



Analysis of *FOXO3a* Gene Polymorphism Associated with Asthma

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Abstract

Single-nucleotide polymorphisms (SNP) represent genetic variation in a human population; 99.9% of the DNA sequence is identical and remaining 0.1% of DNA contains sequence variants. Around ten million SNPs are identified post human genome project. Identification of SNPs associated with disease phenotype has diagnostic potential and may predict drug response. Several technologies genotype thousands of SNPs simultaneously, for instance, genotyping arrays, gene-chip arrays, bead array technology, pyrosequencing, TaqMan approach, etc., however Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) remains a simplest, reliable, and cost effective method. In PCR-RFLP, a fragment that is to be analyzed for a mutation is amplified from genomic DNA using PCR, digested using appropriate restriction enzyme and then separated by gel electrophoresis. Sets of primer are used to amplify a fragment of interest and are designed in such a way that it flanks the polymorphic site and is located in such a way as to generate uneven sized fragments upon restriction endonuclease cleavage of the amplified PCR products. *FOXO3a* plays a key role in maintaining immunoregulation and is associated with several inflammatory diseases. Here, we report the PCR-RFLP protocol developed for detection of polymorphism in *FOXO3a* gene (rs13217795) by amplifying a 667 bp fragment followed by restriction endonuclease analysis using *PagI* to obtain RFLP patterns.

Key words *FOXO3a*, Asthma, rs13217795, SNP, Polymorphism, PCR-RFLP

1 Introduction

FOXO3a, a member of the forkhead class “O” (FOXO) transcription factor family which controls a broad range of biological processes such as DNA damage repair, apoptosis, and cell cycle regulation. Recent articles have established involvement of *FOXO* gene in development, growth, stress resistance, apoptosis, cell cycle, immunity, metabolism, and aging [1–3]. We for the first time investigated an association between single-nucleotide polymorphism (SNP) of the *FOXO3a* (rs13217795, C > T transition) gene and asthma and found that rs13217795 possibly contributed to T cells hyperactivity in Indians [4]. Also meta-analysis revealed significant association between the *FOXO3a*; rs13217795

polymorphism and longevity in terms of the C vs. the T allele (OR = 1.27, 95% CI = 1.10–1.46, $P = 0.001$) and was gender specific [4]. Polymorphisms are inherited differences found among the individuals in more than 1% of normal population. Hence further exploration of the role of the *FOXO3a* gene in human longevity and other aging phenotypes is required in different populations. We have previously used Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique to detect SNP and insertion or deletion (Indels) of bases in the human genome [5, 6]. PCR-RFLP-based analysis is a prominent technique for genotyping of species-specific variations. Important advantages of the PCR-RFLP technique include low cost and lack of requirement for advanced instruments. In addition, the design of PCR-RFLP analyses generally is simple and can be achieved using publicly available programs specially to identify appropriate restriction enzymes and designing of primers. This chapter describes the approach used to detect rs13217795 polymorphism in human *FOXO3a* gene using the PCR-RFLP technique.

2 Materials

Prepare all solutions using ultrapure water (ultrapure water is prepared by purifying deionized water, having sensitivity of 18 M Ω at 27 °C) and molecular grade reagents. Prepare all reagents and store at room temperature, unless indicated otherwise.

2.1 Equipment

1. Thermocycler.
2. Hot water bath with temperature controller.
3. Electrophoresis unit with power pack.
4. UV cabinet/ gel documentation system.

2.2 PCR-RFLP

1. Purified human DNA [7].
2. Primers.
3. *Taq* Polymerase (3 U/ μ L).
4. *Taq* Polymerase buffer A (Tris with 15 mM MgCl₂).
5. dNTP mix (10 mM, 2.5 mM each dNTPs).
6. DNA ladder (100 bp).

Store above reagents at –20 °C

7. Agarose.
8. Ethidium bromide (EtBr) (10 mg/mL) (*see Note 1*).
9. Gel-loading dye.
10. Tris—borate—EDTA buffer (5 $\times$ -TBE) (1.1 M Tris; 900 mM Borate; 25 mM EDTA; pH -8.3) (*see Note 2*).

11. Restriction enzyme (*see* **Note 3**).
12. Sterile deionized water.

3 Methods

3.1 Identification of Restriction Enzyme and Primer Design

1. The single-nucleotide polymorphism (SNP); rs13217795 in *FOXO3a* gene is selected target in this method (*see* **Note 4**).
2. Obtain available sequence of selected SNP through NCBI using Reference SNP (refSNP) number, in this protocol it is *rs132177995*.
3. Save the latest sequence in FASTA format.
4. Go to restriction enzyme site.
(Examples: 1. Webcutter 2.0: <http://bio.lundberg.gu.se/cutter2/>
2. NEBcutter V2.0: <http://www.labtools.us/nebcutter-v2-0/>).
5. Enter the sequence or upload the FASTA format and make restriction enzyme analysis in database.
6. Choose the best fitted restriction enzyme (*see* **Note 5**).
7. Once the best enzyme is chosen, design a primer pair using software tools such as Gene Runner, Primer3, etc. In the present method the designed primer amplifies the 667 bp region sequence in *FOXO3a* gene (rs132177995, C > T transition) Fig. 1.
8. Analyze the specificity of primers using BLAST search and PCR (*see* **Note 6**).

