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ORIGINAL INVESTIGATION

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Susceptibility gene for non-obstructive azoospermia located near HLA-DR and -DQ loci in the HLA class II region

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Abstract The technical developments and expanded indications for testicular sperm extraction (TESE) with intracytoplasmic sperm injection (ICSI) provide great advantages for patients with non-obstructive azoospermia. Such success, however, also means that genetic abnormalities in non-obstructive azoospermia can be transmitted to the next generation, demonstrating the importance of being able to understand the genetic background of non-obstructive azoospermia. We have previously reported that human leukocyte antigens (HLA)-A33 and -B44 in the HLA class I region and the HLA-DRB1*1302 allele in the HLA class II region are linked to susceptibility to non-obstructive azoospermia in Japanese men. However, strong linkage of HLA-DRB1*1302 with HLA-A33 and -B44 is also evident in the Japanese population. Thus, uncertainty prevails as to whether the HLA class I or class II molecule is more directly associated with non-obstructive azoospermia. In the present study, we performed association analysis with 21 polymorphic microsatellite markers identified near the HLA genes to map the gene involved in the development of non-obstructive azoospermia more precisely. Microsatellite markers located in the HLA class I region or the class III region showed no statistically significant association with this disorder, although once again the HLA-A33 and -B44 alleles showed a significant association. In contrast, some of the microsatellite markers in the HLA class II region and at the HLA-DRB1 and -DQB1 loci displayed strong associations with non-obstructive azoospermia. Taken together, our previous and present data suggest that the critical region for development of non-obstructive azoospermia is near the HLA-DRB1 and -DQB1 segments in the HLA class II region.

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Introduction

Infertility affects about 15% of all couples attempting pregnancy, with male factors being responsible in approximately half the cases. About 15%–20% of infertile men exhibit azoospermia (Matsumiya et al. 1994), which may be caused either by the failure of spermatogenesis or by obstruction of the seminal tract. Congenital dysfunction in spermatogenesis, referred to as non-obstructive azoospermia, is probably a result of genomic abnormalities. Today, even men who have non-obstructive azoospermia of presumed genetic origin are considered to possess some testicular spermatozoa as demonstrated by testicular sperm extraction (TESE; Schoysman et al. 1993; Silber et al. 1995a, 1995b, 1997; Devroey et al. 1995; Schlegel 1999). The technical developments and expanded indications for TESE with intracytoplasmic sperm injection provide great advantages for patients. Such success, however, also means that genetic abnormalities in non-obstructive azoospermia can be transmitted to the next generation, indicating the importance of our being able to understand the genetic background of non-obstructive azoospermia.

Studies carried out in the 1990s in patients with non-obstructive azoospermia have implicated a deletion in the long arm of the Y chromosome (Yq11); the corresponding gene locus is known as the azoospermia factor (AZF; Ma et al. 1993; Reijo et al. 1995; Vogt et al. 1995; Chandley and Cooke 1994). Fewer than 15% of patients with non-obstructive azoospermia, however, are thought to have these Yq microdeletions (Reijo et al. 1995, 1996; Girardi et al. 1997; Vogt et al. 1997). Accordingly, X-chromosomal or autosomal genes are also likely to be in-

