicism (*P*=0.0071, Fisher's exact test).

Next, we analyzed if there was any relationship between the age at diagnosis and the presence of mosaicism (high or low) or true heterozygosity in each group of patients. As shown in Figure 3B, there was not differences between the age at diagnosis in the group of bilateral patients with high-level mosaicism or true heterozygosity (10.44±8.44 months (n=9) vs 10.71±12.01 months (n=76) respectively, mean±SD). By contrast, the age at diagnosis was higher in unilateral cases with high level mosaicism when compared with unilateral patients with true-heterozygous mutations and this difference was nearly significant (18.27±11.11 months (n=11) vs 10.47±8.82 months (n=15) respectively, mean±SD, *P*=0.0571, T-test). There was no differences in the age at diagnosis between unilateral cases with low level mosaicism and no mutation in blood DNA, although in this case the number of cases in the group of low

level mosaicism was very small (27.67±8.02 months (n=3) vs 24.17±11.86 months (n=12) respectively, mean±SD).

Finally, we analyzed the differences in the age at diagnosis among the three group of patients: i) bilateral patients in which a mutation was detected by Sanger sequencing in blood DNA (including high-level mosaicism and true heterozygous), ii) unilateral patients in which a mutation was detected by Sanger sequencing in blood DNA (including high level mosaicism and true heterozygous) and iii) unilateral patients in which no mutation was detected by Sanger sequencing in blood DNA (thus including low level mosaicism cases and cases with no mutation) (Figure 3C). There was no differences between bilateral and unilateral cases in which a mutation was detected by Sanger sequencing in blood DNA (10.68±11.64 months (n=85) vs 13.77±10.41 months (n=26) respectively, mean±SD). However, there was differences highly significant between the group of unilateral cases in which no mutation was detected by Sanger sequencing in blood (24.87±11.04 months, n=15, mean±SD) and the other two groups of patients in which a mutation was detected by Sanger sequencing in blood DNA (P=0.0026 and P=0.0001 vs unilateral and bilateral groups respectively).

DISCUSSION

This study is a comprehensive re-analysis of a large cohort of 153 consecutive retinoblastoma patients using next generation sequencing. In all of these patients, pathogenic mutations were previously identified in blood or tumor DNA using standard screening strategies (Sanger sequencing, MLPA, etc..). We detect high level mosaicism in 14% of sporadic bilateral and 38% of sporadic unilateral patients in whom a pathogenic mutation was detected in blood DNA by Sanger sequencing. In addition, mosaicism was detected in 19% of sporadic unilateral patients in which no mutation was detected by Sanger sequencing in blood DNA. Our study suggests that a significant percentage of retinoblastoma patients are actually mosaics that were overlooked by the use of less sensitive techniques. This finding has important consequences in the management of patients and families, since these can benefit from the new information provided by these studies.

The first systematic study designed to assess the frequency of mosaicism in RB patients was performed by Sippel et al.[11]. In that study, mosaicism was estimated to occur in 10% of patients. However, that study was conducted before the next generation sequencing era and thus it is not surprising that as new techniques of massive sequencing have been incorporated higher percentage of mosaicism have been observed. For example, Chen et al.,[18] analyzed a group of sporadic unilateral patients that were negatives by Sanger sequencing. They observed low level mosaicism in only 4 out of 70 patients analyzed (6%). By contrast, we have observed low level mosaicism in 3 out of 16 unilateral patients (19%) in which no mutations were detected by Sanger sequencing in blood DNA, but in which mutations were identified in tumor DNA.

