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## Correlation between *RB1* germline mutations and second primary malignancies in hereditary retinoblastoma patients treated with external beam radiotherapy

Amélie Chaussade<sup>1</sup>, Gaël Millot<sup>2</sup>, Constance Wells<sup>3</sup>, Hervé Brisse<sup>4</sup>, Marick Laé<sup>5</sup>, Alexia Savignoni<sup>6</sup>, Laurence Desjardins<sup>7</sup>, Rémi Dendale<sup>8</sup>, François Doz<sup>1,9</sup>, Isabelle Aerts<sup>1</sup>, Irène Jimenez<sup>1</sup>, Nathalie Cassoux<sup>7,9</sup>, Dominique Stoppa Lyonnet<sup>3,9,10</sup>, Marion Gauthier Villars<sup>3</sup> and Claude Houdayer<sup>3,9,10\*</sup>

1 Département d'oncologie pédiatrique, adolescents jeunes adultes, Institut Curie

2 UMR 3244, centre de recherche de l'Institut Curie, Paris, France

3 Pôle de Biopathologie, Service de Génétique, Institut Curie, Paris, France

4 Département d'Imagerie Médicale, Institut Curie, Paris, France

5 Pôle de Biopathologie, Service de Pathologie, Institut Curie, Paris, France

6 Département de Biostatistiques, Institut Curie, Paris, France

7 Département d'oncologie chirurgicale, service d'Ophthalmologie, Institut Curie, Paris , France

8 Radiation Oncology Department,, Institut Curie, Paris, France

9 Université Paris Descartes, Paris, France

10 INSERM U830, centre de recherche de l'Institut Curie, Paris, France

\*Corresponding author:

Claude Houdayer

e-mail: [claud.houdayer@curie.fr](mailto:claud.houdayer@curie.fr)

Retinoblastoma (Rb) results from biallelic inactivation of the *RB1* gene. Hereditary Rb patients *i.e* germline carriers of a *RB1* mutation also have a risk of developing subsequent malignant neoplasms (SMN) such as osteosarcomas. This SMN risk is maximized by external beam radiotherapy treatments (EBRT), which is why these treatments are now avoided. Nevertheless, EBRT is still a matter of great concern, as EBRT-treated patients are in their adulthood and SMNs remain the major cause of death for patients.

To decipher the relationship between *RB1* genotype and SMN development in EBRT treated patients, we conducted a retrospective study in a cohort of 160 irradiated hereditary Rbs with fully resolved *RB1* mutational status. Median follow-up was 22 years [1-51] and median age of patients was 27 years old [7-53]. Among these 160 Rb patients, 120 did not develop any SMN (75%) and 40 developed SMNs (25%). The age at which EBRT is given (*i.e.* before or after the age of 12 months) was not correlated to SMN development ( $p=0.6$ ). We didn't find any difference in *RB1* mutation type between patients with or without SMN, neither could we detect any linkage between mutation type and SMN location, SMN type and age at diagnosis. Interestingly, among 13 carriers of a *RB1* low penetrance mutation, 3 of them developed sarcomas, a rare tumor that cannot be attributed to the general population.

Our study cannot explain why a *RB1* mutation leads or not to a SMN but demonstrated that EBRT patients with a low penetrance mutation remain at risk of SMN and should be cautiously monitored.

**Keywords:** external beam radiotherapy; hereditary retinoblastoma; germline mutation; second malignant neoplasm; genotype phenotype correlation

## INTRODUCTION

Retinoblastoma (Rb) [MIM 180200] is the most common pediatric intraocular neoplasm, with an incidence rate ranging approximately from 1/15000 to 1/20000 births. This malignant childhood tumor develops from the retina and results from biallelic inactivation of the *RB1* tumor suppressor gene. The *RB1* gene is located on chromosome band 13q14 and encodes a 105kDa tumor suppressor protein involved in the control of cell cycle regulation and many different cellular processes (Dyson, 2016).

Retinoblastoma can be unilateral *i.e.* one eye is affected (60% of cases) or bilateral *i.e.* both eyes are affected (40% of cases) and is mainly sporadic (90% of cases). In the remaining 10% there is a family history of Rb with at least 2 affected cases. It is widely admitted that all familial forms and bilateral cases carry a germline mutation and are therefore considered as “hereditary Rbs”. In hereditary Rbs, a *RB1* germline mutation is associated with a somatic mutation in a single cell of the retina that develops into a tumor. Importantly enough, germline carriers of a *RB1* mutation have a 90% risk of developing Rb (high penetrance) but also have a greater risk of developing subsequent malignant neoplasms (SMN), such as osteosarcomas, soft tissue sarcomas, melanomas and brain tumors, while survivors with nonhereditary Rb don’t have any increased risk of SMN (Kleinerman et al., 2005; Moll et al., 1997; Temming et al., 2017). Hence, the *RB1* germline mutation is a major risk factor for SMNs.

Up to the 80’s, treatments mainly consisted of surgery by enucleation, external beam radiotherapy (EBRT) and brachytherapy. It appeared that a combination of a *RB1* germline mutation plus external beam radiotherapy maximizes the risk of SMNs, especially in the irradiation field, with a 36% (95% CI: 31 to 41%) cumulative incidence for developing a new cancer at 50 years after diagnosis of Rb (Kleinerman et al., 2005; Temming et al., 2017). Of

note, the combination of chemotherapy plus EBRT showed a significantly increased risk of SMNs compared to treatment with EBRT alone (hazard ratio 1,31; 95% CI: 1.02 to 1.68) (Wong et al., 2014). For all these reasons, first-line treatments now avoid EBRT and mainly rely on systemic chemotherapy associated to a local treatment or enucleation for advanced stages thus leading to an increased survival rate above 95% in developed countries (Broaddus et al., 2009).

Nevertheless, external beam radiotherapy is still a matter of great concern for patients, as EBRT-treated patients are in their adulthood and SMNs remain the major cause of death for hereditary Rb patients (Kleinerman et al., 2012). Unfortunately, the reason why some hereditary Rb patients who received EBRT develop SMNs while others did not remains an open question. One previous study addressed the relationship between *RBI* genotype and SMN risk in 199 survivors of hereditary Rb and suggested an increased risk for carriers of a specific nonsense mutation (Dommering et al., 2012), a finding not confirmed in another following study on 488 Rb patients (Temming et al., 2015). However, in both studies, only a fraction of patients received EBRT. To fill this gap, we designed a study that focuses on genotype / SMN relationship in a cohort of 160 irradiated hereditary Rbs with fully resolved mutational status. This large-scale study provides marked homogeneity in the clinical and genetic management of EBRT-treated patients, as all patients were exclusively identified and followed at the same centre, thereby offering reliable clinical and biological information.

