The −308 G>A SNP of TNFA is a factor predisposing to chronic rhinosinusitis associated with nasal polyposis in aspirin-sensitive Hungarian individuals: conclusions of a genetic study with multiple stratifications

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Abstract

Single nucleotide polymorphisms (SNPs) of the tumour necrosis factor alpha (TNFa) gene (TNFA) have been extensively studied and shown to be associated with an increased risk of the development of various chronic inflammatory diseases. Inflammation has been demonstrated to play a central role in the pathogenesis of chronic rhinosinusitis (CRS), and TNFA is a key pro-inflammatory cytokine with important functions in these processes. In order to determine whether the well-known TNFA −308 G>A SNP has a role in a genetic predisposition to CRS in the Hungarian population, we analyzed our genomic collection containing control and CRS patient samples in a case–control study, and compared the genotype and allele frequencies. There was no significant difference in the observed genotype or allele frequencies between the controls and the total CRS group. However, after careful stratification of the patient group on the basis of the observed clinical symptoms, we found a significantly higher carriage rate of the rare A allele-containing genotypes among the CRS patients with nasal polyposis (NP) who also exhibited sensitivity to aspirin (acetylsalicylic acid, ASA+). It is concluded that genetic variants of the TNFA gene may affect the risk of CRS in a clinically well-defined group of CRSNP+ASA+ patients in the Hungarian population. Our results also emphasize that the group of CRS patients is not homogenous in that patients exhibiting different clinical symptoms exist. Their carried genetic predisposing factors, and as a result, the exact molecular events leading to the development of various forms of CRS, may also differ.

Keywords: chronic rhinosinusitis, nasal polyposis, single nucleotide polymorphism, TNFA −308 G>A

Introduction

Chronic rhinosinusitis (CRS) is characterized by persistent inflammation of the nasal and paranasal mucosa (1). CRS is present when any combination of the major symptoms, including nasal congestion or blockage, loss of smell, rhinorrhea, post-nasal drip and facial pain or pressure occurs for an extended period of time (over 12 weeks) despite various treatment...
attempts. It is a clinically heterogeneous disease frequently associated with other airway diseases, such as asthma, allergy, chronic obstructive pulmonary disease and bronchiectasis (2–6).

Nasal polyposis (NP) is characterized by non-granulomatous inflammatory tissue extensions of the mucosal surface lining the sinonasal cavities (7). CRS can occur either with (CRSNP+) or without (CRSNP-) the appearance of NP. A special group of CRSNP+ patients exhibits a marked sensitivity to aspirin (acetylsalicylic acid, ASA) or other non-steroidal anti-inflammatory drugs. In such sensitive individuals (ASA+), consumption of even a small dose of ASA can cause bronchial constriction, rhinorrhea and shock symptoms related to a non-IgE-mediated pharmacological hypersensitivity reaction (8–10).

The pathogenesis of CRS is a complex process in which several self and environmental factors have been implicated (1, 11). Anatomic features of the upper airway that cause mucous stagnation inside the sinuses, bacterial and fungal infections and abnormal immune regulation have been shown to play an important role in the pathogenesis of the disease (12–14). Apart from these, individual genetic factors have a significant role in making certain individuals susceptible to the development of CRS (15, 16).

Aberrant immune regulation often results in chronic inflammation, thus the different molecules (cytokines, chemokines and anti-microbial peptides) that are playing an important role in these processes have been suggested to be involved in the pathogenesis of CRS, too. One such candidate is the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα), the presence of which has already been detected in various cellular components of the polypoid tissues of CRS patients (17, 18). This has led to the suggestion that genetic polymorphisms affecting the regulation and/or function of the gene encoding the TNFα cytokine (TNFA) may play important roles in the genetic predisposition to CRS (19, 20).

