



3.4) was found for D16S3098 (3). Later, the linkage to type 1 disease on chromosome 16q22-q24 was replicated in the extended data set of 767 US and UK affected families, with MLS of 3.93 for D16S3098 marker, suggesting a role of this region in predisposition to type 1 diabetes (4). On the other hand, genome-wide scans in Scandinavians (5) and the Dutch (6) failed to show a linkage of this region to the disease.

Interestingly, the T1D susceptibility locus on chromosome 16q22-q24 partially overlaps with the susceptibility loci mapped for other autoimmune disorders, such as celiac disease (7) and asthma (8), suggesting that this genomic region could harbor a common but unknown susceptibility gene for autoimmunity. Association studies of *NQO1*, a positional candidate gene for susceptibility, located 20 cM centromeric from D16S3098 and encoding NAD(P)H-quinone oxidoreductase 1 [EC 1.6.99.2], have produced inconsistent results (9-12). The susceptibility locus contains another likely positional candidate gene, *ICSBP1*, situated on 16q24.1 14 cM telomeric from D16S3098. The *ICSBP1* gene encodes interferon consensus sequence binding protein 1, a transcription factor of the interferon regulatory family, which is mainly expressed in cells of the immune system and plays a key role in the maturation of macrophages (13). No polymorphic markers within this gene have yet been evaluated for association with an autoimmune disease.

Since linkage studies in different populations for the 16q22-q24 locus showed conflicting results, it would be interesting to examine whether this locus contributes to susceptibility to T1D in Russian affected patients. We evaluated a set of polymorphic genetic markers, which are situated on chromosome 16q21-q24 and include single nucleotide polymorphisms (SNPs), located within the *NQO1* and *ICSBP1* genes, in Russian type 1 dia-

betic families but failed to find a significant linkage and association of these markers with T1D.

## Patients and Methods

### Patients

We studied 114 Russian simplex families, each containing both parents and two siblings (one affected with T1D (proband), diagnosed before the age of 17, and one non-diabetic sibling). Sixteen simplex families were collected from the Samara Diabetic Centre, the others being recruited from the Endocrinology Research Center in Moscow. Ninety-eight Russian multiplex families, containing 108 affected full sib pairs, were recruited from the Endocrinological Research Center. Of these 98, 93 families each included both parents and 2 affected siblings, whereas the remaining 5 families each contained both parents and 3 diabetic children. In simplex and multiplex families, all parents had no diagnosed T1D. Informed consent was obtained from all subjects before participation in this study. The research protocol was approved by the Ethics Committee of the Endocrinology Research Centre and performed in accordance with the principles of the Helsinki Declaration.

Diabetes was diagnosed according to the criteria defined by the American Diabetes Association (14). T1D was classified according to the presence of ketosis, low body mass index, and the need for insulin treatment. In all subjects, diagnosis of the disease was confirmed by the presence of at least one of the two major islet autoantibodies: GAD65 antibodies and/or anti-tyrosine phosphate-like molecule (ICA512) antibodies (15,16). C-peptide levels were measured in the blood serum of patients using a commercially available radioimmunoassay (Medipro AG, Teufen, Switzerland) (17). Hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>) measurements were performed using high-performance liquid chromatography (DIAMAT, BIO-RAD, Hercules, CA, USA). Immunological and

**Table 1.** Clinical and biochemical characteristics of affected and non-affected children in Russian diabetic families

Characteristic	Multiplex families (n=98)		Simplex families (n=114)	
	affected sibs (n=201)		probands (n=114)	non-affected sibs (n=114)
Male/female ratio	107/94		66/48	53/61
Age, years (range)	16.5±5.7 (4-27)		19.3±4.5 (7-29)	21.1±6.3 (5-31)
Duration of diabetes, years (range)	8.3±3.2 (2.5-19)		9.5±4.3 (1.1-25)	
Insulin dose, U/kg (range)	1.07±0.03 (0.93-1.15)		1.15±0.05 (0.9-1.24)	
Body mass index, kg/m <sup>2</sup> (range)	17.3±1.7 (14.2-21.6)		18.5±2.2 (13.5-22.5)	23.0±2.5 (13.1-25.1)
HbA <sub>1c</sub> , % (range)	9.1±2.4 (5.5-13.9)		9.8±2.2 (5.2-13.5)	6.5±1.4 (4.4-8.6)
Basal serum C-peptide, pmol/mL (range)	0.23±0.04 (0.11-0.36)		0.25±0.04 (0.11-0.33)	0.41±0.04 (0.35-0.55)
GAD65 antibodies-positive patients, No. (%)	156 (77)		100 (88)	
ICA512 antibodies-positive patients, No. (%)	145 (72)		81 (71)	