## PATIENTS AND METHODS

### Patients

*Institut Curie* is a reference center for Rb management in France. Patients with suspected Rb are referred to the Institute throughout France to confirm the diagnosis and continue medical care. Initial diagnosis is made by fundus examination and further confirmed by histologic criteria when treatment involves enucleation. The subsequent malignant neoplasm diagnosis is confirmed by histologic analyses on the tumor biopsy or by a radiologist for the brain tumors' diagnosis. Pediatric counseling is provided after the initial diagnosis to align on the severity of the disease and to start the treatment. The medical treatment decision is made by a multi-disciplinary team, which typically includes pediatricians, ophthalmologists and pathologists. All Rb patients are offered genetic counseling and mutational analysis of the *RBI* gene in constitutional and tumor DNA. Among all Rb patients diagnosed between 1965 and 2011 in the Institute, we identified 165 Rb patients with a germline *RBI* mutation who received external beam radiotherapy. All these patients are being followed from their initial diagnosis of Rb. To properly study the genotype/SMN correlation, 5 putative mosaic carriers *i.e.* unilateral non familial cases were excluded from analysis. As a result, our cohort was made of 160 bilateral and/or familial cases (Figure 1).

Appropriate individual written consent for genetic analysis was obtained from all participating patients or their legal guardians and this study was approved by the GTT, *Groupe Thematique Transverse* “retinoblastoma” of the *Institut Curie*.

### Treatment

The administered treatment was the reference treatment at the time of the diagnosis *i.e.* external beam radiotherapy with a 45Gy tumor dose to the affected eye. The irradiation protocol depended on the need to irradiate 1 or 2 eyes. In the case of bilateral ocular

irradiation, two opposite photon fields (Cobalt or X Rays) with high energy were used. In the case of unilateral ocular irradiation, one lateral electron beam associated with one anterior electron beam were most often used. Chemotherapy treatment differed from patient to patient according to evolution. The different chemotherapy drugs used were notably anthracyclines, alkylating agents, platinum salts, and topoisomerase inhibitors.

### **Mutation analysis**

Germline mutational screening was performed by different approaches depending on the year of analysis *i.e.* a combination of Denaturing High Performance Liquid Chromatography, Sanger sequencing and Quantitative Multiplex PCR of Short fluorescent Fragments or Multiplex Ligation Probe Amplification analyses for point mutation and gross alteration screening, respectively (Dehainault et al., 2004; Houdayer et al., 2004). Next generation sequencing was used for more recent analyses (Collet et al., 2015).

Splice mutations were confirmed using RNA extracted from lymphoblastoid cell lines and following RT PCR with primers flanking the deleted exon(s). Puromycin treatment was also used in some cases to inhibit nonsense mediated decay (NMD), as previously described (Houdayer et al., 2008).

### **Penetrance definition**

Germline carriers usually develop bilateral or multifocal tumors. However, some rare families exhibit low penetrance and variable expressivity of the disease because bilaterally affected, unilaterally affected, and unaffected mutation carriers are known to coexist. Low penetrance mutations lead to a reduced amount of wild type pRb or a partially functional mutant pRb (Harbour, 2001). For this study and according to previous literature, promoter mutations, nonsense mutations occurring in exons 1 and 2, previously described missense mutations, in

frame splice mutations or large deletions leaving pocket domains intact were considered as low penetrance mutations (Lohmann and Gallie, 2004; Taylor et al., 2007). Whole gene deletions are also known to be associated with low penetrance when both copies of the survival gene *MED4* are deleted (Dehainault et al., 2014). As *MED4* status was available, these whole gene deletions encompassing *MED4* were considered as low penetrance mutations.

### **Statistical methods**

The R environment was used for all the analyses. Statistical significance was set to  $P \leq 0.05$ . Two-tailed tests were systematically used. Type I error was not controlled by any procedure of correction (exploratory statistical analysis). Qualitative variables were compared using the Fisher exact test. Quantitative variables distributed in classes of a qualitative variable were compared using Mann-Whitney-Wilcoxon and Kruskal-Wallis tests.

## RESULTS

### **Patients' characteristics and general considerations**

Characteristics of the 160 patients are listed according to family history of Rb, sex, age, laterality, treatment by chemotherapy, type of SMN, age at diagnosis, date of death or last follow-up, mutation site and type, mutation description and penetrance (Table 1 and supplemental Table). Median follow-up was 22 years [1-51] and median age of patients was 27 years old [7-53]. This cohort is composed of 92 males (57.5%) and 68 females (42.5%), sex ratio=1.35. Forty-five cases were familial (28.1%), 113 cases were sporadic (70.6%) and for the last 2 cases this information was not known.

All patients received external beam radiotherapy and 84 patients (52,5%) also received chemotherapy. Among these 160 Rb patients, 120 did not develop any SMN (75%) and 40 developed SMNs (25%). The proportion of patients who also received chemotherapy was not statistically different between the former and the latter group *i.e.* 64/120 (53.3%) and 20/40 (50%), respectively. As opposed to previous findings (Abramson et al., 1998), we didn't find any correlation between the age at which EBRT is given (*i.e.* before or after the age of 12 months) and the development of SMN ( $p=0.6$ ).