3.2 PCR RFLP

To optimize the PCR reaction and condition for RFLP, set up the PCR using 50–150 ng DNA, 5–15 pmol of primers, 30–45 PCR cycles, and 58–62 °C of annealing temperature. The PCR reaction and conditions optimized by us are mentioned in Tables 1 and 2.

1. Perform the PCR reaction for isolated DNA using designed primer pairs (Table 1) [3].
2. Carry on the PCR reaction as mentioned in Table 2. The total volume of PCR reaction was 20 µL.
3. Thermal cycling parameters for PCR reaction is mentioned in Table 3.
4. The amplified products are examined in 2% w/v agarose gel (Fig. 2).
5. Prepare the restriction mixture on ice for digesting 10 µL of PCR product as indicated in Table 4 (*see* **Note 7**).

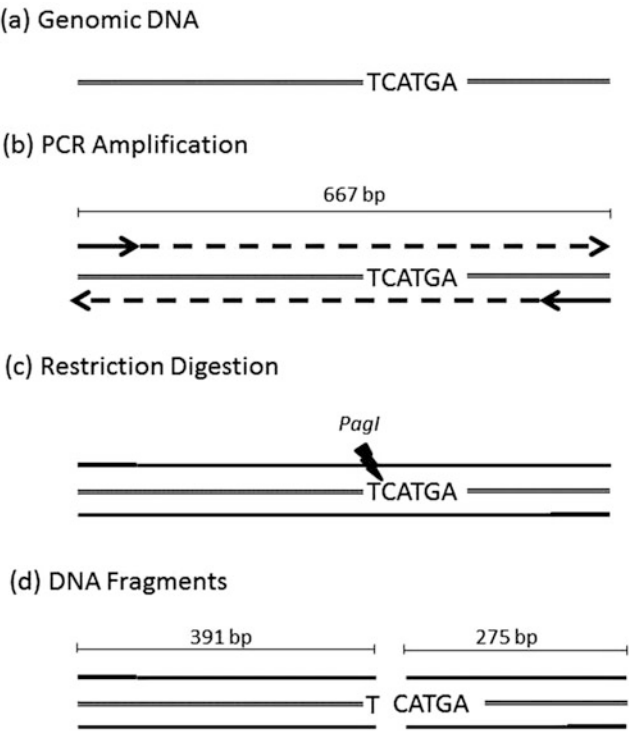


Fig. 1 Schematic representation of restriction site for *PagI* intergenic region sequence (667 bp) to detect rs13217795 polymorphism (not to scale)

Table 1
Technical data for SNP analysis in human FOXO3a gene

Gene name	SNP	Primers	GC content (%)	Annealing temperature (°C)	Restriction enzyme	Amplicon size (bp)
FOXO3a	rs13217795	F: 5'- CTCCTTGGTTCAGTTTGGTG - 3' R: 5'- ATGAGTGAAGATGGAAGTAAGC - 3'	52.6% 40.9%	62 °C	<i>PagI</i>	667 bp

6. Incubate at 37 °C for 15 min using Thermalcycler or water bath.
7. Prepare 2% w/v agarose gel for analysis of digested samples.
8. After incubation, mix 20 µL of the digested samples with loading dye. Slowly load the sample mixture into the wells of the submerged gel under TBE using a disposable micropipette.
9. Run the agarose gel at 90 V for 2 h 30 min. Examine the gel using UV cabinet/gel documentation system and photograph the gel showing the RFLP patterns (Fig. 3).
10. Analyze the restriction profiles as mentioned in Table 5.

Table 2
PCR reaction

Reaction components	Volume (effective concentration)
DNA template (100 ng)	0.5 μ L
Taq Polymerase buffer A	3 μ L
dNTPs (10 mM)	1 μ L
Primers (10 pmol)	0.3 μ L each
Taq DNA Polymerase (3 U/ μ L)	0.3 μ L
Deionized water	14.6 μ L
Total	20 μ L

Table 3
PCR condition

Steps	Temperature	Time
1. Initial denaturation.	95 °C	5 min
2. Denaturation.	95 °C	30 s
3. Annealing.	62 °C	30 s
4. Extension.	72 °C	1 min
Go to step 2 and repeat 35 times		
5. Final Extension	72 °C	5 min
Hold at 25 °C		

