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Journal of Steroid Biochemistry & Molecular Biology 92 (2004) 465–468
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

A rapid and simple method for detection of Asn363Ser polymorphism of the human glucocorticoid receptor gene

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Received 6 April 2004; accepted 17 August 2004

Abstract

Asn363Ser polymorphism of the human glucocorticoid receptor has been detected in approximately 4% of the population and it has been associated with several diseases and pathologic conditions. Here we describe a new, simple and cost-effective allele-specific PCR method for a rapid screening of this polymorphism. When compared to currently used PCR-based restriction fragment length polymorphism (RFLP) and direct DNA sequencing methods, the new allele-specific PCR method showed 100% accuracy for the detection of Asn363Asn and Asn363Ser genotypes. The feasibility of these methods were tested in 301 patients, including 47 patients with postmenopausal osteoporosis in whom the frequency of Asn363Ser polymorphism was similar to that found in control subjects (4.3% versus 4.4%).

Keywords: Allele-specific PCR; Genetic polymorphism; Glucocorticoid receptor

1. Introduction

Glucocorticoid receptor, a member of the steroid receptor superfamily, mediates the effect of endogenous and exogenous glucocorticoids. Its gene is located on the long arm of chromosome 5 (5q31), and it consists of nine exons [1]. Three functional and structural domains of the glucocorticoid receptor have been described [2]; the first is located in the amino-terminal region and it spans the first 439 amino acids, the second is the DNA-binding domain, which involves amino acids 440–495, and the third is the hormone-binding region, formed by amino acids 496–777.

Recent studies suggest that certain variants of the glucocorticoid receptor gene may alter glucocorticoid sensitivity and may, therefore, play a role in several disorders of the human organism. Both germline and somatic mutations of the glucocorticoid receptor gene have been described; some of them have been associated with the glucocorticoid resistance syndrome while others may contribute to alterations of glucocorticoid sensitivity in different tissues [3]. Among the several gene variants, Asn363Ser (ATT-GTT) polymorphism [4] has been studied most extensively and its presence has been documented in 1.2–6.8% of the Caucasian populations [5–10]. As shown in recent studies, this polymorphism seems to be associated with an increased body mass index, increased cholesterol levels, coronary artery diseases, and with a tendency towards lower bone mineral density, without changes in glucocorticoid hormone levels of the affected subjects [8,9,11,12].

In earlier studies, Asn363Ser polymorphism was studied using polymerase chain reaction (PCR) followed by single strand conformation polymorphism (SSCP) [8] or digestion with Tsp 509I restriction enzyme [9], or direct sequencing of the corresponding exon of the glucocorticoid receptor gene. Here we describe a new, simple and cost-effective allele-specific method for detection of Asn363Asn and Asn363Ser genotypes, which can be easily applied to large-scale DNA samples handled in population-based studies.

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doi:10.1016/j.jsbmb.2004.08.005

2. Subjects and methods

2.1. Subjects

Three hundred and one Hungarian patients with different disorders (84 men, 217 women, age range 19–84 years), including 47 patients with postmenopausal osteoporosis were tested for the Asn363Ser polymorphism of the glucocorticoid receptor gene. Written informed consent was obtained from all subjects. The study was approved by the local Ethical Committee of Semmelweis University.

2.2. Methods

DNA was isolated from peripheral blood leukocytes using the DNA Isolation Kit for Mammalian Blood (Boehringer Mannheim Corp., Indianapolis, IN, USA). Restriction fragment length polymorphism (RFLP) was performed in all patients. Of the 301 patients, 66 were tested with allele-specific PCR and 34 with direct DNA sequencing of exon 2 of the glucocorticoid receptor gene.

2.3. PCR/RFLP

PCR and enzymatic digestion conditions described by Lin et al. [9] and forward and reverse oligonucleotide primers reported by Koper et al. [13] were used (2/4F: 5′-CCAGTAATGTAACACTGCCCC-3′, 2/4R: 5′-TTCGACCAGGGAAGTTCAGA-3′) (Fig. 1). The PCR protocol included 10 cycles of 1 min each at 94, 65 and 72 °C, followed by 15 cycles for 1 min each at 94, 60 and 72 °C, then 20 cycles for 1 min each at 94, 58 and 72 °C. The reaction was completed with a final extension step at 72 °C for 30 min (MJ Research PTC 100 ThermoCycler, Waltham, USA). The reaction mixture consisted of 200 ng of DNA in a final volume of 50 μl, containing 0.3 μmol/l of each oligonucleotide primer (Invitrogene Life Technologies, Glasgow, UK), 10 mmol/l Tris–HCl, 2.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 0.2 mmol/l deoxynucleotide triphosphate, 0.5 U Taq polymerase (Pharmacia Biotech, Uppsala, Sweden) and 5% glycerol.

