EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Relationship between CYP2E1 Polymorphism and Increase of ALT Activity during Therapy of Patients with Pulmonary Tuberculosis

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Association of *CYP2E1* polymorphism with ALT activity increase was studied in patients with pulmonary tuberculosis receiving therapy by intermittent and daily protocols. The greatest increment of ALT activity in the group receiving therapy by intermittent protocol was seen in the patients with *CYP2E1*7632TA* genotype. In patients with wild homozygotic *1C/1C* (*6/6*) genotype, ALT activity significantly increased, but remained within the normal range (p=0.048). In the group on daily regimen, activity of ALT increased significantly in patients with all genotypes identified. A more pronounced elevation surpassing the median of the upper threshold of ALT norm was observed in patients with *7632TA* genotype (p=0.0051) and in patients with *7632TA* or *-71GT* or *1C/1D* genotypes in combinations with wild type alleles by other detected polymorphisms (p=0.0277). Detection of the *CYP2E1* gene *7632T>A* polymorphism was found to be the most informative test for prediction of the hepatotoxic reactions during therapy for tuberculosis.

Key Words: *genetic polymorphism; cytochrome P450 2E1; tuberculosis; hepatotoxicity; alanine aminotransferase*

The incidence of hepatotoxic reactions during therapy for tuberculosis reaches 47% and depends on many factors, primarily the therapeutic protocol, race, gender, age, body weight, and alcohol consumption [5]. Metabolism of antituberculous drug (ATD) is often responsible for drug hepatotoxicity. N-Acetyltransferase 2 (NAT2), amidase, and cytochrome P450 2E1 (CYP2E1) are involved in isoniazid metabolism. The quantity of isoniazid metabolites (hydrazine and acetylhydrazine) depends on the ratio of NAT2 and amidase activities proportion [9]. A relationship between *NAT2* slow acetylation phenotypes and hepatotoxicity under conditions of ATD therapy has been demonstrated [9]. Studies of amidase are difficult because the gene encoding the enzyme involved in isoniazid metabolism is not identified. Acetylhydrazine is oxidized by CYP2E1 with the formation of such hepatotoxins as acetylhydrazine, acetylonium ion, acetyl radical, or ketene [12], which damage cell macromolecules, initiate the development of oxidative stress, and eventually cause hepatocyte death [13]. *CYP2E1* polymorphism manifests by changes in the protein expression and activity [10], which prompted us to study the relation-

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ship between *CYP2E1* polymorphism and increase in ALT activity in patients with pulmonary tuberculosis during the therapy.

MATERIALS AND METHODS

Patients with pulmonary tuberculosis (*N*=145; residents of the Novosibirsk region, not relatives; treated at Clinical Department of Novosibirsk Institute of Tuberculosis) were divided into 2 groups receiving therapy by different protocols. Group 1 patients (*N*=75) received ATD in the intermittent regimen twice a week. Isoniazid (12 mg/kg) and rifampicin (10 mg/kg) were injected intravenously by drip infusion, streptomycin (16 mg/kg) intramuscularly, pyrazinamide (25 mg/kg) and ethambutol (20 mg/kg) were administered orally. Group 2 patients (*N*=70) received daily oral isoniazid (10 mg/kg), pyrazinamide (25 mg/kg), and rifampicin (10 mg/kg); streptomycin (16 mg/kg) was injected intramuscularly. Serum ALT was measured on admission and 1 month after the start of therapy or earlier (after manifestation of hepatotoxicity symptoms). Patients with ALT activity >50 U/liter on admission were excluded from the study.

Isolation of DNA from the whole blood was carried out by the standard phenol-chloroform method. The *CYP2E1* polymorphisms were denoted according to the current nomenclature (http://www/cypalleles.ki.se/cyp2e1.htm) except the rs2515641 polymorphism, denoted as Phe421Phe (*C/T*) [3]. The polymorphism of the number of tandem repeats (VNTR) was evaluated as described previously [4], substitutions *-1293G>C* (*Pstl* polymorphism) and *-1053C>T* (*Rsal* polymorphism) by the previously described method [8], correcting the parameters of the PCR cycle for optimization for the laboratory equipment (Terzik, DNA Technology).