The TNFA locus is located within the highly polymorphic major histocompatibility III region on chromosome 6 (6p21.3). There are many single nucleotide polymorphisms (SNPs) within this gene, especially in its 5′ regulatory region, often exhibiting associations with the pathogenesis of certain chronic inflammatory and immune-mediated diseases (21). One of them, the TNFA -308 G>A (rs1800629) is a relatively frequent SNP in Caucasian populations, which have already been investigated in the context of CRS (22–25). The results of these studies led to the postulation of a correlation between the carriers of the rare A allele and CRS, and also CRSNP+, in Turkish and US Caucasian populations, whereas the same association was not detected in Canadian individuals.

In order to establish whether the TNFA -308 G>A SNP is involved in a genetic predisposition to CRS in the Hungarian population, we performed case–control genetic studies. Our results indicate that the rare A allele, or alternatively, a linkage group carrying this allele may be a genetic predisposing factor in the Hungarian population, but this association is restricted to the ASA+ group of CRSNP+ patients.

Methods

Study population and ethics

Buccal swab samples were obtained from a total of 544 Caucasian individuals (169 controls and 375 patients) from various otorhinolaryngology centres in Hungary (University of Szeged; Semmelweis University, Budapest; Pécs University; Borsod-Abauj-Zemplén County Hospital and University Teaching Hospital, Miskolc; and St John’s and North-Buda Hospitals of the Municipality of Budapest). The ages of the recruited individuals were between 18 and 72 years. Patients were selected after trained medical professionals had established the diagnosis of CRS. Exclusion criteria included the presence of signs of acute upper airway infection, known malignancy or any other general disease, infectious disease, primary or acquired immune deficiency, dialysis, any chronic autoimmune diseases, rhinitis medicamentosa or odontogenic sinusitis. The control group comprised individuals without any medical history of CRS and with none of the above-described diseases and conditions.

The study was approved by the Hungarian Research Ethics Committee. All participating subjects gave their written consent before sample collection. The study was performed in accordance with the principles stated in the Declaration of Helsinki and its later revisions.

Polymorphism analysis

Genomic DNA was obtained from buccal swab samples by using the QIAGEN EZ1 DNA Investigator Kit (QIAGEN, Germany).

Patient and control samples were genotyped for the TNFA -308 G>A SNP by the PCR-RFLP (Restriction Fragment Length Polymorphism) method, as described previously (26). Briefly, a short 106-bp genomic sequence around the –308 position of the TNFA promoter was amplified by using the following primers: 5′-GAG GCA ATA GGT TTT GAG GGG CAT-3′ and 5′-Ggg aca cac AAG CAT CAA GG-3′. Restriction analysis of the resulting fragments was performed with an NcoI enzyme (Fermentas, Vilnius, Lithuania). Electrophoresis of the digested PCR products was performed on a 2% agarose gel (Lonza, Rockland, ME, USA). To visualize DNA fragments, gels were stained with GelRed (Biotium, Inc., Hayward, CA, USA).

Statistical analysis

Statistical analysis was carried out on the various groups of patients and controls according to the rules of case–control allelic association study designs. Genotype and allele frequencies were calculated by determining the percentage of individuals carrying the different genotypes and the percentage of carried alleles in each group. For the statistical analyses, the dominant genotype model was applied, where the group of rare-allele carriers (GA + AA) was compared with the group of non-carriers (GG). Statistical significance of the associations, odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated using the SPSS software (Version 17, SPSS, Chicago, IL, USA).

Hardy–Weinberg equilibrium (HWE) was calculated using a web-based calculator (http://www.otge.org/software/hwemr-calc.shtml) (27).

Literature search

A PubMed search was performed and all the currently available reports investigating the possible relationship between...
CRS (with or without NP) and the TNFA -308 G>A SNP in different populations were reviewed.

Results

Demographic analysis

The clinical characteristics of the study participants are presented in Table 1. Altogether 169 controls (84 males and 85 females) and 375 CRS patients (204 males and 171 females) were genotyped for the TNFA -308 G>A SNP. The mean ages of the controls and the cases were 43.1 and 48.8 years, respectively. Aspirin sensitivity (ASA+) was detected in 18.4% (n = 60) of the CRSNP+ patients. None of the controls was aware of ASA sensitivity (Tables 1 and 2). We also analyzed whether our study population was in HWE for the studied genetic variant. We did not detect any deviation from the HWE neither in the controls nor in our patient group (Table 1).

