Association of the tumor necrosis factor -308 A/G promoter polymorphism with Tourette syndrome

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Summary

Several lines of evidence suggest that certain subtypes of obsessive-compulsive and tic disorders might be pediatric manifestations of post-streptococcal autoimmunity caused by cross-reactive auto-antibodies. As tumor necrosis factor (TNF) is known to play a seminal role in coordinating the humoral immune response, TNF gene polymorphisms have been proposed as genetic risk factors both in obsessive-compulsive disorder (OCD) and Tourette syndrome (TS). The aim of present study was to investigate two TNF promoter polymorphisms (-238 A/G: rs361525 and -308 A/G: rs1800629) on the genetic susceptibility to OCD and TS in a child psychiatric sample (102 OCD and 117 TS patients). In the case-control setup the genotype and allele frequencies were compared to a control group from the general population (n=405). As a control child psychiatric sample, 194 children with attention deficit hyperactivity disorder were also genotyped. Our results revealed that the TNF -308 G-allele was more frequent in children with TS compared to controls (90.2% vs 84.8%, p=0.037). For confirmation of this genetic association a family based analysis, the Transmission Disequilibrium Test was used, which showed preferential transmission of the G-allele to TS patients (nominal p-value 0.011). Moreover, this allele was also transmitted more frequently to children with tic symptoms (nominal p-value 0.039). No association was found between OCD or obsessive, compulsive symptoms and the studied TNF polymorphisms. Based on these findings, the TNF -308 G-allele can be associated with Tourette syndrome, highlighting the potential pathophysiological role of TNF dysregulation.

Introduction

Obsessive-compulsive disorder (OCD) affects approximately 2% of the child and adolescent population (Boileau, 2011). It is characterized by intrusive thoughts (obsessions) that are expected to be relieved by repetitive acts (compulsions). The obsessions and compulsions are time consuming, and significantly interfere with the person's normal routine, occupational functioning, or social activities (American Psychiatric Association, 2013). In half of the cases the symptom onset is below age 19 (Kessler *et al*., 2005). Twin studies indicate the importance of separating childhood-onset OCD, because the observed genetic influence on OCD symptoms is much higher in children (ranging from 45% to 65%) compared to adults (where the heritability estimates are between 27-47%) (van Grootheest *et al*., 2005). Tourette syndrome (TS) is less frequent with a prevalence rate of 1% in school-age children. It is part of the tic-spectrum disorders, which affect 6-12% of children (Singer, 2005). TS is characterized by multiple motor tics and at least one phonic tic, and is rarely present without comorbid conditions, such as OCD and/or attention deficit hyperactivity disorder (ADHD) (Singer, 2005). As the involuntary movements and vocalizations in TS, the obsessions and compulsions in OCD, or the impulsivity in ADHD might reflect inhibitory control problems in the basal ganglia-thalamo-cortical circuits (as proposed by Casey *et al*., 2001), we aimed to study genetic risk factors in these three basal ganglia related neuropsychiatric disorders.

The term PANDAS (pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections) was coined by Swedo and co-workers (1998) to describe a subset of neuropsychiatric disorders that are suspected to be corollaries of streptococcal infections. This novel though debated neuropathological category encompasses some OCD and TS cases as well as other entities from the tic spectrum disorders, assigning a causative role to auto-reactive post-streptococcal antibodies in the pathogenesis (Martino *et al*., 2009). Infections by group A beta-hemolytic Streptococcus might cause immune dysregulation, resulting in the production of primarily anti-streptococcal anti-M antibodies that elicit various inflammatory diseases such as polyarthritis (acute rheumatic fever) aggravated by endocarditis. These antibodies can also cross-react with certain epitopes in the central nervous system including glycolytic enzymes such as pyruvate kinase, aldolase C, and neuron-specific enolase (Dale *et al*., 2006). Structural and functional impairment of the basal ganglia and corpus striatum by these auto-antibodies might manifest either in Sydenham chorea (chorea minor) or PANDAS. On the other hand, little is

known on the pathomechanism of neuronal dysfunctions, albeit there are data indicating that self-reactive antibodies detected in chorea minor (the cerebral manifestation of acute rheumatic fever) might induce the activation of calcium/calmodulin dependent protein kinase II (Kirvan *et al.*, 2003). Importantly, the tics - as involuntary movement symptoms - are present in the majority of PANDAS cases, indicating the impairment of basal ganglia in the pathogenesis.