Subject number	Mutation type	Mutation description	Expected consequence	Site	Penetrance	Age/ <u>age of death</u> <sup>a</sup>	Sex	Familial RB	CH	EBRT < 12 months	Type of SMN $\beta$	Age at diagnosis of SMN (years)
1	Missense	c.1981C>T	p.(Arg661Trp)	20	Low	<u>24</u>	M	Yes	Yes	No	Soft tissue sarcoma	21
2	<b>Inframe deletion</b>	<b>c.1197_1214del</b>	<b>p.(Leu400_Asn405)del</b>	<b>12</b>	<b>High</b>	<b>35</b>	<b>M</b>	<b>No</b>	<b>No</b>	<b>Yes</b>	<b>Soft tissue sarcoma</b>	<b>28</b>
3	Inframe deletion	c.1420_1451del	p.(Ser474Valfs*8)	15	High	<u>14</u>	M	No	No	No	Soft tissue sarcoma	10
4	Inframe deletion	c.1972_1986del	p.(Ala658_Leu662)del	20	High	38	F	No	Yes	No	Osteosarcoma	33
5	Nonsense/frameshift	c.86dup	p.(Glu30*)	1	Low	40	M	No	NA	Yes	Soft tissue sarcoma	35
6	Nonsense/frameshift	c.321_322del	p.(Val108*)	3	High	30	M	No	No	Yes	Soft tissue sarcoma	25
7	Nonsense/frameshift	c.352del	p.(Thr118Leufs*7)	3	High	15	M	No	Yes	No	Brain tumor	11
8	Nonsense /frameshift	c.585G>A	p.(Trp195*)	6	High	26	M	No	No	Yes	Soft tissue sarcoma	19
9	Nonsense/frameshift	c.763C>T	p.(Arg255*)	8	High	33	M	Yes	NA	NA	Brain tumor	29
10	<i>Nonsense/frameshift</i>	<i>c.958C&gt;T</i>	<i>p.(Arg320*)</i>	10	<i>High</i>	27	<i>F</i>	<i>Yes</i>	<i>No</i>	<i>No</i>	<i>Osteosarcoma, Osteosarcoma</i>	18,20
11	Nonsense/frameshift	c.958C>T	p.(Arg320*)	10	High	<u>14</u>	F	No	Yes	Yes	Soft tissue sarcoma	6
12	Nonsense /frameshift	c.1072C>T	p.(Arg358*)	11	High	<u>20</u>	F	No	Yes	Yes	Brain tumor	19
13	Nonsense/frameshift	c.1197del	p.(Leu400*)	12	High	27	F	No	No	Yes	Soft tissue sarcoma	22
14	Nonsense /frameshift	c.1333C>T	p.(Arg445*)	14	High	26	F	No	No	Yes	Soft tissue sarcoma	21
15	<b>Nonsense/frameshift</b>	<b>c.1399C&gt;T</b>	<b>p.(Arg467*)</b>	<b>15</b>	<b>High</b>	<b>28</b>	<b>F</b>	<b>No</b>	<b>No</b>	<b>Yes</b>	<b>Breast phyllode tumor</b>	<b>17</b>
16	<b>Nonsense/frameshift</b>	<b>c.1430del</b>	<b>p.(Leu477Argfs*18)</b>	<b>16</b>	<b>High</b>	<b>22</b>	<b>F</b>	<b>No</b>	<b>Yes</b>	<b>NA</b>	<b>Soft tissue sarcoma</b>	<b>18</b>
17	Nonsense/frameshift	c.1467dup	p.(Ala490Argfs*3)	16	High	26	M	No	Yes	Yes	Osteosarcoma, brain tumor	16,22

Subject number	Mutation type	Mutation description	Expected consequence	Site	Penetrance	Age/ <u>age of death</u> <sup>a</sup>	Sex	Familial	CH	EBRT < 12 months	Type of SMN <sup>ß</sup>	Age at diagnosis of SMN (years)
<b>18</b>	<b>Nonsense/frameshift</b>	<b>c.1666C&gt;T</b>	<b>p.(Arg556*)</b>	<b>17</b>	<b>High</b>	<b>26</b>	<b>M</b>	<b>No</b>	<b>Yes</b>	<b>No</b>	<b>Soft tissue sarcoma</b>	<b>20</b>
<b>19</b>	<i>Nonsense/frameshift</i>	<i>c.1666C&gt;T</i>	<i>p.(Arg556*)</i>	<i>17</i>	<i>High</i>	<u><i>16</i></u>	<i>M</i>	<i>No</i>	<i>NA</i>	<i>No</i>	<i>Osteosarcoma,</i> <i>Osteosarcoma,</i>	<i>7,15,16</i>
<b>20</b>	Nonsense/frameshift	c.1633G>T	p.(Glu545*)	17	High	35	M	No	Yes	Yes	Soft tissue sarcoma	7
<b>21</b>	Nonsense/frameshift	c.1654C>T	p.(Arg552*)	17	High	23	M	No	Yes	Yes	Osteosarcoma	15
<b>22</b>	Nonsense/frameshift	c.1735C>T	p.(Arg579*)	18	High	40	F	No	Yes	Yes	Soft tissue sarcoma	25
<b>23</b>	<b>Nonsense/frameshift</b>	<b>c.1735C&gt;T</b>	<b>p.(Arg579*)</b>	<b>18</b>	<b>High</b>	<b>36</b>	<b>M</b>	<b>No</b>	<b>No</b>	<b>NA</b>	<b>Osteosarcoma</b>	<b>13</b>
<b>24</b>	<i>Nonsense/frameshift</i>	<i>c.1735C&gt;T</i>	<i>p.(Arg579*)</i>	<i>18</i>	<i>High</i>	<u><i>28</i></u>	<i>M</i>	<i>No</i>	<i>Yes</i>	<i>Yes</i>	<i>Squamous cell carcinoma, Soft tissue</i>	<i>17,25</i>
<b>25</b>	<b>Nonsense/frameshift</b>	<b>c.1847delinsTT</b>	<b>p.(Lys616Ilefs*37)</b>	<b>19</b>	<b>High</b>	<b>24</b>	<b>M</b>	<b>No</b>	<b>Yes</b>	<b>No</b>	<b>Soft tissue sarcoma</b>	<b>18</b>
<b>26</b>	Nonsense/frameshift	c.2664del	p.(Ser888Argfs*17)	25	High	<u>22</u>	F	No	Yes	NA	Soft tissue sarcoma	20
<b>27</b>	Large rearrangement	exon3-17del	NA	3-17	High	<u>13</u>	F	Yes	No	Yes	Osteosarcoma	13
<b>28</b>	Splice	c.658C>G	p.(Leu220Val)	7	High	34	F	No	No	Yes	Osteosarcoma	13
<b>29</b>	Splice	c.862-10T>C	In frame exon 9 skipping	8-9	Low	31	M	Yes	Yes	Yes	Osteosarcoma	10
<b>30</b>	Splice	c.939G>T	In frame exon 9 skipping	9	High	<u>18</u>	M	No	NA	NA	Soft tissue sarcoma	17
<b>31</b>	Splice	c.1049+3A>G	Out of frame exon 10 skipping	10	High	31	F	Yes	Yes	Yes	Soft tissue sarcoma, brain tumor	15, 25
<b>32</b>	Splice	c.1215+1G>A	Out of frame exon 12 skipping	12	High	<u>22</u>	F	Yes	Yes	No	Soft tissue sarcoma	20
<b>33</b>	<b>Splice</b>	<b>c.1215+1G&gt;A</b>	<b>Out of frame exon 12 skipping</b>	<b>12</b>	<b>High</b>	<b>53</b>	<b>M</b>	<b>Yes</b>	<b>No</b>	<b>NA</b>	<b>Soft tissue sarcoma</b>	<b>46</b>
<b>34</b>	Splice	c.1333-2A>G	In frame exon 14 skipping	13	High	<u>20</u>	M	Yes	Yes	No	Soft tissue Sarcoma	19