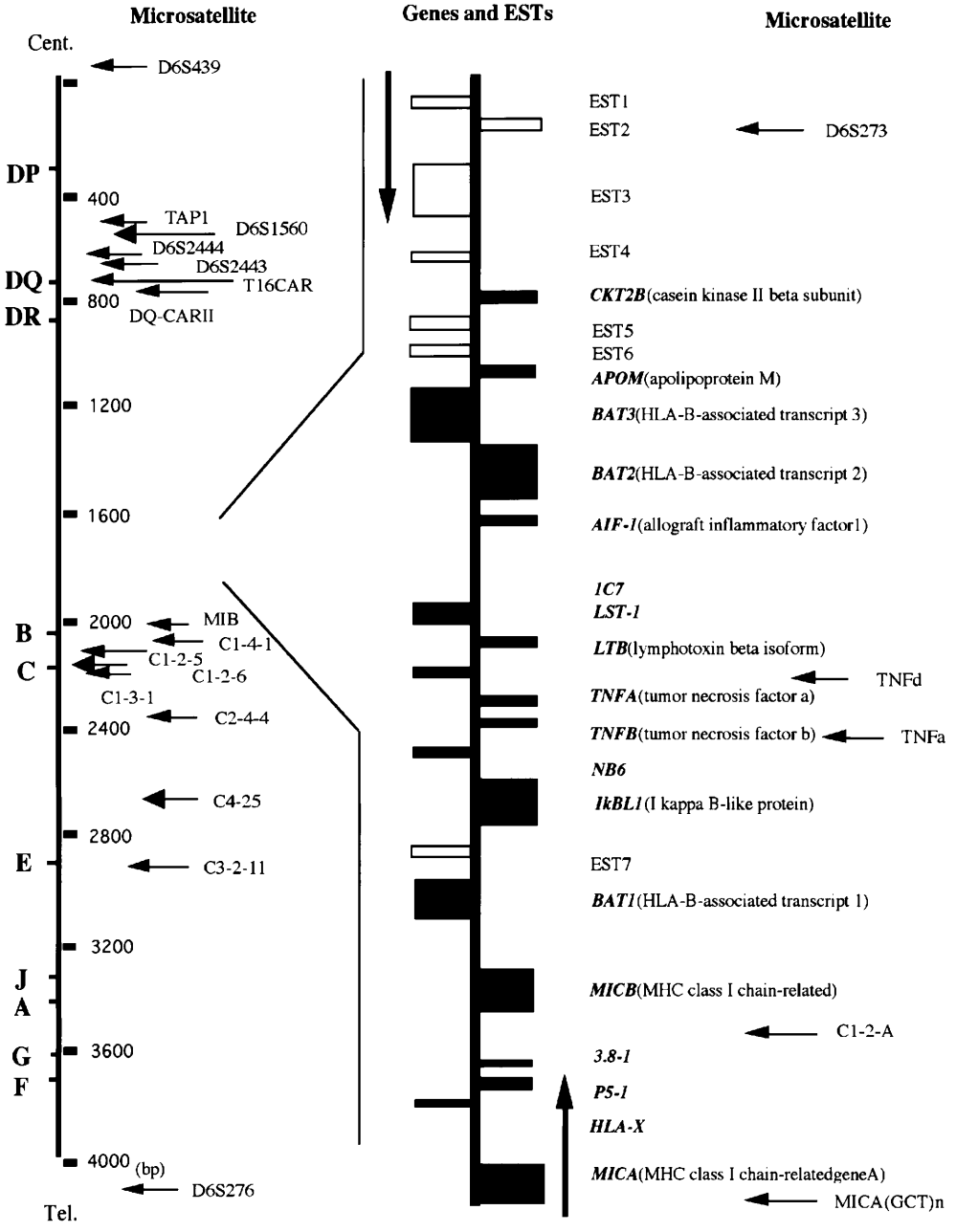

Fig. 1 Gene map around a susceptibility locus for non-obstructive azoospermia in the HLA region (*EST* expressed sequence tag). The location of all microsatellite markers employed in this study is displayed *left* with a physical scale (in kb) around the HLA region. Among them, the D6S273 and D6S439 microsatellite markers are located on the most telomeric and the most centromeric ends, respectively

volved. In the mouse, the T/t alleles are located within the murine homolog of the major histocompatibility (MHC) complex on chromosome 17, and several genes that are expressed during spermatogenesis have been cloned within them (Lader et al. 1989; Ha et al. 1991; Mazarakis et al. 1991). This evidence suggests a genetic basis of MHC-associated effects on fertility.

