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Polymorphisms at the TNF Locus in Chinese Han Population

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ABSTRACT: One hundred sixty-four unrelated healthy individuals from Chinese Han population were investigated in order to define the distribution of eight polymorphic loci within the tumor necrosis factor (TNF) gene cluster and determine their relationship between the high polymorphic microsatellite TNFa, b, d, and other elements. The cloning and sequencing for five microsatellites were simultaneously done. In this study, the distribution of TNF alleles apparently vary from other ethnic groups. A new allele was detected and confirmed. It should be emphasized that a very strong association between TNFd8 and TNFe4 is reported and d8e4 haplotype appears to be specific to the population studied. In addition, five extended haplotypes were established in this population: a6b5c1d8e4TNF308-1TNF-βNco1-1TNFAspH1-2, a2b1c2d5e1TNF308-1TNF-βNco1-2TNFAspH1-2, a11b4c1d4e3TNF308-1TNF-βNco1-2TNFAspH1-1, a10b4c1d4e3TNF308-1TNF-βNco1-2TNFAspH1-1, and a2b3c1d2e3TNF308-2TNFAspH1-2. Data suggest that important ethnic differences may exist and that it is a necessary initiative for further research. *Human Immunology 63, 71–75 (2002).* © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: TNF-α; TNF-β; gene polymorphism; haplotype; HLA

ABBREVIATIONS

TNF	tumor necrosis factor
HLA	human leukocyte antigen
PCR	polymerase chain reaction
MHC	major histocompatibility complex
RFLP	restriction fragment length polymorphism

INTRODUCTION

The gene for tumor necrosis factor-α (TNF-α) and lymphotoxin-α (TNF-β), referred to as the TNF locus, are tandemly arranged within a 7-kilobase region in the major histocompatibility complex (MHC) 250 kb centromeric to human leukocyte antigen (HLA-B) and 340 kb telomeric to the C2/Bf complex on the short arm of chromosome 6 [1]. The TNF gene is 12 kilobases in length and contains several areas. A NcoI restriction fragment length polymorphism (RFLP) at position TNF-308 in the promoter of the TNF-α gene, a NcoI and an AspH1 RFLP in the first intron of the TNF-β gene have been identified [2–4]. Five highly polymorphic microsatellites have also received particular attention [5]. Several studies have reported that TNF individual alleles correlated with TNF secretion from activated monocytes [6, 7]. Furthermore Stüber *et al.* [8] have demonstrated that TNFB2 homozygote patients with a diagnosis of severe sepsis displayed higher plasma TNF-α concentrations. The TNF-308 site has also been described that was related to increased transcription level of the TNF mRNA [9]. These findings suggest that TNF polymorphisms may play a role in the pathogenesis of many autoimmune, infectious, and neoplastic diseases. TNF alleles are in linkage disequilibrium with alleles of both MHC class I and class II genes. Previously, Wilson *et al.*

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[10] revealed a very strong association between the TNF-308 and HLA-A1, B8, and DR3 alleles. In addition the association between TNFa, TNFb, and TNFc have also been investigated in four European populations [11]. A few population/sample-specific haplotypes have been established, suggesting that haplotype studies can be a useful tool for discriminating between different ethnic groups. In the present study healthy individuals from Chinese Han population were investigated. We extensively determined the frequencies of the various alleles at eight polymorphic sites within the TNF locus and examined the relationship of other polymorphic elements to the three highly polymorphic microsatellites TNFa, TNFb, and TNFd. It is necessary to the further study of the possible involvement of the alleles in pathologic processes in our population.

MATERIALS AND METHODS

Subject Selection and Extraction of Genomic DNA

The subjects of this study were 164 unrelated healthy individuals from Chinese Han population in Hubei province. Genomic DNA was obtained from venous blood following the procedure described previously, with minor modifications [12].

TNF Polymorphism Typing

Five microsatellites were amplified using a single-step polymeras chain reaction (PCR) with primers described by Udalova *et al.* [5]. TNF microsatellite alleles were typed by a 6% polyacrylamide 0.4-mm sequencing gel followed by silver nitrate staining. Fragments were sized using DNA markers and simultaneously typed with known alleles derived from the cloned PGEM-T vector. The RFLP sites in the first intron of TNF-β marked by AspH1 and Nco1 were analyzed using primers and conditions described by authors [3, 4, 13], except that BSIHKA1 was used (an isoschizomer of AspH1 [New England Biolabs, Beverley, MA, USA]). The digested products electrophoresed in 0.1% ethidium bromide-stained 1.5% agarose gels. Similarly, the TNF-308 polymorphism was performed by PCR amplification and Nco1 digestion [2]. The digested products were separated on a 10% nondenaturing polyacrylamide gel. Alleles were visualized as described for TNF microsatellite typing.

