ABSTRACT

Background: Adenosine deaminase (ADA) structural gene consists of 12 exons. A number of common variants have been found within the coding and intronic region of the gene: the possible role of this variability on ADA function are unknown. In the present study we reexamined our data on a set of immune related diseases [Type 1 Diabetes (T1D), Rheumatoid Arthritis (RA), Endometriosis (ENDO) and Non Diabetic Coronary Artery Diseases (NDCAD)] investigating with a new approach the relevance of ADA genetic variability on susceptibility to these disorders.

Methods: Three single nucleotide polymorphisms were considered, namely Taq1 site (ADA1, nt 4050-4053, exon 1), Pst1 site (ADA2, nt 19465-19470, intron 2) and MLu NI (ADA6, nt 31230-31235, exon 6). We reanalyzed the data on 199 children with T1D, on 79 subjects with RA, on 98 women with ENDO, on 136 NDCAD and on 246 healthy blood donors. ADA genotype was determined by restriction fragment length polymorphism (RFLP) analysis. The subjects were classified according to the number of homozygous genotypes for the more frequent alleles at the three loci. All subjects were from White population of Rome.

Results: All diseases show a strong excess of subjects with the more frequent genotype in all loci as compared to controls suggesting a protective effect of heterozygosity within the ADA gene.

Conclusions: The data suggest that the combination of polymorphic sites within the ADA gene influences the susceptibility to some immune related disorders and calls for further studies on the relationship between ADA gene structure and immune functions.

KEYWORDS: ADA gene; Coronary Artery Disease; Endometriosis; Rheumatoid Arthritis; Type 1 Diabetes

INTRODUCTION

The ADA gene is composed of 12 exons and is localized on 20q13.11 [1]. Differences among normal sequences have been found both within the coding and intronic regions of gene and a study on linkage disequilibrium has shown areas of weak associations and those of strong associations within pairs of internal markers [2,3].

The presence of a Taq1 site (ADA1) (nt 4050-4053) at exon 1 corresponds to a functional variation due to a substitution of asparagine for aspartic acid at codon 8.
This mutation represents the basis of the biochemical polymorphism described by Spencer et al [4] with two common alleles \( ADA^1 \) and \( ADA^2 \).

Adenosine deaminase catalyzes the irreversible deamination of adenosine to inosine, contributing to the regulation of adenosine concentration in body fluids. Adenosine is a local hormone that influences blood flow and platelet aggregation, increases insulin sensitivity in adipocytes [5-7] and decreases insulin sensitivity in muscle fibers [8,9]. Decreased concentration of adenosine weakens T cell activation while its high concentration increase the activity of adenosine receptors strengthening T cell activation [10,11]. Adenosine is a cardio protective agent [12] and impairment of adenosine related signal transduction contributes to chronic heart failure [13].

\( ADA \) acts also as an ecto-enzyme [14,15] contributing to degradation of extracellular adenosine [11] and to the signal transduction through interaction with CD26 and A1R [16].

In the present study we investigated the possible influence of allelic combination within the \( ADA \) gene on the susceptibility to a set of immune related disorders. Three single nucleotide polymorphisms \( ADA^1, ADA^2 \) and \( ADA^6 \) were examined [17]. The position of polymorphic sites within \( ADA \) gene and the restriction enzymes are presented in table 1. \( ADA^1 \) is an exonic site involved in the regulation of enzymatic activity. \( ADA^2 \) is an intronic polymorphism and \( ADA^6 \) is a synonymous substitution, therefore they do not change the protein sequence but could influence tissue specific expression. Moreover, \( ADA^2 \) and \( ADA^6 \) polymorphisms may be markers of DNA sequences responsible for functional variation in the enzymatic activity and ecto enzymatic functions.

**Table 1.** Polymorphic sites studied within the \( ADA \) gene

<table>
<thead>
<tr>
<th>Name</th>
<th>Position within the gene</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ADA^1 )</td>
<td>Exon 1 nt 4050-4053</td>
<td>Taq1</td>
</tr>
<tr>
<td>( ADA^2 )</td>
<td>Intron 2 nt 19465-19470</td>
<td>Pst1</td>
</tr>
<tr>
<td>( ADA^6 )</td>
<td>Exon 6 nt 31230-31235</td>
<td>MluN1</td>
</tr>
</tbody>
</table>

**MATERIAL AND METHODS**

We studied 199 children with Type 1 Diabetes (T1D), 79 subjects with Rheumatoid Arthritis (RA), 98 women with endometriosis, 136 non diabetic subjects with Coronary Artery Disease (CAD) and 246 healthy blood donors (Controls). All subjects were from the White population of Rome. Data on these subjects have been previously reported [18-22]. An analysis on the role of combination of \( ADA^1, ADA^2 \) and \( ADA^6 \) loci, however, has not been previously performed. Informed verbal consent was obtained from the patients or from their mothers to participate in the study that was approved by the Council of our Department. The collection of data was performed a few years ago before the establishment of an Ethical Committee. All procedures followed were in accordance with the ethical standards and with the Helsinki Declaration of 1964 and its later amendments. \( ADA \) genotypes were determined by PCR. Genomic DNA was extracted from venous blood samples collected in NaEDTA using the procedure of Kunkel [23]. PCR amplification was carried out according to Hirschorn [17] as previously described [18].

