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Study of Polymorphic Variants C190T, G191A, G857A, and 859Del of the *NAT2* Gene by the Method of Restriction Fragment Length Polymorphism

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New methods of restriction fragment length polymorphism analysis were developed to distinguish polymorphic variants C190T, G191A, G857A, and 859Del of the *NAT2* gene located close to each other.

Key Words: *NAT2; genetic polymorphism; mutation detection; restriction fragment length polymorphism analysis (RFLP analysis)*

Arylamine-N-acetyltransferase (EC 2.3.1.5) is an enzyme for biotransformation of xenobiotics, which catalyzes N-acetylation and O-acetylation of aromatic and heterocyclic amines and hydrazines [6]. *NAT2* polymorphism is phenotypically manifested in the existence of rapid and slow acetylators in the population and is characterized by significant interethnic differences. There are 53 alleles of human *NAT2* gene. Each allele contains one or several nucleotide exchanges, of them 27 exchanges were identified. They are located in the single coding exon of *NAT2* (http://N-acetyltransferasenomenclature.lousville.edu). Molecular and epidemiological studies are performed to reveal the association between polymorphic variants of the *NAT2* gene and multifactor diseases. The pathogenesis of these diseases is related to the effect of xenobiotics, which serve as a substrate for N-acetyltransferase. In the majority of these studies, polymorphic variants are detected by means of restriction fragment length polymorphism analysis (RFLP analysis) [1,2,4]. When the polymorphic variant is detected by the disappearance of a recognition site, this method allows us to identify mutational variability in several nucleotides that form the endonuclease recognition site (but not in a particular position). Polymorphism cannot be localized. Several polymorphisms can occur close to each other in the primary gene sequence. They should be detected by restriction endonuclease, which exhibits the formation (not disappearance) of a new recognition site under conditions of polymorphism. Polymorphic variants G191A and G857A were detected by means of RFLP analysis with restriction endonuclease MspI [3] and endonuclease BamHI [7], respectively. Previous studies showed that polymorphic variants C190T and 859Del appear close to the above mentioned variants [1,8]. Therefore, it is necessary to develop new methods for the detection of these polymorphisms by RFLP analysis.

This work was designed to develop new methods of RFLP analysis for the detection and distinction of polymorphic variants C190T, G191A, G857A, and 859Del of the *NAT2* gene.

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MATERIALS AND METHODS

Experiments were performed with the bank of 167 DNA samples (Program "Public Health of Siberia"). DNA was isolated from whole peripheral blood samples by the standard method of extraction with phenol chloroform. Blood samples were stabilized with EDTA in a final concentration of 50 mM. The *NAT2* gene fragment was amplified with oligonucleotide primers Nat-Hu 14 and Nat-Hu 16 [7] to obtain a 1000-b.p. product. The reaction mixture consisted of 60 mM Tris-HCl (pH 8.5 at 25°C), 1.5 mM $MgCl_2$, 25 mM KCl, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, 50 μM of each deoxynucleoside triphosphate, 0.2 μM of each primer, and 20 U/ml Taq polymerase. Amplification was performed under the following conditions: 1 min at 94°C; 1 min at 56°C; 1 min at 72°C; 4 min, initial denaturation; and 6 min, chain elongation.

Restriction endonucleases were selected with the primary sequence of the *NAT2* gene (GenBank, Acc. number AY331807.1) using Dnasis and VectorNTI softwares (Table 1). Enzymatic hydrolysis of the amplified DNA fragment was performed for 3 h under optimal conditions for restriction endonucleases. Hydrolysis products were separated by electrophoresis with 2% agarose gel in Tris-acetate buffer. After electrophoresis, DNA bands were visualized by staining with ethidium bromide. They were identified in transmitted UV light by the comparison with marker enzymes. Experiments were performed with restriction endonucleases BamHI, HinfI, and MspI (SibEnzim).

