A conserved linkage group on chromosome 6, the 8.1 ancestral haplotype, is a predisposing factor of chronic rhinosinusitis associated with nasal polyposis in aspirin-sensitive Hungarians

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ABSTRACT

Inflammation plays a central role in the pathogenesis of chronic rhinosinusitis (CRS), and TNFα is a key pro-inflammatory cytokine in the pathogenesis of this disease. In our previous studies, we showed that the TNFA -308A allele is a genetic predisposition factor in a subgroup of aspirin-sensitive (ASA+) CRS patients suffering from nasal polyps (NP) in the Hungarian population.

To determine whether the TNF -308A allele or the presence of a complex, extended ancestral haplotype (8.1AH) located on chromosome 6 is responsible for the previously observed genetic effect, we performed a case-control study for examining the frequency of 8.1AH carriers in controls and in subgroups of CRS patients.

Our novel observations demonstrate that the presence of the 8.1AH may be responsible for the development of severe forms of CRS (CRSwNP, ASA+) and strengthen the clinical observation that CRS patients can be classified into clinically and genetically different subgroups.

1. INTRODUCTION

Chronic rhinosinusitis (CRS) is caused by the persistent inflammation of the nasal and paranasal mucosa, usually lasting for an extended period of time (over 12 weeks) despite various treatment attempts [1,2]. It is a clinically heterogeneous disease, and its major symptoms include nasal congestion or blockage, loss of smell, rhinorrhea, post-nasal drip and facial pain or pressure. CRS patients may or may not develop nasal polyps NPs (CRSwNP and CRSsNP, respectively), which are nongranulomatous inflammatory tissue extensions of the mucosal surface lining the sinonasal cavities [1,3]. Some patients within the CRSwNP group also exhibit a marked sensitivity to aspirin (acetylsalicylic acid) or nonsteroidal anti-inflammatory drugs (ASA+). These patients may experience the following when they consume aspirin: bronchiolar constriction, rhinorrhea and shock symptoms related to a non-IgE-mediated pharmacological hypersensitivity reaction [4,5,6].

The pathogenesis of CRS is complex, and several inherent and environmental factors have been implicated. Recent studies indicate that this disease is also polygenic [1,7,6].

Abnormal immune regulation and the resulting chronic inflammation are important factors in the etiology of the disease [8,9]. The pro-inflammatory cytokine tumor necrosis factor α (TNFα) has been shown to play a role in CRS pathogenesis. The presence of elevated levels of this protein was detected in various cellular components of polypoid tissues, including the epithelium, endothelium, fibroblasts, macrophages, lymphocytes and, most importantly, eosinophils [10,11]. These observations suggest that genetic polymorphisms affecting the regulation and/or function of this cytokine may play an important role in the genetic predisposition to CRS. One particular single nucleotide polymorphism (SNP), the TNFA -308 G/A, which is located in the regulatory region of the gene, has been studied in various ethnic groups affected with CRS; however, the results obtained to date are contradictory [12,13,14,7,15]. To clarify the role of this SNP in the genetic predisposition to CRS in the Hungarian population, we previously conducted a case-control study to systematically analyze the distribution of various genotypes in controls and in clinically well-defined groups within the CRS cohort. We found an association only between the rare TNFA -308A allele and the ASA+ group of CRSwNP patients, suggesting that this allele is associated with the development of the most severe forms (CRSwNP, ASA+) of this disease [15].

It has been suggested that the TNFA -308 G/A SNP may affect the regulation of the *TNFA* gene [16,17]. Other results indicate that the rare A allele might also be part of an extended linkage group on the short arm of chromosome 6, called the 8.1 ancestral haplogroup

(8.1AH). This multigene haplotype is ~4.7 million nucleotides in length and includes a majority of the human major histocompatibility complex (MHC) genes, as well as non-MHC genes, altogether over 250 loci. The exact function of the 8.1AH is currently unknown, but this particular combination of alleles has been suggested to predispose the development of a number of chronic inflammatory and autoimmune diseases [18].