PCR products were digested with the Tsp 509I restriction enzyme (New England Biolabs, Beverly, USA) at 65 °C for 3 h. This reaction yielded fragments of 135, 73, 70, 60 and 19 bp for the wild type allele (Asn636), and 135, 92, 70 and 60 bp for the mutant allele (Ser363). The fragments were separated by agarose gel (3.5%) electrophoresis, and visualized by ethidium bromide staining. Because of the small difference between the size of the 60, 70 and 73 bp fragments, two bands could be seen in wild type homozygous (Asn363Asn) samples (of which the first corresponded to the 60, 70 and 73 bp fragments and the second was a 135 bp fragment), whereas three bands could be seen in heterozygous (Asn363Ser) samples (70 bp (corresponding to 60, 70 and 73 bp fragments), 92 bp and 135 bp fragments) (Fig. 2a).

2.4. Allele-specific PCR

In addition to the original forward and reverse oligonucleotide primers described by Koper et al. (Fig. 1), allele-specific reverse oligonucleotide primers with different 3′-terminal nucleotide sequences were designed (Invitrogene Life Technologies). Primer sequences were 363W: 5′-ATCCTTGGCACCTATTCCAAT-3′ (corresponding to the wild type Asn allele) and 363M: 5′-ATCCTTGGCACCTATTCCAAC-3′ (corresponding to the mutant Ser allele). Each reaction mixture consisted of 200 ng of DNA in a final volume of 50 μl, containing 0.5 μmol/l of the forward oligonucleotide primer, 0.25 μmol/l each of the two reverse oligonucleotide primers (2/4R for amplification of a control fragment and an allele-specific 363W or 363M primer), 10 mmol/l Tris–HCl, 2.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 0.2 mmol/l deoxynucleotide triphosphate, 0.5 U Taq polymerase (Pharmacia Biotech) and 5% glycerol. After a denaturation step for 5 min at 95 °C, the PCR protocol included 35 cycles for 1 min each at 95, 63 and 72 °C, followed by a final extension step of 10 min at 72 °C. This reaction yielded a control fragment of 357 bp in each tube and a specific fragment of 306 bp in those tubes where the allele (Asn or Ser) corresponding to the specific primer (363W or 363M) was present. The fragments were separated by agarose gel

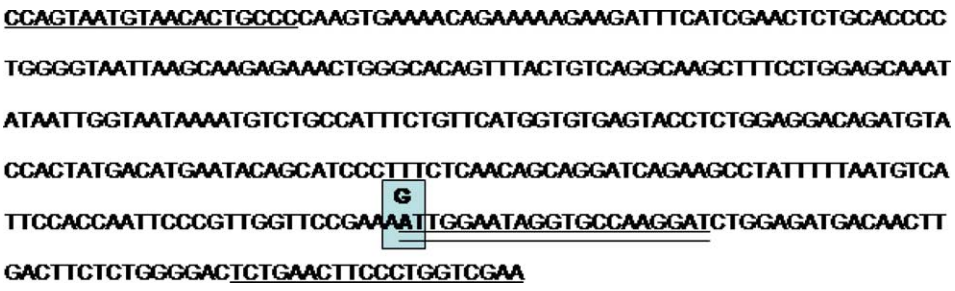

Fig. 1. Nucleotide sequence of the PCR-amplified fragment of exon 2 of the human glucocorticoid receptor gene (GenBank accession number: M73816). The highlighted AAT sequence is corresponding to the examined polymorphic site (codon 363, nucleotide 1220). Wild type sequence, AAT coding for asparagine; mutant sequence, AGT coding for serine. Single underlined sequences indicate the position of non-specific forward and reverse primers (2/4F, 2/4R) described by Koper et al. [13]. Double underlined sequence indicates the position of allele-specific reverse primers (363W and 363M) differing in their terminal 3′-nucleotide, corresponding to the A (wild type) or G (mutant) variant.

