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Detection of Arg72Pro Polymorphism of the Tumor Suppressor Gene (*TP53*) by a Rapid One-step Tetra-primer Amplification Refractory Mutation System-PCR

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ABSTRACT

The Tumor Protein p53 gene (*TP53*), known as the guardian of the genome, encodes the p53 protein that plays an important role in cell cycle and in maintenance of the genome stability by preventing mutations. The transition from G to C at codon 72 of the *TP53* gene represents a non-synonymous single nucleotide polymorphism (Arg72Pro) that has been extensively genotyped for association with a wide variety of cancers. A new method for Arg72Pro detection is described based on the tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR), with a single PCR to discriminate both alleles. Two primers that amplify a common amplicon independently of the allele considered are combined in a single PCR reaction with two specific primers, differing in the 3' base. In the presence of Pro/Pro or Arg/Arg, amplification occurs both in the general amplicon and in the specific allele; in the presence of Arg/Pro three different amplicons are produced. Validation by PCR-RFLP revealed 100% accordance for genotype assignment. The method was successfully applied for genotype determination of the Arg72Pro in a novel population from Aleppo, Syria, with 51% homozygous for the Arg allele and 12% homozygous for the Pro Allele.

Key words: Single nucleotide polymorphism, tumor protein p53, RFLP, rs1042522, human polymorphism, polymerase chain reaction

INTRODUCTION

The human *TP53* tumor suppressor gene is located on chromosome 17p13 and encodes a 53 kDa nuclear phosphoprotein, which plays a central role in many cellular processes, such as cell-cycle control, DNA repair and apoptosis (Levine, 1997). Since *TP53* plays an important role in cell cycle regulation and in maintenance of genome stability by preventing mutations, it is often referred to as the guardian of the genome (Matlashewski *et al*., 1984). Loss or mutation of *TP53* is probably the commonest single genetic change in cancer (Olivier *et al*., 2009; Petitjean *et al*., 2007a; Malkin, 2001; Fisher, 2001; El-Deiry, 2003).

A common *TP53* polymorphism at codon 72 of exon 4 (dbSNP ID: rs1042522), designated as Arg72Pro, has been reported to modify the risk and/or prognosis of many types of cancers, such as colorectal, esophagus, stomach, ovary, cervix, bladder, breast, prostate and lung cancer (Zehbe *et al*., 2001; Lee *et al*., 2000; Belyavskaya *et al*., 2006; Costa *et al*., 2008; Noma *et al*., 2004;

Chen *et al.*, 2000; Weston *et al.*, 1992; Perfumo *et al.*, 2006; Agorastos *et al.*, 2004; Olivier *et al.*, 2005; Petitjean *et al.*, 2007b; Pietsch *et al.*, 2006). This polymorphism derives from a single-nucleotide substitution at codon 72, where either CCC encodes proline or CGC encodes arginine, resulting in a non-conservative change. This polymorphism is located in a proline-rich domain of p53, which is known to be important for the growth suppression and apoptotic functions (Pietsch *et al.*, 2006). Evidence is emerging indicating that the Arg allele and the Pro allele of the *TP53* codon 72 polymorphism are not equivalent in biochemical property and function (Thomas *et al.*, 1999).

The most common method used to date for Arg72Pro genotype determination is the polymerase chain reaction based-restriction fragment length polymorphism method (PCR-RFLP) (Lee *et al.*, 2000; Belyavskaya *et al.*, 2006; Costa *et al.*, 2008). The method is relatively slow, since it comprises several steps, including PCR, restriction enzyme digestion of the PCR product and gel electrophoresis. Polymerase Chain Reaction-Single Strand Conformational Polymorphism (PCR-SSCP) was also used for the detection of Arg72Pro (Noma *et al.*, 2004).

The aim of the present study was to develop a rapid single-step mismatch PCR method using tetra-primer Amplification Refractory Mutation System (ARMS) for the detection of Arg72Pro, which is probably the most extensively studied single nucleotide polymorphism in cancer research. We have also applied the method to characterize the distribution of this polymorphism in a novel population from Aleppo, Syria.