Substitutions *-71G>T*, *7632T>A* (*Dral*), and Phe421Phe (*C/T*) were evaluated by our original method. In order to evaluate the *-71G>T* polymorphism, a 476 b.p. fragment was synthesized by PCR. The reaction mixture (30 μl) contained 4.5 mM $MgCl_2$, 0.32 mM dNTP, 1 U Taq DNA polymerase (Sibenzyme), 0.4 μM forward (5'-ACCCCACGTTCTTAACTATGG) and 0.4 μM reverse (5'-CTTCAATTCCAACTGGAAGAGG) primers, and 50-100 ng genome DNA. The PCR was carried out as follows: primary denaturation for 3 min at 95°C; 35 cycles according to the following protocol: denaturation at 95°C for 50 sec, annealing at 61°C for 15 sec, and elongation at 72°C for 30 sec; final elongation at 72°C for 2 min. Amplification products were subjected to hydrolysis by *BstDEI* restriction endonuclease (Fig. 1, *a*).

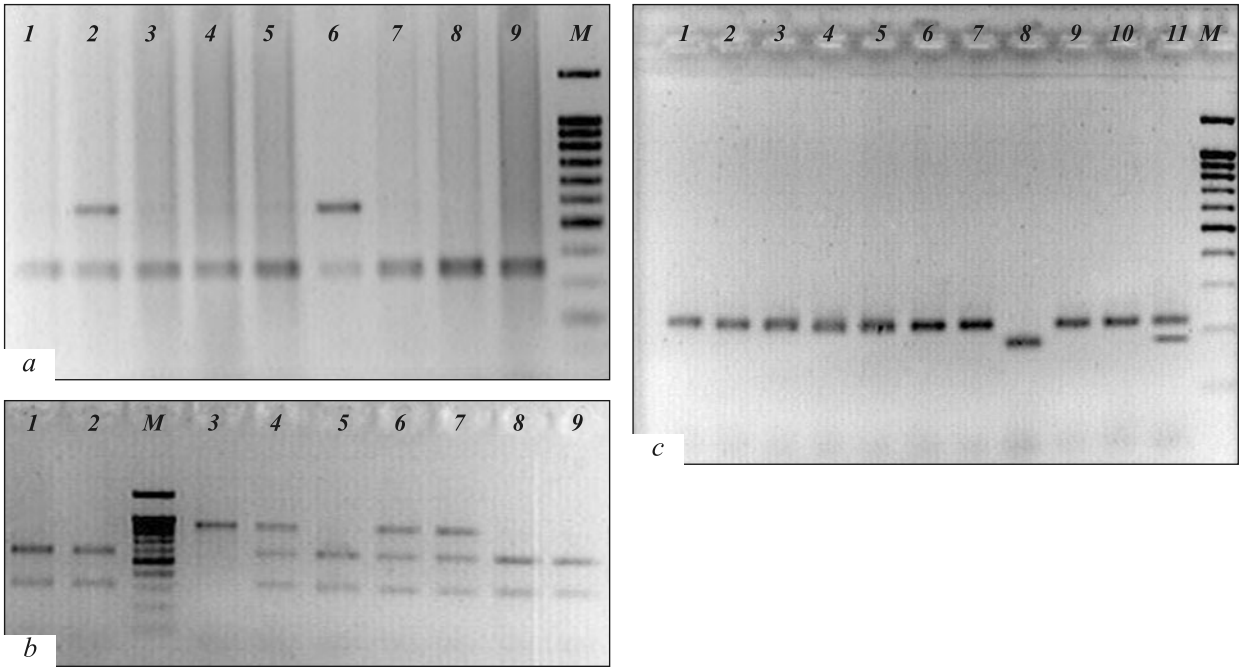

Fig. 1. Gene *CYP2E1* polymorphisms *-71G>T*, *7632T>A*, and Phe421Phe (*C/T*). *a*) *-71G>T* polymorphism. Tracks *1*, *3-5*, *7-9* contain 249 and 227 b.p. fragments and correspond to genotype *-71GG*; tracks *2*, *6* contain 249, 227, and 476 b.p. fragments and correspond to genotype *-71GT*; 2% agarose gel. *b*) *7632T>A* polymorphism. Tracks *1*, *2*, *5*, *8*, *9* contain fragments of 318, 572, and 39 b.p. and correspond to genotype *7632TT*; tracks *4*, *6*, *7* contain fragments of 318, 572, 890, and 39 b.p. and correspond to genotype *7632TA*; track 3 contains fragments of 890 and 39 b.p. and corresponds to genotype *7632AA*; 2% agarose gel. *c*) Phe421Phe (*C/T*) polymorphism. Tracks *1-7*, *9*, *10* contain fragments of 214 and 37 b.p. and correspond to genotype *CC*; track 11 contains fragments of 214, 179, 35, and 37 b.p. and corresponds to genotype *CT*; track 8 contains fragments of 179, 35, and 37 b.p. and corresponds to genotype *TT*; 3% agarose gel. *M* is 100-1000 b.p. molecular weight marker with a 100 b.p. step and an extra 1500 b.p. band in all gels.

For studies of polymorphism *7632T>A*, a 929 b.p. fragment was amplified. The reaction mixture (30 µl) contained 2.83 mM $MgCl_2$, 0.15 mM dNTP, 0.75 U Taq DNA polymerase (Sibenzyme), 0.333 µM forward (5'-TTGTGGTCTTAAGGCTCGTC) and 0.333 µM reverse (5'-CTTTTGTCTATATTGGAACCTCCTC) primers, and 40-80 ng genome DNA. The PCR was carried out by the following protocol: primary denaturation at 95°C for 3 min; 35 cycles according to the following protocol: denaturation at 95°C for 50 sec, annealing at 64°C for 15 sec, and elongation at 72°C for 1 min; final elongation at 72°C for 2 min. Amplification products were subjected to hydrolysis with *Dral* restriction endonuclease (Fig. 1, *b*).

