

Excess of high activity monoamine oxidase A gene promoter alleles in female patients with panic disorder

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A genetic contribution to the pathogenesis of panic disorder has been demonstrated by clinical genetic studies. Molecular genetic studies have focused on candidate genes suggested by the molecular mechanisms implied in the action of drugs utilized for therapy or in challenge tests. One class of drugs effective in the treatment of panic disorder is represented by monoamine oxidase A inhibitors. Therefore, the monoamine oxidase A gene on chromosome X is a prime candidate gene. In the present study we investigated a novel repeat polymorphism in the promoter of the monoamine oxidase A gene for association with panic disorder in two independent samples (German sample, $n = 80$; Italian sample, $n = 129$). Two alleles (3 and 4 repeats) were most common and constituted >97% of the observed alleles. Functional characterization in a luciferase assay demonstrated that the longer alleles (3a, 4 and 5) were more active than allele 3. Among females of both the German and the Italian samples of panic disorder patients (combined, $n = 209$) the longer alleles (3a, 4 and 5) were significantly more frequent than among females of the corresponding control samples (combined, $n = 190$, $\chi^2 = 10.27$, $df = 1$, $P = 0.001$). Together with the observation that inhibition of monoamine oxidase A is clinically effective in the treatment of panic disorder these findings suggest that increased monoamine oxidase A activity is a risk factor for panic disorder in female patients.

INTRODUCTION

Panic disorder is an anxiety disorder characterized by panic attacks, anticipatory anxiety and phobic avoidance with a

life-time prevalence of 1–3% and a female:male ratio among affected of 2:1 (1,2). Co-morbidity with other psychiatric disorders, e.g. major depression, is common. Family and twin studies suggest a genetic contribution to the pathogenesis of panic disorder with an estimated heritability of 46% (3,4).

Segregation analyses failed to demonstrate a definitive Mendelian inheritance (5) and no major gene locus could unambiguously be identified by a genome-wide linkage study (6). This has led to the hypothesis that the pathogenesis of panic disorder is multifactorial and oligo- or polygenic, with each gene contributing only a small portion of the variance. Association studies, which are better suited to discover such susceptibility genes with minor effects, therefore appear to be the method of choice to search for panic disorder genes. They have provided, in addition to a series of negative results, the first positive results awaiting confirmation in independent samples (7,8).

Candidate genes for these association studies have been chosen on the basis of our knowledge of the molecular mechanism of drugs effective in the treatment of panic disorder or in provoking panic attacks, e.g. the fact that serotonin re-uptake inhibitors are effective drugs (9) led to the study of a functional serotonin transporter gene polymorphism (7,10) and the fact that the adenosine receptor antagonist caffeine induces panic attacks (11), to the study of adenosine receptor genes (8).

Monoamine oxidase A inhibitors, like other antidepressants, are effective treatments in panic disorder making the monoamine oxidase A gene one of the classical candidate genes (12). The monoamine oxidase A gene has been localized on chromosome Xp (13). Gene and promoter structure, as well as regulation of expression, have been extensively characterized (14–17). Several mutations and polymorphisms of the monoamine oxidase A gene have been previously described (13,18–20). The recent discovery of a new polymorphism in the promoter of the gene (21) led us to characterize it functionally using a luciferase reporter gene assay and to investigate it for association with panic disorder in two independent samples.

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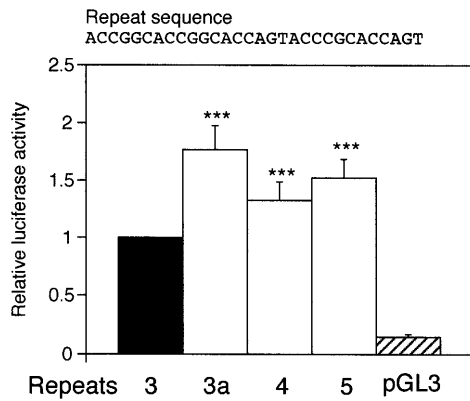


Figure 1. Transcriptional activity of monoamine oxidase A gene promoter repeat constructs transfected into human neuroblastoma SH-SY5Y cells. Data are mean (\pm SD) relative luciferase activities of four independent experiments in triplicate. Constructs with 3a, 4 and 5 repeats were significantly more active than constructs containing 3 repeats (***) $P < 0.0001$, one way ANOVA followed by Fisher's protected least significant difference test).