MATERIALS AND METHODS

Subjects: The population studied consisted of 92 white healthy unrelated subjects living in Aleppo, Syria (50 males, 42 females, Mean age 41±10). The participants were not previously diagnosed with cancer. All subjects were native Syrians. Informed consent was obtained from each subject. Blood samples were collected in EDTA tubes and anonymously coded and stored.

DNA isolation: Genomic DNA was isolated from 200 μL venous blood using a spin column format kit (EuroGold™ Blood DNA mini kit, Euroclone, Italy). The procedure was carried out according to the manufacturer recommendations.

Quantification of Genomic DNA: Quantification of DNA was performed using V-650 UV-VIS spectrophotometer (Jasco, Japan). A260/A280 ratios were in the range of 1.8-1.9 indicating high purity Genomic DNA extract. Extraction yield was in the range of 4-8 μg per 200 μL blood.

Tetra primer ARMS-PCR analysis: Four primers were used in a single PCR reaction (schematic illustration in Fig. 1). Two primers, P1 and P2 (Table 1), were designed to amplify a 281 bp band which served as a control for the success of amplification. Two specific primers, P3 and P4, with complementary 3'-terminal nucleotide to the corresponding polymorphism, were introduced (Table 1). To enhance the specificity, a destabilizing mismatch was incorporated at the 3rd nucleotide from the 3'-terminus in each primer.

The PCR reaction was carried out in a total volume of 30 μL, containing 50-100 ng of genomic DNA as template, 0.125 μM of P1 and P2, 0.25 μM of P3 and P4, 0.2 mM of each dNTP, 2.4 mM

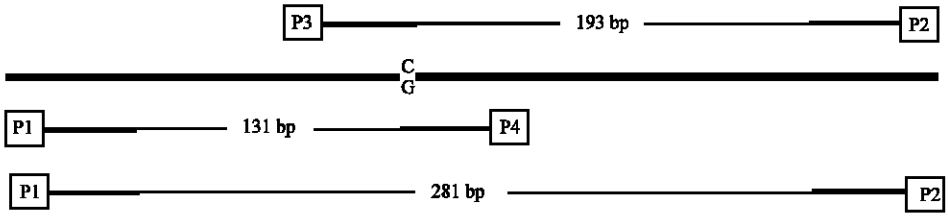

Fig. 1: Schematic illustration of the expected amplicons in ARMS analysis. Primers P2 and P1 always amplify a common region spanning the Arg72Pro pylomorphism. The amplification of a 193 or 131 bp band indicates the presence of either the Proline or Arginine allele, respectively

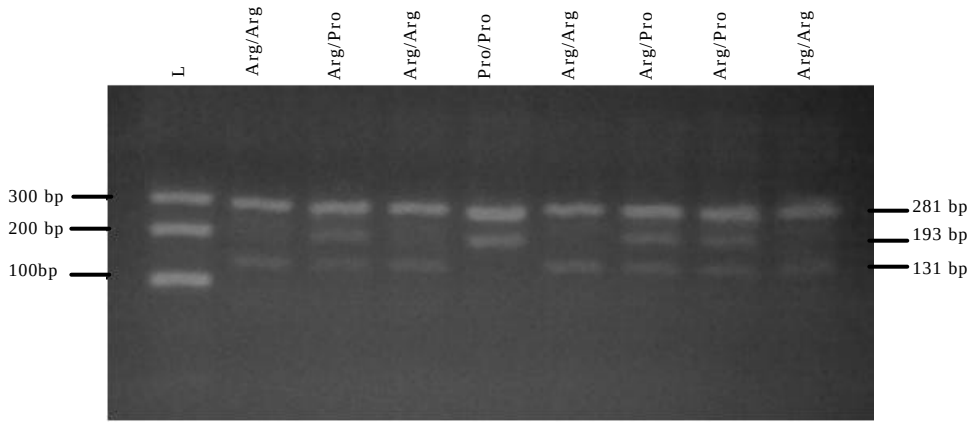

Fig. 2: Agarose gel electrophoretogram of the *TP53* Arg72Pro polymorphism analysis by tetra-primer ARMS-PCR for 8 different subjects. L: DNA ladder

