**Molecular study of the retinoblastoma in western Algerian population. Research of gene Rb mutations at the constitutional level.**

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**Background:** Retinoblastoma is a malignant intraocular tumour which generally reaches the child with a frequency from 1/15000 to 1/20000. Antioncogene Rb changes (chromosome 13q14.2), are at the origin of this cancer. Two allele’s mutations of this gene are required for retinoblastoma development. The aim of this study is research and identification of mutations able to affect the gene Rb at the constitutional level. **Methods:** Study concern 61 patients. The twenty-seven exons and promoter of Rb gene were amplified by PCR, with 15 exons studied by DGGE (Denaturing Gel Gradient Electrophoresis) and 12 by SSCP (Single Strand Conformation Polymorphism). These techniques allow selecting cases for sequencing. **Results:** Sequencing results gave nineteen different variations bases, including seven exonic changes: five nonsens mutations located in exons 1,7,8,18 and 23 and two misense mutations in exons 19 and 20. These changes remain minority compared to the twelve intronic changes whose possible implication remains to be elucidated. Polymorphisms found in exons 2,3,4,11 and 17 were already described in the literature. **Conclusion:** Neomutations detection is important because it allows both the early treatment of children with the mutated gene that screening asymptomatic carriers at risk of transmitting the disease to their offspring.

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**Introduction**

Retinoblastoma is a malignant neuro-epithelial origin, with an average incidence of one case every 15.000-20.000 live births (1). This cancer usually affects children in the first months of life. There are two forms, the hereditary form in which the first event is germinal and second somatic. For the sporadic form, the two events are somatic. Mutations affecting antioncogene Rb (chromosome 13q14.2) (2), are the cause of this cancer. The mutations of both alleles of this gene are required for the survey of retinoblastoma.

The Rb gene is part of the Rb/E2F pathway that plays a role in the development of retinoblastoma. It is at the middle of the cell cycle in the transition G1 / S. The protein encoded by Rb (*p*Rb) exists in two forms: phosphorylated, inactive form and a non- phosphorylated, active form (3). The last form has the essential property to bind E2F transcription factor and thus block the progression of S phase (4).

The aim of the present study is to identify mutations that may affect the Rb gene at the constitutional level. This would help in the first to understand the molecular pathology of retinoblastoma and second to the early identification of a person witch present a risk, asymptomatic carriers and possibly prenatal diagnosis.

**Material and methods**

**Material**

Study included 61 unrelated patients with unilateral or bilateral sporadic retinoblastoma, recruited at the ophthalmology clinic of "front de mer" in Oran and pediatric ophthalmology department of the EHS Canastel. Oran. The DNA of patients were designated by Ru or Rb letters, respectively, for unilateral and bilateral forms followed by a number

**Methods**

Extraction of genomic DNA was performed from whole blood using the NaCl technique (5). The 27 exons of the RB gene were amplified by PCR (Lille, France) (6). Twenty-seven primer pairs were used for amplification of the promoter and 27 exons Rb gene components. Exons 15 and 16 were amplified together. The choice of primers (7) was done in intronic parts flanking each exon to cover with all primers, most of the RB gene (Table 1).

Analysis of the Rb gene at the constitutional level was performed by DGGE (Denaturant Gradient Gel Electrophoresis) (8) for 15 exons and SSCP (Single Strand Conformation Polymorphism) (9) for the remaining 12 exons. Both techniques enable screening of the index case to sequence (ABI 3130, Applied Biosystems Foster City, Californie USA) (10). Sequence analyzes were performed using the Seqscanner of applied biosystem (http://marketing.appliedbiosystems.com/mk/get/SSS\_login?isource=fr\_E\_Pg\_Prod\_AB\_Gbl\_SeqScan\_20050920) and Multalin (<http://multalin.toulouse.inra.fr/multalin/>).

**Results and Discussion**

Constitutional analysis of 61 DNA patients after study by DGGE (Figure 1) and SSCP (Figure2), revealed 19 different variations bases, in 14 exons: 1, 2, 3, 4, 7, 8, 9, 11, 12, 17, 18, 19, 20 and 23. The result of sequencing followed by "Multalin" analysis (Figure 3) gave: **exon 1:** G→ A transition in position 91pb of exon 1, Glu31Stop, **exon 2:** C → T transition 76 bp intron2, **exon 3 :** C → T transition 12pb, intron3 ; A → G transition 37 bp, intron3, C → T transition 45pb, intron3; **exon 4:** G → T transversion 23pb, intron4; **exon7:** deletion of 4pb position 24 between codons 7 and 10 in exon7 (Patient Ru14 given as example in Figures 2 and 3) transition G → A 78pb, intron7; **exon 8:** C → T transition 45pb, exon 8, arg225stop; **exon 9:** transition T → C-29pb, intron 8, C → A transversion -15 bp intron 8 (RB7 Patients and Ru42 given as example in Figure 1), **exon 11:** A → C transversion 41pb, intron11; **exon 12:** G → A transition 1pb, intron 12 ; **exon 17:** del A-56, intron16; **exon 18:** C → T transition 40bp, exon 18, arg578stop; **exon 19:** C → T transition 61pb, intron19; G →C transversion 80pb, exon19, Ala635Pro; **exon 20:** T →A transversion 1pb, exon20, Val654Glu; **exon 23:** deletion of 7 bp in exon 23 stop codon position 809.