The information that we obtained from tumors, allow us to analyze specific mutations by ultradeep sequencing in blood, which may explain why we found a higher percentage of mosaicism in this group of patients. In any case, the differences observed may also be due to the differences in the number of patients studied. In addition, Chen et al.,[18] detected low level mosaicism in 6 out of 20 patients with bilateral retinoblastoma (30%), where Sanger sequencing excluded the presence of mutations. However, it should be taken in account that the group of bilateral patients studied in Chen et al., is not comparable to the group studied by us. While Chen et al., analyzed 20 sporadic retinoblastoma patients in which standard screening resulted negative, we analyzed 107 sporadic retinoblastoma patients in which Sanger sequencing identified previously a pathogenic mutation in blood DNA. These differences explain the differences observed between both studies. Thus, the study carried out by Chen et al., is a good estimate of the percentage of low level mosaicism that can be found in bilateral patients, while our study provides a good estimate of the percentage of high level mosaicism observed in these bilateral patients. In other study, Amitrano et al.,[19] re-analyzed by NGS 9 bilateral and 1 unilateral patients previously classified as heterozygotes after Sanger sequencing. They observed high level mosaicism in 2 out of 9 bilateral (22%) patients and in the only unilateral patient studied. In our case, we observed high level mosaicism in 14% (14/100) and 38% (11/29) of bilateral and unilateral patients, respectively. The differences observed in the percentage of mosaic cases among the different studies can be explained by the different sizes of the cohorts studied.

In our study, we have observed that high level mosaicism was significant more frequent in unilateral (38%) than in bilateral (14%) patients in which standard

Sanger sequencing proved the existence of a blood mutation. The biological interpretation of this observation is unknown although it is tempting to speculate that in high level mosaicism the number of retinal cells with one RB1 mutation will be lower than in true heterozygosity cases, making more infrequent the appearance of the one second hit in cells with mono-allelic inactivating mutation. This should result in a lower probability of arising cells with biallelic inactivation of the *RB1* gene. In addition, this observation suggests that patients with high level mosaicism will develop unilateral retinoblastoma more likely, which is an important information to consider during genetic is counseling. Our results also show a relationship between the level of mosaicism and the age at diagnosis, particularly in unilateral cases. Thus, age at diagnosis was significant higher in unilateral cases with low-level mosaicism or no mutation than in the group of unilateral retinoblastoma patients with high level mosaicism or true heterozygous mutations. This information could also be relevant in the management of patients.

The most obvious importance of identifying mosaicism is its implication for genetic counseling. While true-heterozygous individuals have 50% probability to pass a mutation to offspring, the probability to transmit the mutation to progeny in mosaic individuals is more difficult to determine. Retinoblastoma families in which the first individual is a mosaic are rare, although several cases have been described[11,21,22]. In any case, the frequency of these cases is far the 15-30% that have been observed in sporadic cases. Although in mosaic patients we cannot accurately determine the risk of transmission to offspring, it seems logical to think, based on the empirical data accumulated, that a high level mosaic patient will have a significantly lower probability of transmitting the

mutation to offspring than a true-heterozygous patient. At the moment, there is not enough data that permit to correlate the percentage of mutant allele detected in blood DNA with the presence of a mutation in germ cells, and thus, only analysis of germ cells, when possible, could provide additional information about the empirical risk of transmitting a mutation to offspring in high level mosaic patients.

Since Sanger sequencing will not allow to distinguish between high level mosaicism and true heterozygosity in many cases, ultra-deep NGS will be necessary to determine accurately the existence or not of high level mosaicism in the patient. Identification of high level mosaicism is important for genetic counselling of progenitors, because mosaic individuals cannot have inherited the mutant allele from their parents and thus these progenitors are not at risk to have offspring with mutations. Identification of mosaicism implicates that it is not necessary to perform genetic testing in the brothers and sisters of the affected member of the family with the consequent benefit from all family members.

Other question that arises from our study is the question about which is the best source of genomic DNA to evaluate somatic mosaicism. Our results indicate that in most patients (7 out of 9) the highest percentages of heterozygosity were observed in DNA isolated from blood, while that DNA isolated form oral mucosa showed in general the lowest heterozygosity values. These differences cannot be attributed to experimental artefacts or differences in DNA quality, since the analysis of two true-heterozygous mutations displayed very similar heterozygosity values among the five different DNA sources analyzed. Although more patients should be analyzed to reach a solid recommendation, we propose that the best way to assess mosaicism can include blood and oral

mucosa DNA, which seem to cover all possible ranges of heterozygosity and moreover, both of these samples are of easy and painless extraction. However, it must be taken into account that in some cases, probably very infrequently, germline mosaicism could be present in one of the progenitors without affecting blood DNA (see for example[11,24]. Thus, the absence of mutations in blood DNA using even very sensitive techniques cannot rule out the possibility that one of the progenitors was a mosaic of the germline.

