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ONLINE RESOURCES

Rapid detection of *RB1* recurrent mutations in retinoblastoma by ARMS-PCR

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Introduction

Retinoblastoma, a relatively rare cancer, is the most common intraocular tumour in childhood (1/15000) which mainly affects children under the age of 6 years (Valverde *et al.* 2005). It is caused by mutations in the *RB1* tumour suppressor gene which occurs in both sporadic and familial forms. In about half of the patients, one mutation is inherited via the germinal cells, while in the remaining cases both mutations occur in the somatic cells. Genetic analysis of *RB1* may have a major impact on genetic counselling of families with retinoblastoma, since preimplantation or prenatal genetic diagnosis could be performed once the mutation was identified (Noorani *et al.* 1996; Richter *et al.* 2003). Further, unaffected at-risk individuals should be regularly monitored with examination under anesthesia; however, if no mutation was identified by genotyping, there is no longer need for follow-up clinical visits.

The *RB1* gene on chromosome 13q14 consists of 27 exons, spanning approximately 180 kb of genomic DNA (Friend *et al.* 1986). So far, a wide range of alterations have been identified in the *RB1* gene (Richter *et al.* 2003; Houdayer *et al.* 2004; Lohmann and Gallie 2004; Valverde *et al.* 2005). Given the large size of *RB1* and absences of hot spots or common mutations in the *RB1*, a full gene analysis is difficult and costly. Therefore, most diagnostic laboratories prefer to carry out exon sequencing approaches which would be expected to detect more than two-third of all mutations in *RB1* (Parsam *et al.* 2009). Nonsense mutations frequently occur in 11 CGA codons (arginine) in the *RB1* gene. These mutations are primarily C to T transition, occurring at CpG dinucleotide and consequently results in a change of CGA to TGA stop codon (R to X mutations). These arginine alterations, together with mutation of the donor splice site in intron 12 account for approximately one-third of all mutations in *RB1* (Cowell *et al.* 1994; Lohmann 1999; Richter *et al.* 2003; Valverde *et al.* 2005; Rushlow *et al.* 2009). Therefore, simple, rapid and reliable methods such as amplification refractory mutation system (ARMS) PCR which could simultaneously analyse a sample for the presence of multiple mutations would be useful. The aim of this study was to develop a multiplex ARMS-PCR method to screen for the most recurrent mutations in *RB1* in patients with retinoblastoma.

Materials and methods

Subjects

To investigate recurrent mutations of *RB1* in retinoblastoma patients, 121 children with sporadic or familial retinoblastoma

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Keywords. retinoblastoma; ARMS-PCR; *RB1* gene; recurrent mutation.

Table 1. Primer sequences, fragment sizes and relevant point mutations of *RB1* gene.

Mutation	Primer name	Sequence	Length (bp)
R251X	251-f-M	F: 5′-TGTTATACCCATTAATGGTTCACCCT-3′	340
	Com8	R: 5′-TCCAGAGTGAGGGAGCTACTCT-3′	
R255X	255-s-M	F: 5′-GTTCACCTCGAACACCCAGTT-3′	322
	Com8	R: 5′-TCCAGAGTGAGGGAGCTACTCT-3′	
R320X	Com10	F: 5′-ATTGCATGCGAACTCAGTGT-3′	186
	320-q-M	R: 5′-CTTTATTTTTAAGATAAATTTCTTCGTATGA-3′	
R358X	358-w-M	F: 5′-TATTTAGTTTTGAAACACAGAGAACACCTT-3′	154
	Com11	R: 5′-CAAATCTGAAACACTATAAAGCCATGAATAAC-3′	
R445X	Com14	F: 5′-ATCCAGGTACTGGACCTACCC-3′	394
	445-v-M	R: 5′-TACAAGCGAACTCCAAGTTTGTATTA-3′	
R455X	Com14	F: 5′-ATCCAGGTACTGGACCTACCC-3′	426
	455-q-M	R: 5′-CTGATTTAAGCATGGATTCCATTACTGA-3′	
R467X	Com14	F: 5′-ATCCAGGTACTGGACCTACCC-3′	872
	467-q-M	R: 5′-GTAAAAAATTTACCTAAAATTTTGAATGGATAATGA-3′	
R551X	Com17 F1	F: 5′-AATAGATATGCCAATGGCTGATAAG-3′	483
	551-q-M	R-5′-TTCCATGATTCGATGTTCACATGA-3′	
R556X	Com17 F2	F-5′-TTTGTCTTTCCCATGGATTCTGAATG-3′	157
	556-q-M	R: 5′-CCATGCAAGGGATTCCATGATTGA-3′	
R579X	Com18	F: 5′-TTTCTAATATAAGCGTTGAAGGTTATACAT-3′	299
	579-q-M	R: 5′-GTGATCAGTTGGTCCTTCTGA-3′	
R787X	Com23	F: 5′-GCAGCTATAATCCAAGCCTAAGAAG-3′	458
	787-q-M	R: 5′-AACTAGGAAACTTGTAAGGGCTTGA-3′	
IVS12+1	Com12	F: 5′-GTGTATTTGAAGATACATTTAACTTGGG-3′	225
	IVS12-j-M	R: 5′-ATTACAATAAATAATGTTTCATATATGGCTTCT-3′	
Control	Forward	F: 5′-CCAAGTGACAAATAGCAAGTGTT-3′	600
	Reverse	R: 5′-AGATATTCTGCAAGTACAATCACATT-3′	