Identified mutations distribution along the gene shows that there is no preferential region of mutations. Among the 7 exonic mutations found: 5 are responsible in the appearance of nonsense mutations and 2 are missense (Table 2). These mutations are minority compared to intronic mutations witch are the most numerous and whose possible involvement remains to be elucidated (Table 3).

Exonic mutations found are causal, since they all lead to the appearance of inactive truncated protein, due to the absence of the region between exons 12 and 27. Indeed, this region encodes a structure called the "binding pocket of oncoproteins." This pocket has two domains, 179 amino acids (codon 393 to codon 571) and 125 amino acids (codon 649-773), separated by an open area of 76 amino acids called "gap" (codon 572 to 648). It serves to hold the transcription factor E2F (11). This region is required for *p*Rb protein in cell cycle regulatory function, and any modification or mutation occurring at this level will cause retinoblastoma. The intron 12 variation base cause an abnormal splicing, it abolishes the splice donor site by change the sequence GT to AT (13). This leads to the production of a longer protein due to intron 12 translate. This variation can affect the protein conformation witch will have impact in its normal regulatory function (14). A study of functional and structural protein *p*Rb is required to confirm the role of this mutation.

Polymorphisms already described in the literature were found in exons 2, 3, 4, 11and 17.

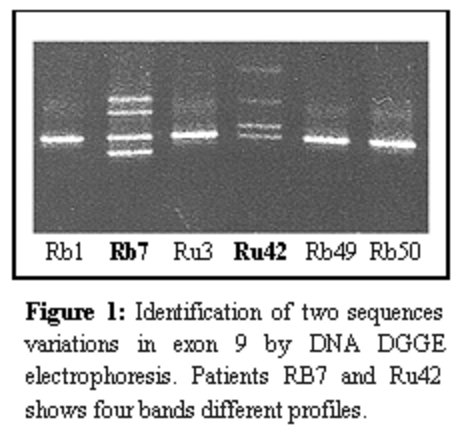
For this study, which involves only sporadic forms of retinoblastoma, we were able to identify mutations in constitutional level that are potentially transferable. This type of event is generally ignored because there is no history of disease in families of index cases. Beyond that, we are well aware of the need to sequence the entire Rb gene to detect mutations that are in the range of from 10 to 15 percent. Our results confirm that because we were able to detect seven mutations in a population of 61 patients with sporadic form of retinoblastoma representing 11.47%.

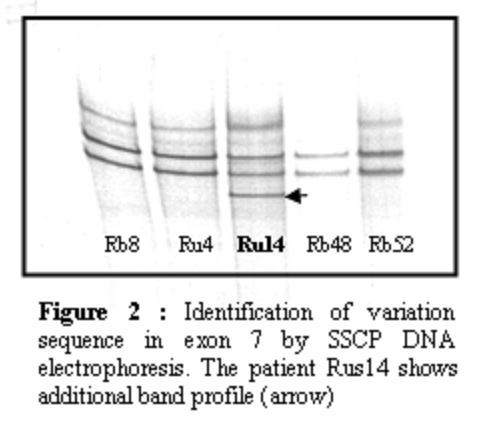
Germinal neomutations detection is important because it allows the prevention and early management of the patient if the disease were to break out. This detection is also important for genetic counseling.

It does allow reassuring branch of family members showing no mutations.

For subjects did not show any molecular defect, it is necessary to explore other signaling pathways may cause retinoblastoma in these cases (15).

Indeed, recent research, demonstrate the existence of genes other than Rb, which may play role in the occurring of retinoblastoma. This work is still ongoing, will in the future a better understanding of the molecular mechanisms that could be causing retinoblastoma (16).