Cloning and Sequencing

The PCR products of five microsatellites were purified and ligated with PGEM-T vector. High efficiency JM 109 competent cells was used in the proces s of transformation. Needed transformants were obtained by blue/white color screening and standard ampicillin selection. Recombinant plasmid DNA were isolated and identified. Sequencing was performed on an ABI 377 DNA sequencer (Applied Biosystems Corp., Foster City, USA).

Statistical Analysis

Allele frequencies were calculated by direct counting. Linkage disequilibrium between TNF loci were calculated from 2×2 contingency tables, which were then analyzed by Chi-square tests and Fisher's exact tests. The corrected p values (p_c) were obtained by multiplying the uncorrected p value ($p < 0.05$ was set for significant level) with the number of alleles observed. Hardy-Weiberg equilibrium were tested in a $2\times n$ analysis using Chi-Square.

RESULTS

TNF Allele Frequencies

The distribution of the various alleles are listed in Table 1. It is noted that a novel TNFd microsatellite allele was detected and designated as TNFd8. The most common TNFa and TNFb alleles were TNFa6 and TNFb5, respectively, which apparently vary from other ethnic groups [14–16]. TNFd6 and TNFe4 alleles were presented at a relatively high frequency compared with that from other ethnic populations, whereas the frequency of TNF308-2 allele was very low. Little difference was found from the previous reported distribution for the TNFc and TNF-AspH1 loci. No deviation from Hardy-Weiberg equilibrium were observed within the TNF locus in our population ($p > 0.05$).

TNFa, TNFb, and TNFd Microsatellites and Other Alleles Within the TNF Locus

The relationships involving TNFa, TNFb, and TNFd microsatellites and their significance levels are summarized in Table 2. The most notable were those involving TNFa2 allele, which was strongly associated with TNFb1 ($\chi^2 = 64.208$, $p_c = 4\times10^{-13}$), TNFc2 ($\chi^2 = 42.951$, $p_c = 3\times10^{-9}$), TNFd2 ($\chi^2 = 36.233$, $p_c = 4\times10^{-7}$), TNFe1 ($\chi^2 = 40.895$, $p_c = 5\times10^{-8}$), and TNF308-2 ($\chi^2 = 31.938$, $p_c = 4\times10^{-6}$) and was moderately linked with TNFb3 ($\chi^2 = 21.420$, $p_c = 4\times10^{-4}$). Similarly, TNFa6 was strongly associated with TNFb5 ($\chi^2 = 75.688$, $p_c = 1\times10^{-17}$), TNFd8 ($\chi^2 = 24.077$, $p_c = 5\times10^{-6}$), and TNF-βNco1-1 ($\chi^2 = 79.977$, $p_c = 5\times10^{-19}$); moderate association with TNFd6 ($\chi^2 = 13.095$, $p_c = 2\times10^{-2}$) and TNFe4 ($\chi^2 = 17.508$, $p_c = 7\times10^{-4}$) were observed. It should be emphasized that very strong linkage equilibrium were observed between TNFb4/TNFAspH1-1 ($\chi^2 = 125.992$, $p_c = 5\times10^{-32}$) and d8/e4 ($\chi^2 = 141.360$, $p_c = 2\times10^{-28}$). Other strong association, such as b3/TNF308-2 ($\chi^2 = 55.141$, $p_c = 7\times10^{-10}$) and b1/e1 ($\chi^2 = 69.232$, $p_c = 2\times10^{-12}$), were also examined. In

TABLE 1 Distribution of TNF alleles at eight polymorphic loci

TNF locus	Allele	Size	Number observed	Percentage
TNFa	a1	97	6	0.0183
	a2	99	52	0.1583
	a3	101	0	0.0000
	a4	103	2	0.0061
	a5	105	13	0.0396
	a6	107	125	0.3811
	a7	109	26	0.0793
	a8	111	2	0.0061
	a9	113	11	0.0335
	a10	115	39	0.1189
	a11	117	31	0.0945
	a12	119	3	0.0091
	a13	121	18	0.0549
TNFb	b1	125	33	0.1006
	b2	126	0	0.0000
	b3	127	37	0.1128
	b4	128	108	0.3293
	b5	129	148	0.4512
	b6	130	0	0.0000
	b7	131	2	0.0061
TNFc	c1	159	69	0.2104
	c2	161	259	0.7896
TNFd	d1	124	2	0.0061
	d2	126	18	0.0548
	d3	128	15	0.0457
	d4	130	116	0.3537
	d5	132	44	0.1341
	d6	134	99	0.3018
	d7	136	0	0.0000
	d8	138	34	0.1037
TNFe	e1	99	21	0.0640
	e2	101	3	0.0091
	e3	103	266	0.8110
	e4	105	38	0.1159
TNF-α 308	1		306	0.9329
	2		22	0.0671
TNF-β Nco1	1		158	0.4817
	2		170	0.5183
TNF-β AspH1	1		101	0.3079
	2		227	0.6921