Production of self-reactive antibodies is probably due to an imbalance of pro-inflammatory cytokines including interleukin 1 and tumor necrosis factor (TNF) that govern the function of B lymphocytes in the humoral response (Alleva *et al.*, 2000). TNF is released from activated monocytes, macrophages (microglia in the central nervous system), as well as T and B lymphocytes (Sarit *et al*., 2012) with profound biological activities including apoptosis induction, inflammation and insulin resistance. Furthermore, several studies found elevated plasma TNF and/or TNF receptor levels in OCD (Fontenelle *et al.*, 2012; Konuk *et al*., 2007), although there is a report on decreased or unchanged cytokine levels as well (Denys *et al*., 2004). No significant alterations in TNF plasma levels have been revealed in TS yet (Gabbay *et al.*, 2009).

The TNF gene (previously known as TNF-alpha, OMIM ID: 191160) is localized to the 6p21.33 locus in the neighborhood of several other TNF cluster genes. The gene spans about 3 kb and encompasses 4 exons. Several inducible transcription factors such as nuclear factor-κB and nuclear factor of activated T cells (NFAT) have been shown to be recruited to the TNF promoter conferring cell type-specific expression on the gene (Falvo *et al.*, 2010). Genetic polymorphisms in the regulatory regions of the TNF gene have been reported to associate with a wide range of inflammatory, immunological, and malignant diseases. Dozens of single nucleotide polymorphisms (SNPs) have been identified to date that might influence plasma TNF levels via modulating its transcription and thereby enhance the susceptibility to the above mentioned illnesses (Qidwai & Khan, 2011). Among them, 2 SNPs have been implicated most frequently in disease pathology: the -238 A/G (rs361525) and the -308 A/G (rs1800629) polymorphisms. The assumption that TNF polymorphisms might associate with pediatric neuropsychiatric disorders mentioned above (OCD, TS, and ADHD) has already been addressed by a number of studies (Hounie *et al.*, 2008; Liu *et al.*, 2011; Drtilkova *et al.*, 2008, respectively). As TNF seems to be a likely candidate gene in post-streptococcal pediatric disorders, we aimed to analyze the genotype distribution of these two important TNF promoter SNPs in children affected by OCD or TS. To assess the specificity of TNF polymorphisms on the genetic susceptibility of OCD and TS, we

also included patients with ADHD, one of the most common basal ganglia related disorders in childhood.

Materials and Methods

The study was designed in compliance with the Helsinki Declaration and was approved by the Hungarian Scientific and Research Ethics Committee of the Medical Research Council (ETT-TUKEB). The patients and their parents, as well as the healthy control subjects provided written informed consent to their participation. Both the clinical and the control samples were ethnically homogenous, of Caucasian origin. A total of 413 children were recruited at the Vadaskert Child and Adolescent Psychiatric Clinic with the major diagnoses of OCD, TS, or ADHD, according to DSM-IV criteria (for the description of the three patient groups see Table 1). The control group consisted of 405 healthy young adults (mean age: 21.26 ± 2.63 years, 51.1% male). Family data was accessible from 105 children (out of the 117 cases) diagnosed with TS. For the family based analyses of tic disorders 264 parents (from 114 trios and 41 duos) were available. The Yale Global Tic Severity Scale (YGTSS) was used to assess the peak tic severity (most severe condition of motor and vocal tic symptoms), and the degree of overall impairment. The three scales produce a total severity score that ranges from 0 to 100 (Leckman *et al.*, 1989).

Genomic DNA was isolated from buccal cells by the DNA purification kit obtained from Gentra (Minneapolis, USA). Genotyping of the -238 A/G (rs361525) and -308 A/G (rs1800629) SNPs was carried out using the C__2215707_10 and C__7514879_10 ABI TaqMan Genotyping Assays, respectively, on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions.