Genetic analysis of the TNFA -308 G>A SNP in the unstratified and stratified groups of CRS patients

The observed genotype and allele frequencies of the TNFA -308 G>A SNP in different populations were reviewed. There had been four previous analyses of the association between CRS (with or without NP) and the TNFA -308 G>A SNP in various ethnic populations (22–25), and compared our data with the results of such investigations (Table 3). Thus, our data indicated that the rare A allele was positively associated exclusively with the development of CRS and NP in the ASA+ patients as the OR for this complex disease was twice as high in individuals who carried the minor A allele in at least one copy (OR = 2.0, at 95% CI: 1.1–3.7 when the observed genotype frequencies were compared and OR = 1.7, at 95% CI: 1.0–2.9 when the allele frequency data were analysed).

Comparison of the results of independent studies analysing the role of the TNFA -308 G>A SNP in various ethnic populations

Next, we stratified the CRS group according to the presence or absence of NP (Fig. 1 and Supplementary Table 2, available at International Immunology Online). We did not detect a statistically significant difference as concerns the observed genotype and allele frequencies in either the CRSNP- (n = 49) or the CRSNP+ (n = 326) cohort relative to the controls (n = 169) (Pearson chi-square test 2x2 table, P = 0.54). Similar results were obtained by comparing the allele frequencies in the groups of controls and patients.

Finally, we stratified the CRSNP+ group to cohorts of patients on the basis of their ASA tolerance (CRSNP-ASA+, n = 266; and CRSNP+ASA+, n = 60) (Fig. 1). The analysis revealed statistically significant differences in the distributions of various genotype and allele frequencies between the CRSNP-ASA+ and control groups (Pearson chi-square test 2x2 table, P = 0.03 and P = 0.04, respectively) (Table 3). Thus, our data indicated that the rare A allele was positively associated exclusively with the observed genotype and allele frequencies in either the CRSNP- (n = 49) or the CRSNP+ (n = 326) cohort relative to the controls (n = 169) (Pearson chi-square test 2x2 table, P = 0.45 and P = 0.39, respectively).

Table 1. Demographic data of the study population

<table>
<thead>
<tr>
<th>Demographic parameter</th>
<th>Controls (n = 169)</th>
<th>CRS (n = 375)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>43.1 (18–69)</td>
<td>48.8 (18–78)</td>
</tr>
<tr>
<td>$\chi^2$ (P-value)*</td>
<td>P &lt; 0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>84 (49.7)/85 (50.3)</td>
<td>204 (54.4)/171 (45.6)</td>
</tr>
<tr>
<td>$\chi^2$ (P-value)*</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>ASA sensitivity</td>
<td>0 (0)</td>
<td>60 (16.0)</td>
</tr>
<tr>
<td>$\chi^2$ (P-value)*</td>
<td>P &lt; 0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>HWE* for the TNFA -308 G&gt;A SNP</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s., statistical comparison was not significant (P > 0.05). *Chi-square analysis with the Pearson correction. *Deviation from the Hardy–Weinberg equilibrium.

Table 2. Stratification of the CRS patient group

<table>
<thead>
<tr>
<th></th>
<th>CRSNP- (n = 49)</th>
<th>CRSNP+ (n = 326)</th>
<th>CRSNP+ASA- (n = 266)</th>
<th>CRSNP+ASA+ (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>48.2 (22–78)</td>
<td>49.1 (18–78)</td>
<td>49.0 (18–77)</td>
<td>46.7 (23–77)</td>
</tr>
<tr>
<td>$\chi^2$ (P-value)*</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>27 (55.1)/22 (44.9)</td>
<td>182 (55.8)/144 (44.1)</td>
<td>158 (59.4)/108 (40.6)</td>
<td>24 (40.0)/36 (60.0)</td>
</tr>
<tr>
<td>$\chi^2$ (P-value)*</td>
<td>n.s.</td>
<td>n.s.</td>
<td>P &lt; 0.05</td>
<td>24 (40.0)/36 (60.0)</td>
</tr>
<tr>
<td>ASA sensitivity</td>
<td>0 (0)</td>
<td>60 (18.4)</td>
<td>0 (0)</td>
<td>60 (100)</td>
</tr>
<tr>
<td>$\chi^2$ (P-value)*</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