were recruited from the Farabi, Rasool-Akram and Mahak hospitals in Tehran, Iran. The study was approved by the Avicenna Research Institute's ethics and human rights committee. The parents and guardians of all patients were consulted and a written informed consent was obtained.

DNA extraction

From each subject, 5 mL of peripheral blood was collected in EDTA containing tube and DNA was extracted by standard salting out protocol (Miller *et al.* 1988).

Designing of primer for ARMS-PCR

Normal and mutant-specific primers were designed for each point mutation, including R251X, R255X, R320X, R358X, R445X, R455X, R467X, R551X, R556X, R579X, R787X and IVS12+1. ARMS-PCR primers were designed according to the approaches used by Ferrie *et al.* (1992). To increase the specificity of ARMS assays, additional mismatches were included in the penultimate bases of mutant ARMS primers. Therefore, purine/pyrimidine mismatches in the penultimate positions were preferentially selected, due to the fact that certain mismatches have a less destabilizing effect. Presence of mismatches at the 3′ end of the mutant-specific primers decreases the amplification of normal DNA sequences to the minimum possible extent because of the destabilizing effect. However, despite this general rule, some primers with purine/pyrimidine mismatches in the penultimate position may amplify both normal and mutant DNA sequences. To overcome this problem, the purine/purine or pyrimidine/pyrimidine mismatches were obligatorily used. On the other hand, to avoid false negative results in the ARMS reactions and to ensure the fidelity of the PCR amplification process, a pair of primers was used as an internal control in each reaction. The internal control primers were selected from chromosomal regions other than chromosome 13. Primer pairs which gave the best results are shown in table 1.

Multiplex ARMS reaction conditions

The ARMS-PCR assays were optimized as three individual multiplex reactions according to the annealing temperature and location of each primer pair (table 2). Each multiplex ARMS-PCR reaction contained: 2.5 μL 10× PCR Buffer (Roche, Berlin, Germany), 3 mM $MgCl_2$, 2.5 mM of each dNTP (Roche), 5 pmol of each relevant primer (table 3), 50 ng template DNA, 1 U *Taq* DNA polymerase (Roche) and sterile distilled water to 25 μL. PCR was performed under the following conditions: an initial denaturation at 94°C for 5 min, followed by 35 amplification cycles of 94°C for 30 s, 57–61°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 5 min (table 2). PCR products were separated on 2% agarose gel containing ethidium bromide and

Table 2. Multiplex ARMS-PCR reactions and the condition of each reaction.

Reaction	Mutation	Exon number	Length (bp)	Annealing temperature
Multiplex 1	R255X	8	322	61
	R358X	11	154	
	R445X	14	399	
	R455X	14	426	
	R467X	15	872	
	Control	–	600	
Multiplex 2	R320X	10	186	57
	R551X	17	483	
	R579X	18	299	
	Control	–	600	
Multiplex 3	R251X	8	340	60
	R556X	17	157	
	R787X	23	458	
	IVS12	12	225	
	Control	–	600	

visualized under UV light. All detected mutations were confirmed by direct sequencing. To ensure the fidelity of each multiplex ARMS-PCR assay, individual reactions were tested on at least 50 different normal controls DNA.

