Candidate-gene approach in fibromyalgia syndrome: association analysis of the genes encoding substance P receptor, dopamine transporter and α1-antitrypsin

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This work was supported by the Kahn Foundation and by a research grant on behalf of the Sacta-Rashi fund.

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Received on November 3, 2008; accepted in revised form on March 12, 2009.

Clin Exp Rheumatol 2009: 27 (Suppl. 56): S33-S38.

Key words: Fibromyalgia, genetics, substance P receptor, dopamin transporter, alpha-1 antitrypsin.

Abbreviations:
FMS: fibromyalgia syndrome
DAT: dopamine transporter
VNTR: variable number tandem repeat
PTSD: post traumatic stress disorder
AA: alpha1-antitrypsin
TACR1: substance P receptor
ADHD: attention deficit hyperactivity disorder

Competing interests: none declared.

ABSTRACT

Background. *Substance P receptor modulates stress, depression, anxiety and pain. Substance P is increased in CSF of fibromyalgia (FMS) patients. We examined the frequency of the substance P receptor (TACR1) 1354 G>C polymorphism in FMS.*

The dopamine transporter (DAT) SLC6A3 3' variable number tandem repeat (VNTR) polymorphism is associated with post traumatic stress disorder (PTSD), a condition with clinical and epidemiological overlap with FMS. We have evaluated the allele frequency of this polymorphism in FMS.

Alpha1-antitrypsin (AAT) deficiency is an autosomal recessive metabolic disease. The PI ZZ phenotype, encoded by the E342K mutation, is associated with emphysema and liver disease, and has been linked with FMS. We have examined the frequency of this mutation in FMS.

Methods. *Eighty-seven Jewish FMS patients participated; 45 of Ashkenazi origin, 32 of non-Ashkenazi origin and 10 of unknown or mixed Jewish origin. Controls consisted of 200 healthy Jewish individuals. Genotyping of the 1354G >C allele in the 3' UTR of TACR1 gene was performed by DdeI restriction analysis, genotyping the SCL6A3 DAT 3' VNTR polymorphism was performed by PCR combined with GeneScan analysis, and the AAT E342K mutation was identified by TaqI restriction analysis.*

Results. *No significant association was found between FMS and the three genetic markers studied here.*

Conclusions. *The current candidate-gene approach study failed to identify significant associations between FMS and three genetic markers with hypothesis-driven clinical relevance. We suggest that a genome-wide association study would be a more fruitful approach for further investigation of the genetic basis of FMS.*

Introduction

Fibromyalgia (FMS) is a common disorder of unknown etiology, which causes considerable morbidity and disability (1). Although not a progressive deforming joint disorder, FMS causes subjective suffering of a magnitude similar to serious inflammatory disorders such as rheumatoid arthritis and is a major cause for loss of work capability and under-performance on a professional and social level (2, 3).

The pathogenesis of FMS remains incompletely understood and while various inflammatory patterns have been identified (4), the condition is generally considered to represent a disorder of central nervous system pain processing (5), rather than an inflammatory or autoimmune disorder.

Currently, there is a paucity of specific treatment for FMS, although various medications including Norepinephrine, a Serotonin reuptake inhibitors (6), and alpha(2)-delta ligands (7) are gaining popularity, as are non–pharmacological approaches such as hydrotherapy, physical therapy etc. (8, 9).

Despite clear evidence of familial aggregation in FMS (10-12), little is known about the genetic basis underlying this syndrome. Sibship analysis has shown a significant genetic linkage of FMS to the HLA region (13), while a twin study demonstrated increased rates of functional disorders such as FMS and irritable bowel disease among monozygotic twin siblings of chronic fatigue patients (14). A handful of candidate genes have been analyzed as carrying possible association with FMS, based on various pathogenetic hypotheses. One polymorphism in the gene coding for a serotonin receptor has been linked with FMS (15, 16). More recent research has linked FMS to a polymorphism in the D4 dopamine receptor gene (17), while less conclusive evidence has been

evoked regarding the link between FMS and the catechol-O-methyltransferase gene polymorphism (18). Most recently, an increased frequency of the minor allele for beta-3 Adrenergic receptors has been demonstrated in individuals with FMS and related syndromes and was associated with pain intensity and measures of hyperalgesia (19).

Taken together, it appears obvious that genetic complexity governs this condition (20).

In the current study we have attempted to establish a possible link between three genetic markers and FMS pathogenesis, namely the tachykinin NK1 receptor (*TACR1*; substance P receptor) 1354G>C polymorphism, the Dopamine Transporter Gene (*DAT1*) polymorphic 40-nucleotide repeat in the 3'UTR area of exon 15 and the Alpha-1 Antitrypsin (*AAT*)^{342}glutamic acidlysine E342K mutation.