clinical characteristics of diabetic and non-affected children are summarized in Table 1.

### DNA Analysis

Genomic DNA was extracted from whole-blood samples collected in disodium EDTA (3 mg/ml), according to the established protocol (18). Microsatellite markers (D16S402, D16S422, D16S504, D16S750, D16S3037, D16S3050, D16S3089, D16S3073, D16S3089, D16S3098, D16S3106, D16S3118, and D16S3140) were chosen from the public databases and analyzed by PCR. Primer sequences were as described in the Genome Database (<http://www.gdb.org>). For each microsatellite marker, one primer was fluorescently labeled with either 6-HEX or FAM (Eurogentec, Seraing, Belgium). The PCR cocktail contained 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.0-1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 0.2 mM each dNTP, 0.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), 5 μM each primers and 100 ng of genomic DNA in a total volume of 10 μL. PCR was carried out on a GeneAmp® PCR System 2700 (Applied Biosystems) at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 55-60°C for 15 seconds and extension at 72°C for 30 seconds, with the final extension at 72°C for 10 minutes. Fluorescence-based genotyping was performed with an ABI PRISM 310 DNA Sequencer (Applied Biosystems) and GeneScan analysis software, Version 3.1.2 (Applied Biosystems).

HLA-DRB1, -DQA1, and -DQB1 alleles were determined by the use of a locus-specific amplification procedure, using a subsequent hybridization of PCR products with a corresponding allele-specific oligonucleotide probe as previously described (19,20).

Single nucleotide polymorphisms (SNPs) located within corresponding positional candidate

genes (*NQO1* and *ICSBP1*) were genotyped in simplex families, using the PCR-restriction fragment length polymorphism (RFLP) approach. The Pro/Ser 187 SNP of the *NQO1* gene was tested using DNA treatment with Hinf I restriction endonuclease, as described earlier (11,21). The Arg/Trp 139 polymorphism of the *NQO1* gene was detected using DNA digestion with Msp I restriction enzyme as described by Sanyal et al (22). Other SNPs were taken from the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>). PCR-RFLP assays for detection of each SNP are described in Table 2. All restriction enzymes used were manufactured in Fermentas (Vilnius, Lithuania). Following digestion, DNA products were separated in a 2% agarose gel with ethidium bromide.

### Statistical Analysis

Multipoint linkage analysis was performed in affected sibling pairs from multiplex families using GENEHUNTER 2.1 software (23). MLS and nonparametric LOD (NPL) values were computed using allele frequencies derived from parents of the affected siblings, assuming the dominance variance exists. Marshfield sex-average map distances were used in the linkage analysis (24). According to criteria for mapping genes involved in complex traits proposed by Lander and Kruglyak (25), a LOD score of 2.2 was considered to indicate a suggestive linkage, whereas a LOD score of 3.6 was assessed as an evidence for significant linkage.