The effect of mosaicism in the *RB1* gene on the predisposition to develop other tumors in adulthood is absolutely unknown. Although retinoblastoma protein plays a key role in the regulation of cell proliferation by controlling the G1/S checkpoint and it is inactivated by different ways in roughly all cancers, germinal mutations in the *RB1* gene predispose to only a few rare cancers, such as osteosarcoma, leiomyosarcoma, leukemia or skin cancer, besides retinoblastoma[25-28]. We hypothesized that the extent of somatic mosaicism could modify the risk to develop determined types of cancer in the adulthood. The results showed in this article are insufficient to test this hypothesis, but it will be interesting to evaluate the occurrence of second tumors in larger cohorts of mosaic patients and larger follow-up. As massive sequencing technologies are being incorporated to the genetic diagnostic laboratories, more cases of mosaicism will be identified. Considering the high sensibility of these technologies, it will be more and more important to define the thresholds that are critical to define low-level and high-level mosaicism. These thresholds should probably be defined in each particular laboratory because technical conditions and bioinformatics analysis can differ from one to another.

In summary, mosaicism is frequent in retinoblastoma patients, particularly in the group of patients with sporadic unilateral disease. Our study also shows that the level of mosaicism correlates with clinical parameters such as disease phenotype and age at diagnosis. In light of these results we recommended to analyze (and reanalyze) the mosaicism status in all retinoblastoma patients with mutation using accurate techniques such as next generation sequencing and take into consideration this information in the management and genetic counseling of retinoblastoma patients and families.

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CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest regarding the publication of this article.

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FIGURE LEGENDS

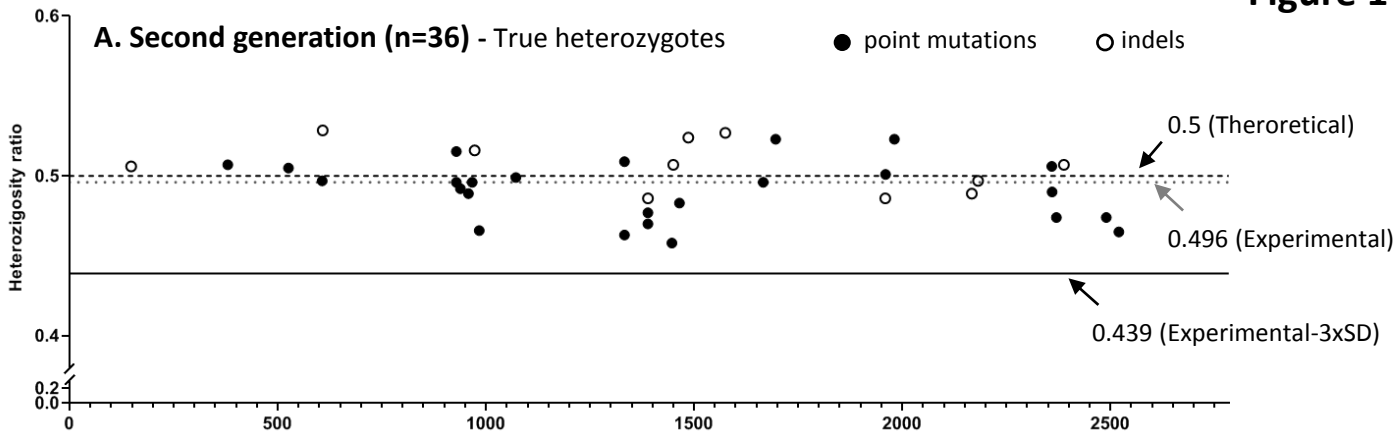
Figure 1. Heterozygosity ratios observed in each patient studied, represented by mutation position. Filled symbols represent point mutations. Open symbols represent indels. **A)** Group of true-heterozygous patients (second generation retinoblastoma) used to calculate the upper threshold for high level mosaicism. Theoretical, experimental and threshold (experimental-3xSD) values are shown. **B)** Heterozygosity ratios observed in sporadic bilateral retinoblastoma patients harboring a mutation detected by conventional sequencing in blood DNA. **C)** Heterozygosity ratios observed in sporadic unilateral retinoblastoma patients, in which a RB1 mutation was identified by conventional Sanger sequencing in blood DNA. **D)** Heterozygosity ratios observed in sporadic unilateral retinoblastoma patients in which two RB1 mutations were identified in tumor DNA but none of them in blood DNA by conventional Sanger sequencing, In these patients, point mutations or indels detected in tumors were analyzed by ultra-deep sequencing in blood DNA. In all graphs, each point represents the heterozygosity ratio observed in the blood DNA sample analyzed (mean of two independent PCR and sequencing reactions per patient).