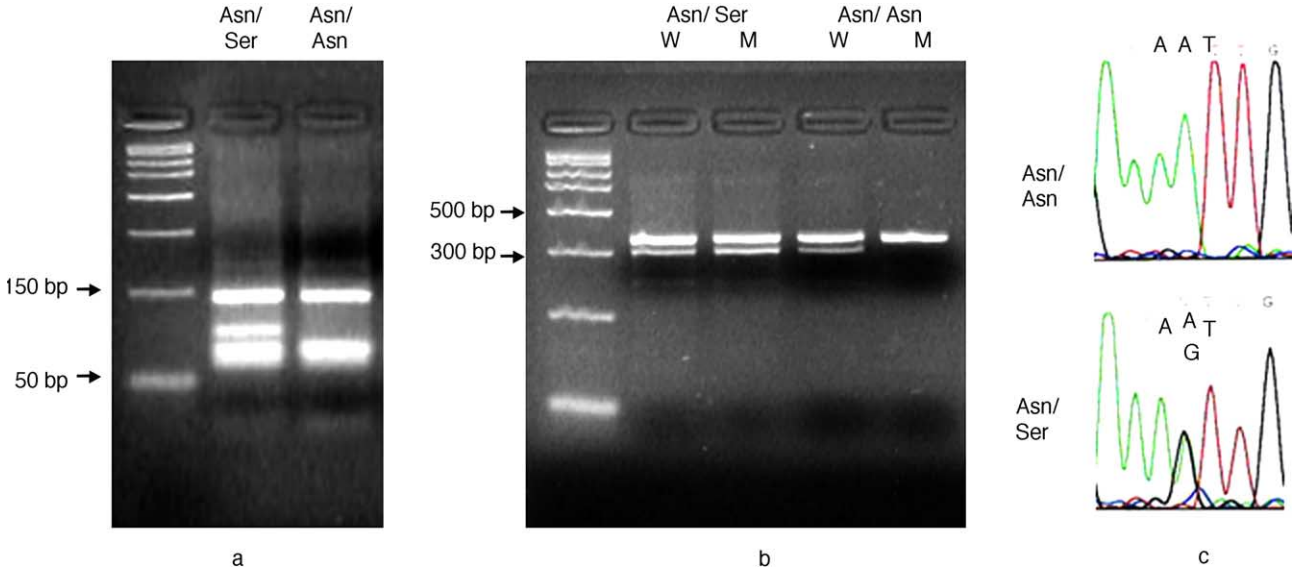

Fig. 2. Agarose gel electrophoresis after restriction enzyme digestion of the PCR-amplified fragment of the glucocorticoid receptor gene (a): lane 1, DNA marker; lane 2, three bands indicating the presence of the polymorphic allele; lane 3, two bands indicating the absence of the polymorphic allele. Allele-specific PCR reaction using the 363W reverse primer specific for the wild type allele and the 363M reverse primer specific for the polymorphic allele (b). A band of 357 bp obtained with 2/4F and 2/4R primers serves as internal control in each reaction. Lanes 2 and 3 illustrate a heterozygous sample with a specific band of 306 bp obtained both with the 363W (W) and the 363M (M) primers. A specific band obtained with the 363W (lane 4), but not with the 363M primer (lane 5) indicates a wild type homozygous sample. Direct sequencing of a wild type homozygous (upper panel) and a heterozygous (lower panel) sample (c).

(3.5%) electrophoresis, and visualized by ethidium bromide staining (Fig. 2b).

2.5. Direct DNA sequencing

For DNA sequencing, PCR reaction using the original forward and reverse oligonucleotide primers described by Koper et al. was performed. The amplified DNA was analyzed on a 2% agarose gel and purified using High Pure PCR Product kit (Roche Diagnostics, Mannheim, Germany). PCR-amplified DNA was sequenced by direct cycle-sequencing using the Big Dye Terminator Cycle-Sequencing kit (Applied Biosystems, Foster City, CA), on an automated sequencer 310 Genetic Analyser from Applied Biosystems (Fig. 2c).

3. Results

Of the 301 patients examined by the PCR/RFLP and/or direct DNA sequencing methods, 27 patients had heterozygous Asn363Ser genotype (27 alleles) and 274 patients showed homozygous wild type (Asn363Asn) genotype. The calculated frequency for the polymorphic Ser allele was 4.5% (27/602 alleles), which corresponds to an average frequency found in other Caucasian populations [5–10]. In a subgroup of 47 patients with postmenopausal osteoporosis, the polymorphic Ser allele was present in the same frequency (4.3%) as measured in control subjects (4.4%).

When compared to the PCR-based RFLP and direct DNA sequencing methods, our new allele-specific PCR method performed in 66 patients (27 heterozygotes and 39 wild type homozygotes) showed 100% accuracy for the detection of Asn363Asn and Asn363Ser genotypes. None of the 301 patients had a homozygous mutant (Ser363Ser) genotype using any of these methods.

4. Discussion

We designed a new method for the detection of Asn363Ser polymorphism of the human glucocorticoid receptor gene with the use of an allele-specific PCR described by Wu et al. [14]. In addition to its 100% accuracy, this new method seems to have several advantages over previously published methods. It is a rapid, simple and cost-effective procedure, which can be easily applied to large-scale DNA samples handled in population-based studies.

For the detection of Asn363Ser polymorphism with our new allele-specific method, one can perform only one PCR reaction using the forward oligonucleotide primer (2/4F) and two reverse primers (2/4R and 363M) designed for the amplification of a larger control fragment and a smaller mutation-specific fragment. This single PCR can distinguish patients who are wild type homozygotes from those who have a homozygous or heterozygous Asn363Ser polymorphism. Considering the low allelic frequency of Asn363Ser polymorphism, the majority of samples indicating wild type homozygotes will require no further examination. For samples that show a specific band (306 bp) together with the control band (357 bp), a second PCR reaction using the wild type specific primer should be performed to examine whether the patient is heterozygous or homozygous for

the polymorphic Ser allele. In average population with an allele frequency of 4%, the genotype of 100 subjects can be precisely determined with only 108 PCR reactions (100 with mutation-specific and eight with wild type primers).

Acknowledgements

This work was supported in part by grants from the National Research and Development Project (Széchenyi Plan, NKFP 1/047 MEDICHEM and NKFP2002-1/A) and by a grant from the Hungarian Scientific Research Fund (OTKA T046508).

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