

# Analysis of SNP profiles in patients with major depressive disorder

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## Abstract

The present study focused on 91 single-nucleotide polymorphisms (SNPs) in 21 candidate genes to find associations with major depressive disorder (MDD). In total, 160 healthy controls and 177 patients with MDD were studied. We applied arrayed primer extension (APEX) based genotyping technology followed by association and haplotype analysis. SNPs in CCKAR, DRD1, DRD2, and HTR2C genes showed nominally significant associations with MDD. None of these associations remained significant after adjustment for multiple testing. Haplotype analysis revealed CCKAR haplotypes to be associated with MDD (global  $p=0.004$ ). More precisely, we found the GAGT haplotype to be associated with increased risk for MDD (OR 7.42, 95% CI 2.13–25.85,  $p=0.002$ ). This haplotype effect remained significant after Bonferroni correction ( $p=0.04$  after Bonferroni's adjustment). Altogether we were able to find some nominal associations, but due to small sample size these results should be taken as exploratory. However, the effect of GAGT haplotype on the CCKAR gene may be considered as increasing the risk for MDD.

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**Key words:** Association, haplotype analysis, major depressive disorder, single-nucleotide polymorphism (SNP).

## Introduction

Mood disorders are among the most prominent causes of disability and the second leading source of disease burden (Merikangas et al., 2002; Murray and Lopez, 1996). Most epidemiological and family studies indicate that the lifetime prevalence of unipolar major depressive disorder (MDD) is between 5% and 10% (Moldin et al., 1991). Suicide has been reported to occur in 10–15% of patients previously hospitalized for depression, a rate of death that is three orders of magnitude greater than that reported for the American population as a whole (Angst et al., 1999; Zubenko et al., 2002). Therefore, MDD is obviously a serious problem for public health.

Family and twin studies demonstrate that genetic factors typically account for 40–50% of the risk for developing MDD (McGuffin et al., 1996). A large number of family studies have demonstrated an increased risk of MDD among relatives of MDD probands, with ~2-fold increased risk in first-degree relatives (Kupfer et al., 1989; McGuffin et al., 1991). However, several reports do not support so high genetic risk for MDD, indicating the importance of environmental factors (Sullivan et al., 2000).

The aim of our study was to screen a set of single-nucleotide polymorphisms (SNPs) for their association with MDD. We defined the genes and their variations which have been previously published in the literature and yielded some (although inconsistent) significant findings, as candidate genes in our study. Genes related to the following neurotransmitter systems were included in the present survey: cholecystokinin (CCK), opioid peptides (OP), serotonin (5-HT) and dopamine (DA). CCK has been extensively studied as

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**Table 1.** Description of single-nucleotide polymorphisms (SNPs) analysed in our study