Subject number	Mutation type	Mutation description	Expected consequence	Site	Penetrance	Age/ <u>age of death</u> <sup>a</sup>	Sex	Familial	CH	EBRT < 12 months	Type of SMN <sup>β,γ</sup>	Age at diagnosis of SMN (years)
35	Splice	c.1345G>A	In frame exon 14 skipping	14	High	45	M	Yes	NA	NA	Soft tissue sarcoma	38
36	Splice	c.1499-1G>A	Out of frame exon 17 skipping	17	High	<u>19</u>	M	No	NA	No	Soft tissue sarcoma	9
37	Splice	c.2520+1G>C	Out of frame exon 24 skipping	24	High	29	F	No	No	No	Soft tissue sarcoma	17
38	Splice	c.2490-28T>C	Out of frame exon 24 skipping	24	High	28	F	No	Yes	No	Osteosarcoma	18
39	<b>Splice</b>	<b>c.2490-26A&gt;C</b>	<b>Out of frame exon 24 skipping</b>	<b>24</b>	<b>High</b>	<b>36</b>	<b>F</b>	<b>No</b>	<b>No</b>	<b>Yes</b>	<b>Uterine trophoblastic tumor</b>	<b>33</b>
40	Splice	c.2489+1G>A	Complex defect (&)	23	High	22	M	No	Yes	Yes	Soft tissue sarcoma	17

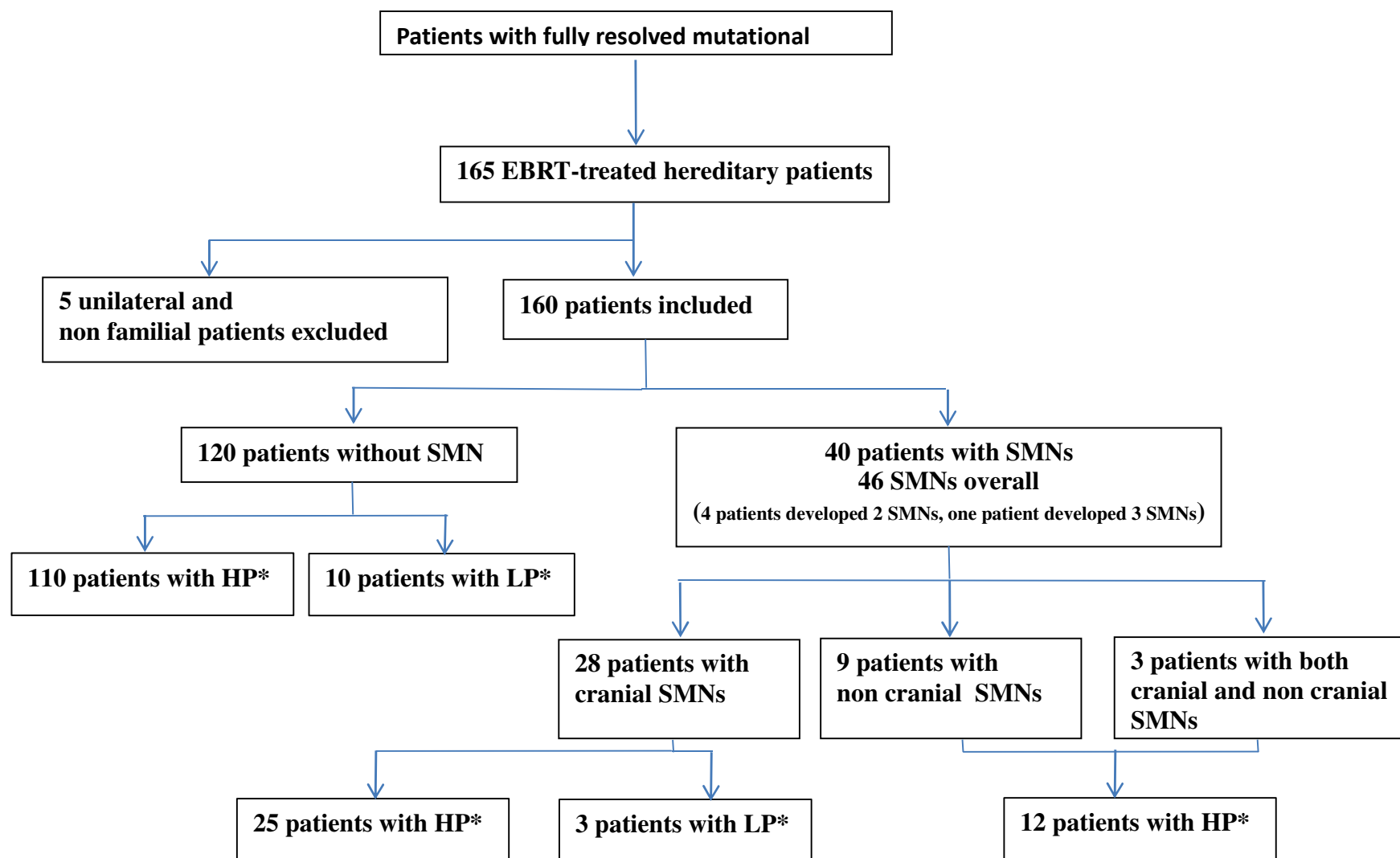
**Table 1. Patients, mutations, treatments and SMNs characteristics.**

α. The age is underlined when the patient is deceased. The underlined ages correspond to the age at which the patient died.

β. The different tumors are listed one after the other in the case of second and third SMNs.

γ. SMN can be located either in irradiated area or at distance from it: cranial SMNs (in irradiated area) are written in black, non cranial SMNs are written in bold and both SMNs in irradiated area and at distance from it are written in italics.

(&) This mutation leads to the synthesis of two abnormal *RBI* transcripts, the first includes the first 11 base pairs of intron 22 with an in frame exon 3 skipping, the second with out of frame exon 23 skipping.