Using the GENEHUNTER software, the transmission disequilibrium test (TDT) was performed in simplex families to identify alleles preferentially transmitted from heterozygous parents to diabetic offspring (26). Chi-square test was used to estimate whether the allele transmission significantly differed from the random pattern (1:1). *P* value (*P<sub>c</sub>*) of less than 0.05, after correction for

**Table 2.** Description of molecular assays to detect SNPs within three candidate genes studied for association with susceptibility to type 1 diabetes mellitus (T1D) in a Russian sample

SNP	dbSNP ID	Gene	Location within the gene (position from the transcription start, bp)	PCR primers, 5'→3'	Annealing temperature, °C ([Mg <sup>2+</sup> ], mM)	Restriction enzyme to digest a PCR product	Digestion temperature, (°C)	Duration of digestion, (hours)	Definition of alleles (length of digestion products, bp*)
A/G	689453	NQO1	exon 2: codon 24 (72)	F:GTCCTTCAACTATGCCATGAAGGC R:CTCCTCTACCTGTGATGTC	65 (1.0)	Pvu II	37	3	G allele: 135 A allele: 109+26
C/T	8052064	ICSBP1	exon 3: codon 138 (414)	F:GATCAGCTCGTCGATTTCAGAGCGAT R:GTGATGCCTCCGTGCCATGTGC	65 (1.0)	Pvu I	37	3	C allele: 11+24 T allele: 141
T/C	11545564	ICSBP1	exon 2: codon 100 (300)	F: CTGGAAGGTTTCATGCCATA R: CACTTGAAGACGAGGTTAC	60 (1.5)	Pvu II	37	3	T allele: 164 C allele: 9+73
C/T	2280378	ICSBP1	intron 6 (16124)	F: GGCTGGAGCAGGGCCTCAGACTCGA R: CATCAGGTGATTGTCGGGAGCC	65 (1.5)	Aat II	37	3	C allele: 88+28 T allele: 116

\*bp - base pair.

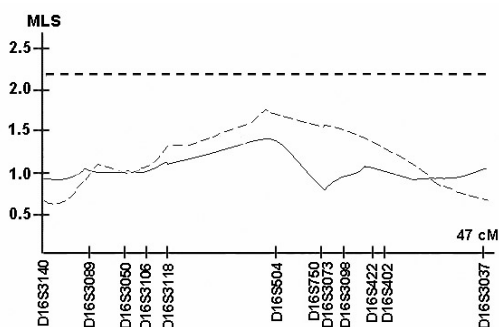
multiple alleles (20), was considered significant. For each microsatellite marker, an overall TDT  $P$  value was also calculated. For each SNP,  $P$  value was multiplied by a total number of alleles tested (6) to obtain a corrected  $P$  value ( $P_c$ ).

## Results

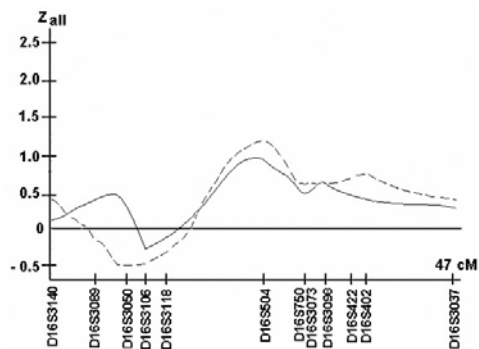
### Multipoint Linkage Analysis

Thirteen microsatellite markers, spanning a distance about 49 cM on chromosome 16q22-q24, were analyzed for linkage with T1D in Russian multiplex families. This genomic region lies between D16S3140 and D16S3037 (situated 74.44 cM and 121.45 cM, respectively, from the p-terminus) and includes a 32-cM susceptibility locus for type 1 diabetes mapped to chromosome 16q22-q24 in UK families (3). Three markers, D16S422, D16S3037, and D16S3098, which showed significant linkage to diabetes in previous studies (3,4), were also evaluated in our investigation.

The peak of linkage (MLS=1.35, NPL=0.91) was found for D16S504 located 7.1 cM apart from D16S3098, a marker that demonstrated maximum strength of linkage to T1D in UK Caucasians (3), but not in our study ( $P=0.18$ ) (Fig. 1 and 2). However, a MLS value observed in our study did not reach a MLS threshold of 2.2 that rep-



**Figure 1.** Multipoint logarithm of odds (LOD) ratio (MLS) plot for type 1 diabetes on chromosome 16q22-q24, obtained using the "estimate" command of GENEHUNTER 2.1, assuming that dominance variance exists, and using allele frequencies from parents of affected sibs in 98 Russian diabetic multiplex families (solid line) and in a subset of 46 multiplex families, carrying the common risk HLA-DR4 haplotype (dashed line). Dotted line indicates the suggestive level of linkage, with a LOD score that is equal or more than 2.2 (25). A scale of genetic distances between the markers is shown in centimorgans, according to the Marshfield sex-average map (24).



