Polymorphisms of TNF microsatellite marker a (TNFa) and HLA-DR-DQ in diabetes mellitus – A study in 609 Swedish subjects.

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Abbreviated title: TNFa in diabetes mellitus

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ABSTRACT:

We explored the importance of the genetic markers; microsatellite TNFa, HLA-DR3-DQ2 and DR4-DQ8 in diabetes mellitus. The studied groups were as follows; autoimmune type 1 (n=63), non-autoimmune type 1 (n=35), latent autoimmune diabetes in adults (LADA; n=54) and non-autoimmune type 2 (n=340) and these patients were compared to 117 healthy controls. HLA genotyping was done with polymerase chain reaction and sequence-specific oligonucleotides. TNFa microsatellites were determined with polymerase chain reaction and fragment size determination. Univariate analysis of these genetic risk factors showed that homozygosity for TNFa2/2 was a significant risk factor for autoimmune type 1 diabetes (odds ratio (OR)=5.82 95% confidence interval (95%CI) 1.97-17.2), for autoimmune negative type 1 diabetes (OR=4.63; 95%CI 1.32-16.2) as well as for LADA (OR=3.90; 95%CI 1.21-12.5). Moreover, heterozygosity for HLA-DR3-DQ2/DR4-DQ8 was an important risk factor for autoimmune type 1 diabetes (OR=16.4; 95%CI 3.60-75) as was DR4-DQ8/x (OR=2.52; 95%CI 1.27-4.98). Heterozygosity for HLA-DR3-DQ2/DR4-DQ8 was a risk factor also for LADA (OR=10.0; 95%CI 2.05-48.9). Neither HLA-DR3-DQ2 nor DR4-DQ8 were risk factors for non-autoimmune type 1 or type 2 diabetes. We concluded that heterozygosity for DR3-DQ2/DR4-DQ8 and to some extent homozygosity for TNFa2/2 were risk factors for autoimmune diabetes irrespective of the clinical classification.

ABBREVIATIONS

GADA glutamic acid decarboxylase antibodies

HLA human leukocyte antigen

IA-2A islet cell antigen-2 antibodies or tyrosine phosphatase antibodies

ICA islet cell antibodies

PCR polymerase chain reaction

LADA latent autoimmune diabetes in adults

NA not applicable

TNFa microsatellite marker a for tumor necrosis factor

TNFα tumor necrosis factor alpha; protein released by cells in the immune system and by

adipocytes

TNFA gene for tumor necrosis factor alpha

INTRODUCTION

The human leukocyte antigen (HLA) region on the short arm of chromosome 6 is the best

genetic risk marker for type 1 diabetes since it accounts for the highest genetic risk. In

particular, the combination of DRB1*03-DQA1*0501-DQB1*0201/DRB1*0401-

DQA1*0301-DQB1*0302 is a known marker for onset of type 1 diabetes at a young age {1-

3}. The locus for tumor necrosis factor alpha (TNFA) is located in the HLA area near the DR

and DQ-region. Five different TNF microsatellite regions denoted a, b, c, d and e have been

described {4-6}. The TNFa microsatellite region is located 3.5kb upstreams of the TNFβ

mRNA start site with 13 different known variants of CA-repeats (98-122bp) {7}. The close

location of this factor to HLA-DR-DQ suggests that certain alleles are in linkage

disequilibrium with each other. Therefore, TNFa could also serve as a marker for diseases

with a known association to HLA-DR-DQ. Several publications have shown that the allele

frequencies of the TNFa microsatellite vary between populations {8-10}. Some TNFa alleles

have shown associations to diseases. For example, TNFa2 and TNFa9 have been reported to

be increased among young type 1 diabetic patients in Japan, whereas no association was

found among type 1 diabetic patients in Italy {7, 11}. Furthermore, TNFa2 has shown an

association to celiac disease {12}. TNFa11 has shown an association both to reumatoid

arthritis {13} and cervical intraepithelial neoplasia {14}. TNFα is an important factor in the

immune system with several known functions. TNFα is released by certain activated cells in

the immune system such as monocytes, macrophages and natural killer cells {15}. The release

of TNFα is known to induce the expression of MHC class II molecules, coded for by the

HLA-DR-DQ- region, on the surface of cells in the immune system {16} and to modulate the

activity of T and B lymphocytes {15}. Overexpression of TNFα has been reported in subjects

with the TNF2 variant in the promotor which is linked to the haplotype HLA-A1-B8-DR3

