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Title: Investigation of genetic predictors of the response to SSRI treatment in patients with panic disorder

Sponsor: Tartu University Hospital Psychiatry Clinic

The study sample comprised 74 adult patients with PD (mean age 33.2 ± 11.6 years; 67.6% of females). Participants were recruited at the psychiatry clinics of the Tartu University Hospital. The diagnosis of PD according to DSM-IV criteria was verified using the Mini International Neuropsychiatric Interview and substantiated by psychiatric history and medical records. Patients with current or past co-morbidity with mood disorders or with other anxiety disorders were allowed to take part in the study, providing PD was the primary diagnosis: however, other problems, such as schizophrenia or other psychotic disorders, severe suicide risk, substance abuse or dependence, organic mental disorder, severe personality disorder, serious unstable medical condition (i.e. endocrine, hepatic, respiratory, and cardiovascular diseases), were excluded. All participants were currently unmedicated at baseline, and did not receive any formal psychological help for at least 3 months before study entry and during the investigation. The Human Studies Ethics Committee of the University of Tartu approved the study protocol, and all participants provided written informed consent.

Treatment and clinical assessment
Patients were treated with 10–20 mg/day of escitalopram for 12 weeks using an open-label non-controlled study design. No other medications were permitted during the treatment period, except for hormonal contraceptives and zolpidem or zopiclone for insomnia and alprazolam for acute anxiety or panic symptoms during the first 6 weeks. At baseline, at two-week intervals, and at the end-point of the study (after the 12-week treatment course) the patients were assessed using the Panic Disorder Severity Scale (PDSS; score range 1⁄40), Clinical Global Impression scale (CGI), and the Hamilton Anxiety Scale (HAM-A; score range 1⁄4 0–42). Possible side-effects were evaluated every 2 weeks using the Toronto Side-effect Scale (TSES).

All patients started treatment with a dose of 10mg/day of escitalopram for the first 4 weeks. Patients who showed at least a 50% decline in the PDSS total score at week 4 continued taking 10mg of escitalopram until the end of the study; the daily dose of escitalopram was increased and maintained at 20mg in patients who demonstrated less than a 50% decrease in PDSS total score at week 4. During each visit, the patients were also asked to report on their regularity of taking the medication. Adherence to treatment was good and as reported by patients, none of them had missed medication for more than 3 days. At the end-point, symptom remission was defined as present, if a patient met all three of the following criteria: score on the CGI improvement scale of 2 or less; PDSS score 7 or less (a cut-off point used in several studies for defining remission); and no panic attacks for at least the past 2

weeks. Patients who did not meet these criteria were classified as non-remitters. According to these criteria, 57 patients were remitters and 17 were non-remitters. Clinical and demographic characteristics of these two groups did not differ significantly at baseline. The severity of the panic symptoms and treatment response were rated blind to the gene expression analysis.

Sample preparation and microarray hybridization

Venous blood was collected directly into PAXgene Blood RNA Tubes (Qiagen) at Baseline and at the end of escitalopram treatment at Week 12. Total RNA was extracted from 2.5 ml of whole blood using the PAXgene Blood RNA Kit (Qiagen, Venlo, The Netherlands). RNA concentration and quality were measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The absorbance ratio of A260/A280 was between 1.87 and 2.26 for all samples included for further analysis. The integrity of individual RNA samples was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) (Schroeder et al., 2006). The RIN (RNA integrity number) values of the RNA samples used for cRNA amplification ranged from 6.4 to 9.7, with only four samples with a RIN value below 7, indicating intact RNA samples. The Illumina® TotalPrep RNA Amplification Kit (Ambion Inc., Austin, TX) was used to amplify the RNA samples for hybridization on Illumina BeadChips. We used the Human-6 v2 and HumanHT-12 v3 BeadChips to determine the gene expression levels. Technical replicates were used to analyze the compatibility of the data from the two different versions of the BeadChips.

Data analysis

The GenomeStudio software (Illumina) was used to merge the data from the two different beadchip versions, resulting in 47,323 probes, which were present on both beadchip versions. Raw data were exported from GenomeStudio and further analyzed using R 3.1.1 software (www.r-project.org). After background correction with the Bioconductor package lumi (Du and Kibbe, 2008), data were quantile normalized and log2 transformed. In total, 20,725 autosomal probes mapping to 14,624 unique genes that had detectable expression signal (detection p-value < 0.05) in >10% of the samples were used in the analyses. Comparisons between remitters and non-remitters in week 0 and 12 were made using t-tests with empirical Bayes correction from the Bioconductor package limma (Wettenhall and Smyth, 2004) treating sample ID as random variable and adjusting for sex, age, sentrix barcode, sample section. We also estimated the interaction effect between time point and remitter status, The fold change was used to determine the direction of changes in average gene expression between the studied groups. Multiple testing correction was done using the Benjamini-Hochberg method to control false discovery rate (FDR) at level = 0.05. Given the sample size of our study that might affect our study power, for hypothesis generation we also considered genes if their expression level was increased or decreased 1.3-fold or more with a p-value ≤ 0.05. Systematic and integrative analyses of gene lists were undertaken using the DAVID bioinformatics resources (Huang et al., 2009). Gene Ontology analysis was performed with the following settings: GOTERM_BP_FAT, GOTERM_CC_FAT, GOTERM_MF_FAT, EASE score 0.05, and medium classification stringency settings. Functional annotation clusters with an enrichment score ≥ 1.3 was considered significant. In addition, KEGG and BIOCARTA pathways with EASE score ≤ 0.05 are shown in the Functional Annotation Tables. To further examine functionally related gene groups, we used the DAVID Gene Functional Classification tool with the similarity threshold set at 0.5.

Results

To identify possible biomarkers for response to escitalopram treatment in patients with PD, we performed differential expression analysis of 20,725 probes mapping to 14,624 unique genes between remitters (n = 57) and non-remitters (n = 17) at baseline and after 12 weeks of treatment with escitalopram.

At baseline we observed at least 1.3-fold difference in expression levels with nominal significance (unadjusted p-value < 0.05) for 29 genes between the two groups. In comparison, after 12 weeks of treatment we identified 22 genes with differential expression with nominal significance, with 11 genes to be found in both comparisons. None of these associations remained significant after correction for multiple testing. We also studied how the effect of interaction between responding to escitalopram and time point on gene expression signatures. We found 11 genes with nominal significance where the effect of the treatment on gene expression levels depended on whether the subject responded to the treatment or were resistant (at least 1.3-fold difference between FC12-weeks and FCbaseline). None of these associations remained significant after correction for multiple testing (see Table 2 for enrichment of informative top-10 genes).

Our findings indicate that there were no major differences in gene expression signatures between PD remitters and non-remitters, throughout the course of escitalopram treatment.