**Figure 2.** Multipoint non-parametric logarithm (NPL) profile for type 1 diabetes on chromosome 16q22-q24, obtained using the "estimate" command of GENEHUNTER 2.1, assuming that dominance variance exists, and using allele frequencies from parents of affected sibs in 98 Russian diabetic multiplex families (solid line) and in a subset of 46 multiplex families carrying the common risk HLA-DR4 haplotype (dashed line). A scale of genetic distances between markers is presented in centimorgans, according to the Marshfield sex-average map (24).

resents suggestive linkage (25). This, suggests a lack of linkage of the studied markers to type 1 diabetes in the Russian family data set. Further linkage analysis of the susceptible HLA-DR4 haplotype (DRB1\*04-DQB1\*0302) was performed in a subset of 46 affected sibling pairs carrying this haplotype. DR4 represents the most common HLA class II risk haplotype for type 1 diabetes in the Russian multiplex family data set (27-29). The HLA-conditioning linkage analysis resulted in increasing the maximum MLS and NPL to 1.77 and 1.22, respectively, for D16S504 marker ( $P=0.11$ ) (Figs. 1 and 2). The MLS value still did not reach the cut-off value of 2.2, showing no significant linkage between markers on 6q22-q24 and T1D in the Russian family data set.

### Transmission Disequilibrium Test

However, a lack of linkage between a marker and disease does not consequently suggest a lack of association of the marker with the disease. Therefore, we estimated transmission of alleles of the peak marker D16S504 and three microsatellites (D16S422, D16S3037, and D16S3098) which displayed a significant relationship to T1D in UK Caucasians (3,4). The TDT analysis was performed in the independent set of 114 Russian simplex families. Among these markers, the significance in preferential transmission of some alleles

**Table 3.** Transmission disequilibrium test of polymorphic markers D16S422, D16S540, D16S3073, and D16S3098 in 114 Russian type 1 diabetic simplex families and in the subset of 42 simplex families, with a proband carrying the predisposing HLA-DR4 haplotype