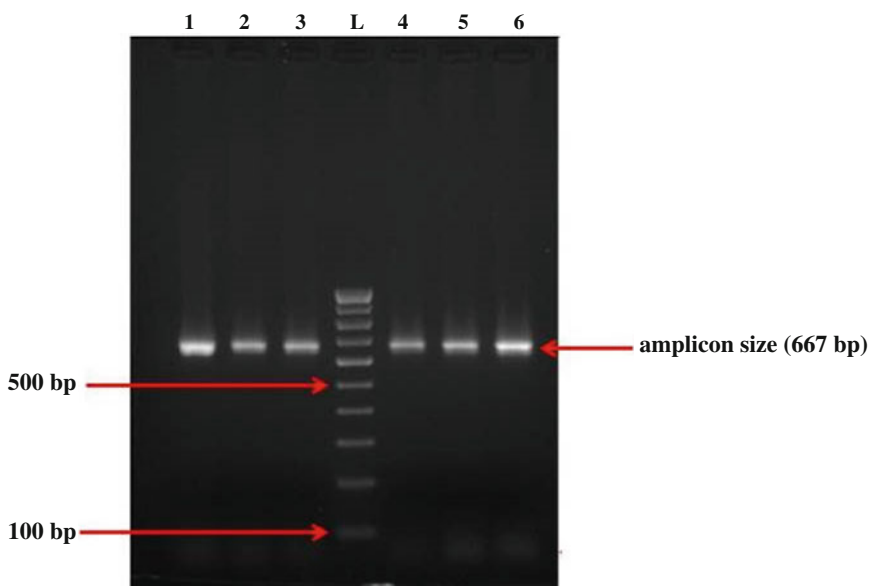
**Fig. 2** Agarose gel picture of desired amplicon

Table 4
RFLP reaction mixture

Reaction components	Volume
PCR reaction mixture	10 µL
Nuclease-free water	14 µL
10× Buffer	5 µL
<i>PagI</i> enzyme	1 µL
Total	30 µL

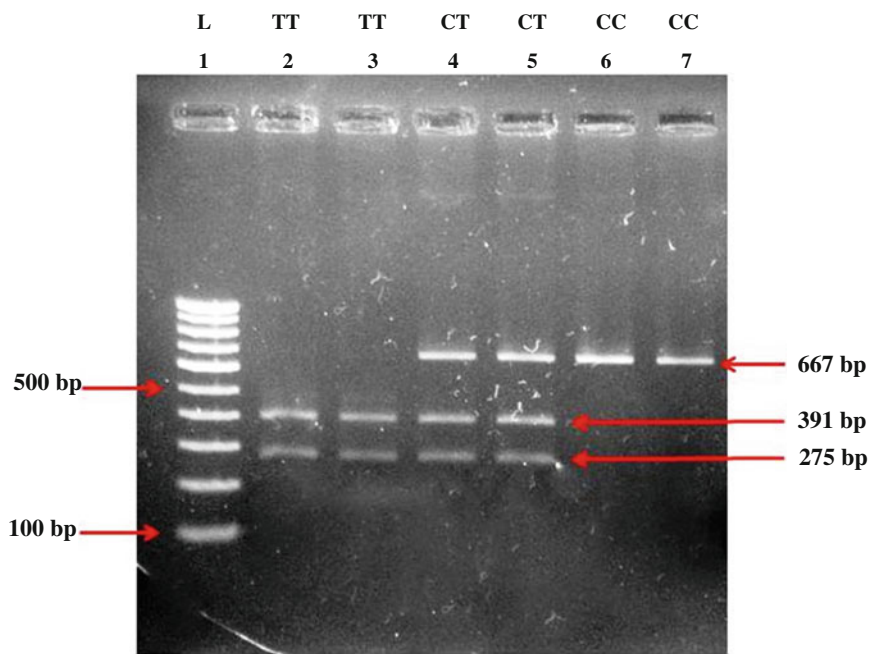


Fig. 3 Electrophoretic analysis showing the restriction profiles

Table 5
Restriction profiles

Band profile	Genotype
667 bp	Homozygous wild type (C/C)
667 bp, 391 bp, 275 bp	Heterozygous wild type (C/T)
391 bp, 275 bp	Mutant type (T/T)

4 Notes

1. Take precautions while handling EtBr, it is highly toxic as it is a carcinogen. Allow gel to cool to 60–70 °C. Add EtBr to 0.5 µg/mL final concentration.
2. Prepare 5× TBE buffer in stock. Use 0.5× TBE buffer for the preparation of agarose gel and electrophoresis buffer, i.e., add 100 mL of 5× TBE buffer in 900 mL of sterile deionized water.
3. It should be selected on the basis of map restriction site analysis using software (refer **step 4** in Subheading 3.1). In this method *PagI* was used as restriction enzyme (New England Biolabs Inc., USA).
4. There are several softwares which help to design PCR-RFLP in which selection of primers and restriction enzymes are incorporated.
5. Restriction fragments smaller than 100 bp should be avoided as they are not clearly resolved on agarose gel. The restriction enzyme should be reliable and cheap.
6. The designed primer pairs should be highly specific and should amplify only the studied species and desired amplicon size should be obtained [in this protocol 667 bp]. No additional or nonspecific bands should be detected.
7. Details like restriction digestion reaction mixture and incubation time is provided by manufacturers in package insert. Make all additions of reaction components on ice. After addition vortex mixture gently and spin the mixture shortly to settle down all droplets on the walls.

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