{17}. The promotor variant TNF2 has been associated to autoimmune diseases such as

aplastic anemia {18}, and also to increased susceptibility for cerebral malaria {19}. Furthermore, the promotor variant TNF2 has also been associated to a more severe course in Non-Hodgkin´s lymphoma {20}. Low production of TNFα has also been associated to autoimmunity for example to systemic lupus erythematosis {21}. Besides, TNFα exerts functions outside the immune system for example as an inhibitor of lipoprotein lipase and as a stimulator of increased glycerol concentration in adipocytes. This route leads to increased resistance to insulin {22-24}. The aim of this study was to examine the importance of microsatellite alleles for the TNFa region and to compare these findings with the known risk factors for type 1 diabetes (HLA-DR3-DQ2 and DR4-DQ8). We wanted to test these factors in autoimmune diabetes with clinical classification of either type 1 or type 2 (LADA) and compare with findings in patients with non-autoimmune diabetes of either type 1 or type 2 diabetes.

MATERIALS AND METHODS

Subjects

This study was focused on adult type 1 diabetic patients in a defined area in the southern part of Sweden. Nevertheless, sporadic cases of type 1 diabetes in children were reported to us from the Childrens Department at Lund University Hospital and these cases were also included. A total of 60 patients out of 1557 patients clinically classified with type 2 diabetes or unclassifiable diabetes from the same area as the type 1 diabetic patients were positive for at least one of islet cell antibodies (ICA), glutamic acid decarboxylase antibodies (GADA) or islet cell antigen-2 antibodies (IA-2A) and were denoted as latent autoimmune diabetes in adults (LADA).

A group of 340 patients clinically classified as type 2 diabetes and aged 50 years or more and negative for beta cell specific autoantibodies were selected from the group of 1557 patients as representatives for classical type 2 diabetes. The clinical classification was done by the treating physician and based on clinical observations such as weight loss and ketoacidosis and reported to us on a standardised form. We invited 119 healthy blood donors from the same geographical area as the diabetic patients to participate in the study. The invited controls signed an informed consent. We received a blood sample from the selected patients and controls for analysis of autoantibodies and genotyping. Complete genotyping was possible in 94% (609/650) of all subjects, 98% (98/100) of type 1 diabetic patients, 90% (54/60) of LADA patients, 92% (340/371) of type 2 patients and in 98% (117/119) of the healthy blood donors. Only patients and controls with complete genotyping are considered in the present report. The type 1 diabetic patients were separated into autoimmune type 1 diabetes or non-autoimmune type 1 diabetes based on the presence or absence of at least one beta cell specific antibody of ICA, GADA or IA-2A. This strategy gave us four different goups of diabetes. Autoimmune type 1 diabetes (n=63; median age 30; range 9-67 yrs), non-autoimmune type 1

diabetes (n=35; median age 50; range 17-89 yrs), latent autoimmune diabetes in adults;

LADA; (n=54; median age 49; range 21-79 yrs) and finally non-autoimmune type 2 diabetes

(n=340; median age 64; range 50-89 yrs). These diabetic patients were compared to the group

with healthy controls (n=117; median age 35: range 19-65 yrs). The strong linkage

disequilibrium within the HLA-system confers that most DR alleles are inherited together

with specific alleles within the DQA1 and DQB1 regions forming a defined DR-DQ type.

Therefore, haplotypes consisting of specific DR and DQA1-DQB1 alleles are reported in the

following way: DR3-DQ2 where DQ2 consists of DQA1*0501-DQB1*0201 and DR4-DQ8

where DQ8 consists of DQA1*0301 and DQB1*0302 and all other haplotypes are referred to

as x. In this study, the importance of the TNFa-alleles compared to HLA-DR3-DQ2 and DR4-

DQ8 were explored for the four aforementioned groups of diabetes.

The linkages between TNFa alleles and HLA-DR-DQ-haplotypes were estimated by Chi-

Square analysis for the respective genotypes in an analysis including all subjects (n=609;

Table 1). This study was approved by the Ethical Committee at Lund University (LU 44-95

and LU 526-00).

Assays for beta cell specific autoantibodies

ICA were analyzed with a double staining technique {25}. The lower limit for positivity for

the pancreas used in this study was 9 JDF-U with a sensitivity of 100% and a specificity of

88% {26}.