addition, some weak association simultaneously existed in our study. The following two-locus haplotypes were found to have frequencies larger than 0.2: TNFa6/b5 (0.3461); TNFa6/TNFβNco1-1 (0.3627); TNFa6/TNFβAspH1-2 (0.3391); TNFb4/c1 (0.3254); TNFb4/d4 (0.2440); TNFb4/TNFβAspH1-1 (0.3020); and TNFb5/TNFβAspH1-2 (0.4102).

Sequencing Results

The sequences of cloned PGEM-T vector comprising TNF microsatellite were analyzed. It was interesting to find that the sequence of TNFb5 allele was completely identical with that from a genebank database. The sequences of TNFa7, TNFc1, TNFd4, and TNFe3 were consistent with that from a genebank database; however, several base exchange were noted as following: TNFa at position 13216 A→G; TNFc 8289 C→G and 8330 G→A; TNFd 961 A→G and 965 G→A; and TNFe 772 A→G. The sequence of new allele TNFd8 was in keeping with the electrophoresis result as above. The difference between TNFd4 and TNFd8 allele was exactly 4 (GA/TC) repeats.

TABLE 2 Association between TNFa, b, d microsatellites and other alleles within the TNF locus

TNF	Δ	Haplotype frequence	*PC*
TNFa2/b1	0.0738	0.0898	4×10^{-13}
TNFa2/b3	0.0435	0.0614	4×10^{-4}
TNFa2/c2	0.0824	0.1157	3×10^{-9}
TNFa2/d2	0.0407	0.0494	4×10^{-7}
TNFa2/e1	0.0479	0.0580	5×10^{-8}
TNFa2/TNF α 308-2	0.0415	0.0512	4×10^{-6}
TNFa6/b5	0.1741	0.3461	1×10^{-17}
TNFa6/d6	0.0713	0.1863	2×10^{-2}
TNFa6/d8	0.0615	0.1010	5×10^{-6}
TNFa6/e4	0.0558	0.1000	6×10^{-4}
TNFa6/TNF β Nco1-1	0.1791	0.3627	5×10^{-19}
TNFa6/TNF β AspH1-2	0.0753	0.3391	1×10^{-1}
TNFa7/b4	0.0382	0.0643	3×10^{-2}
TNFa7/d4	0.0393	0.0673	2×10^{-2}
TNFa7/TNF β Nco1-2	0.0336	0.0747	3×10^{-1}
TNFa7/TNF β AspH1-1	0.0438	0.0682	2×10^{-3}
TNFa9/b3	0.0202	0.0239	2×10^{-2}
TNFa9/c2	0.0150	0.0221	1×10^{0}
TNFa10/b4	0.0454	0.0845	5×10^{-2}
TNFa10/ TNF β Nco1-2	0.0635	0.1251	1×10^{-4}
TNFa10/ TNF β AspH1-1	0.0373	0.0739	3×10^{-1}
TNFa11/b4	0.0608	0.0919	4×10^{-6}
TNFa11/d4	0.0416	0.0750	4×10^{-2}
TNFa11/ TNF β Nco1-2	0.0436	0.0925	4×10^{-2}
TNFa11/ TNF β AspH1-1	0.0525	0.0816	3×10^{-4}
TNFa13/b4	0.0351	0.0532	1×10^{-3}
TNFa13/d6	0.0356	0.0525	5×10^{-2}
TNFa13/ TNF β AspH1-1	0.0525	0.0816	1×10^{-3}
TNFb1/c2	0.0677	0.0888	3×10^{-9}
TNFb1/e1	0.0523	0.0587	2×10^{-12}
TNFb3/c2	0.0435	0.0672	4×10^{-3}
TNFb3/d2	0.0441	0.0503	1×10^{-10}
TNFb3/d3	0.0316	0.0367	1×10^{-5}
TNFb3/ TNF α 308-2	0.0460	0.0535	7×10^{-10}
TNFb4/c1	0.0654	0.3254	6×10^{-1}
TNFb4/d4	0.1275	0.2440	1×10^{-8}
TNFb4/ TNF β AspH1-1	0.2002	0.3020	5×10^{-33}
TNFb5/d8	0.0512	0.0980	3×10^{-3}
TNFb5/e4	0.0418	0.0941	2×10^{-1}
TNFb5/ TNF β AspH1-2	0.0980	0.4102	4×10^{-3}
TNFd3/c2	0.0329	0.0425	2×10^{-4}
TNFd4/ TNF β Nco1-2	0.0883	0.2716	1×10^{-2}
TNFd5/e1	0.0428	0.0514	5×10^{-7}
TNFd5/ TNF β Nco1-2	0.0438	0.1133	3×10^{-1}
TNFd8/e4	0.0909	0.1029	2×10^{-28}
TNFd8/TNF β Nco1-1	0.0550	0.1050	2×10^{-4}