Human MHC encompasses 3.6 Mb and is divided into three regions: class II (1.1 Mb), class III (0.7 Mb), and class I (1.8 Mb). The whole region extends from the centromere to the telomere on chromosome 6 (6p21.3; MHC Sequencing Consortium 1999). More than 100 expressed non-human leukocyte antigen (HLA) genes are present in the HLA region, although the function of most of these remains uncertain (MHC Sequencing Consortium 1999; Shiina et al. 1999; Fig. 1). A prominent characteristic of the HLA genes is a marked degree of polymorphism, and the HLA antigens are considered important factors in the susceptibility to some disorders. MHC-associated effects on fertility as described in mice have also been suggested for humans (Gill 1994), because the spermatogenic genes have been identified within or close to the human HLA class II region in analogy to the murine T/t complex (Ragoussis et al. 1992; Tirosvoutis et al. 1995). Significant differences of the HLA class II allele and haplotype frequencies between males with normal spermatogenesis and males with infertility have been reported (van der Ven et al. 2000). Likewise, we have linked the HLA-A33, -B44, and -DR13 antigens, and the HLA-DRB1*1302 allele to susceptibility to non-obstructive azoospermia in

Japanese men (Miura et al. 1998; Tsujimura et al. 1999). In addition, we have proposed linkage disequilibrium as a possible way by which some HLA antigens and corresponding genes could be linked to the susceptibility to non-obstructive azoospermia; if the gene responsible for azoospermia is located near a certain locus of the HLA molecule, the linkage should be genetically maintained in a population (White et al. 1984). Therefore, a considerable need exists to investigate which HLA class I and II regions have a genuine association with non-obstructive azoospermia, and whether a candidate gene predisposing to azoospermia exists on chromosome 6.

In the present study, we have performed association analysis with 21 polymorphic microsatellite markers identified near the HLA genes by large-genome sequencing of the HLA class I, II, and III regions in order to map the gene involved in non-obstructive azoospermia more precisely.

Materials and methods

Subjects

We studied 67 infertile Japanese men with non-obstructive azoospermia. This diagnosis was confirmed by vasography and by a Johnsen score of less than 7 in a biopsy specimen from the testis (Johnsen 1970). We excluded from study any patient with chromosomal abnormalities or obstructive azoospermia. Unrelated healthy Japanese donors (n=248, male and female) were studied as controls. All samples were collected after obtaining informed consent.

Genomic DNA extraction

Genomic DNA was extracted by using a salting-out procedure after treatment of peripheral blood leukocytes with proteinase K, as described elsewhere (Miura et al. 1998; Tsujimura et al. 1999).

Genotyping for microsatellite allele

To determine the number of repeat units of 21 microsatellite loci exhibiting polymorphisms near the HLA class I, II, and III regions, we synthesized unilateral primers by labeling the 5'-ends with a fluorescent regent, 6-FAM, HEX, or TET (PE Biosystems, Foster City, Calif.; Fig. 1). Polymerase chain reaction (PCR) primers and conditions for amplifying C3-2-11, C4-2-25, C2-4-4, C1-3-1, C1-2-6, C1-2-5, C1-4-1, MIB, MICA-TM(GCT)$_n$ (i.e., MHC class-I-chain-related gene A transmembrane region), and C1-2-A were as described elsewhere (Ota et al. 1997; Tamiya et al. 1998, 1999). The PCR primers for D6S276, TNFa, TNFd, D6S273, DQ-CARII, T16CAR, D6S2443, D6S2444, D6S1560, TAP1, and D6S439 and the PCR conditions were comparable to those previously reported (Foissac and Cambon-Thomsen 1998). PCR-amplified products were denatured for 5 min at 100°C, mixed with formamide-containing stop buffer, and applied together with a size standard (GS500 TAMRA; PE Biosystems) to lanes of a 4% polyacrylamide denaturing gel containing 8 M urea. Gels were run in a model 377 automated DNA sequencer (PE Biosystems). Fragment sizes were determined automatically by using GeneScan software (PE Biosystems).

Statistical analysis

Gene (allele) frequencies were estimated by direct counting. The significance of differences in the distribution of alleles between patients with non-obstructive azoospermia and healthy controls was tested by the χ^2 method with a continuity correction and by Fisher's exact probability test (P test). P was corrected (Pc) by multiplication by the number of microsatellite alleles observed at each locus. A value of Pc less than 0.05 was accepted as indicating statistical significance. To control for the effect of linkage disequilibrium between loci, the Mantel-Haenszel weighted odds ratio (OR) and 95% confidence intervals (CI) were calculated (Mantel and Haenszel 1950).