**Fig. 1. Flow chart showing the number of patients included (n=160) and excluded.**

\*LP= low penetrance mutation, HP=High penetrance mutation

Depending on the tumor location, the 40 patients with SMNs were splitted in three groups.

### Subsequent malignant neoplasms (SMNs)

Among the 40 hereditary Rb patients who developed SMNs, 23 were males (23/40, 57.5%) and 17 females (17/40, 42.5%), sex ratio=1.35. These 40 patients made up a total number of 46 tumors: 35 patients developed one SMN (35/46, 76.1%), 4 developed second and third SMNs (4/46, 8.7%) and at last one Rb patient developed a second, a third and a fourth SMN (Table 1). SMN types were mainly soft tissue sarcomas (25/46, 54.3%), osteosarcomas (13/46, 28.3%) and brain tumors (5/46 patients, 10.9%). Type, number and age at diagnosis of SMN were not statistically different between familial and bilateral sporadic cases (with  $p=0.7$ ,  $p=0.6$  and  $p=0.2$ , respectively). Twenty-eight patients developed a cranial SMNs (28/40, 70%), nine patients developed a SMN at distance from irradiate areas (9/40, 22.5%) and 3 developed both types (3/40, 7.5%). In addition, for 2 patients the second and a third tumor where both located in irradiated area. The split of the different types of mutation per SMNs and per groups of patients is shown Tables 2 and 3.

Type of <i>RBI</i> mutation	Number of patients	Number of tumors	Type of SMN*			
			Soft tissue sarcoma	Osteosarcoma	Brain tumor	Others
Nonsense/frameshift	22	27	13	8	4	2
Splice	13	14	9	3	1	1
Large rearrangements	1	1	0	1	0	0
Missense mutations	1	1	1	0	0	0
Inframe deletions	3	3	2	1	0	0
<b>Total</b>	<b>40</b>	<b>46</b>	<b>25</b>	<b>13</b>	<b>5</b>	<b>3</b>

**Table 2. Type of SMNs according to *RBI* mutation type.**

Others: one malignant breast tumor, one squamous cell carcinoma and one uterine trophoblastic tumor.

(\*)All the 46 tumors were taken into account.

Type of <i>RBI</i> mutation	Number of cases	Number of cases without SMN	Number of cases with SMN	Number of cases with cranial SMN	Number of cases with SMN at a distance from irradiated areas
<b>Nonsense and frameshift</b>	92(57.5%)	70(58.3%)	22(55%)	17*(54.8%)	8*(66.7%)
<i>Low penetrance mutation</i>	5	4	1	1	0
<b>Splice mutation</b>	41(25.6%)	28(23.3%)	13(32.5%)	10(32.3%)	3(25%)
<i>Low penetrance mutation</i>	1	0	1	1	0
<b>Large rearrangements</b>	16(10%)	15(12.5%)	1(2.5%)	1(3.2%)	0
<i>Low penetrance mutation</i>	1	1	0	0	0
<b>Missense mutation</b>	5(3.1%)	4(3.3%)	1(2.5%)	1(3.2%)	0
<i>Low penetrance mutation</i>	3	2	1	1	0
<b>Promoter mutation</b>	3(1.9%)	3(2.5%)	0	0	0
<i>Low penetrance mutation</i>	3	3	0	0	0
<b>Inframe deletion</b>	3(1.9%)	0	3(7.5%)	2(6.5%)	1(8.3%)
<i>Low penetrance mutation</i>	0	0	0	0	0
<b>Total</b>	<b>160</b>	<b>120</b>	<b>40</b>	<b>31</b>	<b>12</b>

**Table 3. Split of the different types of mutation per groups of patients: total SMN, no SMN, cranial SMN, SMN at distance from irradiated areas.**

Benign tumors were not taken into account. The number of low penetrance mutations is italicized for each type of mutation.

(\*) 3 patients with a nonsense/frameshift mutation had both cranial and non cranial SMN.

In all but 3 cases, SMNs were linked to a high penetrance mutation (Table 3). We found that no patient with a promoter mutation developed a SMN and only one out of 16 patients with a large rearrangement developed a SMN. On the other hand, the 3 patients with an inframe deletion developed a SMN. Splice mutations, which accounted for 25.6% of patients, reached 32.5% of SMN. For nonsense/frameshift mutations, the proportions are similar, with 57.5% and 58.3% of patients and SMN, respectively. **In any case, the series is too small to point out a specific mutation type in SMN development.** No statistical difference was found on the type of mutation between patients who developed cranial SMNs and patients who developed SMNs at distance from an irradiated area ( $p=0.13$ ).

Five patients, all carriers of an out of frame high penetrance mutation, developed a second or third SMN, highlighting the risk for predisposed patients (Table 1). Among these 5 patients, 2 developed second and third cranial SMNs whereas 3 developed both cranial and non-cranial SMNs. Twelve patients died from SMN consequences and median age of death was 19.5 years [13- 28] whereas all patients free of SMN are alive today ( $p=2 \times 10^{-8}$ ) further highlighting the burden of SMNs. With regards to genotype, we didn't detect any linkage between the age of death and the type of mutation ( $p=0.25$ ) or penetrance ( $p=0.24$ ).

### **Penetrance and SMN**

Due to the number of different mutation classes and the acknowledged low penetrance class of mutations in Rb, we regrouped mutations by penetrance type (high and low) before analysis. Among the 13 patients with a low penetrance mutation, 3 (23.1%) developed a SMN (2 soft tissue sarcomas and an osteosarcoma) and among the 147 carriers of a high penetrance mutation, 37 (25.2%) developed a SMN. We didn't find any difference in high and low penetrance mutations between patients with or without SMN (3/10 vs. 37/110,  $p=0.87$ ), neither could we detect any linkage between penetrance and SMN location, SMN type and age at diagnosis of SMNs.

### **Parental origin**

Since a correlation between the gender of the transmitting carrier and Rb penetrance in low penetrance mutation carriers was recently described (Eloy et al., 2016), we searched for the parental origin of the mutant allele and this information was available in 38 cases. With regards to high penetrance mutations, 15 patients received the mutant allele from their mother and 4 developed a SMN whereas 19 patients received the mutant allele from the father and 3 developed a SMN ( $p=0.68$ ). With regards to low penetrance mutations, numbers are too small

to reach any significance: 1 patient received the mutant allele from the mother and did not develop any SMN whereas 3 patients received the mutant allele from the father and 2 developed a SMN (Table 4).