Allele length, bp*	All simplex families (n=114)										Simplex families with the HLA-DR4 haplotype (n=42)										
	proband					non-affected sibs					proband					non-affected sibs					
	T†	NT‡	$\chi^2_1$	P	$P_c$	T†	NT‡	$\chi^2_1$	P	$P_c$	T†	NT‡	$\chi^2_1$	P	$P_c$	T†	NT‡	$\chi^2_1$	P	$P_c$	
D16S504																					
268	19	24	1.16	0.28		25	18	2.28	0.13		11	14	0.72	0.40		15	10	2.0	0.16		
274	1	2	0.67	0.41		1	2	0.67	0.41		0	0	0.00	1.00		0	0	0.00	1.00		
276	11	12	0.09	0.77		14	9	2.17	0.14		5	6	0.18	0.67		5	6	0.18	0.67		
280	29	16	7.51	0.01	>0.05	18	27	3.60	0.06		16	9	3.92	0.05	>0.05	10	15	2.0	0.16		
282	11	16	1.85	0.17		13	14	0.07	0.79		3	6	2.00	0.16		5	4	0.22	0.64		
Overall (df=4)			5.63	0.23				4.39	0.36				3.41	0.49				2.20	0.70		
D16S3073																					
176	4	9	3.84	0.05	>0.05	8	5	1.38	0.24		3	4	0.29	0.59		4	3	0.29	0.59		
178	17	19	0.22	0.64		18	18	0.00	1.00		6	10	2.00	0.16		9	7	0.50	0.48		
180	40	26	5.94	0.01	>0.05	29	37	1.94	0.16		17	10	3.63	0.06		12	15	0.67	0.41		
182	24	31	1.78	0.18		30	25	0.91	0.34		10	12	0.36	0.55		11	11	0.00	1.00		
Overall (df=3)			5.89	0.12				2.12	0.54				3.14	0.37				0.73	0.87		
D16S3098																					
157	33	36	0.61	0.43		39	30	2.35	0.13		13	13	0.00	1.00		15	11	1.23	0.27		
159	18	24	1.71	0.19		22	20	0.19	0.66		9	11	0.40	0.53		10	10	0.00	1.00		
161	12	12	0.00	1.00		10	14	1.33	0.25		4	7	1.64	0.20		6	5	0.18	0.67		
163	21	15	2.00	0.16		14	22	3.56	0.06		11	7	1.78	0.18		7	11	1.78	0.18		
165	3	0	6.00	0.01	>0.05	2	1	0.67	0.41		1	0	2.00	0.16		0	1	2.00	0.16		
Overall (df=4)			4.99	0.29				4.05	0.40				2.91	0.57				2.60	0.63		
D16S422																					
188	4	8	2.67	0.10		7	5	0.67	0.41		2	2	0.00	1.00		2	2	0.00	1.0		
200	40	31	2.28	0.13		34	37	0.25	0.62		18	12	2.4	0.12		11	19	4.26	0.04	>0.05	
202	8	5	1.38	0.24		5	8	1.38	0.24		3	3	0.00	1.00		4	2	1.33	0.25		
206	10	10	0.00	1.00		12	8	1.60	0.21		5	7	0.67	0.41		6	6	0.00	1.00		
208	17	26	3.77	0.05		21	22	0.05	0.83		8	11	0.95	0.33		12	7	2.63	0.10		
210	1	0	2.00	0.16		1	0	2.00	0.16		0	0	0.00	1.00		0	0	0.00	1.00		
Overall (df=5)			6.95	0.22				2.98	0.70				1.99	0.85				4.1	0.54		

\*bp - base pair.

†T - transmitted.

‡NT - not transmitted.

**Table 4.** Transmission disequilibrium test of SNPs within the NQO1 and ICSBP1 in 114 Russian type 1 diabetic simplex families and in the subset of 42 simplex families, with a proband, carrying the predisposing HLA-DR4 haplotype

Gene	SNP	Allele	All simplex families (n=114)										Simplex families with the HLA-DR4 haplotype (n=42)									
			proband					non-affected sibs					proband					non-affected sibs				
			T*	NT†	$\chi^2_1$	P	$P_c$	T*	NT†	$\chi^2_1$	P	$P_c$	T*	NT†	$\chi^2_1$	P	$P_c$	T*	NT†	$\chi^2_1$	P	$P_c$
NQO1	A/G 72	Ser	16	18	0.24	0.62		17	17	0	1.0		9	6	1.2	0.27		10	5	3.33	0.068	
NQO1	Arg/Trp 139	Trp	53	44	1.67	0.2		47	50	0.19	0.66		25	19	1.64	0.2		20	24	0.73	0.39	
NQO1	Pro/Ser 187	Ser	49	41	1.42	0.23		46	44	0.09	0.77		26	20	1.57	0.21		18	28	4.35	0.037	>0.05
ICSBP1	C/T 414	T	22	20	0.19	0.66		23	19	0.76	0.38		9	10	0.11	0.74		9	10	0.11	0.74	
ICSBP1	T/C 300	C	9	4	3.85	0.05	>0.05	5	8	1.38	0.24		6	2	4.0	0.046	>0.05	4	4	0	1.0	
ICSBP1	C/T 16124	T	59	47	2.72	0.10		50	56	0.68	0.41		31	27	0.55	0.46		26	32	1.24	0.27	

\*T - transmitted.

†NT - not transmitted.

from parents to diabetic offspring was preliminary observed (Table 3). However, after correction for multiple alleles, no significance was observed. Further conditioning for the predisposing HLA-DR4 haplotype could also not reveal the significance in preferential allele transmission from parents to probands in the subset of 42 simplex families, each including a proband with the DR4 haplotype (Table 3). Hence, these results showed the lack of association between the studied microsatellite markers and T1D in the Russian simplex families.