In conducting these investigations, analyses were performed on the entire study population as well as on the Ashkenazi Jewish population only, which is a relatively genetically homogeneous population advantageous for studying possible associations between genetic variations and complex diseases (21).

Materials and methods

Study population

The study population included unrelated 87 Jewish Israeli FMS patients (81 women, 6 men) treated at the department of rheumatology, Tel-Aviv Sourasky medical center. Patients were enrolled consecutively and were not selected for any clinical or familial features. The recruitment interval spanned two and a half years, from March 2003 to September 2005. All patients were examined on recruitment and fulfilled ACR criteria for FMS classification (22). All patients underwent a detailed interview to disclose ancestry (Ashkenazi or non-Ashkenazi), disease symptoms, rheumatologic or functional somatic co-morbidities, family history and treatment. Forty-five patients were of full Ashkenazi origin, 32 were non-Ashkenazi (predominantly Sephardic) origin and 10 of undetermined or mixed origins.

Control DNA samples included 200 randomly sampled from anonymous young healthy Jewish individuals, aged 20-45, mostly women (150 Ashkenazi and 50 non-Ashkenazi). The institutional and national supreme Helsinki committees for genetic studies approved the study and all participants gave written informed consent.

Genotyping and mutation detection

Genomic DNA was isolated from peripheral blood using standard protocols.

FMS patients were screened for *TACR1* mutations at the 3' UTR of the gene using the following primers: forward 5' TGACCTGCCTCCCTTCATGCATGG 3' and reverse 5' CTTCCGGGTCCGCAGCTGTGCTGC 3'.PCR reactions were performed using FastStart Taq Polymerase according to manufacturer recommendation (Roche diagnostics, Mannheim, Germany) using a Biometra PCR system (Biometra GmbH, Gottingen, Germany). DNA alterations were analyzed using a WAVE DHPLC apparatus (Transgenomic Inc., Omaha, NE), as described previously (45). Samples with variant heterochromatograms were sequenced using the BigDye Terminator Chemistry (Applied Biosystems, Foster City, CA) and analyzed using an automated ABI Prism 310 Genetic Analyzer (Applied Biosystems). Genotyping of the *TACR1* 1354G >C mutation was performed using *Dde*I restriction enzyme (New England Biolabs, Beverly, MA). The wildtype 321 bp allele was digested to 191 and 130 bp fragments. In the presence of this mutation, the 191 bp fragment was further digested to 99 and 92 bp fragments.

Genotyping of the *SCL6A3 DAT1* 40bp 3' VNTR polymorphism was performed by PCR amplification of a specific fragment from the 3' UTR of the gene using the following primers: forward 5' FAM-TGTGGTGTAGGGAACGGCCTGAG 3' and reverse 5' CTTCCTGGAGGTCACGGCTCAAGG 3'. The length of the amplified product was determined using ABI Prism 310 Genetic Analyzer and analyzed using GeneScan Analysis Software version 3.1.2 (Applied Biosystems).

Genotyping of the *AAT* E342K mutation was performed by PCR amplification of a 162 bp fragment using the following primers: forward 5' ATAAGGCTGTGCTGACCATCGTC 3' and reverse 5' TTGGGTGGGATTCACCACTTTTC 3' followed by TaqI restriction enzyme analysis (New England Biolabs). The wildtype allele was digested to 140 and 22 bp fragments. In the presence of this mutation the digestion is abolished to yield an uncut 162 bp fragment.

Statistical analysis

The odds ratio (OR) and confidence interval (CI) were calculated as an estimation of risk among mutation carriers. Results were analyzed using the EpiInfo 2000 (http://www.cdc.gov/epiinfo) or SPSS software V. 15 (SPSS Inc., Chicago, IL). To determine significant differences in the frequency of genetic variations between patients and controls Chi square test was performed with $p<0.05$ considered as statistically significant.

Results

Using DHPLC screening, a novel 1354G>C variation was detected in the 3' UTR of the *TACR1* gene (Fig. 1). The frequency of this novel germline mutation was determined in 87 FMS patients and 200 controls. Twenty-nine of the 87 FMS patients (33.3%) and 55/200 controls (27.5%) carried the 1354C variant, including two patients and four controls who were homozygous carriers (Table IA). The genotype distributions (Table IA) and allele frequencies (Table IB) of this variation did not differ significantly when comparing all FMS patients to controls ($p=0.57$ and $p=0.39$ respectively). Similar results were also obtained when comparing only the Ashkenazi patients and controls ($p=0.147$ and $p=0.117$, respectively), although the relative risk for FMS in carriers of the *TACR1* 1354C allele was higher [OR 1.72 (CI: 0.89-3.33), not significant].