The GADA and IA-2A were analyzed with radioimmunoprecipitating assays {27-29}. The

lower limits were 21.2 WHO-U/ml for the GADA assay and 14.7 WHO-U/ml for the IA-2A

assay. The sensitivity for the GADA assay was 70% and for the IA-2A assay 50%, both

assays had a specificity of 100% in the Diabetes Standardization Program {30}.

TNFa genotyping

Genomic DNA was extracted from frozen white blood cells using standard procedures with digestion of proteins with Proteinase K and salting out of the DNA with 6 mol/L sodium chloride. Total amount of DNA was measured at 260 nm and purity was checked at 260/280 nm.

The TNFa microsatellite region was amplified with a single step PCR using 5´CCTCTCTCCCCTGCAACACACA3´ as forward primer and 5´GCCTCTAGATTTCATCCAGCCACAG 3´ as reverse primer. The reverse primer was labelled with agents 6-HEX, 6-FAM, or TET (Amersham-Pharmacia Biotech, Uppsala, Sweden). In brief, the 25.0 µl reaction contained 250 ng of genomic DNA, 30 pmol of each primer, 8 nmol of each DNTP (Promega, Madison, WI, USA), 2.5 µl PCR-buffer (1x) and 2.5 U of the Ampli-Taq Gold DNA polymerase (Applied Biosystems/Roche, Branchburg, NJ, USA) and went through an initial denaturation step at 96°C followed by 34 cycles starting with a denaturation step at 95°C for 40s, annealing at 60°C for 40s and strand synthesis at 72°C for 40s with a final extension at 72°C for 7 minutes. After amplification, the number of base-pairs in the PCR-fragments were determined using a DNA sequencer (ABI-Prism 373, Perkin-Elmer, Norwalk, CT, USA). An internal size marker (500-TAMRA; Perkin-Elmer) was added to each sample to allow accurate determination of the fragment sizes. The different alleles of the TNFa microsatellite correspond to fragment sizes as follows; TNFa1-98bp, TNFa2-100bp, TNFa3-102bp, TNFa4-104bp, TNFa5-106bp, TNFa6-108bp, TNFa7-110bp, TNFa8-112bp, TNFa9-114bp, TNFa10-116bp, TNFa11-118bp, TNFa12-120bp, TNFa13-122bp.

HLA-DR and HLA-DQ typing

The second polymorphic exons of DQA1, DQB1, and DRB1 regions of the HLA system were amplified using PCR with specific primers for each region. For the separate amplification reactions 250 ng of genomic DNA was used. The amplified products were dotted onto nylon membranes under denaturing conditions {31}. Membranes were hybridazed with sequence specific oligonucleotides, the 3´end labelled with ^{32}P-deoxy-CTP, and washed under specific stringency conditions for the respective probe (eight for the DQA1-region, 11 for the DQB1-region, and 16 for the DRB1-region) {32} before exposure to x-ray film.

Statistical analyses

The allele frequencies of TNFa were estimated by direct counting (total number of alleles = two times the number of individuals in the respective group). The different genotypes for TNFa were grouped as TNFa2/2, TNFa2/x and x/x where x denotes any other allele than TNFa2. These TNFa genotypes were compared univariately as odds ratios (OR) and 95% confidence intervals (95%CI) to estimate the importance of homozygosity or as single factors (heterozygosity) for autoimmune type 1 diabetes, LADA, non-autoimmune type 1 diabetes or non-autoimmune type 2 diabetes. The importance of HLA-DR3-DQ2/x, DR4-DQ8/x and DR3-DQ2/DR4-DQ8 were also analysed univariately for the four different groups of diabetes. In the logistic regression analysis, diabetes or not diabetes was the dependent variable, and the independent factors tested for their separate risk measured as OR were TNFa2/2, TNFa2/x, DR3-DQ2/x, DR4-DQ8/x and DR3-DQ2/DR4-DQ8. Confidence intervals (95%CI) below or above 1.0 were considered as significant. A CI above 1 represents increased risk, whereas a CI below 1 represents decreased risk. Frequencies were compared with the Chi-square test. P-values less than 0.05 were considered as significant. The statistical calculations were done with the Statistical Package for Social Sciences (SPSS), version 11.03 for Mac OS 10 (SPSS, Inc, Chicago, Il, USA) and MedCalc version 7.4 for Windows.