In the current work, we examined whether the previously observed association between the TNFA -308A allele and the ASA+ group of CRSwNP patients is due to the presence of this particular allele or due the presence of the complete 8.1AH. In a case-control study the distribution of three additional marker SNPs (advanced glycosylation end product-specific receptor, AGER -429T/C; heat shock protein 1B, HSP70-2 -1267A/G; lymphotoxin alpha LTA +252A/G) was analyzed using our genetic collection. These data were combined with the previously published TNFA -308G/A SNP results for the same study group [15]. Based on previous analysis of this chromosomal region in the Hungarian population, the 8.1AH was confirmed when at least one rare allele is present for all four SNPs [19].

Our results indicate that it is most likely the presence of the 8.1AH, and thus the combination of multiple alleles, that is responsible for the development of severe forms of CRS.

2. MATERIALS AND METHODS

2.1 Study population and ethics

A total of 544 Caucasian individuals (375 patients and 169 controls) were enrolled to the study from various otorhinolaryngology centers in Hungary (the University of Szeged; Semmelweis University, Budapest; University of Pécs; Borsod-Abaúj-Zemplén County Hospital and University Teaching Hospital, Miskolc; and St. John's and North-Buda Hospitals, Budapest). The age range of recruited individuals was 18–72 years. Patients were selected after trained medical professionals had established the diagnosis of CRS based on the recent European guidelines [1]: besides in addition to the available clinical history and symptomatology, nasal endoscopy and paranasal sinus CT scans were performed all patients to identify the presence of nasal polyps. Aspirin intolerance was diagnosed determined based on the available medical history. Exclusion criteria included the presence or 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 was comprised of individuals who had no medical history of CRS and who did not exhibit any of the exclusion criteria. Demographical data of the study group are summarized in Supplementary Table 1.

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.

2.2 Polymorphism analyses

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

Patient and control samples were genotyped for the chosen SNP markers using restriction fragment length polymorphism analysis (PCR-RFLP) of PCR-amplified fragments. The selected marker SNPs (AGER -429T/C, HSP70-2 -1267A/G, LTA +252A/G) together with the previously reported TNFA -308 G/A [15] were successfully analyzed for 157 control and 353 patient samples. The applied primers and the reaction conditions are summarized in Supplementary Table 2. After digestion with the relevant enzyme (Fermentas, Vilnius, Lithuania), electrophoresis of the digested PCR products was performed on a 2% agarose gel (Lonza, Switzerland). To visualize the DNA fragments, gels were stained with GelRed (Biotium, Inc., Hayward, USA). TNFA -308 G/A genotypes of the same study group have been published previously [15].

2.3 Statistical analysis

Statistical analysis was carried out on the data from various groups of patients and controls according to the rules of case–control allelic association study design. First, genotype and allele frequencies for the AGER -429T/C (rs1800625), HSP70-2 -1267A/G (rs1061581) and LTA +252A/G (rs909253) SNPs 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 three most common genetic models (dominant, co-dominant, recessive) were evaluated for all the studied SNPs. Based on the obtained results, we decided that the dominant genotype model was the best (smallest p values) for the mode of inheritance in these cases, where the group of rare-allele-carriers (homozygotes and heterozygotes together) was compared to the group of non-carrier homozygotes using a χ^2 test (2x2 table). To avoid systematic false positive (type I) statistical errors caused by the large number of comparisons we performed in the dataset, the conservative Bonferroni correction was also applied.

The control group was in Hardy-Weinberg equilibrium for all studied SNPs.

8.1AH carriers, a total of 510 samples, were identified individually when genotypes were successfully identified for all four SNPs by counting the simultaneous presence of the rare TNFA -308A, AGER -429C, HSP70-2 -1267G, LTA +252G (A/C/G/G) alleles in heterozygotic or homozygotic form at each position. The number of 8.1AH carriers and non-carriers in both the control and the patient groups was analyzed using the χ^2test (2x2 table).

Statistical calculations were performed using the SPSS software (Version 17, SPSS, Chicago, USA), whereas power calculations were performed using the 'R' software package (http://www.R-project.org).

3. RESULTS

3.1 Demographic and clinical analysis

The demographic and clinical characteristics of the study participants are presented in Supplementary Table 1.

