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

Lotfi Louhibi[1, 2], Amina Mama Boubekeur[2], Khadidja Mahmoudi[3], Rym Khadidja Abderrahmane[2], Fatima Zohra Nehili[4], Abdellah Boudjema[2], Meriem Aberkane[5], Nadhira Saidi-Mehtar[2].

1 – Faculty of Science, department of Biotechnology, University of Oran Es- Senia, Oran, Algeria
2 - Faculty of Science, department of Applied Molecular Genetics, University of Science and Technology of Oran, Mohamed Boudiaf, Oran, Algeria
3 - Pediatric Ophtalmology Service « pediatric Hospital EHS of Canastel » Oran, Algeria
4 - Ophtalmology Service, Ophtalmology Clinic « Front de mer » CHU Oran, Algeria
5 - Faculty of Medecine, University of Oran Es-sénia, Oran, Algeria.
Lotfi Louhibi: Email adress: louhibi@yahoo.com. Phone : +213665003191, ful postal address: University of Science and Technology Mohamed Boudiaf, El mnaouar 1505 USTO-MB 31000 Oran, Algeria.

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.
[Lotfi Louhibi, Amina Mama Boubekeur, Khadidja Mahmoudi, Rym Khadidja Abderrahmane, Fatima Zohra Nehili, Abdellah Boudjema, Meriem Aberkane, Nadhira Saidi-Mehtar. **Molecular study of the retinoblastoma in western Algerian population. Research of gene Rb mutations at the constitutional level.** *Cancer Biology* 2013;3(2):1-7]. (ISSN: 2150-1041). http://www.cancerbio.net. 1

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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).

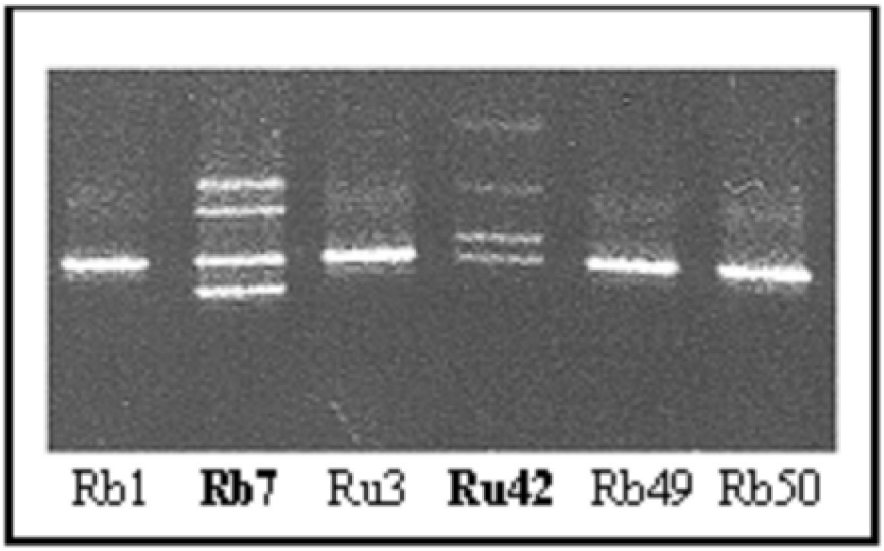

Figure 1: Identification of two sequences variations in exon 9 by DNA DGGE electrophoresis. Patients RB7 and Ru42 shows four bands different profiles.