Results

This association study investigating susceptibility to non-obstructive azoospermia by using 21 polymorphic mark-

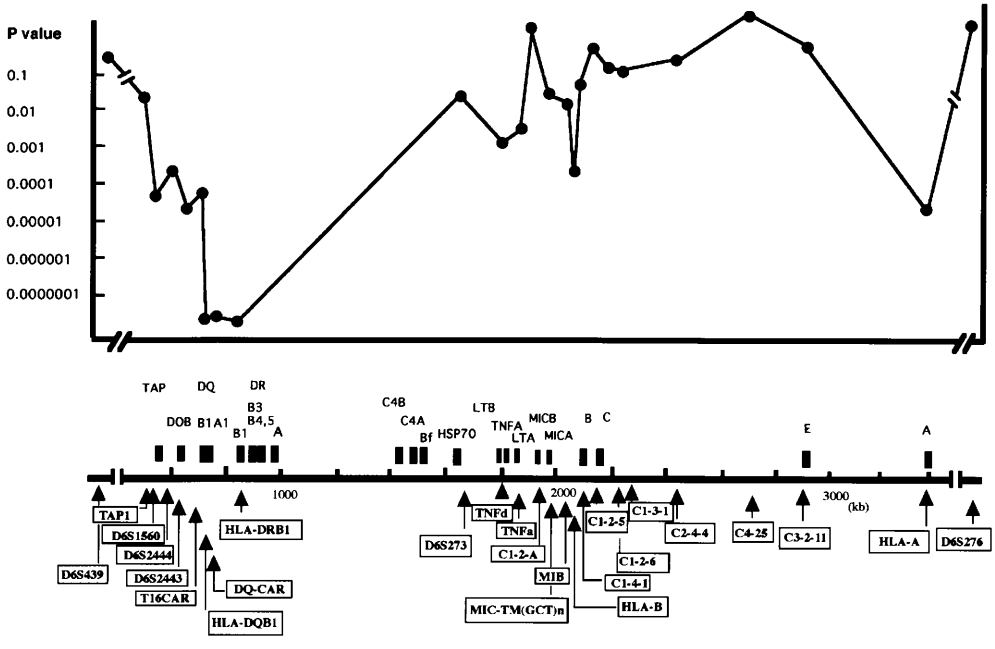

Fig. 2 Non-obstructive azoospermia susceptibility gene mapping by association analysis with the genetic markers in the HLA region. *P* values obtained by the association test between the control and the patient groups are displayed with the location of genetic markers used for mapping. The gene map *bottom* shows the location of those genetic markers (*boxed*); representative genes are indicated by *black boxes* in the HLA region

Table 1 Statistically significant alleles assoicated with azoospermia disease in Japanese pateints (*RR* relative risk)

Marker	No of alleles	Allele	Control (n=248)	Patient (n=67)	RR	χ^2	*P*	*Pc*
HLA-A	10	A33	33(13.3%)	24(35.8%)	3.64	18.04	0.000022	0.0002
HLA-B	21	B44	36(14.5%)	22(32.8%)	2.88	11.78	0.00018	0.0037
Cl-2-5	18	218	48(19.4%)	23(34.3%)	2.18	6.77	0.0092	0.17
HLA-DRB1	21	1302	31(12.5%)	29(43.3%)	5.34	32.42	0.000000012	0.00000026
DQ-CARII	11	207	28(11.3%)	27(40.3%)	5.30	30.80	0.000000029	0.00000031
HLA-DQB1	11	0604	29(11.7%)	28(41.8%)	5.42	32.24	0.000000014	0.00000015
T16CAR	14	229	31(12.5%)	22(32.8%)	3.42	15.59	0.000079	0.0011
D6S2443	11	203	31(12.5%)	23(34.3%)	3.66	17.69	0.000026	0.00029
D6S2444	10	148	65(26.2%)	29(43.3%)	2.15	7.34	0.0067	0.067
D6S1560	16	247	22(8.9%)	18(26.9%)	3.77	15.40	0.000087	0.0014