RESULTS

Four alleles (3, 3a, 4 and 5 30-bp repeats) of the monoamine oxidase A gene promoter polymorphism were observed in both samples. An additional rare fifth allele (2 30-bp repeats) was detected in the Italian sample. The 3a allele contained 3 repeats plus 18 bp (CCAGTACCGGCACCGGCA) of the repeated motif (Fig. 1). The most frequent allele in the control samples was the 4 allele with 61 (German sample) and 57% (Italian sample), respectively, followed by the 3 allele with 36 (German sample) and 40% (Italian sample). Both constituted 97% of all alleles observed in both samples (Table 1).

Functional characterization demonstrated a significantly higher activity ($P < 0.0001$, one way ANOVA followed by Fisher's protected least significant difference test) in a luciferase assay in human neuroblastoma SH-SY5Y cells of the constructs containing the longer 3a, 4 and 5 repeat alleles versus the construct containing the 3 repeat allele (Fig. 1). For a comparison between controls and patients, two allele groups were formed on the basis of the functional characterization. The short allele group(s) contained all alleles with 3 repeats (plus the 2 repeat allele in the Italian sample), the long allele group (l) contained all alleles with 3a, 4 and 5 repeats (Table 1).

Table 1. Allele and genotype frequencies of the monoamine oxidase A gene promoter polymorphism in patients with panic disorder versus controls

| Sample | | Alleles | | | | | Genotypes | | | | |
|------------------------------|---------|---------|-----|----|-----|---|------------|----------------------|--------------------------|---------------------------|--|
| | | 2 | 3 | 3a | 4 | 5 | 2+3 (s, %) | 3a+4+5 (l, %) | With 2 or 3 (s/s+s/l, %) | Only 3a, 4 and 5 (l/l, %) | |
| German | | | | | | | | | | | |
| Panic disorder ($n = 80$) | Males | 0 | 11 | 0 | 18 | 0 | 11 (38) | 18 (62), $P > 0.99$ | | | |
| | Females | 0 | 22 | 2 | 77 | 1 | 22 (22) | 80 (78) $P = 0.03$ | 17 (33) | 34 (67) $P = 0.07$ | |
| | Total | 0 | 33 | 2 | 95 | 1 | 33 (25) | 98 (75) $P = 0.06$ | 28 (35) | 52 (65) $P = 0.15$ | |
| Control ($n = 80$) | Males | 0 | 11 | 1 | 17 | 0 | 11 (38) | 18 (62) | | | |
| | Females | 0 | 36 | 0 | 63 | 3 | 36 (35) | 66 (65) | 26 (51) | 25 (49) | |
| | Total | 0 | 47 | 1 | 80 | 3 | 47 (36) | 84 (64) | 37 (46) | 43 (54) | |
| Italian | | | | | | | | | | | |
| Panic disorder ($n = 129$) | Males | 0 | 13 | 0 | 34 | 0 | 13 (28) | 34 (72) $P = 0.46$ | | | |
| | Females | 1 | 48 | 1 | 112 | 2 | 49 (30) | 115 (70) $P = 0.01$ | 41 (50) | 41 (50) $P = 0.05$ | |
| | Total | 1 | 61 | 1 | 146 | 2 | 62 (29) | 149 (71) $P = 0.01$ | 54 (42) | 75 (58) $P = 0.05$ | |
| Controls ($n = 110$) | Males | 1 | 13 | 0 | 25 | 1 | 14 (35) | 26 (65) | | | |
| | Females | 2 | 59 | 0 | 77 | 2 | 61 (44) | 79 (56) | 46 (66) | 24 (34) | |
| | Total | 3 | 72 | 0 | 102 | 3 | 75 (42) | 105 (58) | 60 (55) | 50 (45) | |
| Combined | | | | | | | | | | | |
| Panic disorder ($n = 209$) | Males | 0 | 24 | 0 | 52 | 0 | 24 (32) | 52 (68) $P = 0.55$ | | | |
| | Females | 1 | 70 | 3 | 189 | 3 | 71 (27) | 195 (73) $P = 0.001$ | 58 (44) | 75 (56) $P = 0.01$ | |
| | Total | 1 | 94 | 3 | 241 | 3 | 95 (28) | 247 (72) $P = 0.002$ | 82 (39) | 127 (61) $P = 0.02$ | |
| Controls ($n = 190$) | Males | 1 | 24 | 1 | 42 | 1 | 25 (36) | 44 (64) | | | |
| | Females | 2 | 95 | 0 | 140 | 5 | 97 (40) | 145 (60) | 72 (60) | 49 (40) | |
| | Total | 3 | 119 | 1 | 182 | 6 | 122 (39) | 189 (61) | 97 (51) | 93 (49) | |