Table 1: Primers used in Arg72Pro *TP53* analysis by tetra primer ARMS-PCR

Primer	Sequence (5'→3')
P1	GCCGTCCCAAGCAATGGATGATT
P2	GGCAACTGACCGTGCAAGTCACAG
P3	AGAATGCCAGAGGCTGCTCC<u>A</u>CC
P4	CTTCTGGTGCAGGGGCCA<u>A</u>GC

Underlined bases indicate the introduced mismatches

of $MgCl_2$, Taq buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl) and 1.3 U of JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, USA). PCR amplification was carried out in a MasterCycler® thermal cycler (Eppendorf, Germany), with an initial denaturation at 94°C for 4 min, followed by 36 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 5 sec and a final extension at 72°C for 5 min. Six microliter of loading buffer was added to the PCR reaction and a 25 μL aliquot was subjected to agarose gel (2%) electrophoresis. The procedure rendered 3 bands in heterozygotes (281, 193 and 131 bp) and 2 bands in homozygotes (Arg/Arg resulting in 281 and 193 bp and Pro/Pro resulting in 281 and 131 bp) (Fig. 2).

Validation by PCR-RFLP analysis: Genotype assignment was validated for 8 samples using the PCR-RFLP method which was carried out as previously reported (Lee *et al*., 2000). The PCR product was digested with BstUI (Fermentas, Lithuania) according to the manufacturer's directions. The digested PCR fragments were separated on 3.5% agarose gels. Genotypes were scored as homozygous for the Pro allele if the 199-bp PCR fragment after digestion remained uncut or homozygous for the Arg allele if after enzyme digestion two bands (113 and 86 bp) were observed. Gels with three DNA fragments (199, 113 and 86 bp) were scored as heterozygotes.

RESULTS AND DISCUSSION

Tetra primer-ARMS PCR analysis: PCR reaction conditions were thoroughly optimized to obtain the highest yield and specificity. The use of a HotStart method was found to be necessary in order to eliminate the formation of primer dimers increasing the yield of the informative amplicons. Figure 2 shows the electrophoretogram of tetra-primer ARMS-PCR products for 8 different genomic DNA samples. The three Arg/Arg, Arg/Pro and Pro/Pro genotypes were clearly distinguished. A common band of 281 bp size as the internal control appeared in all the cases and the presence of either the Arg or Pro variant rendered an additional fragment of 131 or 193 bp, respectively. Analysis by PCR-RFLP proved the validity of the proposed method.

RFLP-PCR has been the traditional method for the detection of Arg72Pro (Lee *et al*., 2000; Belyavskaya *et al*., 2006; Costa *et al*., 2008). The several steps associated with the use of this method, especially restriction enzyme digestion, increase analysis time. By contrast, the proposed method involves a single PCR step. The PCR-SSCP method (Noma *et al*., 2004) is difficult to implement and may require re-optimization. By contrast, the proposed method is simple, robust and demands low level of expertise.

The speed, simplicity, ease of use, cost-effectiveness and high accuracy of the proposed method make it suitable for the analysis of very large number of samples, which is a demand for reliable case-control and population genetic studies.

Genotype distribution of Arg72Pro polymorphism in a sample population from syria: A total of 92 subjects living in Aleppo were genotyped for the Arg72Pro polymorphism, which is known to be differentially distributed among different populations. Among the 92 subjects genotyped, 47 (51%) were homozygous for the Arg allele, 11 (12%) were found to be homozygous for the Pro allele and 34 (37%) were found to be heterozygous. The genotype distribution was found to be in Hardy-Weinberg equilibrium ($\chi^2 = 1.49$; df = 2; p = 0.47) (Table 2).

Table 2: Genotype and allele distribution for Arg72Pro *TP53* polymorphism in a syrian population

	Genotype			Alleles	
	AA	AP	PP	A	P
Cases (n = 92)	47	34	11	128	56
Frequencies (%)	51	37	12	69.6	30.4

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