Results

In the current study, 121 unrelated Iranian retinoblastoma patients were analysed for the presence of 12 recurrent mutations in *RB1*. Among these, 56 patients were unilateral and 65 patients had bilateral tumours. The mean age of patients was 17.6 months, and it was 21.7 and 14.4 months for unilateral and bilateral patients, respectively. ARMS-PCR reactions were performed for all samples and mutations were identified in 30 (24.8%) patients (figure 1; table 3). Among the cases, two (out of 56) and 28 (out of 65) point mutations (3.6% versus 43.1%) were found in patients with unilateral and bilateral forms, respectively. According to our results,

Table 3. Mutations detected using new multiplex ARMS-PCR.

Mutation	Bilateral patients	Unilateral patients
p.R251X	2	0
p.R255X	1	0
p.R320X	2	0
p.R358X	2	0
IVS12+1G>T	0	2
p.R445X	5	0
p.R455X	1	0
p.R467X	3	0
p.R551X	2	0
p.R556X	2	0
p.R579X	4	0
p.R787X	4	0
Sum	$28/65 = 43\%$	$2/56 = 3.5\%$

the mutation detection rate in bilateral patients was significantly higher than unilateral ones ($P < 0.02$). All above-mentioned mutations were confirmed by direct sequencing. However, in one sample with a R787X mutation sequencing result was clearly normal.

Discussion

Rapid and cost-effective methods for mutation screening are significant factors for diagnostic laboratories (Noorani *et al.* 1996). Today, mutations are detected by an increasing number of methods, ranging from genomic restriction fragment length polymorphism (RFLP) to next generation whole genome sequencing approaches. However, each of these techniques has its own advantages and limitations. In many genetic disorders, the responsible mutations can be detected easily, because there are relatively few common mutations in specific ethnic populations. In contrast, in some single gene disorders, for example, retinoblastoma a wide variety of different mutations spread throughout the gene are implicated. This makes mutation analysis very difficult, and therefore a technique which could simultaneously detect multiple mutations would be useful (Valverde *et al.* 2005; Rushlow *et al.* 2009; Ahani *et al.* 2011).

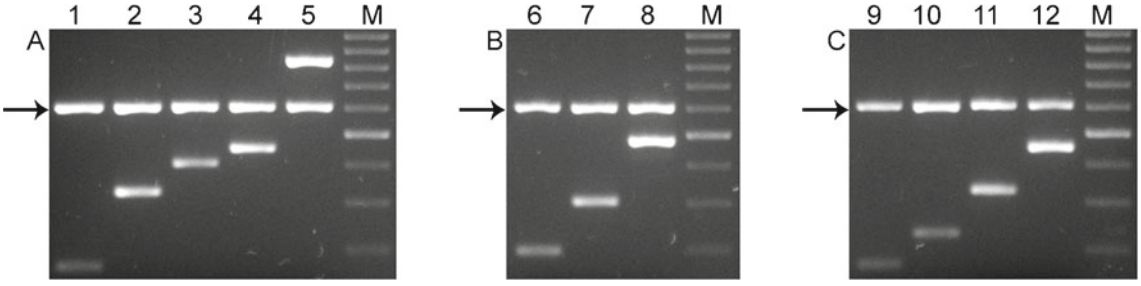

Figure 1. Mutations detected by ARMS-PCR assay. A, B and C depict the results of multiplex ARMS-PCR reactions 1, 2 and 3, respectively. (1) R358X, (2) R255X, (3) R445X, (4) R455X, (5) R467X, (6) R320X, (7) R579X, (8) R551X, (9) R556X, (10) IVS12+1, (11) R251X, (12) R787X; M, marker size 100 bp. The arrows show internal control bands.