SPSS 17.0 for Windows (IBM Corporation, Armonk, NY, USA)
was used for the case-control and tic severity analyses. Deviation from the Hardy-Weinberg equilibrium was calculated by comparing expected and observed genotypes (chi-square analyses with df = 1). In the family based analysis the Transmission Disequilibrium Test (TDT) was used to calculate allele transmission from heterozygote parents using the formula TDT $\chi^2 = (b-c)^2/(b+c)$ where b is the number of times when heterozygous parents transmit the risk allele to an affected offspring and c is the number of times that they transmit the other allele (Spielman *et al.*, 1993). The adjusted significance level for multiple comparisons was calculated by the false

discovery rate (Benjamini *et al.*, 2001). The *in silico* transcription factor binding analysis was performed using the Alibaba 2.1 transcription factor binding prediction software (http://www.gene-regulation.com/) and the TRANSFAC database.

Results

The genotype and allele frequencies of the two TNF promoter polymorphisms in the patient and control groups are presented in Table 2. No significant deviation from the Hardy-Weinberg equilibrium was detected in either the patient or control groups. The ADHD group served as a control child psychiatric sample (with possible basal ganglia involvement). Neither of the TNF polymorphisms were associated with ADHD or OCD, only the TS group showed higher G-allele frequency at the -308 A/G SNP ($\chi^2 = 4.33$, df = 1, $p = 0.037$).

Since there was no AA homozygote at the -238 A/G (rs361525) SNP in the patient groups, the AA and AG genotypes were grouped together in the chi-square analyses. For the same reason, we could test only the recessive model at the risk calculation using the G-allele as the risk allele, and the AA + AG vs GG grouping system. Similarly, the odds ratio (OR) calculation at the -308 A/G (rs1800629) SNP was meaningful only in the AA + AG vs GG setting (see Table 2 for ORs). These genotype-wise analyses did not yield any significant result; we could observe only a tendency at the -308 A/G SNP in the TS group.

To confirm the possible genetic association between the -308 G-allele and TS, parents of TS patients were genotyped for the -308 A/G SNP and the Transmission Disequilibrium Test (TDT) was carried out. In the available 105 TS families the allele transmission could be unambiguously determined at 40 heterozygote parents: the A-allele was transmitted 12 times, whereas the G-allele was transmitted 28 times (TDT $\chi^2 = 6.4$, $p = 0.011$). The comorbid conditions, sex, and age of this reduced TS sample (n = 105, 87.6% male, mean age 12.17 ± 2.99, comorbid ADHD 37.1%, OCD 24.8%, anxiety 23.8%, CD 7.6%, LD 16.2%) did not differ significantly ($p > 0.1$) from the total TS sample (n = 117).

Next, we wanted to check if the same association would exist in a broader sense for tic disorders. Therefore, from our sample of 413 child psychiatric patients 186 children with tic symptoms - independently of the main diagnosis - were analyzed for the -308 A/G SNP (for description of this patient group see Table 1). With genotype frequencies of AA 1.1%, AG 19.9%, GG 79%,

and allele frequencies of A 11%, G 89% among the children with tic symptoms, the case-control analyses (allele-wise: $\chi^2 = 3.7$, df = 1, $p = 0.054$, and genotype-wise (AA + AG vs GG): $\chi^2 = 2.6$, df = 1, $p = 0.107$) showed similar tendencies. The family analysis showed preferential transmission of the -308 G-allele in the available 155 families of children with tic symptoms (34 times transmission vs 19 times non-transmission, TDT $\chi^2 = 4.25$, $p = 0.039$). Using the Yale Global Tic Severity Scale (YGTSS) scores, however, the GG genotype group did not have significantly more severe tic symptoms compared to the AG genotype group using either the specific or the overall tic severity scores in the analysis of variance (motor tic: AG 12.63 ± 4.74 vs GG 13.90 ± 4.98, $p = 0.235$; vocal tic: AG 9.96 ± 5.89 vs GG 10.07 ± 5.96, $p = 0.935$; impairment: AG 21.78 ± 8.90 vs GG 23.43 ± 11.13, $p = 0.478$; total score: AG 45.11 ± 17.39 vs GG 47.38 ± 20.46, $p = 0.599$).

Since the TS and tic symptom present groups were overlapping, we set the number of analyses to four in this sample (corresponding to the association analyses of -238 A/G SNP & TS, -308 A/G SNP & TS, -308 A/G SNP & tic symptom present group, and -308 A/G SNP & tic symptom severity scores) at the multiple testing correction with the false discovery rate. With the adjusted significance level at $\alpha = 0.05$, only the TS family based association result stayed significant ($p < 0.0125$). Taken together, these findings indicate a potential association of the TNF -308 G-allele with TS, but not necessarily with tic symptoms.