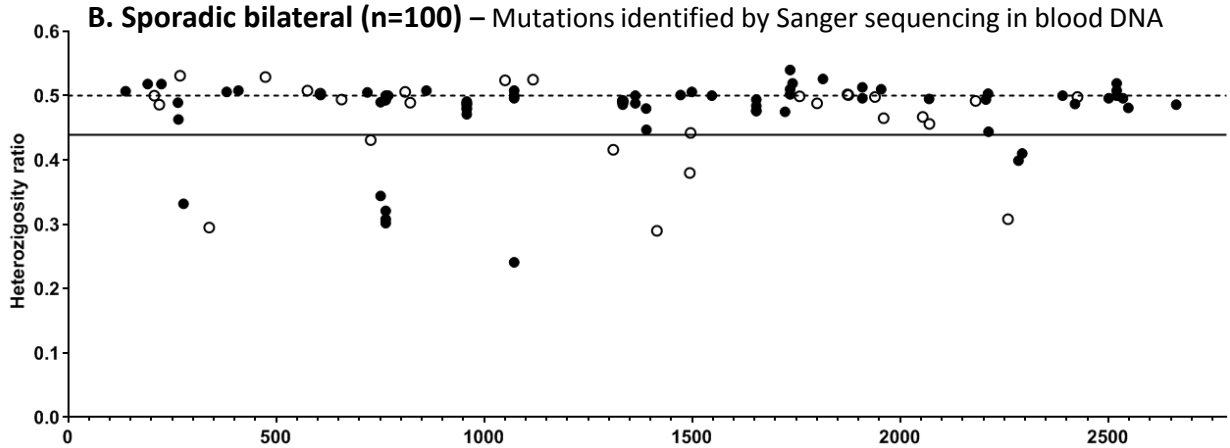
Figure 2. Heterozygosity ratios observed in isolated DNA from five different tissues (blood, saliva, oral mucosa, urine and hair) in a group of patients with high-level mosaicism and low-level mosaicism. Two true heterozygous patients (patients 25 and 33) harboring the same mutations that two patients with high-level mosaicism (patients 142 and 139 respectively) were included as control to discard any possible artefact related with the source of DNA. Each point