CRSNP+−−, chronic rhinosinusitis occurring with (+) or without (−) nasal polyposis, respectively; CRSNP+ASA−/ASA+, chronic rhinosinusitis occurring with nasal polyposis and with (+) or without (−) aspirin sensitivity; n.s., the result of the statistical comparison is not significant (P > 0.05). *Chi-square analysis with the Pearson correction.
when the distributions of various genotypes and alleles were compared in the control and the overall CRS patient groups, nor when we performed the analysis on the control and CRSNP⁺ groups.

We also assessed the frequencies of ASA⁺ patients within the CRSNP⁺ cohorts in the previous studies. In the Canadian and one of the Turkish studies, this group was relatively numerous (38.8 and 34.0%, respectively), whereas in our case, the proportion of ASA⁺ patients (18.4%) was more similar to the data reported on the US Caucasians (16.4%) and the other cohort of Turkish patients (14.6%). This may reflect differences in the inclusion criteria applied to select the patient and control cohorts from the different populations.

We also noted that the frequencies of rare A allele-containing genotypes varied greatly in the controls of the different ethnic groups, which was in agreement with previous, similar case-control investigations. Moreover, one of the Turkish studies did not mention whether the studied cohort was in HWE (22), whereas the other Turkish study could not demonstrate HWE within their controls (24).

**Discussion**

CRS is a multifactorial disease of unknown etiology (28) in which yet undefined external and internal factors initiate innate and adaptive immune responses in the sinonasal cavities (1, 14, 16). During these processes, elevated expressions of various cytokines (e.g. TNFα and IL-1) play important roles (17, 18), e.g. in the initiation of local inflammation, a process of great importance during the clearance of attacking pathogens (29, 30). However, the extensive activation of these mechanisms...
Table 4. Comparative analysis of the previously published genetic studies investigating the association of the TNFA −308 G>A SNP and CRS

<table>
<thead>
<tr>
<th>Percent of GA+AA in controls</th>
<th>Percent of ASA− in CRSNP+ patients</th>
<th>Association detected</th>
<th>Controls in HWE</th>
<th>Analysed population</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.7%</td>
<td>14.6%</td>
<td>Yes</td>
<td>No</td>
<td>Turkish</td>
<td>Erbek (2007) (24)</td>
</tr>
<tr>
<td>11.5%</td>
<td>16.4%</td>
<td>Yes</td>
<td>Yes</td>
<td>US Caucasian</td>
<td>Bernstein (2009) (23)</td>
</tr>
<tr>
<td>—</td>
<td>38.8%a</td>
<td>No</td>
<td>Yes</td>
<td>Canadian</td>
<td>Mfuna-Endam (2010) (25)</td>
</tr>
<tr>
<td>6.7%</td>
<td>34.0%</td>
<td>Yes</td>
<td>No data</td>
<td>Turkish</td>
<td>Batikhan (2010) (22)</td>
</tr>
<tr>
<td>27.8%</td>
<td>18.4%</td>
<td>Yes</td>
<td>Yes</td>
<td>Hungarian</td>
<td>Present data</td>
</tr>
</tbody>
</table>

ASA−: aspirin sensitivity; CRSNP+: chronic rhinosinusitis occurring with nasal polyposis.

*aEstimated value based on the reported proportions of NP− and ASA− patients in the overall CRS cohort. Association was detected only between the rare A allele and the ASA− group of CRSNP+ patients.

can also result in the establishment of chronic inflammation and the subsequent development of NP.