DNA samples from all 87 FMS patients and 199 Ashkenazi and non-Ashkenazi controls were genotyped for the dopamine transporter *DAT1* 40bp VNTR. The frequencies of the different 40bp repeat genotypes are detailed in Table IIA. Of note, the frequency of the 10/10 repeat genotype was lower in FMS samples compared to controls

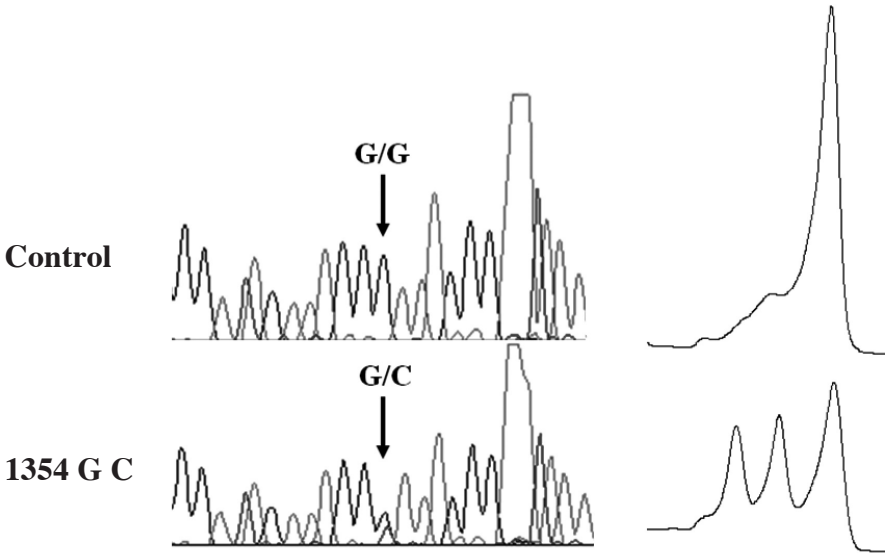

Fig. 1. The 1354G>C mutation in the 3' UTR of *TACR1* gene. Detection of the *TACR1* 1354G>C variant by DHPLC (right) a nd sequence (left) analyses. The arrow indicates the site of sequence alteration.

(23.0% and 31.7%, respectively), although not significantly (p=0.21). Table IIB presents the allele frequency of the most common alleles, the 9 and 10 repeats in Ashkenazi and non-Ashkenazi FMS patients and controls. No difference in the allele frequency of the 9 and 10 repeats was observed between the two groups (p=0.709).

Finally, none of the 87 FMS patients harbored the *Alpha-1 Antitrypsin* (*AAT*) E342K mutation.

Discussion

The current candidate-gene approach study failed to identify significant associations between FMS and three genetic markers with hypothesis - driven clinical relevance.

Our results did not demonstrate significant differences in frequency of the 1354G>C polymorphism in *TACR1* between patients and controls, nor were there significant differences in frequencies when only Ashkenazi patients and controls were analyzed. Similarly, no significant difference between the frequencies of the *DAT1* 40bp VNTR repeat genotypes was demonstrated.

Substance P, an 11 amino acid peptide neurokinin, carries diverse roles in the possible pathogenetic neurotransmitter in FMS. Several studies have confirmed the finding of elevated CSF levels of substance P in FMS (23-25). Nonetheless, substance P alterations cannot be definitively attributed an etiologic role and could represents an epiphenomenon. Several lines of evidence however make substance P an attractive candidate. In a murine model of dopamine-β hydroxylase gene knock-out and noradrenalin deficiency, Jasmin *et al.* have demonstrated that NK1 receptor mediates both hyperalgesia and morphine resistance (26). Restoring noradrenalin CNS levels normalised both the nociceptive threshold and morphine efficacy. A similar effect was also achieved by substance P antagonists, leading the investigators to conclude that when unopposed by noradrenalin, substance P acts on NK1 receptors to mediate chronic hyperalgesia. This insight is intriguing in the context of FMS, highly characterised by hyperalgesia, and particularly in view of the current role attributed to noradrenalin reuptake inhibitors in FMS treatment

Table I A. Frequencies of the *TACR1* 1354G>C genotype variations in Ashkenazi and non-Ashkenazi FMS patients and controls.