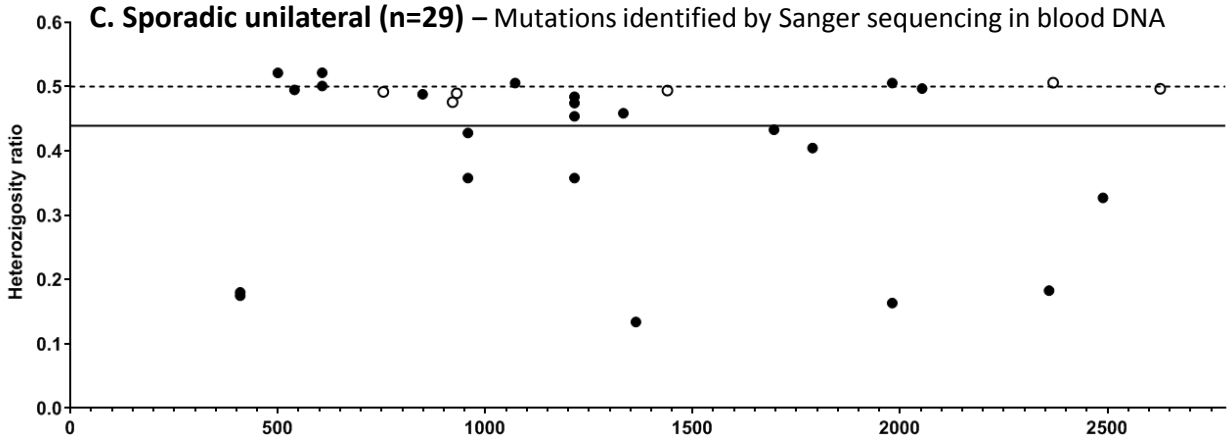
represents the heterozygosity ratio observed in each DNA sample analyzed (mean of two independent PCR and sequencing reactions per tissue and patient).

Figure 3. Genotype-phenotype relationship. **A)** Percentage of mosaicism observed in the different groups of patients studied. Differences statistical significant were observed between sporadic bilateral and sporadic unilateral patients with high-level mosaicism (Fisher´s exact test). **B)** Relationship between age at diagnosis and presence of mosaicism in each group of patients (Student's T test). **C)** Relationship between age at diagnosis and presence of high-level mosaicism/heterozygous and low-level mosaicism/no mutation. Age at diagnosis in the group of sporadic unilateral retinoblastoma with low-level mosaicism/no mutation was significant higher when compared to the groups of patients with high-level mosaicism/heterozygous (Student´s T test).

Figure 1


A. Second generation (n=36) - True heterozygotes
point mutations
indels
0.5 (Theroretical)
0.496 (Experimental)
0.439 (Experimental-3xSD)
Heterozigosity ratio


B. Sporadic bilateral (n=100) – Mutations identified by Sanger sequencing in blood DNA
Heterozigosity ratio


C. Sporadic unilateral (n=29) – Mutations identified by Sanger sequencing in blood DNA
Heterozigosity ratio

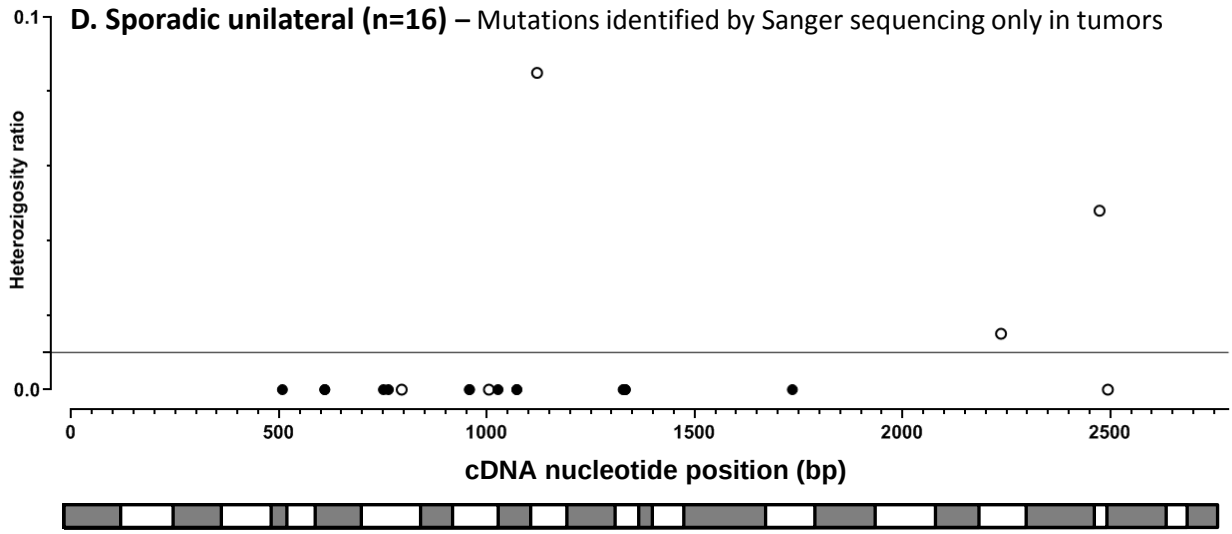

D. Sporadic unilateral (n=16) – Mutations identified by Sanger sequencing only in tumors
Heterozigosity ratio
cDNA nucleotide position (bp)
RB1 exons