Panic disorder and control samples were compared using χ^2 analysis. Groups (s and l) were formed on the basis of the functional analysis (Fig. 1).

Significantly more individuals had longer alleles (3a, 4 or 5) among female patients with panic disorder as compared with female controls in the German ($\chi^2 = 4.72$, $df = 1$, $P = 0.03$) as well as in the Italian ($\chi^2 = 6.13$, $df = 1$, $P = 0.01$) sample (Table 1). Similarly, genotypes with only long alleles showed a trend to be more frequent among female panics in the German sample ($\chi^2 = 3.26$, $df = 1$, $P = 0.07$) and were significantly more frequent among females in the Italian sample ($\chi^2 = 3.81$, $df = 1$, $P = 0.05$), when compared with their respective controls. Combining the two samples, significance increased considerably ($\chi^2 = 10.27$, $df = 1$, $P = 0.001$ for the allele distribution and $\chi^2 = 6.41$, $df = 1$, $P = 0.01$ for the genotype distribution).

No significant difference was observed between male panic patients and male controls in both samples. This is reflected in a decrease in the significance level when the complete samples are compared ($\chi^2 = 9.63$, $df = 1$, $P = 0.002$ for the allele distribution and $\chi^2 = 5.62$, $df = 1$, $P = 0.02$ for the genotype distribution). A partial explanation may be the fact that an increase in genotypes with only long alleles was observed among males versus females in the combined control sample ($\chi^2 = 9.52$, $df = 1$, $P = 0.002$), more so than in the combined panic sample ($\chi^2 = 3.40$, $df = 1$, $P = 0.07$). However, no significant difference was seen when the allele distribution was compared between males and females in the combined control sample ($\chi^2 = 0.33$, $df = 1$, $P = 0.56$) as well as in the combined panic sample ($\chi^2 = 0.70$, $df = 1$, $P = 0.40$).

DISCUSSION

The major finding of this study is the significant excess of functionally more active monoamine oxidase A gene promoter alleles in female patients with panic disorder of two independent samples. The difference between males and females can be tentatively explained in two ways. The male subsamples are rather small and the power to display significant difference of the magnitude found in females is very low (~14%). Thus, it is possible that an association may have been missed. Alternatively, gender differences in the genetic background of panic disorder cannot be excluded. A recent clinical trial using artificial neural networks indeed suggests a better response to moclobemide, a reversible monoamine oxidase A inhibitor, in women with prominent anxiety symptoms as compared with men (22).

While one advantage of association studies is their enhanced power to detect genes with small effects, their major disadvantage is the increased likelihood of false positive results (23,24). For example, a previously reported association between monoamine oxidase A gene polymorphisms with bipolar affective disorder (25) could not be replicated by several other groups (26,27). One way to reduce the probability of first order errors is to look for replication in an independent sample. The fact that the original finding in the German sample could be confirmed in the Italian sample renders it less likely that the observed association between long alleles of the monoamine oxidase A gene promoter polymorphism and panic disorder is a false positive, but does not exclude it. Therefore, verification in haplotype relative risk samples with family-based internal controls allowing for linkage disequilibrium tests is needed (28,29).