Table 2 Association of haplotype A33 with the azoospermia patients after stratification for the effect of HLA-DRB1*1302 (*OR* odds ratio, *95%CI* 95% confidence interval)

DRB1*1302	A33 negativity		A33 positivity		Weighted OR	χ^2	*P*	95%CI
	Control	Patients	Control	Patients				
Present	8	10	23	19	1.25	0.12	0.72	0.56<1.25 (OR)<2.79
Absent	189	33	10	10				

Table 3 Association of haplotype DRB1*1302 with the azoospermia patients after stratification for the effect of HLA-A33 (*OR* odds ratio, *95%CI* 95% confidence interval)

HLA-A33	DRB1*1302 negativity		DRBl*1302 positivity		Weighted OR	χ^2	*P*	95%CI
	Control	Patients	Control	Patients				
Present	10	5	23	19	3.59	11.08	0.00087	1.63<3.59 (OR)<7.95
Absent	189	33	8	10				

ers near HLA class I, II, and III regions together with the HLA-A, -B, DRB1 and DQB1 loci demonstrated, in the HLA region, the presence of three segments that showed significantly low *P* (*Pc*) values (Fig. 2). One of these segments was near the HLA class II region with extremely low *P* (*Pc*) values for the -DRB1 and -DRQ1 loci and for DQCARII. Table 1 lists all alleles showing statistically significant associations with non-obstructive azoospermia in this Japanese population. All alleles for each microsatellite marker were named according to their amplified fragment length, except for the GCT repetitive polymorphism in the MICA gene (Mizuki et al. 1997a; Ota et al. 1997). The most significant associations with non-obstructive azoospermia were observed in the DQB1 allele (DQB1*0604; χ^2=32.24, *Pc*=1.5×10^{-7}), the DRB1 allele (DRB1*1302; χ^2=32.42, *Pc*=2.6×10^{-7}), and allele 11 of the DQCARII microsatellite marker, which is located between the DRB1 and DQB1 loci (χ^2=30.80, *Pc*=3.1×10^{-7}; Table 1, Fig. 2). No allele at the various loci specifically decreased in patients compared with controls. Strong linkage disequilibrium between HLA-A33 and DR13 has been noted in a Japanese population. To determine which locus, viz., HLA-A or HLA-DRB1, is pathogenically related to non-obstructive azoospermia, the association of HLA-A33 with non-obstructive azoospermia was examined after stratification of patients according to the possible confounding effect of HLA-DRB1*1302. This was estimated by calculation of the Mantel-Haenszel weighted OR. No significant association was observed for the HLA-A33 allele (Table 2; 0.56<95%CI<2.79). In contrast, DRB1*1302 showed an extremely strong association with non-obstructive azoospermia even after stratification of patients for a possible confounding effect of HLA-A33 (Table 3; 1.63<95%CI<7.95). In addition, we could not find any homozygotes for high risk alleles.

Discussion

Microdeletion in the Y chromosome (AZF), the best-known genetic abnormality associated with non-obstructive azoospermia, is present in fewer than 15% of patients with this reproductive defect (Reijo et al. 1995, 1996). Additional genetic causes therefore are suspected. The relationship between non-obstructive azoospermia and genomic factors other than AZF, especially gene(s) located on the X or autosomal chromosomes, has not been clarified (Saxena et al. 1996; Yen et al. 1996). Recently, Olsen et al. (2001) reported a candidate gene for human male infertility at the 6p21.3 position of chromosome 6, corresponding to HLA region, according to digital differential display techniques. We previously reported that HLA-A33 and HLA-B44 in the class I region, and HLA-DR13 and the HLA-DRB1*1302 allele in the class II region were linked