RESULTS

Polymorphic variant G191A of the *NAT2* gene was described previously [3]. Endonuclease MspI was used for the detection of this variant by RFLP analysis. The MspI recognition site in a particular region of the wild-type allele includes nucleotides 189-192. Disturbances in the recognition site are observed under polymorphism in any of 4 nucleotides (Fig. 1, *a*). Polymorphic variant C190T was discovered in 2000. Hence, some samples with the MspI polymorphism could include the C190T polymorphism. These features can introduce large errors into the results of genotyping. Polymorphisms in C190T (Arg64Trp) and G191 (Arg64Gln) are followed by amino acid exchanges, which has a variety of functional manifestations [5,9]. Since changes in the fragment formed during hydrolysis with MspI occur in both these polymorphisms, this restriction endonuclease can be used for polymorphism screening to reduce the time and cost of measurements. We developed a new approach to identify these polymorphisms. The original amplified samples with the MspI polymorphism were subjected to hydrolysis with restriction endonuclease Bse1I. A new recognition site for this endonuclease was formed in the 190T allele. In the absence of a new recognition site for Bse1I, the study was performed with endonuclease Bst2UI. This endonuclease was characterized by a new recognition site in the 191A allele (Fig. 1, *a*). Sometimes, the study is directed toward identification of a certain polymorphism. Endonucleases Bse1I and Bst2UI should be used to detect the 190T and 191A alleles, respectively. Primary screening with endonuclease MspI is not required. In this sample, there were no individuals with the MspI polymorphism (Fig. 1, *b*). These data illustrate the absence of polymorphism in positions 190 and 191. The proposed approach is of considerable significance in studying the population with high frequency of the MspI polymorphism (*e.g.*, African-American population) [3].

Similar results were obtained for polymorphisms of G857A and 859Del in the *NAT2* gene.

TABLE 1. Restriction Endonucleases for the Detection of Polymorphic Variants of the *NAT2* Gene by RFLP Analysis

Polymorphism	Restriction endonuclease	Length of hydrolysis fragments	
		wild-type allele	polymorphic allele
C190T	MspI	93, 93, 814	93, 907
	Bse1I	227, 62, 18, 693	188, 39, 62, 18, 693
G191A	MspI [3]	93, 93, 814	93, 907
	Bst2UI	204, 273, 367, 156	186, 18, 273, 367, 156
G857A	BamHI [7]	854, 146	1000
	HinfI	467, 137, 396	467, 137, 249, 147
859Del	BamHI	854, 146	1000
	AspS9I	480, 520	480, 373, 147

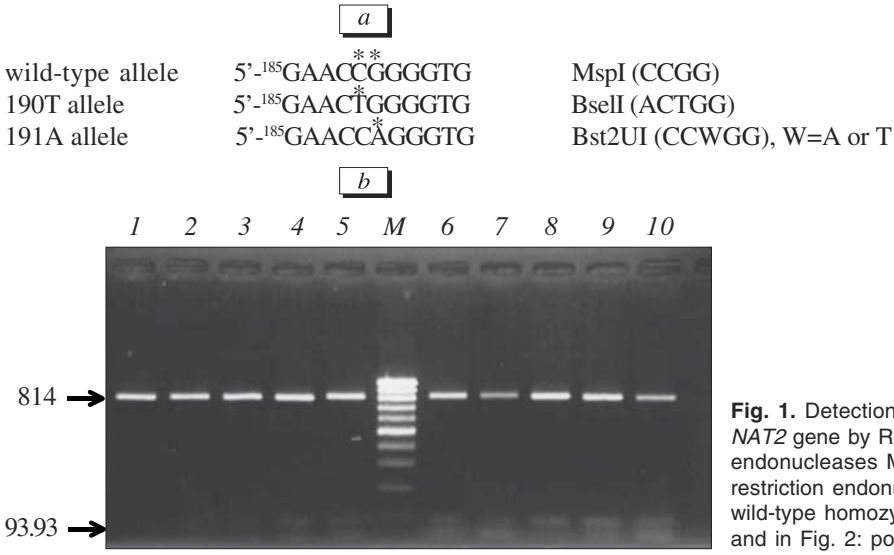

Fig. 1. Detection of polymorphisms in C190T and G191 of the *NAT2* gene by RFLP analysis. *a*) recognition sites for restriction endonucleases MspI, Bse1I, and Bst2UI; *b*) electrophoresis of restriction endonuclease MspI hydrolysis products. Bands *1-10*, wild-type homozygote; *M*, size marker of DNA fragments. Here and in Fig. 2: position of polymorphisms is shown by asterisks.