A 251 b.p. fragment was synthesized by PCR for studies of Phe421Phe (*C/T*) polymorphism. The reaction mixture (30 µl) contained 4.5 mM $MgCl_2$, 0.32 mM dNTP, 1 U Taq DNA polymerase (Sibenzyme), 0.467 µM forward (5'-TGTATGACAACCAA GAATTTCCTG) and 0.467 µM reverse (5'-GT-CATGTTGTCAATAGAAACAGGG) primers, and 50-100 ng genome DNA. The PCR protocol was as follows: primary denaturation at 95°C for 3 min; 40 cycles according to the following protocol: denaturation at 95°C for 40 sec, annealing at 64°C for 15 sec, and elongation at 72°C for 16 sec; final elongation at 72°C for 2 min. Amplification products were subjected to hydrolysis with *Tru9I* restriction endonuclease (Fig. 1, *c*).

Analysis of correspondence of the genotype distribution to Hardy–Weinberg equilibrium was carried out using Hwe_win software (M. Freidin, Tomsk, 1999). Other calculations using nonparametrical statistics methods were carried out using Statistica 9.1 software. The significance of ALT elevation in the course of therapy was evaluated by the Wilcoxon test. The differences were significant at $p<0.05$.

RESULTS

Genotype distribution corresponded to Hardy–Weinberg equilibrium for all the studied polymorphisms. The incidence of alleles and genotypes (Table 1) was about the same as in other Europeoid populations of Russia [1] and all over the world [10].

Only few cases of ALT activity elevation above the upper threshold level were found in the group of patients receiving therapy by the intermittent protocol. Analysis of these values in patients with different *CYP2E1* genotypes showed a significant, though within the normal range of values, elevation of serum ALT activity in the carriers of the homozygotic wild type allele *1C/1C* (*6/6*) genotype. This attests to very high activity of CYP2E1 encoded by the wild type allele in isoniazid metabolism and generation of reactive metabolites. A more pronounced elevation of ALT activity, in some cases above its upper threshold level, was found in the patients with *7632TA* genotype. The level of statistical significance was however not attained because of low number of patients in this group (Table 2).