<b>Parental origin</b>	<b>High mutations penetrance</b>		<b>Low penetrance mutations</b>	
	Maternal	Paternal	Maternal	Paternal
<b>Number of transmitted alleles</b>	15	19	1	3
<b>Number of SMNs</b>	4	3	0	2

**Table 4. Parental origin and SMNs for high and low penetrance mutations.**

## DISCUSSION

Our study reports a homogeneous cohort of hereditary Rb patients with fully resolved mutational status and who were all treated using external beam radiotherapy, with or without additional chemotherapy. Finding a genotype-subsequent malignant neoplasm correlation has never been as important as today since SMNs are now the leading cause of mortality for survivors of hereditary Rbs submitted to EBRT. Hence, deciphering the relationship between *RBI* genotype and SMN development in EBRT treated patients might shed light on cancer risk and guide the patients' medical supervision.

We confirmed that the majority of SMNs were soft tissue sarcomas and osteosarcomas (Kleinerman et al., 2007). On the other hand, despite the fact that epithelial cancers are usually reported, we only found one occurrence of skin cancer (squamous cell carcinoma) and no melanoma, bladder or lung cancer (MacCarthy et al., 2009). A plausible explanation is that the median follow up in our study was only 22 years, whereas epithelial cancers are being observed after 20 years of follow up (Marees et al., 2008).

A recent and large study on 1015 Rb patients by Kleinerman and co-workers (Kleinerman et al., 2012) evaluated the influence of family history and suggested that familial Rb patients were at slightly higher risk of SMN compared to bilateral sporadic cases. Melanoma would account for this slight increase in risk. Our own results, on a smaller but more homogeneous series, don't support these findings as number, type and age at diagnosis of SMNs were not statistically different between familial and sporadic cases. This is a logical finding as the risk entailed by the mutation should be the same once the said mutation is constitutional and not mosaic. Here may lay one explanation for these discrepancies, as mosaicism cannot be excluded in Kleinerman's study. With regards to melanoma, we didn't find any case in our series for reasons of follow up duration (see above) and also because this increased melanoma

risk is probably linked to the presence of shared genetic determinants cosegregating with the *RBI* mutation in a subset of Rb families as previously described for lipomas (Genuardi et al., 2001). As a result our sampling (42 familial cases) is too low to expect any detection of this risk.

Although our series is too small to allow a precise determination of the correlation between mutation type and SMN phenotype, the following can be observed. High penetrance mutations are at high risk of SMN, further supporting existing data (Temming et al., 2015)(Dommering et al., 2012). The five patients who developed a third tumor all carried a high penetrance mutation, strengthening the fact that these *RBI* carriers should be carefully monitored. With regard to low penetrance mutations, a previous study reported a statistically significantly decreased risk of second primary malignancy for patients with a low penetrance mutation (HR : 0.19; 95% CI 0.05-0.81, p=0.025) (Dommering et al., 2012). We cannot confirm these findings as our own results didn't show any significant difference for SMN occurrence between carriers of high penetrance or low penetrance mutations. This could be attributed to a smaller sample size (160 vs. 199 in Dommering's study) but the fact is that in our context of EBRT-treated patients, among 13 carriers of a low penetrance mutation, 3 of them developed sarcomas in irradiated area, a rare tumor that cannot be attributed to the general population. Low penetrance mutations are responsible for a lower rate of Rb (Harbour, 2001), but it seems that once a low penetrance mutation carrier patient has developed Rb, he remains at risk for SMN. Consequently these patients cannot be reassured and should benefit from the same monitoring as carriers of high penetrance mutations.

With regards to SMN in or out irradiated area, we were unable to find any linkage between mutation type and SMN location. Similarly, the mutation is not predictive of SMN type as the same mutation can result in no SMN or SMN of different types. This is a general

phenomenon in genetics, possibly involving tissue specific factors, and environment and modifier genes. Gender of the transmitting parent was also analyzed, in order to see if transmission of the maternal allele could be linked to a less severe phenotype. No difference emerged from high penetrance mutations, in accordance with what is seen for the primary tumor (Eloy et al., 2016). For low penetrance mutation carriers, the numbers were too small to draw any conclusion but a slight trend is worthy of further investigation in larger series.

Overall, at this point in time, it cannot be explained why the same mutation may lead or not to a SMN and mutation type cannot be used to predict type or location of SMN. One notable finding is that EBRT patients with a low penetrance mutation appear at risk of SMNs and should therefore be cautiously monitored.

## FUNDINGS

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## Supplemental Table

Subject number	Mutation type	Mutation description	Expected consequence	Site of the mutation	Penetrance	Age	sexe	Familial	CH	EBRT<12 months
1	Missense	c.1463C>A	p.(Ala488Glu)	15	Low	16	F	No	Yes	No
2	Missense	c.1687T>C	p.(Trp563Arg)	17	High	36	M	No	No	No
3	Missense	c.2132T>A	p.(Ile711Lys)	21	High	32	F	Yes	Yes	No
4	Missense	c.1981C>T	p.(Arg661Trp)	20	Low	24	M	Yes	No	Yes
5	Nonsense/frameshift	c.54_79del	p.(Glu19Profs*3)	1	Low	24	M	Yes	No	Yes
6	Nonsense/frameshift	c.191del	p.(Leu64*)	2	Low	37	M	Yes	No	No
7	Nonsense/frameshift	c.178_185delTTATG TCA	p.(Leu60Glufs*47)	2	High	31	M	Yes	No	Yes
8*	Nonsense/frameshift	c.178_185delTTATG TCA	p.(Leu60Glufs*47)	2	High	31	M	Yes	No	Yes
9	Nonsense/frameshift	c.220_221insAG	p.(Ala74Arg*4)	2	Low	15	M	No	Yes	No
10	Nonsense/frameshift	c.193A>T	p.(Lys65*)	2	Low	28	M	No	Yes	Yes
11	Nonsense/frameshift	c.297G>A	p.(Trp99*)	3	High	26	F	No	No	No
12	Nonsense/frameshift	c.321_322del	p.(Val108*)	3	High	41	F	No	Yes	No
13	Nonsense/frameshift	c.324del	p.(Asp109Thrfs*2)	3	High	25	M	No	No	Yes
14	Nonsense/frameshift	c.557dup	p.(Asn186Lysfs*17)	6	High	32	M	No	No	Yes
15	Nonsense/frameshift	c.565_572del	p.(Leu189Lysfs*11)	6	High	31	F	No	No	Yes
16	Nonsense/frameshift	c.660_661del	p.(Val222Profs*2)	7	High	20	F	No	Yes	No
17	Nonsense/frameshift	c.713del	p.(Pro238Hisfs*26)	7	High	22	M	No	No	Yes
18	Nonsense/frameshift	c.751C>T	p.(Arg251*)	8	High	38	M	Yes	No	Yes
19	Nonsense/frameshift	c.724_725dup	p.(Val243Glnfs*23)	8	High	33	M	No	No	No
20	Nonsense/frameshift	c.751C>T	p.(Arg251*)	8	High	30	F	No	No	Yes
21	Nonsense/frameshift	c.751C>T	p.(Arg251*)	8	High	27	M	No	No	Yes
22	Nonsense/frameshift	c.751C>T	p.(Arg251*)	8	High	26	M	No	Yes	No
23	Nonsense/frameshift	c.751C>T	p.(Arg251*)	8	High	23	M	No	Yes	Yes
24	Nonsense/frameshift	c.763C>T	p.(Arg255*)	8	High	30	M	No	No	Yes