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**Table 1:** List of sequence amorce of Rb gene

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| --- |
| **Exon/ Promoter** **Sequence** |
| Promoteur CTGGACCCACGCCAGGTTTC  GTTTTGGGCGGCATGACGCCTT  Exon1 CCGGTTTTTCTCAGGGGACGTTG  TTGCGCCCGCCCTACGCACAC  Exon 2 (35GC)TTTCACAGTAGTGTTATGTG  ATTTCCTCTGGGTAATGG  Exon 3 (40GC)CAGTTTTAACATAGTATCCA  ATACACTTTCATAACGGCTC  Exon 4 (35GC)AGTAGTGATTTGATGTAGAG  TGAGCTAACATTAAAAGGGA  Exon 5 (35GC)CTACTATGACTTCTAAATTACG  CAAGATGTTTGAGATTATTCC  Exon6 TCTATTATGCATTTAACTAAGG  (30GC)GAGTACCAGAATTATAGGAAC  Exon 7 (25GC)ATACTCTACCCTGCGATTTT  CATTTGTTGTATTTTGACCC  Exon 8 TATCCTTCTAATGAAACCTA  GCTCATAACAAAAGAAGTAA  Exon 9 TTGACACCTCTAACTTACCC  (40GC)ACAATTATCCTCCCTCCACA  Exon 10 ACCTCACTTTTAGATAGACC  (45GC)GTTATAGGACACACAATTCACA  Exon 11 (40GC)GCTGGGTCATCTATTTTCTA  ACCTGGCCTTCAATATATA  Exon 12 GAGGCAGTGTATTTGAAGAT  AATGGATAAACGGAACGAGT  Exon 13 (50GC)CTTATGTTCAGTAGTTGTG  CCTATGTGTTCCTTTATTAC  (50GC)CTTATGTTCAGTAGTTGTG  Exon 14 CCATATTTGTAAGAAGGGT  (45GC)GATGATCTTGATGCCTTGAC  Exon 15-16 ATTCAATGCTGACACAAATAAGGTT  TTCTCCTTAACCTCACACTATCC  Exon 17 GCTATTTCCTATGAGTCCGT  GAGTTCTTGTTTATCCCTTA  Exon 18 (50GC)GACTTTTAAATTGCCACTGT  ATTCCCTACAGTTTCTTTAT  Exon 19 AGGCAGTAATCCCCAGGAAAAGCCA  CACAGAGATATTAAGTGACTTGCCC  Exon 20 TTCTCTGGGGGAAAGAAAAGAGTGG  AGTTAACAAGTAAGTAGGGAGGAGA  Exon 21 TCCTGGATAATTGAGCCTTG  (45GC)CCTTATCTTTCCAATTCTAT  Exon 22 (50GC)TAATCCAAGCCTAAGAAGTA  GATACTTTTGACCTACCCTG  Exon 23 TCTAATGTAATGGGTCCACCAAAAC  CATCTTGCGTTGCTTAAGTCGTAAA  Exon 24 TAAAACTAAGAGACTAGGTGAGTAT  TAGATTTGGGTAGGAAAAAAATCTC  Exon 25 ATATTTGGTCCAATGAAGCAGAAAATT  TGATGCTATGTATTTTTTCAGTGGT  Exon 26 AACCACTGTATTTTGTGAGAACCAC  TGAATGTGGTCAAGCAATGT  Exon 27 TCTAGCTATTTGAATATGCA  (46GC)AAATCTTGTGTAAATCCTGCC |

**Table 2:** Exonic mutations of Rb gene

|  |
| --- |
| Exon n° Position of the mutation Consequences |
| 1 Transition G→A, 91 Glu 31 Stop  7 del 4pb codons 7-10, 24pb Codon Stop 212    8 Transition C→T, 45pb Arg225Stop  18 Transition C→T, 40pb Arg578Stop  19 Transversion G→C, 80pb Ala635Pro  20 Transversion T→A, 1pb Val654Glu  23 del 7pb, codon 23 et 27 Stop 809 |

**Table 3:** Intronic mutations of Rb gene

|  |
| --- |
| Amplified region exon n° Position of the mutation |
| Exon 2 transition C→T, 76pb  Exon 3 transition T→C, 12pb intron3  Exon3 transition A→G, 37pb intron3  Exon 3 transition C→T, 45pb intron3  Exon 4 transversion G→T, 23pb intron4  Exon 7 transition G→A, 78pb intron7  Exon 9 transition T→C, -29pb intron8  Exon 9 transversion C→A, -15pb intron8  Exon 11 transversion A→C, 41pb intron11  Exon 17 delA -56pb intron16  Exon 12 transition G→A, 1pb intron12  Exon 19 transition T→C 61pb |

Inactivation of both alleles of Rb gene represents the tip of an iceberg of event that determines retinoblastoma development, progression and severity. It is well established that mutations in the Rb gene alone are not sufficient to trigger retinoblastoma. An increasing number of studies have suggested that the epigenetic modifications are associated with retinoblastoma development (17, 18).

**Conclusion**

The situation is different depending on whether a sporadic case with a first event or a germinal case with only somatic mutations.

For patients with a constitutional anomaly Rb gene, an investigation is crucial to distinguish whether they are carriers of the deleterious allele but are asymptomatic or whether it simply a neomutations. For individuals with no constitutional mutation, we can confirm a non- transmissible retinoblastoma and the investigation of non affected family members is not need.

In retinoblastoma, as in most cancers, the prognosis depends on early diagnosis. The early identification of mutation allows a rapid management, continuous monitoring and thereby contribute to the prevention of disease.

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