to non-obstructive azoospermia in Japanese men (Miura et al. 1998; Tsujimura et al. 1999). All relative risks (RR) were very high (4.0, 8.4, 5.9, and 8.2, respectively), indicating that the molecules could be useful markers for this disorder. However, strong linkage of the HLA-DRB1*1302 allele with HLA-A33 and -B44 was evident in a previous study of a Japanese population (Hashimoto et al. 1994). Thus, which of the HLA class I and class II molecules are associated with non-obstructive azoospermia has been uncertain. The class III region also contains many expressed genes, showing the highest gene density in the human genome (MHC Sequencing Consortium 1999; Browning and McMichael 1996). Many genes located at the telomeric end of the class III region are involved in immune and inflammatory responses, such as HSP70, TNFA, LTA (TNFB), and LTB. We have therefore previously proposed that linkage disequilibrium could be involved in associations between some HLA antigens and genes and susceptibility to non-obstructive azoospermia. In addition, the expression of HLA antigens during spermatogenesis has been documented (Martin-Villa et al. 1996). Some of the human homolog of murine T/t complex genes, which are expressed in testis, are also close to the human MHC complex (Ragoussis et al. 1992; Tirosvoutis et al. 1995). Therefore, the aim of the present study has been to investigate whether HLA class I or II is associated with non-obstructive azoospermia, and whether a gene responsible for this disorder exists in the HLA region. Tamiya et al. (1998, 1999) have identified informative polymorphic microsatellites with dinucleotide to pentanucleotide repeats within a large-scale genome sequence corresponding to the entire class I region, extending from the MICB (MHC class I chain-related gene B) gene to the HLA-F gene (Mizuki et al. 1997b; Shiina et al. 1998, 1999). In addition, several other known polymorphic microsatellites have been located in the HLA class II and class III regions (Foissac and Cambon-Thomsen 1998). Here, we have selected 21 microsatellites distributed from the centromeric to the telomeric end of the 3.6-Mb HLA region, together with DRB1 and DQB1 alleles, to perform a systematic association analysis of non-obstructive azoospermia to define the critical region for susceptibility.

Our data indicate that neither the microsatellite markers located in the HLA class I region (C1–2-A, MICA-TM(GCT)$_n$, MIB, C1-4-1, C1-2-5, C1-2-6, C1-3-1, C2-4-4, C4-25, and C3-2-11) nor those in the class III region (D6S273, TNFd, and TNFa) show any statistically significant association with the disorder, although the HLA-A33 and -B44 alleles reveal statistically significant associations as in our previous reports. No significantly associated alleles have been found for two microsatellite markers flanking the HLA region, viz., D6S439 on the centromeric side and D6S276 on the telomeric side. In contrast, some of the microsatellite markers in the HLA class II region (D6S1560, D6S2444, D6S2443, T16CAR and DQ-CARII) and the HLA-DRB1 and -DQB1 loci display very strong association with non-obstructive azoospermia according to our χ^2 analysis (Table 1; Fig. 2). Figure 2 illustrates correlations between the *P* values and these polymorphic markers; the *Pc* values of the -DRB1 and -DQB1 loci and DQCARII are extremely low. The lowest *P* value is observed at the DRB1 locus (DRB1:1302, $P=1.2\times10^{-7}$), confirming our previous finding. Calculation of the Mantel-Haenszel weighted OR has shown that a significant association of DRB1*1302 is retained, even after patient stratification for a possible confounding effect of HLA-A33, which suggests that associations of HLA-DRB1*1302 with non-obstructive azoospermia are independent of HLA-A33. Considered together, our previous and present data suggest that the critical region for development of non-obstructive azoospermia is near the HLA-DRB1 and -DQB1 segments in the HLA class II region.

In conclusion, a candidate gene on chromosome 6 (6p21.3) appears likely to be involved in some cases of non-obstructive azoospermia. Whether this gene is located in the narrow segment between the HLA-DQB1 and -DRB1 loci remains uncertain. Although further investigation is necessary to clarify whether HLA-DRB1*1302 or -DQB1*0604 are genuinely associated with non-obstructive azoospermia, this report is the first to indicate the possibility of a gene responsible for non-obstructive azoospermia being located on chromosome 6.

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