RB1 is a relatively large gene, and to date many different mutations have been identified in all its exons. The presence of multiple unique mutations together with the absence of mutational hot spots in the *RB1* gene, making genetic testing complex and challenging, and accordingly, the entire coding region and flanking sequences should be analysed. However, some recurrent mutations in 11 CGA codons of *RB1* have been reported by several investigators (Cowell *et al.* 1994; Lohmann 1999; Houdayer *et al.* 2004; Bamne *et al.* 2005; Taylor *et al.* 2007; Rushlow *et al.* 2009; Chen *et al.* 2010; Ahani *et al.* 2011). Most sporadic bilateral patients carry a constitutional mutation. In the literature, the mutation detection rate for sporadic bilateral and unilateral patients are 60–90% and 10–20%, respectively. The frequency of constitutional mutations in unilateral patients is lower than bilateral, since they may have mosaic mutations which cannot not be detected in blood samples (Richter *et al.* 2003; Houdayer *et al.* 2004; Valverde *et al.* 2005).

In the current study, a simple ARMS-PCR method was designed for rapid screening of the recurrent mutations in *RB1*. One hundred and twenty-one patients were analysed by ARMS-PCR and totally 12 different mutations were detected in 30 patients (24.8%). Mutation detection rates in the unilateral and bilateral cases were 3.6% (2/56) and 43.1% (28/65), respectively. There was a significant difference in mutation detection rate between the bilateral and unilateral retinoblastoma, which could be explained by different penetrance of germ-line mutations. Based on previous studies, some *RB1* mutations such as nonsense mutations have a complete penetrance, which usually lead to bilateral multifocal retinoblastoma. In contrast, mutations that disrupt splicing or alter promoter function as well as missense mutations that occur outside the pocket domains of pRb, have a reduced penetrance (Lefevre *et al.* 2002; Valverde *et al.* 2005; Taylor *et al.* 2007; Zhang *et al.* 2008; Abouzeid *et al.* 2009). These types of mutations usually result in unilateral retinoblastoma which may be unifocal or multifocal. Similarly, in two unilateral cases from this study, mutation in the donor splice site of intron 12 (IVS12+1) was detected; whereas, all the 28 detected mutations in bilaterals were nonsense (table 3).

To our knowledge, PCR-based methods for detection of common mutations in *RB1* have been reported in only few studies (Richter *et al.* 2003; Rushlow *et al.* 2009). The importance of PCR-based techniques for mutation detection in retinoblastoma has recently been shown by Rushlow *et al.* (2009). They found mutations in 92.6% of the cases by a combination of sequencing (for detection of point mutations) and deletions/duplications analysis of *RB1*. Moreover, additional mutations in the cases with clearly normal sequencing results were found by PCR-based methods, so the detection rate increased to 94.8%. This could be explained by low-level mosaicism; when only low percentages of lymphocytes carry a mutation, depending on the proportion of mutated cells, it would be difficult to detect a mutation by direct sequencing. High normal background in sequencing results due to the presence of normal cells within the sample can mask the presence of mutations. PCR-based mutation detection methods have a relatively high sensitivity, so that in samples with low-level mosaicism mutant alleles can be specifically detected. Similarly, we found a R787X mutation by ARMS-PCR assay, whereas the sequencing result for exon 23 of *RB1* in this patient was clearly normal.

Recurrent mutations in *RB1* in 20–40% of patients (in this study, 43% of bilateral patients) could be screened by ARMS-PCR, which can be considered as a rapid and cost-effective technique. As mentioned above, the entire coding region should be screened to detect other mutations in *RB1*. However, some relatively large rearrangements, including deletions and duplications, ranging from single exon to whole gene deletion, account for approximately 10–20% of the total mutations in *RB1*. Nowadays, such large rearrangements can be detected by multiplex ligation-dependent probe amplification (MLPA). By a combination of MLPA and ARMS-PCR assays in routine genetic analysis, mutation detection rate may increase to 30–50%. Therefore, these approaches can detect *RB1* mutations in about half of the patients within a short time. If no mutations were detected by ARMS-PCR and MLPA, the whole coding region should be sequenced.

In conclusion, we suggest that ARMS-PCR as a simple, reliable, rapid, and cost-effective technique, could be used in the first step of genetic analysis in patients with retinoblastoma.

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