TABLE 1. Incidence of *CYP2E1* Alleles and Genotypes in Tuberculosis Patients (*N*=145) in the Novosibirsk Region

Polymorphism	Allele/genotype	N	Incidence, %
Number of tandem repeats in promotor site	*1A* (5 repeats)	1	0.35
	1C (6 repeats)	284	97.93
	1D (8 repeats)	5	1.72
	1A/1A	0	0
	1A/1C	1	0.7
	1A/1D	0	0
	1C/1C	139	95.9
	1C/1D	5	3.4
	1D/1D	0	0
-1293G>C (PstI)	*G*	285	98.3
	C	5	1.7
	GG	140	96.6
	GC	5	3.4
	CC	0	0
-1053C>T (RsaI)	C	285	98.3
	T	5	1.7
	CC	140	96.6
	CT	5	3.4
	TT	0	0
-71G>T	G	271	93.4
	T	19	6.6
	GG	126	86.9
	GT	19	13.1
	TT	0	0
7632T>A (DraI)	T	266	91.7
	A	24	8.3
	TT	122	84.1
	TA	22	15.2
	AA	1	0.7
Phe421Phe *(C/T)*	C	255	87.9
	T	35	12.1
	CC	113	77.9
	CT	29	20.0
	TT	3	2.1

TABLE 2. Time Course of ALT Activity during Treatment of Patients with Different *CYP2E1* Genotypes

Polymorphism	Genotype	*N*	Incidence, %	ALT: median (interquartile intervals)		*p*
				on admission	1st month of therapy	
Intermittent therapy						
Number of tandem repeats	*1C/1C* (*6/6*)	63	95.5	17 (12-27)	20 (14-34)	0.0488
	1C/1D (*6/8*)	2	3.0	15.5 (9-22)	11.5 (6-17)	0.1791
	1A/1C (*5/6*)	1	1.5	10	27	-
-1293G>C and *-1053C>T* (*RsaI/PstI*)	*-1293GG/-1053CC*	64	97.0	16 (12-26)	18 (13.5-34)	0.0566
	-1293GC/-1053CT	2	3.0	22.5 (18-27)	25.5 (23-28)	0.6547
-71 G>T	GG	56	84.8	15.5 (12-23)	18 (13-32.5)	0.1060
	GT	10	15.2	27.5 (12-45)	22.5 (17-52)	0.2622
7632T>A (*DraI*)	TT	58	87.9	16 (12-23)	17.5 (13-32)	0.1697
	TA	8	12.1	22.5 (10-34.5)	30.5 (20-51)	0.1415
Phe421Phe (*C/T*)	CC	50	75.8	16 (12-23)	19 (13-32)	0.1790
	CT	13	19.7	18 (11-45)	23 (15-42)	0.1261
	TT	3	4.5	22 (7-41)	17 (6-80)	1.00
Total		66	100.0	16.5 (12-27)	19 (14-34)	0.0521
Daily therapy						
Number of tandem repeats	*1C/1C* (*6/6*)	51	94.4	18 (12-24)	27 (19-52)	0.00002
	1C/1D (6/8)	3	5.6	16 (11-25)	32 (25-65)	0.1088
	1A/1C (5/6)	0	0.0	-	-	-
-1293G>C and *-1053C>T (RsaI/PstI)*	-1293GG/1053CC	52	96.3	18 (12-24.5)	26 (19.5-50.5)	0.00001
	-1293GC/1053CT	2	3.7	11 (11-11)	165.5(40-291)	0.1797
-71 G>T	GG	47	87.0	18 (11-24)	25 (19-52)	0.00005
	GT	7	13.0	19 (14-25)	32 (25-176)	0.0280
7632T>A (DraI)	TT	44	81.5	18 (12.5-26)	24.5 (19-39.5)	0.0004
	TA	10	18.5	13.5 (11-19)	57.0 (32-78)	0.0051
Phe421Phe *(C/T)*	CC	41	75.9	18 (11-23)	25 (19-48)	0.0002
	CT	13	24.1	18 (14-24)	28 (25-83)	0.0064
	TT	0	0.0	-	-	-
Total		54	100.0	18 (12-24)	27.5 (20-52)	0.000005

Serum ALT levels increased significantly in the group receiving daily therapy in patients with all the studied *CYP2E1* genotypes (Table 2). Importantly, 75% quartile values (in one quarter of all examined patients) were above the upper threshold of normal ALT values. The parameter remained within the normal range only in patients with homozygotic *CYP2E1* genotypes by the wild type alleles *Phe421Phe* (*CC*) and *7632TT*. The median ALT values surpassed the upper threshold value (p=0.0051) in the patients with the *7632TA* genotype.