3.2 Genetic analysis of the AGER -429T/C (rs1800625), HSP70-2 -1267A/G (rs1061581) and LTA +252A/G (rs909253) SNPs in the unstratified and stratified groups of CRS patients

Initially, we compared the distribution of various genotypes for the studied AGER -429T/C, HSP70-2 -1267A/G and LTA +252A/G SNPs between the controls and the unstratified group of CRS patients, but no statistically significant differences were detected (Supplementary table 3).

Subsequently, we stratified the CRS group according to the presence or absence of NPs (CRSwNP and CRSsNP groups). We did not detect a statistically significant difference with respect to the observed genotype and allele frequencies for the analyzed SNPs when comparing these two groups to the control cohort (Supplementary table 4).

Finally, we stratified the CRSwNP group to cohorts of patients based on their ASA sensitivity (CRSwNP, ASA– and CRSwNP, ASA+). The statistical analysis revealed differences in the distributions of various genotype and allele frequencies between the CRSwNP, ASA+ and control groups in case of the AGER -429T/C and the LTA +252A/G SNPs, but only the latter remained significant after the Bonferroni correction. No significant differences were detected for the Hsp-2 -1267A/G polymorphism (Table 1).

3.3 Haplotype analysis

We generated haplotypes using the presented data and combined them with the previously published TNFA -308 G/A SNP analysis results [15]. Carriers of the 8.1AH were identified as individuals simultaneously carrying rare alleles for all four studied SNPs either in heterozygotic or homozygotic form. Next, we established three categories within the control and patient cohorts: carriers of the TNFA -308 A allele (either in heterozygotic or in a homozygotic form) that was not in the context of the 8.1AH; carriers of the rare TNFA -308 A allele that was in the context of the 8.1AH; and individuals who did not carry either the TNFA -308 A allele or the 8.1AH. We performed a statistical analysis and compared the distribution of individuals within these categories. Our results indicated statistically significant associations comparing the control *vs.* the CRS and the control *vs.* the CRSwNP, ASA+ groups. Borderline significances were noted between the control *vs.* the CRSsNP and the control *vs.* the CRSwNP, ASA– groups, but these did not remain significant after Bonferroni correction (Table 2). Power calculations determined that the study population was sufficiently large, and the applied test was suitable to accurately and reliably detect the reported associations between the control and the CRSwNP, ASA+ patient group (power of detection: 0.99).

3.4 Genetic analysis of the AGER -429T/C, HSP70-2 -1267A/G, LTA +252A/G and TNF -308G/A SNPs in the non-8.1AH carrier cohort of controls and patients

To determine whether any alleles of the four SNPs have an effect on the genetic predisposition to CRS by themselves, we repeated all the previously described polymorphism analyses including only the 8.1AH non-carriers in each group, to disregard the combined effect of this large linked chromosomal segment. None of the comparisons in this analysis revealed any statistically significant differences (Supplementary Tables 5, 6 and 7).

4. DISCUSSION

CRS is a multifactorial chronic inflammatory disease of the sinonasal cavities. Both genetic and environmental factors contribute to the pathogenesis of the disease. It is clear that incorrectly regulated innate and adaptive immune events play a major role in the development of CRS and that, during these events, the expression of various pro-inflammatory cytokines (TNFα and IL-1α) become elevated [10,20]. Overactivation of these factors can cause massive local inflammation and, while these processes are known to clear various microbial pathogens [21,22], they can be deleterious and ultimately lead to the development of NPs.

These conclusions suggest that genetic factors affecting the regulation and/or function of these pro-inflammatory cytokines may increase the risk of developing various forms of CRS. The results from genetic studies investigating a well-known SNP (-308G/A) located in the promoter region of the *TNFA* locus in Turkish [12,14], Canadian [7], US Caucasian [13] and Hungarian individuals [15] are conflicting. However, our earlier analysis and comparison of the available data seem to provide at least two explanations. On one hand, differences in study design and the selection criteria for recruiting patients and controls may account for the observed differences. On the other hand, differences in the linkage groups associated with different TNFA alleles in various populations may also lead to the noted discrepancies [15].