Subject number	Mutation type	Mutation description	Expected consequence	Site of the mutation	Penetrance	Age	sexe	Familial	CH	EBRT<12 months
25	Nonsense/frameshift	c.772_776del	p.(Asn258Glnfs*11)	8	High	22	M	No	Yes	Yes
26	Nonsense/frameshift	c.774_780dup	p.(Ala261Glnfs*12)	8	High	32	M	No	No	No
27	Nonsense/frameshift	c.958del	p.(Arg320Aspfs*12)	10	High	17	F	No	Yes	No
28	Nonsense/frameshift	c.958C>T	p.(Arg320*)	10	High	16	M	Yes	Yes	No
29*	Nonsense/frameshift	c.964G>T	p.(Glu322*)	10	High	38	M	Yes	Yes	Yes
30	Nonsense/frameshift	c.958C>T	p.(Arg320*)	10	High	37	F	No	No	Yes
31	Nonsense/frameshift	c.958C>T	p.(Arg320*)	10	High	26	F	No	No	Yes
32	Nonsense/frameshift	c.1000del	p.(Arg334Aspfs*15)	10	High	35	M	NA	NA	Yes
33	Nonsense/frameshift	c.1000del	p.(Arg334Aspfs*15)	10	High	24	F	No	No	No
34	Nonsense/frameshift	NA	p.(Arg358*)	11	High	41	F	No	No	Yes
35	Nonsense/frameshift	c.1073C>T	p.(Arg358*)	11	High	25	F	No	No	No
36	Nonsense/frameshift	c.1073C >T	p.(Arg358*)	11	High	24	F	No	Yes	Yes
37	Nonsense/frameshift	c.1156dup	p.(Met386Asnfs*9)	12	High	33	M	No	No	Yes
38	Nonsense/frameshift	c.1235_1236del	p.(Lys412Argfs*15)	13	High	30	F	No	No	No
39	Nonsense/frameshift	c.1258A>T	p.(Lys420*)	13	High	23	M	No	Yes	No
40	Nonsense/frameshift	c.1262delATAinsT	p.(Asp421Valfs*2)	13	High	39	M	Yes	No	No
41	Nonsense/frameshift	c.1262delATAinsT	p.(Asp421Valfs*2)	13	High	35	F	Yes	No	Yes
42	Nonsense/frameshift	c.1306C>T	p.(Gln436*)	13	High	27	M	No	Yes	No
43	Nonsense/frameshift	c.1388C>G	p.(Ser463*)	14	High	13	M	Yes	Yes	No
44	Nonsense/frameshift	c.1388C>G	p.(Ser463*)	14	High	35	M	Yes	Yes	Yes
45	Nonsense/frameshift	c.1399C>T	p.(Arg467*)	15	High	13	F	No	Yes	No
46	Nonsense/frameshift	c.1399TC>T	p.(Arg467*)	15	High	28	F	No	No	No
47	Nonsense/frameshift	c.1421+29del	p.(Ser474Thrfs*4)	15-16	High	28	F	No	No	NA
48	Nonsense/frameshift	c.1420delA	p.(Ser474Alafs*4)	15-16	High	9	M	Yes	Yes	Yes
49	Nonsense/frameshift	c.1467C>A	p.(Cys489*)	16	High	24	F	No	Yes	Yes
50	Nonsense/frameshift	c.1494T>G	p.(Tyr498*)	16	High	13	M	No	Yes	No
51	Nonsense/frameshift	c.1443_1449dup	p.(Met484Phefs*11)	16	High	20	F	Yes	No	No
52	Nonsense/frameshift	c.1654C>T	p.(Arg552*)	17	High	14	F	No	Yes	No
53	Nonsense/frameshift	c.1654C>T	p.(Arg552*)	17	High	7	M	No	Yes	No
54	Nonsense/frameshift	c.1654C>T	p.(Arg552*)	17	High	27	F	No	No	Yes