RESULTS

General findings of the genetic risk factors in different groups of diabetic patients

The microsatellite allele TNFa2 seems to be in linkage disequilibrium with in particular HLA-DR3-DQ2 and to some extent DR4-DQ8 since TNFa2 was present in 97% (31/32) of the subjects with the genotype HLA-DR3-DQ2/DR4-DQ8 (df=2; χ^2=12.8; p<0.005).

Furthermore, TNFa2 was found in 87% (111/128) of the subjects with the genotype HLA-DR3-DQ2/x (df=2; χ^2=81.6; p<0.001) (Table 1).

TNFa2 was the most frequent allele in all groups of patients and in controls. The highest allele frequency was found in LADA-patients (0.4444), followed by autoimmune type 1 diabetes (0.3810), non-autoimmune type 1 diabetes (0.3714), controls (0.3120) and finally non-autoimmune type 2 diabetes (0.2882) (Table 2). The other 12 allele frequencies for TNFa were similar in all four groups of diabetic patients and healthy controls (Table 2).

At least one of HLA-DR3-DQ2 and/or DR4-DQ8 were found in most patients with autoimmune type 1 diabetes (81%; 51/63). The finding of one or both high-risk genotypes (DR3-DQ2 and/ or DR4-DQ8) was significantly more frequent in autoimmune type 1 diabetes compared with LADA-patients (63%; 34/54; p=0.049), non-autoimmune type 2 (43%; 147/340; p<0.001) and also compared with controls (48%; 56/117; p<0.001) but not compared with non-autoimmune type 1 diabetes patients (63%; 22/35; p=0.83). The finding of at least one of HLA-DR3-DQ2 and/or DR4-DQ8 did not differ significantly between controls and the three other groups of diabetes.

TNFa2 either in homozygosity (TNFa2/2) or heterozygosity (TNFa2/x) was found in about half of the patients and controls, but did not differ significantly between the different groups of patients or controls. The highest frequency was found in LADA patients (72%; 39/54), followed by non-autoimmune type 1 (60%; 21/35), controls (58%; 68/117), autoimmune type 1 diabetes patients (56%; 35/63) and finally non-autoimmune type 2 (49%; 166/340).

Risk estimates for the genetic risk factors for the four different groups of diabetes

Autoimmune type 1 diabetes

Three factors were found to be significant for autoimmune type 1 diabetes in the univariate risk analysis. Most important for autoimmune type 1 diabetes was HLA-DR3-DQ2/DR4-DQ8 (OR=16.4; 95%CI 3.60-75.0) followed by homozygosity for TNFa2/2 (OR=5.82; 95%CI 1.97-17.2) and finally DR4-DQ8/x (OR=2.52; 95% CI 1.27-4.98). Conversely, absense of HLA-DR3-DQ2 and/or DR4-DQ8 conferred protection (OR=0.246; 95%CI 0.105-0.446) (Table 3). When the same risk factors were entered into the multiple logistic regression analysis, heterozygosity for HLA-DR3-DQ2/DR4-DQ8 yielded an even higher risk value (OR=48.2; 95%CI 8.04-289) as did DR4-DQ8/x (OR=6.79 95%CI 2.75-16.8). In the logistic regression analysis, also HLA-DR3-DQ2/x reached significance (OR=3.36; 95%CI 1.18-9.62), whereas TNFa2/2 was no longer significant (Table 4).

Latent autoimmune diabetes in adults

Two factors were found to be significant for LADA in the univariate risk analysis. The highest OR for LADA was found for heterozygosity for HLA-DR3-DQ2/DR4-DQ8 (OR=10.0; 95%CI 2.05-48.9) followed by homozygosity for TNFa2/2 (OR=3.90; 95%CI 1.21-12.5) (Table 3). In the multiple logistic regression analysis, only heterozygosity for HLA-DR3-DQ2/DR4-DQ8 remained significant (OR=7.59; 95%CI 1.38-41.8) (Table 4).

Non-autoimmune type 1 diabetes

The univariate analysis identified homozygosity for TNFa2/2 as a genetic risk factor for non-autoimmune type 1 diabetes (OR=4.63; 95%CI 1.32-16.2) (Table 3). None of the studied risk factors were significant when entered into the multiple logistic regression analysis (Table 4).