The majority of the *CYP2E1* polymorphisms studied are associated with functional manifestations. The *7632T>A* substitution (allele **6*) leads to a decrease in

TABLE 3. Time Course of ALT during Therapy of Patients with Different Combinations of *CYP2E1* Genotypes

Genotype combination	*N*	Incidence, %	ALT: median (interquartile interval)		*p*
			on admission	during month 1 of therapy	
Intermittent treatment					
7632TA, -1293GG, -1053CC	6	9.1	23 (8-35)	41.5 (17-52)	0.1730
7632TA or *-71GT,* or *1C/1D*, with *-1293GG, -1053CC*	14	21.2	27.5 (9-41)	28 (16-52)	0.1981
7632TA or *-71GT,* with *1C/1C, -1293GG, -1053CC*	12	18.2	33.5 (10-43)	37.5 (16.5-53)	0.0995
7632TA or *-71GT*, or *1C/1D*, with wild type for the rest polymorphisms	4	6.1	23 (10-34.5)	41.5 (24.5-52)	0.2733
Total	66	100.0	16.5 (12-27)	19 (14-34)	0.0521
Daily therapy					
7632TA, -1293GG, -1053CC	8	14.8	16.5 (11-21)	57 (28.5-76.5)	0.0117
7632TA or *-71GT*, or *1C/1D*, with *-1293GG, -1053CC*	18	34.6	17 (12-23)	34.5 (25-75)	0.0003
7632TA or *-71GT*, with *1C/1C, -1293GG, -1053CC*	11	20.4	17 (12-21)	49 (25-78)	0.0058
7632TA or *-71GT*, or *1C/1D*, with wild type for the rest polymorphisms	6	11.1	14 (11-19)	62 (25-78)	0.0277
Total	54	100.0	18 (12-24)	27.5 (20-52)	0.000005

enzyme activity *in vivo* [10] and hence, hence, lover ATD toxicity can be expected. However, similarly as in our study, the heterozygotic *7632TA* genotype was a hepatotoxicity risk factor in the Indian population [2]. Conjugated substitutions *-1293G>C* and *-1053C>T* (allele **5B*) led to reduction of the enzyme activity *in vivo* [10] and in combination with substitution *7632T>A* (allele **5A*) to a lesser induction of the enzyme *in vivo* [10] and hence, can be expected to reduce the toxicity. The wild type allele *1293GG* is really a risk factor for ATD-induced hepatotoxicity [6]. Gene *CYP2E1* containing 8 repeats in the promotor region is stronger induced under conditions of ethanol consumption and obesity in comparison with the gene containing 6 repeats in the promotor region [10], which also can augment the toxicity in some cases. Substitution *-71G>T* can modify the toxicity through regulation of *CYP2E1* expression. Activity of *CYP2E1* promotor is suppressed by IFN-γ. This mechanism does not work: IFN induces no suppression in case of G for T substitution [11]. Hence, if the toxicity is linked with immune reactions, inflammation, and secretion of IFN-γ, allele *-71T* stimulates the toxicity as a result of high expression.

With due consideration for these facts, we analyzed the combinations of genotypes presumably associated with increase of toxic reactions during therapy for tuberculosis. The increase of ALT activity in the group of patients receiving therapy by the intermittent protocol did not reach statistically significant values for any of the genotype combinations (Table 3). However, the patients with genotypes *7632TA* or *-71GT* or *1C/1D* without mutant alleles for the rest polymorphisms developed a more pronounced elevation of ALT activity in comparison with the entire group, in more than 25% patients this parameter surpassing the upper threshold level (Table 3). In the group on daily regimen, the increase in ALT activity above the upper threshold of normal was detected in more than half of the patients with this genotype combination (Table 3).

Hence, the results indicate that the most severe toxic effects of ATD were observed in patients with the heterozygotic *CYP2E1*7632TA* genotype and in patients with *7632TA* or *-71GT* or *1C/1D* genotypes in combinations with wild type alleles for the rest studied polymorphisms. During daily therapy (a predominant protocol for antituberculous therapy in Russia), ALT activity in more than half of patients with these genotypes increased above the upper threshold of normal within the very first month of therapy. This fact indicates an informative value of these genotypes

for prediction of hepatotoxic reactions by the results of genetic testing.

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