In addition, we evaluated by TDT analysis 6 SNPs located within two positional candidate genes, *NQO1* and *ICSBP1*, on chromosome 16q22-q24 (3 SNPs in each gene). No significant difference in the allele transmission was shown, suggesting that these markers are not associated with T1D in the family data set, even when stratified for the common HLA risk haplotype (Table 4).

## Discussion

We tested 12 microsatellite markers, located within the putative T1D susceptibility locus

on chromosome 16q22-q24, but found no significant evidence for their linkage and association with the disease in the Russian family data set. Additionally, we examined 6 polymorphic nucleotide substitutions from two positional candidate genes, ICSBP1 and *NQO1*. The ICSBP1 gene was chosen because of the crucial role of its protein product in the differentiation and maturation of macrophages, antigen-presenting dendritic cells, and Langerhans cells (13,30). NAD(P)H-quinone oxidoreductase 1, a product of the *NQO1* gene, detoxifies quinones derived from the oxidation of phenolic metabolites of benzene. The enzyme is therefore involved in chemoprotection and plays a role in antioxidant defense via generation of antioxidant forms of ubiquinone and vitamin E (31). Since the oxidative stress is likely to be involved in  $\beta$ -cell destruction (32), *NQO1* is considered as a candidate gene for T1D susceptibility.

Among 3 SNPs tested in the *NQO1* gene, 2 (Pro/Ser 187 and Arg/Trp 139) represent functionally relevant amino acid substitutions. Ser187 and Trp139 molecular variants of NAD(P)H-quinone oxidoreductase 1 are shown to be less active than the wild-type enzyme (21,33). Both common *NQO1* polymorphisms have been widely evaluated for association, with a variety of diseases, including type 1 diabetes. However, no association of the codon 187 dimorphism with diabetes was found in affected Danish families (11). We also showed the lack of association between Pro/Ser 187 and Arg/Trp 139 variants of the *NQO1* gene and T1D in Russian simplex families (Table 4).

We could not find reports of the relationship between genetic variations within another positional candidate gene for susceptibility to T1D on chromosome 16q24, ICSBP1, and any complex trait or disease have been presented yet. In this study, therefore, we first analyzed 3 SNPs in the ICSBP1 gene for relation to T1D, but found no association with the disease.

Our data are consistent with the results of genome-wide scans for susceptibility to type 1 diabetes in Scandinavians (5) and the Dutch (6), showing no evidence for the T1D susceptibility locus in 6q22-q24,

In various populations, the evidence for linkage to T1D has been reported for more than 20 loci (1). However, the majority of originally identified loci was not reproduced in later and larger genome-wide scans (3,5,34-36). This is particularly

characteristic for minor susceptibility loci and could reflect genetic heterogeneity of type 1 diabetes in diverse ethnic groups (5). The susceptibility locus on 16q22-q24 seems to represent a locus with minor or intermediate genetic effect, for which a sib risk ( $\lambda_s$ ) of 1.6 was obtained in UK families (3). Thus, it is not surprising that this susceptibility locus on the chromosome 16q22-q24 was not found in the Russian family data set.

Complex and heterogeneous genetic background is a typical feature of multifactorial disorders, which include a variety of autoimmune, metabolic, and neurological diseases. For example, whole-genome screens for loci predisposing to autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and others, conducted in different populations identified discrete chromosomal regions potentially harboring susceptibility genes. However, with the exception of the HLA locus on 6p21, no single locus generated overwhelming evidence of linkage (37,38). These results suggest a complex genetic etiology, including multiple genes of small to moderate effect and probable genetic heterogeneity.

Ethnic differences, where the proportion of individuals with a particular gene variant is different between human populations, also influence the genetic effects of complex diseases (39). Differentiated environments and life-styles in geographically separated populations greatly affect quantitative genetic effects by enhancing or negating contribution of genes responsible for the complex trait. So, studies in separate populations should help in our understanding of genetic heterogeneity of complex disorders, such as type 1 diabetes, by revealing common and ethnically specific genetic variants responsible for susceptibility or resistance to the disease.

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