Figure 2

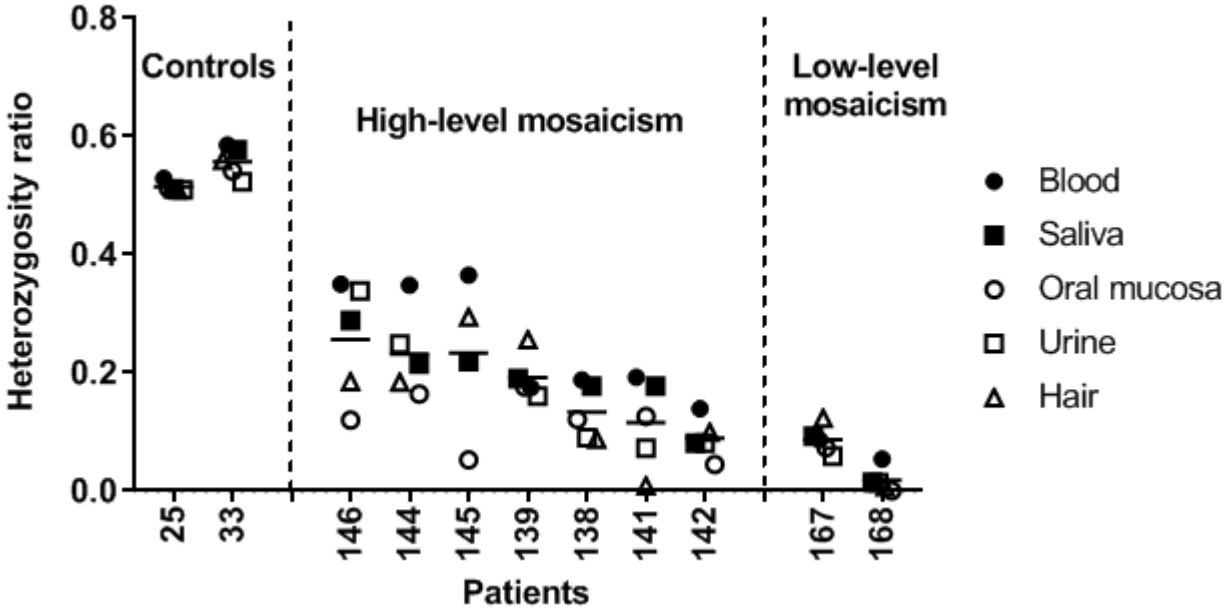

Heterozygosity ratio
0.8
0.6
0.4
0.2
0.0
Controls
High-level mosaicism
Low-level mosaicism
25
33
146
144
145
139
138
141
142
167
168
Patients
Blood
Saliva
Oral mucosa
Urine
Hair