Non-autoimmune type 2 diabetes

None of the studied risk factors were significant for non-autoimmune type 2 diabetes, neither when studied as univariate factors or when entered into the multiple logistic regression analysis. TNFa2/x was found to be protective in the univariate analysis (OR=0.571; 95%CI 0.374-0.873), but not in the multiple logistic regression analysis (Table 3 and 4).

DISCUSSION

In this study we have shown that heterozygosity for HLA-DR3-DQ2/DR4-DQ8 was the most important risk factor for autommune diabetes in adult age. It was highly significant both for classical autoimmune type 1 diabetes (OR=16.4) and for LADA (OR=10.0). We could also demonstrate that homozygosity for TNFa2/2 was a significant risk factor for autoimmune type 1 diabetes (OR=5.82), for LADA (OR=3.90) and also for non-autoimmune type 1 diabetes (OR=4.63). One interesting aspect of the results is the additional risk conferred by TNFa2/2 in non-autoimmune type 1 diabetes where DR3-DQ2 and DR4-DQ8 are less prevalent.

Despite the finding of homozygosity for TNFa2 (TNFa2/2) as a risk factor for autoimmune diabetes and non-autoimmune type 1 diabetes, the finding of TNFa2 per se did not confer an increased risk for any of the different types of diabetes.. Previously, TNFa2 has shown an association to type 1 diabetes with onset in childhood but not to type 1 diabetes in adults in a Japanese population {7}. Moreover, TNFa12 has been shown to be associated to LADA in the Japanese population {33}. In contrary to our findings, none of the TNFa alleles showed an association to type 1 diabetes in an Italian population {11}.

It seems likely that TNFa2 contributes to an increased risk for autoimmune diabetes (type 1 or LADA) due to its linkage to the HLA-DR-DQ region since TNFa2 was found in most patients with the susceptible genotypes HLA-DR3-DQ2 and/or DR4-DQ8. We could not detect a linkage disequilibrium between the second most common allele TNFa6 and HLA-DR3 and/ or DR4-DQ8 (data not shown). Furthermore, TNFa2 was found to be significant in the univariate analysis, but not in the multivariate analysis and this finding indicate that the contribution of the TNFa region is weaker compared to the contribution of the HLA-DR-DQ-region. This finding is in accordance with a previous report where patients with type 1 diabetes and heterozygous for DR3/DR4 had higher frequencies of the TNFa2 allele compared to controls {34}.

TNFa2 has been reported to be the most frequent allele of the TNFa alleles in the backgound population found in about 30% or more of healthy subjects in the Swedish population {14, 35}, as well as in the Danish population {34}.

There seems to be a biological importance of the different TNFa microsatellite alleles, since it has been shown that the secreted levels of TNFα from cultured peripheral blood monocytes was high in subjects homozygous for TNFa2 {34}. There has been conflicting results regarding the importance of the levels of TNFα in relation to type 1 diabetes. One study showed that children with type 1 diabetes as well as first degree relatives had increased levels of TNFα, both circulating levels, and released from stimulated cultured peripheral monocytes {36}. Another study showed that lower levels of TNFα were released from cultured unstimulated pheripheral monocytes in type 1 diabetic children and first degree relatives compared to in healthy subjects {37}.

It is difficult to dissect the specific contribution of TNFa2 for the development of type 1 diabetes from that of HLA-DR3-DQ2 and/or DR4-DQ8 due to the linkage disequilibrium and the fact that other autoimmune diseases with an increased frequency of TNFa2 also have the same predisposing alleles within HLA as type 1 diabetes such as myasthenia gravis {35} and celiac disease {12}. Perhaps, TNFa2 contributes with an additive effect. In the present study, the type 1 diabetes associated markers HLA-DR3-DQ2 and/or DR4-DQ8 were not associated to non-autoimmune diabetes, neither clinically classified as type 1 nor as type 2. Despite this fact, homozygosity for TNFa2 was found to be a risk factor for non-autoimmune type 1 diabetes. None of the TNFa microsatellite alleles were associated to non-autoimmune type 2 diabetes.

In conclusion, homozygosity for TNFa2 is a risk factor for both classical autoimmune diabetes with onset in childhood and for LADA. This risk is likely due to the linkage disequilibrium to HLA-DR3-DQ2 and DR4-DQ8. Heterozygosity for HLA-DR3-DQ2/DR4-

DQ8 is an important risk factor both for classical autoimmune diabetes in adults and for

LADA, but not for non-autoimmune type 1 diabetes or non-autoimmune type 2 diabetes.

Acknowledgements

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