	Patients and origins				Controls and origins		
Genotype	Ashkenazi n=45*	Non- Ashkenazi n=32	Undetermined and mixed n=10	Total n=87	Ashkenazi n=150*	Non-Ashkenazi n=50	Total n=200
GG	28	22	8	58	115	30	145
GC	16	9	2	27	32	19	51
CC	1	1	–	2	3	1	4

n: number of individuals tested; *1354G/C genotype frequencies in Ashkenazi FMS patients *vs*. Ashkenazi controls, p=0.147.

B. Allele frequencies of the *TACR1* 1354G/C in Ashkenazi and non-Ashkenazi FMS patients and controls.

	Patients and origins				Controls and origins		
Alleles	Ashkenazi	Non- Ashkenazi	Undetermined and mixed	Total	Ashkenazi	Non-Ashkenazi	Total
G	72	53	18	143	262	79	341
C	18	11	2	31	38	21	59
total	90	64	20	174	300	100	400

1354G/C allele frequencies in Ashkenazi FMS patients *vs*. Ashkenazi controls: OR=1.72 (0.89 <OR> 3.33) p=0.117 (Yates corrected).

Table II. *DAT1* 40bp VNTR genotypes in FMS patients and controls.

A.

Number of repeats	Patients n=87 (%)	Controls n=199 (%)
9/9	9 (10.3)	29 (14.6)
9/10	52 (59.8)	101 (50.8)
10/10	20 (23.0)	63 (31.7)
9/11	2 (2.3)	1 (0.5)
10/11	3 (3.4)	2 (1.0)
6/10	1 (1.1)	–
7/10	–	1 (0.5)
8/10	–	1 (0.5)
8/9	–	1 (0.5)

B.

Total number and frequencies (brackets) of the most common *DAT1* 40bp alleles, the 9- and 10-repeat alleles, in 87 FMS patients and 199 controls.

Population	9 repeat allele	10 repeat allele
FMS patients	72 (0.41)	96 (0.55)
Controls	161 (0.40)	231 (0.58)

(6). Alterations in chronic nociception have been observed in another model based of NK1 receptor knock-out mice (27). Thus, the windup effect, whereby repetitive stimulation of un-myelinated C-fibers leads to an increased response to subsequent C-fiber input, has been found to be totally lacking in NK1 knockout mice. Although FMS definitely comprises more than a simple windup effect to external noxious stimulation, the possibility that alterations in the NK1 receptor gene may bear relevance for the generalised response to pain appears tantalising.

The tachykinin NK1 receptor (TACR1; substance P receptor) is widely distributed in many areas throughout the central and peripheral nervous systems, as well as in the gastrointestinal, genitourinary, cardiovascular and respiratory systems (28). The use of NK1 receptor antagonists has been regarded as holding hope for the treatment of diverse clinical conditions, including depression and anxiety (29), irritable bowel syndrome (30) and cystitis (31). Disappointingly, the use of a substance P antagonist has not proven effective in FMS, though this class of medications is yet considered to hold promise for this indication (32).

In view of this background, in the current study, we attempted to evaluate the possibility that alterations in the NK1 gene may be more common among FMS patients than among control individuals. As noted above, no statistically significant difference between patients and controls was observed, regarding the frequency of the novel 1354G>C polymorphism. Notably, this polymorphism is located in the 3' UTR area, which is a non–coding region of the gene. As a result, it is not straightforward to determine what effect a polymorphism at this location may have on the expression and /or function of the NK1 receptor. Nonetheless, it is well established that 5' and 3' untranslated regions contain sequence motifs crucial for many aspects of gene regulation and expression. Further research is necessary in order to clarify the possible role of such polymorphisms in disease pathogenesis.

Similarly to TACR1, DAT1 appeared to be a plausible candidate in the search for genetic markers in FMS due to its central role in regulation of dopamine reuptake. FMS has been linked with reduced presynaptic dopamine activity, as demonstrated on positron emission tomography (33) and dopaminergic agents have been used in the treatment of FMS (34). An association has been described between FMS and the dopamine D4 receptor exon III repeat polymorphism with a relationship to novelty seeking personality traits (35) as well as with altered dopamine D2 receptor function (36).