The G857A polymorphism (Gly286Glu) was first revealed in the *NAT2* gene. Endonuclease BamHI was used for the detection of this polymorphism by RFLP analysis [7]. BamHI has a single recognition site (nucleotides 856-861) in the sequence of the wild-type allele (Fig. 2, *a*). Recent studies showed that the 859Del polymorphism is accompanied by a reading frame shift [1]. Polymorphisms of G857A and 859Del cannot be distinguished with endonuclease BamHI. It was impossible to differentiate these polymorphisms by RFLP analysis. Changes in the fragments formed during hydrolysis with BamHI occured in the presence of polymorphism in 857 or 859. Hence, this restriction endonuclease can be used for screening. Endonuclease HinfI should be used after the disappearance of a restriction site for BamHI. This endonuclease is characterized by a new recognition site in the 857A allele. Sometimes, the disappearance of a recognition site for BamHI is not accompanied by the formation of a new recognition site for HinfI. Under these conditions it is necessary to hydrolyze the original amplified product with restriction endonuclease AspS9I, which has a new recognition site in the 859Del allele.

Restriction analysis of all samples with the BamHI polymorphism revealed the formation of a new recognition site during hydrolysis with HinfI. Our results illustrate the G857A polymorphism of these samples. However, the 859Del polymorphism

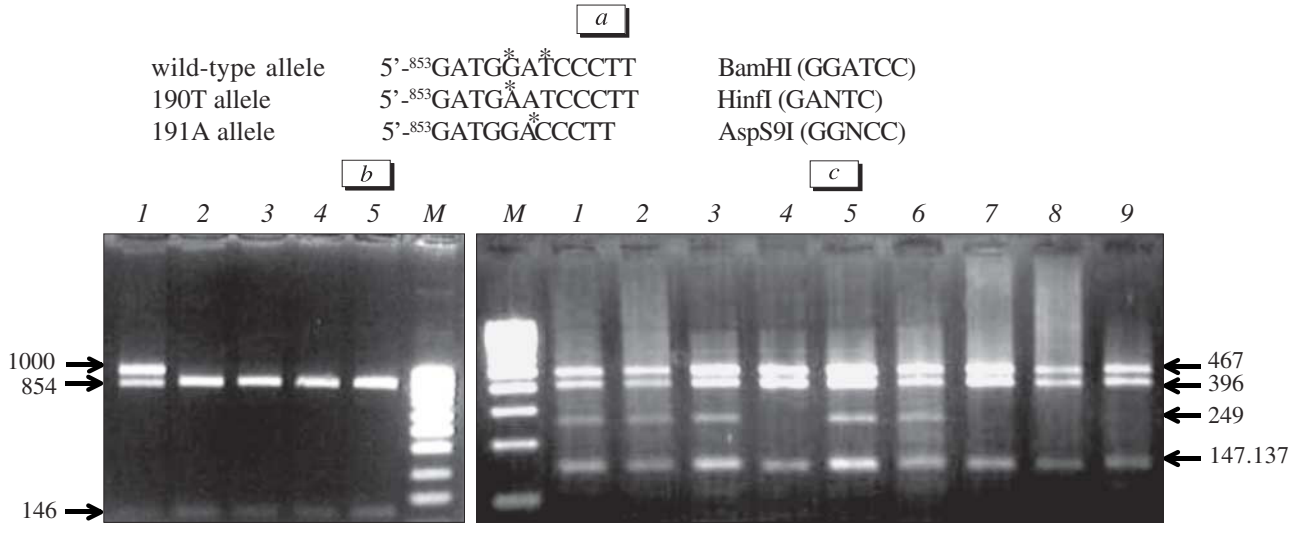

Fig. 2. Detection of polymorphisms in G857A and 859Del of the *NAT2* gene by RFLP analysis. *a*) recognition sites for restriction endonucleases BamHI, HinfI, and AspS9I; *b*) electrophoresis of restriction endonuclease BamHI hydrolysis products. Bands *2-5*, wild-type genotype; band *1*, estimated BamHI polymorphism in the heterozygous state; *c*) electrophoresis of restriction endonuclease HinfI hydrolysis products. Bands *4* and *7-9*, wild-type genotype. Bands *1-3*, *5*, and *6*: 857AG genotype.

is not characteristic of study sample. Typical hydrolysis electrophoretograms are shown in Fig. 2, *b*, *c*.

The proposed methods of RFLP analysis allow us to distinguish polymorphic variants C190T, G191A, G857A, and 859Del of the *NAT2* gene that are located close to each other.

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