The three intragenic polymorphisms spanning over about 28 Kb have a known molecular basis and include the presence/absence of a Taq1 site (\( ADA^1 \), nt 4050-4053, exon 1), of a Pst1 site (\( ADA^2 \), nt 19465-19470, intron 2) and a MluNI site (\( ADA^6 \), nt 31230-31235, exon 6). The PCR volume was 25 µl containing 100ng of DNA, 1.5mM di MgCl2, 2.5 reaction buffer, 5pmoles of prymar, 50 mM dNTP, 2U of Supertherm DNA polymerase. Thirty cycles were performed using a DNA Thermal Cycler. 7µl of each reaction was digested in 2U of the specific enzyme according to the manufacturer’s direction. Each digestion was resolved in 3% agarose gel in Tris acetate / EDTA buffer at Ph 8.0. Following electrophoresis the gel was stained with ethidium bromide and the fragments were visualized by U.V..

The alleles corresponding to the presence and absence of restriction site were assigned as allele *1 and allele *2 respectively.

Table 2 shows the scheme by which we classified the subjects according to the combination of \( ADA^1, ADA^2 \) and \( ADA^6 \) genotypes. Subjects carrying three common genotypes were ranked 3 while those carrying only genotypes with the rare alleles were ranked 0.

Determination of genotypes was made in groups of twenty including cases and controls to eliminate errors due to incomplete enzymatic digestion.

Chi-square test of independence was performed using the SPSS package [24].
TABLE 2. SCHEME SHOWING THE CLASSIFICATION OF SUBJECTS ACCORDING TO THE COMBINATIONS OF ADA1, ADA2, AND ADA6 GENOTYPES.

<table>
<thead>
<tr>
<th>ADA1</th>
<th>ADA2</th>
<th>ADA6</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>C</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>R</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>C</td>
<td>C</td>
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<tr>
<td>C</td>
<td>R</td>
<td>C</td>
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<tr>
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<td>R</td>
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<tr>
<td>R</td>
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<td>R</td>
<td>1</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>R</td>
<td>0</td>
</tr>
</tbody>
</table>

C= Homozygous for the common allele;  
R=Homozygous or heterozygous for the rare allele

Table 3. Distribution of total number of common ADA1, ADA2 and ADA6 genotypes in T1D, in Rheumatoid Arthritis, in Endometriosis, in CAD non diabetic and in blood donors (controls).

<table>
<thead>
<tr>
<th>Controls (A)</th>
<th>T1D (B)</th>
<th>Rheumatoid Arthritis (C)</th>
<th>Endometriosis (D)</th>
<th>CAD non diabetic (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 common genotypes</td>
<td>20.7%</td>
<td>30.8%</td>
<td>36.4%</td>
<td>46.2%</td>
</tr>
<tr>
<td>2 common genotypes</td>
<td>36.2%</td>
<td>41.5%</td>
<td>33.8%</td>
<td>27.7%</td>
</tr>
<tr>
<td>1 common genotype</td>
<td>37.0%</td>
<td>22.1%</td>
<td>28.6%</td>
<td>26.2%</td>
</tr>
<tr>
<td>No common genotypes</td>
<td>6.1%</td>
<td>5.5%</td>
<td>1.3%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Total n°</td>
<td>246</td>
<td>195</td>
<td>77</td>
<td>65</td>
</tr>
</tbody>
</table>

Chi square test of independence \( \chi^2 \) df p

| A vs B | 13.324 | 3 | 0.004 |
| A vs C | 10.413 | 3 | 0.015 |
| A vs D | 19.847 | 3 | 0.0002 |
| A vs E | 28.010 | 3 | 0.00003 |
| B vs C vs D vs E | 17.190 | 9 | 0.046 |

Fig 1. The relationship between ADA gene heterozygosity and relative risk of immune related diseases.
RESULTS

Table 3 shows the distribution of the number of homozygous genotypes for the more frequent ADA₁, ADA₂ and ADA₆ alleles in controls and in the immune related diseases considered. All diseases show a statistically significant difference as compared to controls with a strong tendency towards an excess of homozygotes for the more frequent allele (rank 3). A border line difference (P=0.046) is observed among the diseases studied. Figure 1 shows the Odds Ratio for the diseases considered in relation to the number of homozygous genotypes for the more frequent allele at the three ADA loci studied. The odds ratio for all diseases is very high in subjects who were homozygous for the more common allele in all ADA loci and decreases with the number of heterozygous loci.

DISCUSSION

The parameter proposed in the present study is an indicator of the number of mutation within the ADA gene and of the degree of heterozygosity. This new analysis is explorative; however, the results suggest that further studies in this area could be rewarding.

T1D and RA are autoimmune diseases. Immunological factors may have an important role in endometriosis [25-26] and in CAD [27-28]; furthermore the role of adenosine deaminase in immune function is well known.

We have previously performed association studies of ADA gene in T1D, in RA and in CAD. In T1D haplotype differences were observed between T1D children and controls [19]. In CAD differences between patients and controls were also observed [21,22]. Only a slight border line association was observed in RA [18].

The present approach is different from that of our previous studies and seems more productive suggesting that the combination of polymorphic sites within ADA gene influences the susceptibility to immune related diseases. The loci investigated could have a role on ADA activity and in turn on adenosine concentration. These loci could also influence the function of ADA as ecto-enzyme, thereby modulating the interaction of ADA with adenosine receptors on the surface of T cells. The association, however, could be due to other loci in linkage disequilibrium with the loci studied.

CONCLUSION

The result suggests that the heterozygosity within the ADA gene is protective against immune related diseases.

AUTHOR CONTRIBUTION

Gloria-Bottini, Magrini, Bottini have conceived the paper
Manca Bitti and Rapini have collected and analyzed the samples and have contributed to statistical analyses
Saccucci and Neri have carried out laboratory analyses
Gloria-Bottini and Bottini have directed the statistical analyses
All Authors have approved the final revision of the paper

REFERENCES