Finally, we performed an *in silico* transcription factor binding analysis using the Alibaba 2.1 transcription factor binding prediction software and the TRANSFAC database. This analysis revealed that the -308 polymorphic site and its 5' and 3' flanking regions contain numerous and partly overlapping Sp1 (specificity protein 1) binding sites, and the -308 G-allele - but not the -308 A-allele - allows binding of upstream stimulatory factors (USF) 1 and 2 to the promoter. USFs are known to elicit transcriptional activation via binding to E-box sequences in promoters; therefore, the presence of the G-allele can contribute to higher transcriptional activity.

Discussion

Research on the possible roles of certain inflammatory cytokines in the background of neuropsychiatric disorders has been a growing field of neuroscience. Several studies established associations between plasma cytokine levels and OCD (Fontenelle *et al*., 2012), autism spectrum disorder (Ricci *et al*., 2013), or depressive disorder (Raedler, 2011). Although little is known on

the penetration of plasma cytokines through the blood-brain-barrier, the presence of cytokine producing macrophages (i.e. microglial cells) in the central nervous system confers pathophysiological relevance on this issue (Sarit *et al*., 2012). Schizophrenia has been found to associate with many cytokines to such extent, that even anti-inflammatory medications have been proposed to combat or prevent this devastating mental illness (Mansur *et al*., 2012). Similarly, the mutual relationship between depression and plasma levels of inflammatory modulators has extensively been investigated (Fagundes *et al*., 2013; Cilan *et al*., 2012).

To address the possible involvement of autoimmune processes in TS and OCD, we analyzed TNF polymorphisms. The ADHD group served as a basal ganglia related but not PANDAS connected patient group. As expected, there was no association between TNF polymorphisms and ADHD. On the other hand, the positive association between TNF -308 A/G SNP and TS suggests a functional significance of TNF in this disorder, which – to our best knowledge – has not been reported yet. Although a similar study has been conducted in a Chinese Han population, no association was reported between the TNF -238 A/G polymorphism and TS (Liu *et al*., 2011), and – unfortunately – the -308 A/G SNP was not investigated.

Concerning the genetic analyses in OCD, our negative findings support the results of Zai *et al*., (2006), but contradict the findings of Lüleyap *et al*., (2012) and Hounie *et al*., (2008). The latter workgroup reported associations between OCD and both TNF -308 (rs1800629) A/G and -238 (rs361525) A/G SNP in a Brazilian population (Hounie *et al*., 2008). Association of the -238 A-allele with OCD has also been confirmed by Cappi *et al*., (2012) using an extended patient population (the number of OCD cases increased from 111 to 183), whereas the association between the -308 A-allele and OCD has not been verified. The -308 A-allele and AA genotype, however, was much more frequent among 45 OCD patients compared to 58 controls in a Turkish study (Lüleyap *et al*., 2012). One of the possible reasons behind the contradictory findings could be the different allele frequencies in the studied populations: the frequency of the minor -308 A-allele was much lower in the Turkish and Brazilian control groups (4.3% and 9%, respectively) compared to Caucasian populations (see the CEU panel with 17.3% (http://www.ncbi.nlm.nih.gov/snp/), the Hungarian control sample with 15.2% (Table 2), or the mainly Caucasian Canadian sample with 13.2% (Zai *et al*., 2006)).

The -308 A/G SNP (rs1800629) is considered a functional polymorphism as Wilson *et al*. (1997) revealed a DNase I hypersensitivity site at the -308 locus, however, the putative transcription

factor binding here has not been identified yet. We performed an *in silico* transcription factor binding analysis which showed that the -308 G-allele enables binding of upstream stimulatory factors 1 and 2 to the promoter. It is tempting to speculate that the -308 G-allele is able to recruit USF, resulting in higher TNF transcription activity and higher cytokine levels; therefore, it can be a risk factor for basal ganglia impairment.