The Dopamine Transporter Gene (*DAT1*) is distributed in various sub-cortical areas of the central nervous system including the ventral mesencephalon, medial forebrain bundle, dorsal and ventral striatum and coincides with several regions of established dopaminergic innervation (37). Dopaminergic imbalance has been implicated in the pathogenesis of various conditions involving central nervous system dysfunction including Parkinson's disease, schizophrenia and attention deficit hyperactivity disorder (38). The *DAT1* protein is coded for by the *SLC6A3* locus (5p15.3) (39). The h*DAT1* gene spans over 64 kb, consisting of 15 exons (40). *DAT1* is the primary mechanism for dopamine reuptake from the synapse in midbrain dopaminergic neurons (41) and the target of psycho-stimulant drugs such as cocaine, amphetamines and methylphenidate. The most commonly studied genetic marker in the *DAT1* gene has been the polymorphic 40-nucleotide repeat in the 3'UTR area of exon 15 (42). Although not involved in amino acid coding, the 3' untranslated region of mRNA has been described as a molecular 'hotspot' for pathology (43) through regulatory effects on nuclear export, polyadenylation status, subcellular targeting and rates of translation and degradation of mRNA. A global survey of allele frequencies for this 40-bp polymorphism has been performed (44), and the 10-repeat allele was found to be most common, except for a Middle Eastern population in which the 9-repeat allele was most frequent.

DAT1 has been implicated in the pathogenesis of attention deficit hyperactivity disorder (ADHD) as well as in the response to treatment with methylphenidate, a DAT1 inhibitor (45). Striking, considerable evidence has linked the *DAT1* gene with Post Traumatic Stress Disorder (PTSD), a syndrome clinically and epidemiologically overlapping with FMS (46-48). In a study examining the possibility of an association between the *DAT1* 3' variable VNTR polymorphism with PTSD, a significant excess of the 9 repeat allele was observed among PTSD patients (49).

We thus chose to examine the frequency of the DAT1 VNTR polymorphism

among FMS patients. Although our random sample of individuals, describing themselves as healthy, were not specifically screened for the presence of FMS, our results do not support the association of any of the *DAT1* 40bp VNTR repeat genotypes or alleles with FMS. Thus, it remains to be studied whether additional genes related to dopaminergic activity will be found to be functional in chronic pain and FMS.

Alpha-1 Antitrypsin (AAT) is a 52kD glycoprotein serine proteinase inhibitor mainly produced by hepatocytes and to a lesser extent by pulmonary epithelial Cells (50). Functional AAT deficiency is responsible for chronic severe pulmonary emphysema and hepatic fibrosis (51). The Z variant of AAT (^{342}glutamic acidlysine, E342K mutation) is characterised in homozygotes by plasma AAT levels of 10% of the normal M allele (52). Following a remarkable case report describing dramatic improvement of FMS symptoms under replacement therapy for AAT deficiency (53), the hypothesis has been raised that AAT deficiency may be responsible for a subset of FMS cases (54). Accordingly, AAT deficiency was presumed to result in an imbalance between tissue inflammatory mediators and anti-inflammatory agents, leading to pain augmentation. Although this hypothesis differs from the more commonly held beliefs regarding the central, non-inflammatory nature of FMS pathogenesis, in view of the profound therapeutic potential of determining a linkage between these two conditions, we investigated the frequency of the *AAT* E342K mutation in FMS patients. Ashkenazi Jews are a relatively genetically homogeneous population who are considered to have derived from a cohort of about 10,000 individuals around the year 1500 (55). This genetic bottleneck, paired with reluctance to marry outside the boundaries of the community, created a population with relatively high genetic homogeneity, thus facilitating the identification of discrete variability which may be more difficult to discern in populations of greater heterogeneity. This approach has been successfully utilised in the past for the identification of genes associated with diverse clinical disorders such as breast cancer (56), colo-rectal cancer (57) and schizophrenia (58), and has previously been used by us for studying a novel founder mutation in the *RNASEL* gene in Ashkenazi prostate cancer patients (21, 59).

Conclusion

Taken together, the lack of significant associations in our study, conducted in a relatively homogeneous population, tends to emphasise the inherent limitations of the current candidate-gene based strategy in the study of FMS, as in other complex and presumably multifactorial conditions. As pointed out previously, previous research has met with some success in this endeavour; nonetheless on the whole our understanding regarding the genetic underpinnings of FMS, a disorder with a strong tendency towards familial aggregation (12), remains insufficient and findings tend to be inconclusive at best. Thus it would appear reasonable to state, that in order to progress further in this regard, new strategies are necessary. As in the case of other complex conditions, a genome-wide screening in FMS (60) would appear to offer the best prospect for achieving significant results which would further our understanding of the biological nature of FMS, as well as illuminating new candidates for more rational treatment in this protean and frustrating condition.

Authors' contributions

J.N.A., M.Y. and A.O.U. designed the study; J.N.A. collected the clinical data and blood samples; A.B.S. performed the DNA extraction and data analysis; J.N.A. wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank the patients for their participation in this study. The assistance of Dr. Helena Yagev-More is gratefully acknowledged.

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