DISCUSSION

Overall, this study presents the first reported Chinese Han population study at the TNF locus. Obviously, differences in allelic distribution exist between our population and other ethnic groups. In particular a novel allele designated TNFd8 was detected at a relatively high frequency (0.1037). Recently Khani-Hanjani *et al.* [17] reported four new polymorphisms designated as alleles a14, b8, b9, and d0, which weren't found in our study. However, their frequencies were rather low. Additionally, TNFe4 is also presented at a high frequency (0.1159). Udalova *et al.* [5] first detected two TNFe4 from 101 cell lines in 1993. Surprisingly, TNFe4 couldn't be found in following population investigation [14, 15]. These new polymorphisms can increase the recognized heterogeneity of this region and may be involved in further disease association. It should be noted that a very strong linkage equilibrium between TNFd8 and TNFe4 is revealed in our data. The association cannot be found in other populations. In view of the specialty of the distribution of TNFd8 and TNFe4 alleles, haplotype TNFd8e4 may be characteristic of our population. Based on maximum likelihood estimate, four most frequent three-locus haplotypes have been described by Crouau-Roy *et al.* [11] in four European populations: TNFa11b4c1, TNFa2b1c2, TNFa6b5c1, and TNFa10b4c1. These haplotypes have also been observed in our study. By analysis of two-locus association, we establish five extended haplotypes that integrate alleles across the TNF locus in our population: TNFa6b5c1d8e4TNF308-1TNFβNco1-1TNFAspH1-2, TNFa2b1c2d5e1TNF308-1TNFβNco1-2TNFAspH1-2, TNFa11b4c1d4e3TNF308-1TNFβNco1-2TNFAspH1-1, TNFa10b4c1d4e3TNF308-1TNFβNco1-2TNFAspH1-1, and TNFa2b3c1d2e3TNF308-2TNFAspH1-2. Previously, Udalova *et al.* [5] indicated several cell lines shared extended TNF haplotypes, such as TNFa6b5c1d3e3, TNFa11b4c1d3e3, TNFa2b1c2d4e1, and TNFa2b3c1d2e3. We found that the differences existing between these haplotypes and our haplotypes are due to the variance of TNFd and TNFe, which suggests that though TNFd and TNFe microsatellites have less polymorphic than TNFa microsatellite, the distribution of TNFd and TNFe alleles in the extended haplotypes can vary widely between different ethnic populations. Nedospasov *et al.* [18] first mapped and characterized TNFa microsatellite, making a nomenclature for it based on its size and variability: [a1 = $(AC/GT)_6$; a2 = $(AC/GT)_7$; · · · a13 = $(AC/GT)_{18}$. Afterwards, no opinion is proposed for it. This study sequenced the cloned PGEM-T vector comprising TNFa microsatelite and revealed that TNFa6 contains 15 (AC/GT) repeats, which is consistent with that from a Genebank database (TNFa2 contains 11 [AC/GT] repeats). The present result do not agree with Nedospasov, so a new nomenclature for TNFa microsatellite should be defined. According to our sequence results, and that from a Genebank database, we make the description that a1 = $(AC/GT)_{10}$; a2 = $(AC/GT)_{11}$; · · · a13 = $(AC/GT)_{23}$. With regard to several base exchange in our sequence result, further studies should be investigated to verify whether they are caused by ethnic difference or other factors. In conclusion, this study defined the distribution of TNF alleles and determined some extended halotypes across the TNF locus. Differences and similarities apparently exist between our population and other ethnic populations. Further study will be done to find more TNF/MHC haplotypes and the association with diseases.

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