Given that the -308 A/G SNP is highly associated with inflammatory diseases such as atopic dermatitis (Behniafard *et al*., 2012), Graves' disease (Kammoun-Krichen *et al*., 2008) and bronchial asthma (Witte *et al*., 2002), several attempts have been made in the past two decades to assign functional significance to this polymorphism. Results from numerous reporter vector based transient transfection assays revealed that the -308 A-allele confers higher transcriptional activity on the TNF promoter. Kroeger *et al*. (1997) found that the -308 A-allele (previously termed TNF2 allele) exhibited twofold transcriptional activation in phorbol ester activated Jurkat cells. Essentially identical results have been published by Wilson *et al*. (1997), and their findings have been corroborated by Sallakci and co-workers (2005), who performed an ELISPOT analysis to demonstrate that the A-allele produced higher TNF expression levels. Baseggio *et al*. (2004) found a protein complex associating only with the -308 A-allele in electrophoretic mobility shift assays but failed to identify it. However, there are equal amount of negative reports observing no differences in the transcriptional activity of these TNF alleles (Brinkman *et al*., 1995-1996; Uglialoro *et al*., 1998; Stuber *et al*., 1995-1996), therefore the functionality of the -308 A/G SNP remains an open question.

In conclusion, our results show no association between TNF promoter polymorphisms and OCD in a Caucasian population. However, we report an association of the TNF -308 A/G SNP (rs1800629) with Tourette syndrome. This finding might assign a specific neuromodulatory role to this seminal inflammatory cytokine in TS among basal ganglia based neuropsychiatric disorders. However, a major drawback of our study is that we did not measure anti-streptococcal antibody titers in patients that prevented us from discriminating between PANDAS and non-PANDAS samples in our TS population. Therefore, this study should be replicated accordingly.

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Table 1. Clinical description of the patient groups

	ADHD (n = 194)	**OCD (n = 102)**	**TS (n = 117)**	**patients with tic (n = 186)**
age	9.41 ± 2.69	15.12 ± 4.19	12.60 ± 3.83	12.46 ± 4.16
sex	88.1% male	69.6% male	88.9% male	86% male
secondary diagnoses				comorbidity
ADHD		14 (13.7%)	44 (37.6%)	89 (47.8%)
OCD	0		32 (27.4%)	60 (32.3%)
tic disorder	42 (21.6%)	28 (27.5%)		
anxiety	29 (14.9%)	47 (46.1%)	30 (25.6%)	53 (28.5%)
conduct disorder	63 (32.5%)	8 (7.8%)	12 (10.3%)	26 (14.0%)
learning disorder	54 (27.8%)	8 (7.8%)	18 (15.4%)	33 (17.7%)

Table 2. Genotype and allele frequencies of TNF polymorphisms in patient and control groups

polymorphism	**control**	**ADHD**	**OCD**	**TS**
-238 A/G (rs361525)				
AA	2 (0.5%)	0	0	0
AG	42 (10.4%)	19 (9.8%)	10 (9.8%)	13 (11.1%)
GG	361 (89.1%)	175 (90.2%)	92 (90.2%)	104 (88.9%)
genotype-wise chi-square *p* [a]		0.689	0.756	0.940
OR (95% CI) in the recessive model (AA + AG vs GG)		0.89 (0.51-1.57)	0.89 (0.43-1.84)	1.03 (0.53-1.98)
A	5.7%	4.9%	4.9%	5.6%
G	94.3%	95.1%	95.1%	94.4%
allele-wise chi-square *p*		0.576	0.664	0.943
-308 A/G (rs1800629)				
AA	13 (3.2%)	9 (4.6%)	3 (2.9%)	0
AG	97 (24.0%)	48 (24.7%)	28 (27.5%)	23 (19.7%)
GG	295 (72.8%)	137 (70.6%)	71 (69.6%)	94 (80.3%)
genotype-wise chi-square *p*		0.653	0.763 [b]	0.075 [b]
OR (95% CI) in the recessive model (AA + AG vs GG)		1.12 (0.76-1.63)	1.17 (0.73-1.88)	0.66 (0.40-1.09)
A	15.2%	17.0%	16.7%	9.8%
G	84.8%	83.0%	83.3%	90.2%
allele-wise chi-square *p*		0.417	0.601	**0.037**

[a] At the -238 A/G (rs361525) SNP the AA genotypes were grouped together with AG genotypes in order to have the expected count more than 5 in every cell in the chi-square test.
[b] One cell (17%) has the expected count less than 5 in the chi-square test.