Figure 3

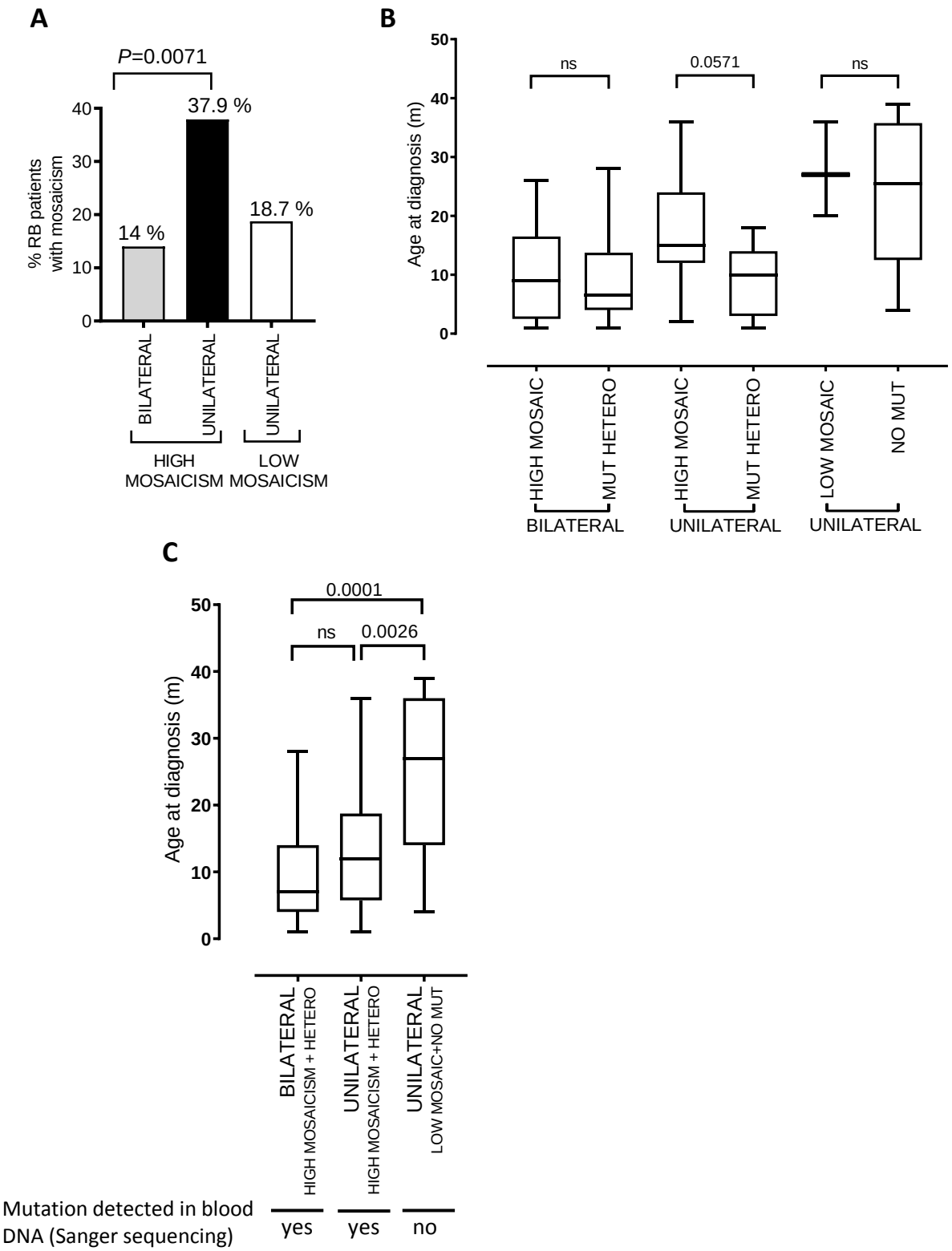

A
P=0.0071
37.9 %
14 %
18.7 %
% RB patients with mosaicism
BILATERAL
UNILATERAL
UNILATERAL
HIGH MOSAICISM
LOW MOSAICISM
B
ns
0.0571
ns
Age at diagnosis (m)
HIGH MOSAIC
MUT HETERO
HIGH MOSAIC
MUT HETERO
LOW MOSAIC
NO MUT
BILATERAL
UNILATERAL
UNILATERAL
C
0.0001
ns
0.0026
Age at diagnosis (m)
BILATERAL
HIGH MOSAICISM + HETERO
UNILATERAL
HIGH MOSAICISM + HETERO
UNILATERAL
LOW MOSAIC+NO MUT
Mutation detected in blood DNA (Sanger sequencing)
yes
yes
no