We aimed to find an explanation for these observed population-related differences and to discriminate whether the rare TNFA -308A allele itself or a well-known linkage group containing this allele, the 8.1 AH, was responsible for the previously observed genetic associations in the CRSwNP, ASA+ group of Hungarians.

Initially, three additional SNP markers in the vicinity of the TNFA -308G/A SNP (AGER -429T/C, HSP70-2 -1267A/G and LTA +252A/G) were analyzed in a case-control study. We examined the genetic associations between the different alleles of these SNPs and the various CRS subgroups. The results of this analysis suggested statistically significant differences in

the distributions of various genotype and allele frequencies between the CRSwNP, ASA+ and control groups for the AGER -429T/C and LTA +252A/G SNPs, although only the latter remained statistically significant after Bonferroni correction.

Next, we combined these data with the previously published TNFA -308 G>A SNP analysis results obtained with the same individuals. The analysis of the 8.1AH was chosen after a careful preliminary literature search. In addition to containing the TNFA -308A allele, the 8.1 AH is the most frequent ancestral haplotype among Caucasians [18]. It is also relatively frequent in the Hungarian population (8–10%, [23]), but its frequency differs among various Caucasian ethnic groups [23]. Based on this information, the 8.1AH appeared to be a good candidate for the explanation of the previously observed genetic differences. Thus, we determined three genotype groups: *i.* carriers of the TNFA -308 A allele (at least in a heterozygote form) that was not in the context of the 8.1AH; *ii.* carriers of the rare A allele that was in the context of the 8.1AH; *iii.* individuals who did not carry either the TNFA -308 A allele or the 8.1AH. The frequency of these alleles was compared in controls and in the various CRS groups. Statistically significant differences were detected when comparing the total CRS, as well as the CRSwNP, ASA+ patients groups to controls. Further analysis of various subgroups within the CRS cohort that are clinically more homogenous suggested that the severity of the disease represented by the various CRS subgroups (CRSsNP > CRSwNP > CRSwNP, ASA+) corresponds to 8.1AH frequency (Table 2).

Finally, we wanted to determine whether any of the studied SNPs act as genetic predisposing or protective factors in the development of CRS. The effect of the 8.1AH was eliminated by repeating all comparisons using only controls and patients not exhibiting 8.1AH (non-8.1AH). No statistically significant results were obtained. Overall, these results strongly imply that the 8.1AH, consisting of a specific combination of alleles located in both MHC and non-MHC genes, is a predisposing factor for the development of the most severe forms of CRS (CRSwNP, ASA–).

The effect of the TNFA -308G>A SNP has been thoroughly investigated in the pathogenesis of a large variety of diseases. The results, however, are often contradictory when different populations are studied. Our results combined with published data clearly suggest that care must be taken when polymorphisms located in the short arm of chromosome 6 are being analyzed. Because large, extended ancestral haplogroups occur frequently, their effect on the pathogenesis of immune-related disorders should be taken into account.

It is not clear what evolutionary benefits resulted in the high frequency of the 8.1AH in various Caucasian populations. In the modern era, however, it seems that the 8.1AH may predispose carriers to more severe reaction to environmental factors, some of which are not yet defined, but causing the development of chronic inflammatory diseases.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

[1] Fokkens, W. J., Lund, V. J., Mullol, J., Bachert, C., Alobid, I.et.al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012, Rhinol. Suppl (2012) 3-298.

[2] Wang, X. and Cutting, G. R. Chronic rhinosinusitis, Adv. Otorhinolaryngol. 70 (2011) 114-121.

[3] Huvenne, W., van, B. N., Zhang, N., van, Z. T., Patou, J.et.al. Chronic rhinosinusitis with and without nasal polyps: what is the difference?, Curr. Allergy Asthma Rep. 9 (2009) 213-220.

[4] Gosepath, J. and Mann, W. J. Current concepts in therapy of chronic rhinosinusitis and nasal polyposis, ORL J. Otorhinolaryngol. Relat Spec. 67 (2005) 125-136.

[5] Klimek, L. and Pfaar, O. Aspirin intolerance: does desensitization alter the course of the disease?, Immunol. Allergy Clin. North Am. 29 (2009) 669-675.