Monoamine oxidase A inhibitors are effective treatments in panic disorder (12). From a functional point of view, it therefore makes sense that an increased monoamine oxidase A activity, as shown for the longer alleles in the luciferase assay, contributes to the pathogenesis of panic disorder. Based on the present study

homozygosity for long alleles in females increases the relative risk versus heterozygosity as well as homozygosity for short alleles in females by factors of between 1.4 and 1.8 (relative risk 1.42, 95% CI = 1.06–1.89 and relative risk 1.77, 95% CI = 1.12–2.81, respectively) (30). This small, but significant effect is quite consistent with the concept of panic disorder as a multifactorial and oligo- or even polygenic disorder (6). Monoamine oxidase A gene variability may thus contribute to various types of psychopathology. Reduced monoamine oxidase A activity has been reported to be associated with impulsiveness and aggressiveness in mice and humans (20,31,32). With regard to panic disorder, future studies will have to address the question to which of its symptoms (panic attacks, anticipatory anxiety or phobic avoidance) increased monoamine oxidase A activity may contribute in particular. It also remains to be investigated if and how other monoamine oxidase A gene variations and polymorphisms interact with the described promoter polymorphism in modulating complex human behaviour and disorders.

MATERIALS AND METHODS

PCR fragments were amplified from genomic DNA using primers MAOAF_{or2} (5'-CCCAGGCTGCTCCAGAAAC) and MAOAR_{ev2} (5'-GGACCTGGGCAGTTGTGC) flanking the polymorphic region located ~1.1 kb upstream of the ATG initiation codon. PCR (40 s at 94°C, 40 s at 59°C, 60 s at 72°C for 35 cycles) was performed in a final volume of 25 µl containing 60 ng of genomic DNA, 10 pmol of each primer, 200 µM of each dNTP [plus 4 µM (R110)dUTP for the Italian sample], 1.5 mM MgCl₂, 75 mM Tris-HCl (pH 9.0 at 25°C), 20 mM (NH₄)₂SO₄, 0.01% Tween 20 and 0.5 U of Taq DNA polymerase (Gibco BRL, Eggenstein, Germany). The PCR products were separated by electrophoresis on a 3% NuSieve agarose gel and visualized by ethidium bromide staining (German sample) or by automated laser fluorescence capillary electrophoresis (Italian sample).

For construct formation, DNA fragments containing alleles of different lengths were PCR amplified using primers ma2 (5'-ATCTCGAGCTTTGGCTGACACGCTCCTG) and ma3 (5'-ATATACGCGTGAACAGCCTGACCGTGGAG). Resulting DNA fragments comprising the monoamine oxidase A gene regulatory region were digested with *Xho*I and *Mlu*I and ligated into pGL3-basic vector (Promega, Mannheim, Germany). All constructs were verified by cycle sequencing. Human neuroblastoma SH-SY5Y cells (2×10^5) were co-transfected with 2 µg of luciferase constructs and 0.5 µg of pRSV40-lacZ to control for transfection efficiency using FuGENE 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). Aliquots of 80 µl of cell extracts were incubated with luciferin reagent (Promega) to measure luciferase activity. In addition, 10 µl of the extracts were tested for β-galactosidase activity and luciferase activities were normalized to β-galactosidase activities using equal amounts of total protein. Four independent experiments in triplicate using different plasmid preparations were performed.

Two samples of patients with panic disorder were investigated in this study. Patients of one sample ($n = 80$) were of German origin and patients of a second sample ($n = 129$) of Italian origin, i.e. both their parents were German and Italian, respectively. Panic disorder was diagnosed as a lifetime condition according to DSM-III-R (1) by taking into account, first, medical records and, second, the structured clinical interview used in the respective centre [SADS-LA and CIDI in Germany and DIS-R in Italy

(33–35)]. The final diagnosis for the consensus diagnosis was assessed by an experienced psychiatrist prior to genotyping. Only patients with predominant panic disorder which occurred primarily in the course of the disorder were included. The German controls ($n = 80$) were unrelated, sex-matched blood donors, while the Italian control group ($n = 110$) included unrelated, unaffected individuals with negative family history for DSM-III-R axis I diagnosis disorders. All patients had given their informed consent.

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