Figure S1

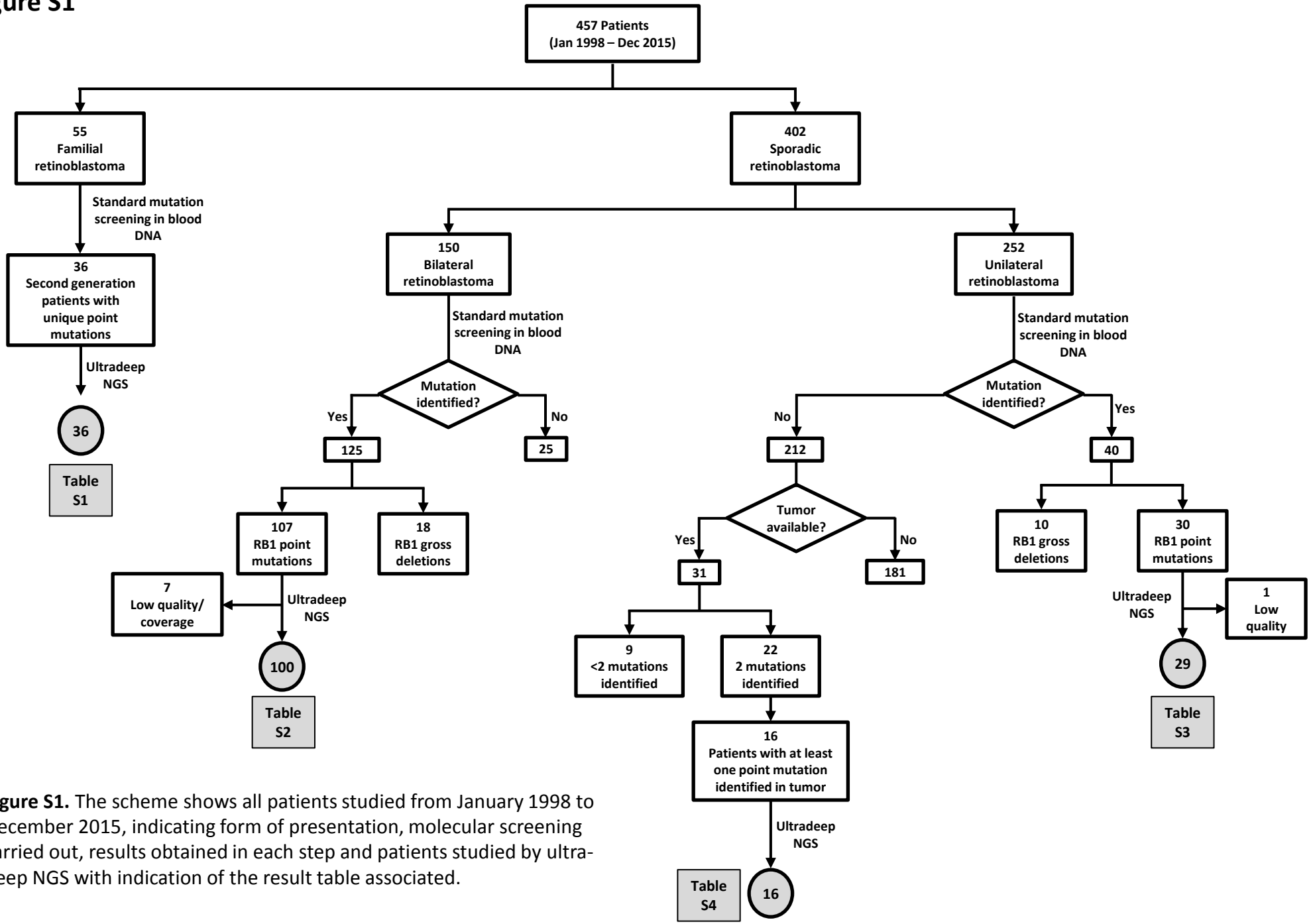


Figure S1. The scheme shows all patients studied from January 1998 to December 2015, indicating form of presentation, molecular screening carried out, results obtained in each step and patients studied by ultra-deep NGS with indication of the result table associated.