[6] Szczeklik, A., Gryglewski, R. J.Czerniawska-Mysik, G. Relationship of inhibition of prostaglandin biosynthesis by analgesics to asthma attacks in aspirin-sensitive patients, Br. Med. J. 1 (1975) 67-69.

[7] Mfuna, E. L., Cormier, C., Bosse, Y., Filali-Mouhim, A.Desrosiers, M. Association of IL1A, IL1B, and TNF gene polymorphisms with chronic rhinosinusitis with and without nasal polyposis: A replication study, Arch. Otolaryngol. Head Neck Surg. 136 (2010) 187-192.

[8] Ooi, E. H., Psaltis, A. J., Witterick, I. J.Wormald, P. J. Innate immunity, Otolaryngol. Clin. North Am. 43 (2010) 473-87, vii.

[9] Tieu, D. D., Kern, R. C.Schleimer, R. P. Alterations in epithelial barrier function and host defense responses in chronic rhinosinusitis, J. Allergy Clin.Immunol. 124 (2009) 37-42.

[10] Bernstein, J. M. The molecular biology of nasal polyposis, Curr. Allergy Asthma Rep. 1 (2001) 262-267.

[11] Lennard, C. M., Mann, E. A., Sun, L. L., Chang, A. S.Bolger, W. E. Interleukin-1 beta, interleukin-5, interleukin-6, interleukin-8, and tumor necrosis factor-alpha in chronic sinusitis: response to systemic corticosteroids, Am. J. Rhinol. 14 (2000) 367-373.

[12] Batikhan, H., Gokcan, M. K., Beder, E., Akar, N., Ozturk, A.et.al. Association of the tumor necrosis factor-alpha -308 G/A polymorphism with nasal polyposis, Eur. Arch. Otorhinolaryngol. 267 (2010) 903-908.

[13] Bernstein, J. M., Anon, J. B., Rontal, M., Conroy, J., Wang, C.et.al. Genetic polymorphisms in chronic hyperplastic sinusitis with nasal polyposis, Laryngoscope 119 (2009) 1258-1264.

[14] Erbek, S. S., Yurtcu, E., Erbek, S., Atac, F. B., Sahin, F. I.et.al. Proinflammatory cytokine single nucleotide polymorphisms in nasal polyposis, Arch. Otolaryngol. Head Neck Surg. 133 (2007) 705-709.

[15] Szabo, K., Kiricsi, A., Revesz, M., Vona, I., Szabo, Z.et.al. 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, Int. Immunol. 25 (2013) 383-388.

[16] Kroeger, K. M., Carville, K. S.Abraham, L. J. The -308 tumor necrosis factor-alpha promoter polymorphism effects transcription, Mol. Immunol. 34 (1997) 391-399.

[17] Wilson, A. G., Symons, J. A., McDowell, T. L., McDevitt, H. O.Duff, G. W. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation, Proc. Natl. Acad. Sci. U.S.A 94 (1997) 3195-3199.

[18] Price, P., Witt, C., Allcock, R., Sayer, D., Garlepp, M.et.al. The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases, Immunol. Rev. 167 (1999) 257-274.

[19] Laki, J., Laki, I., Nemeth, K., Ujhelyi, R., Bede, O.et.al. The 8.1 ancestral MHC haplotype is associated with delayed onset of colonization in cystic fibrosis, Int. Immunol. 18 (2006) 1585-1590.

[20] Lennard, C. M., Mann, E. A., Sun, L. L., Chang, A. S.Bolger, W. E. Interleukin-1 beta, interleukin-5, interleukin-6, interleukin-8, and tumor necrosis factor-alpha in chronic sinusitis: response to systemic corticosteroids, Am. J. Rhinol. 14 (2000) 367-373.

[21] Kato, A. and Schleimer, R. P. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity, Curr. Opin. Immunol. 19 (2007) 711-720.

[22] Takeuchi, O. and Akira, S. Pattern recognition receptors and inflammation, Cell 140 (2010) 805-820.

[23] Toth, E. K., Kocsis, J., Madaras, B., Biro, A., Pocsai, Z.et.al. The 8.1 ancestral MHC haplotype is strongly associated with colorectal cancer risk, Int. J. Cancer 121 (2007) 1744-1748.