Hereditary genetic factors appear to be important in the genetic predisposition to CRS (15, 16), and it was also proposed in the literature that polymorphisms that participate in the regulation and/or function of pro-inflammatory cytokines may be involved in the pathogenesis of the disease. In this respect, polymorphisms of the promoter region of the TNFA locus have already been analyzed in Turkish (22, 24), Canadian (25) and US Caucasian (23) individuals, with special attention to the −308 G>A SNP. The results of these investigations are conflicting, however, for reasons that are currently unknown.

In order to investigate whether the well-known and relatively frequent TNFA −308 G>A promoter polymorphism predisposes carrier individuals to the development of CRS in the Hungarian population, we performed a case–control study to compare the frequencies of various genotypes and alleles in control individuals and in CRS patients. The results of the previous studies combined with our data led us to categorize our patients into clinically more homogeneous subgroups. Detailed analysis of these indicated that the proportion of individuals carrying the rare A allele differed between the controls and the CRSNP+ASA− group (P = 0.03 in the comparison of the genotypes and P = 0.04 for the allele frequencies), and also comparing the CRSNP+ASA− versus the CRSNP+ASA+ groups (P = 0.03 in the comparison of the genotypes and P = 0.04 for the allele frequencies). These data suggest that the CRSNP+ASA+ subgroup is a genetically unique entity within the group of CRS patients, at least in the studied Hungarian population, in which the rare A allele of the studied TNFA −308 SNP can be regarded as a genetic susceptibility factor. This correlates well with the available clinical data, suggesting that this cohort of patients is a clinically distinct group that often demonstrates extreme resistance to therapy and in whom the incidence of relapses after surgical removal of the polypoid tissues is very high.

Next, we compared our results with the already published data, investigating the role of the TNFA −308 SNP in the genetic predisposition to CRS in different populations (22–25), but this turned out to be somewhat challenging. The reason, however, for the differences is not clear. Differences in study design and selection criteria utilized to recruit patients and controls can offer only a partial explanation. Another important factor to be considered is the ethnicity of the study populations. It has been noted earlier both by ourselves and by other researchers that the frequency of the rare A allele-containing genotypes varies greatly already in the general population in different ethnic groups (31, 32). Discrepancies can also be observed with regard to whether a given −308 A allele predisposes the carrier individuals to the same diseases in different ethnic populations (31, 32). Thus, comparison of all these data with our current results strongly argues for the need of multicentric, systematic clinical and/or genetic investigations performed according to consensus patients’ inclusion/exclusion criteria. Such investigations would help us to identify clear population-related differences, e.g. in the percentage of NP− or ASA− individuals among the CRS cohort or in the inherited genetic predisposing or protective factors.

The differences observed in various studies of CRS can formally also suggest that it is maybe not the A allele of the TNFA −308 G>A SNP itself that is responsible for the observed associations. Alternatively, an extended linkage group on the short arm of chromosome 6 around the TNFA locus may rather be responsible for the genetic predisposition to the investigated disease. This, together with the existence of population-specific linkage groups, could explain the observed population-related differences.

In our study, the gathered clinical and genetic data suggest that the overall group of CRS patients is indeed heterogeneous. Many differences have been described in the clinical characteristics of these patients (severity of inflammation, sensitivity to ASA), and severe cases often exhibit extreme treatment resistance and the recurrence of symptoms after the surgical resection of polypoid tissues. All these data suggest that not only the genetic predisposing factors but also the actual disease pathogenesis at the molecular level may differ in the various CRS subgroups.

Limitations of the current study arise from the moderate size of the cohort that was used for the analyses (169 controls and 375 CRS patients). We believe, however, that in the era of robust genome-wide association studies carefully designed classical case–control studies of such moderately sized study cohorts can still have their significance. Such targeted studies can investigate the relevance of the observed associations reported by large-scale analyses. Also, because of their relative ease and less cost-effective nature they can be used to detect population-related differences, as well as discover special cohorts within larger patient groups differ in terms of...
the exact disease pathogenesis. Information gathered from such studies can be used to develop more sophisticated patient inclusion/exclusion criterion systems in order to generate homogenous study groups for future high-throughput analyses.

Supplementary data

Supplementary data are available at International Immunology Online.

Funding

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