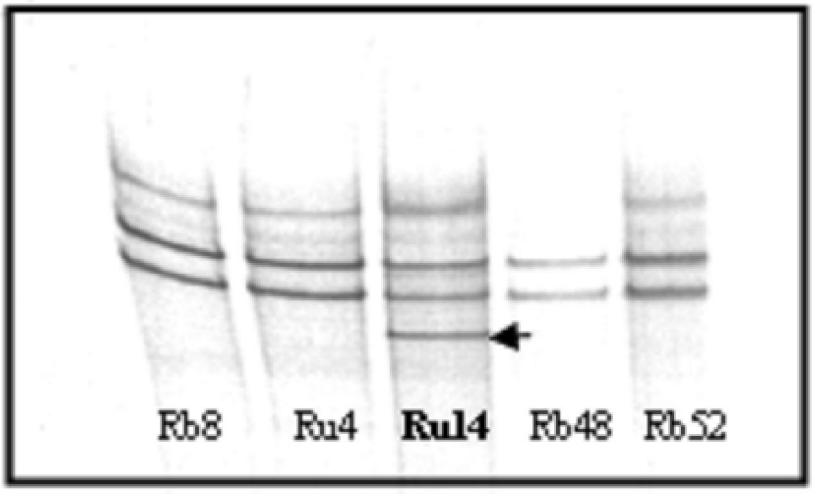

Figure 2 : Identification of variation sequence in exon 7 by SSCP DNA electrophoresis. The patient Rus14 shows additional band profile (arrow)

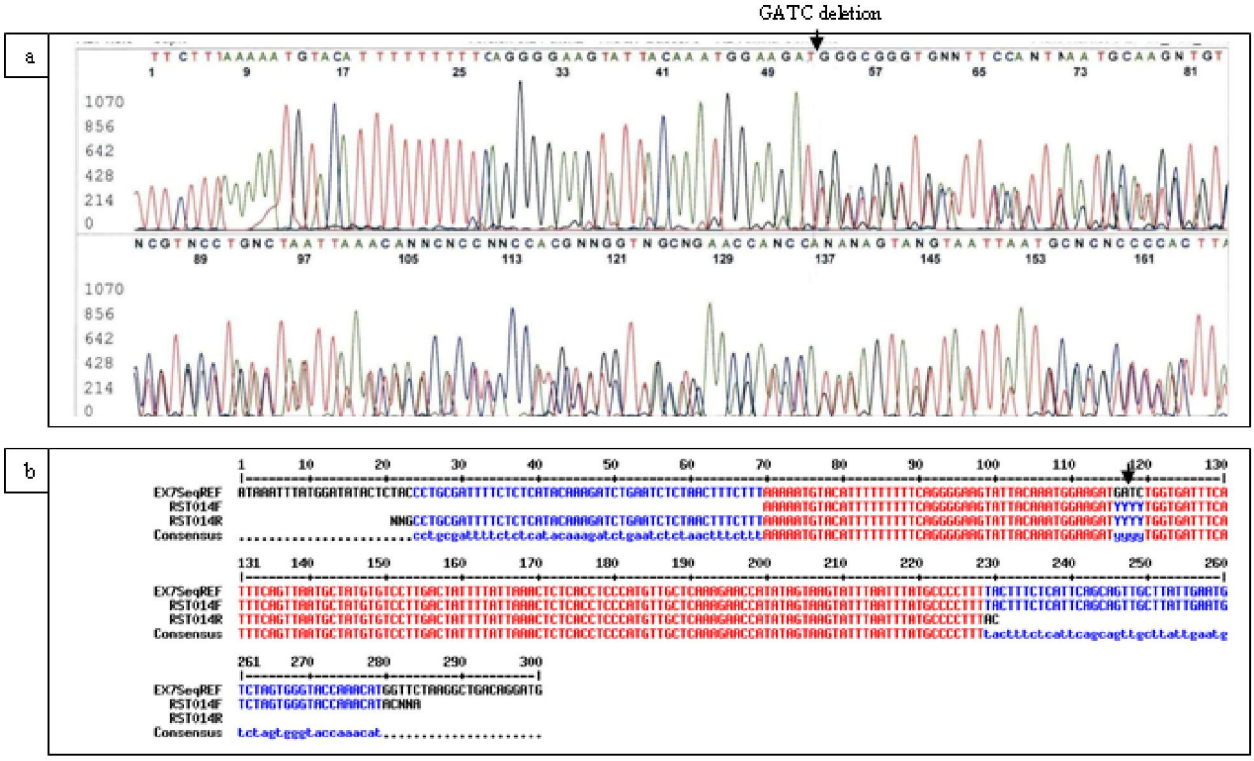

Figure 3: Patient Ru14 DNA sequencing shows a heterozygous deletion in exon7 of GATC bases in codons 7-10 (arrow).

a) Frame shift mutation. Heterozygous deletion revealed by peaks overlapping.

b) Deletion confirmed by the two strands alignment (arrow).

Table 1: List of sequence amorce of Rb gene

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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