Subject number	Mutation type	Mutation description	Expected consequence	Site of the mutation	Penetrance	Age	sexe	Familial	CH	EBRT <12 months
55	Nonsense/frameshift	c.1654C>T	p.(Arg552*)	17	High	21	F	No	Yes	Yes
56	Nonsense/frameshift	c.1666C>T	p.(Arg556*)	17	High	13	M	No	Yes	No
57	Nonsense/frameshift	c.1666C>T	p.(Arg556*)	17	High	13	M	No	Yes	No
58	Nonsense/frameshift	c.1666C>T	p.(Arg556*)	17	High	17	F	No	Yes	Yes
59	Nonsense/frameshift	c.1735C>T	p.(Arg579*)	18	High	22	F	No	Yes	No
60	Nonsense/frameshift	c.1887_1888del	p.(Glu629Aspfs*23)	19	High	32	F	Yes	No	Yes
61	Nonsense/frameshift	c.1959dupA	p.(Val654Serfs*14)	19	High	10	M	No	Yes	No
62	Nonsense/frameshift	c.1861_1862del	p.(Arg621Cysfs*31)	19	High	26	M	No	No	Yes
63	Nonsense/frameshift	c.2053C>T	p.(Gln685*)	20	High	27	F	No	Yes	Yes
64	Nonsense/frameshift	c.2059_2062del	p.(Thr687Cysfs*8)	20	High	10	M	Yes	Yes	Yes
65	Nonsense/frameshift	c.2139delA	p.(Val714*)	21	High	21	M	No	Yes	No
66	Nonsense/frameshift	c.2172_2182del	p.(Val725Glnfs*22)	21	High	16	M	No	Yes	No
67	Nonsense/frameshift	c.2209del	p.(Glu737Argfs*7)	21	High	18	M	No	Yes	Yes
68	Nonsense/frameshift	c.2236G>T	p.(Glu746*)	22	High	26	F	No	No	No
69	Nonsense/frameshift	c.2239_2242dup	p.(Glu748Glyfs*4)	22	High	40	M	Yes	Yes	Yes
70	Nonsense/frameshift	c.2239_2242dup	p.(Glu748Glyfs*4)	22	High	31	F	Yes	No	Yes
71	Nonsense/frameshift	c.2236_2242dup	p.(Glu748GlyFs*5)	22	High	24	M	No	Yes	Yes
72	Nonsense/frameshift	c.2273C>A	p.(Ser758*)	22	High	22	M	No	Yes	Yes
73	Nonsense/frameshift	c.2359C>T	p.(Arg787*)	23	High	17	M	No	Yes	No
74	Nonsense/frameshift	c.2359C>T	p.(Arg787*)	23	High	39	M	No	NA	Yes
75	Large rearrangement	exon[1-6]del	NA	1-6	High	35	F	NA	NA	NA
76	Large rearrangement	exon[1-17]del	NA	1-17	High	23	F	No	Yes	No
77	Large rearrangement	exon[3-6]del	NA	3-6	High	15	M	No	Yes	No
78	Large rearrangement	exon6del	NA	6	High	14	M	No	Yes	No
79	Large rearrangement	exon12del	NA	12	High	11	F	Yes	Yes	Yes
80	Large rearrangement	exon12del	NA	12	High	11	F	Yes	Yes	Yes
81	Large rearrangement	exon[8-9]del	NA	8-9	High	25	M	Yes	Yes	Yes
82	Large rearrangement	exon[9-11]del	NA	9-11	High	40	M	Yes	No	No
83	Large rearrangement	exon[9-11]del	NA	9-11	High	35	F	Yes	Yes	Yes

Subject number	Mutation type	Mutation description	Expected consequence	Site of the mutation	Penetrance	Age	sexe	Familial	CH	EBRT< 12 months
84	Large rearrangement	exon[18-23]del	NA	18-23	High	29	F	No	Yes	Yes
85	Large rearrangement	exon[21.22]del	NA	21-22	High	43	F	No	No	Yes
86*α	Large rearrangement	13qdel	NA	13qdel	Low	27	M	Yes	No	No
87 β	Large rearrangement	whole gene del	NA	-	High	38	F	No	NA	No
88 β	Large rearrangement	whole gene del	NA	-	High	31	F	No	No	No
89 β	Large rearrangement	whole gene del	NA	-	High	29	F	No	No	Yes
90	Splice	c.501-1G>A	NA	5	High	29	F	No	No	Yes
91	Splice	c.501-1G>A	NA	5	High	28	F	No	Yes	Yes
92	Splice	c.607+1G>A	NA	6	High	25	F	No	Yes	Yes
93	Splice	c.607+1G>C	NA	6	High	20	M	No	Yes	No
94	Splice	c.719-9C>G	Out of frame exon 8 skipping	8	High	41	M	Yes	Yes	Yes
95	Splice	c.861+2T>C	NA	8	High	34	M	No	Yes	Yes
96	Splice	c.1049+2delT	Out of frame exon 10 skipping	10	High	28	M	No	No	Yes
97	Splice	c.1215+1G>A	Out of frame exon 12 skipping	12	High	38	F	No	No	Yes
98	Splice	c.1215+1G>A	Out of frame exon 12 skipping	12	High	36	M	Yes	No	Yes
99	Splice	c.1215+1G>A	Out of frame exon 12 skipping	12	High	28	F	No	No	No
100	Splice	c.1215+1G>A	Out of frame exon 12 skipping	12	High	23	F	Yes	Yes	No
101	Splice	c.1332G>C	In frame exon 13 skipping	13	High	12	F	No	Yes	No
102	Splice	c.1332+1G>A	In frame exon 13 skipping	13	High	25	F	Yes	No	Yes
103	Splice	c.1332+1G>A	In frame exon 13 skipping	13	High	23	M	No	Yes	Yes
104	Splice	c.1333-2A>G	In frame exon 14 skipping	13-14	High	31	M	Yes	No	Yes
105	Splice	c.1389+4A>G	In frame exon 14 skipping	14	High	23	F	No	Yes	Yes
106	Splice	c.1389+5G>A	In frame exon 14 skipping	14	High	22	M	No	Yes	Yes
107	Splice	c.1498+5G>A	Out of frame exon 16 skipping	15-16	High	42	M	Yes	No	Yes
108	Splice	IVS18+1G>A	NA	18	High	33	F	No	No	Yes
109	Splice	c.1960+1G>A	In frame exon 19 skipping	19	High	7	M	No	Yes	No

Subject number	Mutation type	Mutation description	Expected consequence	Site of the mutation	Penetrance	Age	sexe	Familial	CH	EBRT< 12 months
110	Splice	c.1960+1del	NA	19-20	High	28	M	No	Yes	Yes
111	Splice	c.2106+1G>C	NA	20	High	12	M	No	Yes	No
112	Splice	c.2211+1G>A	exon 21 skipping	21	High	20	M	No	Yes	No
113	Splice	c.2211+1G>C	exon 21 skipping	21	High	16	M	No	Yes	No
114	Splice	c.2211+1G>A	exon 21 skipping	21	High	9	M	No	Yes	No
115	Splice	c.2211+5G>T	exon 21 skipping	21	High	43	M	Yes	No	No
116	Splice	c.2490-1398A>G	p.(Arg830Serfs*3)	23	High	23	M	Yes	Yes	No
117	Splice	c.2663+1G>A	exon 25 skipping	25	High	24	M	No	Yes	No
118	Promoter	c.-196A>G	NA	Promoter	Low	39	M	Yes	No	Yes
119	Promoter	c.-197G>A	NA	Promoter	Low	27	M	No	No	Yes
120	Promoter	c.-193T>G	NA	Promoter	Low	25*	F	Yes	No	Yes

**Supplemental table. Characteristics of the 120 patients with no SMN.**

(\*)Unilateral Rb cases

(<sup>α</sup>) deletion involving *MED4*

(<sup>β</sup>) deletion without *MED4* involvement



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