| Gene name<br>(abbreviation)                               | Gene and SNP                          | Position<br>from ATG | Location     | db SNP rs #            | Allele 1  | Allele 2                    | Function | Allele 1<br>frequency |
|---|---------------------------------------|----------------------|--------------|------------------------|-----------|-----------------------------|----------|-----------------------|
| Cholecystokinin<br>(CCK)                                  | CCK –45                               | CCK –1172            | 3p22–p21.3   | rs1799923              | C         | T                           | 5'-UTR   | 0.89                  |
|   | CCK 1270                              | CCK –9               | 3p22–p21.3   | rs754635               | C         | G                           | 5'-UTR   | 0.85                  |
|   | CCK 6662                              | CCK 5386             | 3p22–p21.3   | rs3774396              | C         | T                           | intron   | 0.98                  |
| Cholecystokinin A<br>receptor (CCKAR)                     | CCKAR –128                            | CCKAR –333           | 4p15.1–p15.2 | rs1800908              | G         | T                           | 5'-UTR   | 0.96                  |
|   | CCKAR 201                             | CCKAR –286           | 4p15.1–p15.2 | rs1799723              | A         | G                           | 5'-UTR   | 0.94                  |
|   | CCKAR 246                             | CCKAR –241           | 4p15.1–p15.2 | rs # n.a.              | G         | A                           | 5'-UTR   | 0.97                  |
|   | CCKAR 608                             | CCKAR 122            | 4p15.1–p15.2 | rs1800856              | G         | A                           | intron   | 0.96                  |
|   | CCKAR 1260                            | CCKAR 773            | 4p15.1–p15.2 | rs1800855              | T         | A                           | intron   | 0.71                  |
|   | CCKAR 1266                            | CCKAR 779            | 4p15.1–p15.2 | rs1800857              | T         | C                           | intron   | 0.76                  |
|   | CCKAR 3849                            | CCKAR 8231           | 4p15.1–p15.2 | rs1805037              | C         | T                           | I296I    | 0.99                  |
|   | Cholecystokinin B<br>receptor (CCKBR) | CCKBR –215           | CCKBR –216   | 11p15.4                | rs1799721 | C                           | A        | 5'-UTR                |
| CCKBR 109   |                                       | CCKBR 109            | 11p15.4      | rs1805000              | C         | T                           | L37F     | 0.93                  |
| CCKBR 1550  |                                       | CCKBR 9962           | 11p15.4      | rs1805002              | G         | A                           | V125I    | 0.92                  |
| CCKBR 2491  |                                       | CCKBR 10907          | 11p15.4      | rs1800843<br>rs8192470 | C         | A                           | Intron   | 0.88                  |
| Dopamine receptor<br>D1 (DRD1)                            | DRD1 –2218                            | DRD1 –2218           | 5q35.1       | rs # n.a.              | T         | C                           | 5'-UTR   | 0.94                  |
|   | DRD1 –2102                            | DRD1 –2102           | 5q35.1       | rs # n.a.              | C         | A                           | 5'-UTR   | 0.93                  |
|   | DRD1 –2030                            | DRD1 –2030           | 5q35.1       | rs # n.a.              | T         | C                           | 5'-UTR   | 0.97                  |
|   | DRD1 –1251                            | DRD1 –1252           | 5q35.1       | rs # n.a.              | G         | C                           | 5'-UTR   | 0.86                  |
|   | DRD1 –800                             | DRD1 –800            | 5q35.1       | rs265981               | T         | C                           | 5'-UTR   | 0.38                  |
|   | DRD1 –94                              | DRD1 –94             | 5q35.1       | rs5326                 | G         | A                           | 5'-UTR   | 0.84                  |
|   | DRD1 –48                              | DRD1 –48             | 5q35.1       | rs4532                 | G         | A                           | 5'-UTR   | 0.44                  |
| Dopamine receptor<br>D2 (DRD2)                            | DRD2 –241                             | DRD2 –50978          | 11q23        | rs1799978              | A         | G                           | 5'-UTR   | 0.78                  |
|   | DRD2 –141                             | DRD2 –50878          | 11q23        | rs1799732              | C         | del                         | 5'-UTR   | 0.84                  |
|   | DRD2 –7054                            | DRD2 –7053           | 11q23        | rs # n.a.              | C         | A                           | 5'-UTR   | 0.92                  |
|   | DRD2 –913                             | DRD2 –913            | 11q23        | rs1079597              | A         | G                           | 5'-UTR   | 0.32                  |
|   | DRD2 –901                             | DRD2 –901            | 11q23        | rs1079598              | C         | T                           | 5'-UTR   | 0.32                  |
|   | DRD2 286                              | DRD2 287             | 11q23        | rs # n.a.              | T         | C                           | intron   | 0.93                  |
|   | DRD2 3625                             | DRD2 3626            | 11q23        | rs2734834              | A         | T                           | intron   | 0.49                  |
|   | DRD2 3785                             | DRD2 3786            | 11q23        | rs1800498              | C         | T                           | intron   | 0.39                  |
|   | DRD2 11924                            | DRD2 11890           | 11q23        | rs1801028              | C         | G                           | S311C    | 0.93                  |
|   | DRD2 11997                            | DRD2 11915           | 11q23        | rs6277                 | T         | C                           | P319P    | 0.94                  |
|   | DRD2 16893                            | DRD2 16891           | 11q23        | rs2234689              | C         | G                           | 3'-UTR   | 0.72                  |
| DRD2 24470  | DRD2 24546                            | 11q23                | rs1800497    | C                      | T         | K713E (in<br>ANKK1<br>gene) | 0.80     |                       |
| Dopamine receptor<br>D3 (DRD3)                            | DRD3 –707                             | DRD3 –710            | 3q13.3       | rs1800828              | G         | C                           | 5'-UTR   | 0.71                  |
|   | DRD3 –343                             | DRD3 –346            | 3q13.3       | rs1800827              | G         | A                           | 5'-UTR   | 0.96                  |
|   | DRD3 25                               | DRD3 25              | 3q13.3       | rs6280                 | A         | G                           | G9S      | 0.69                  |
| Dopamine receptor<br>D4 (DRD4)                            | DRD4 –1217                            | DRD4 –1216           | 11p15.5      | rs # n.a.              | G         | del                         | 5'-UTR   | 0.62                  |
|   | DRD4 –809                             | DRD4 –808            | 11p15.5      | rs936461               | G         | A                           | 5'-UTR   | 0.80                  |
|   | DRD4 –768                             | DRD4 –767            | 11p15.5      | rs4987058              | G         | A                           | 5'-UTR   | 0.86                  |
|   | DRD4 –616                             | DRD4 –615            | 11p15.5      | rs747302               | C         | G                           | 5'-UTR   | 0.68                  |
|   | DRD4 –521                             | DRD4 –521            | 11p15.5      | rs1800955              | C         | T                           | 5'-UTR   | 0.41                  |
|   | DRD4 –376                             | DRD4 –376            | 11p15.5      | rs916455               | C         | T                           | 5'-UTR   | 0.96                  |
| Dopamine receptor<br>D5 (DRD5)                            | DRD5 1481                             | DRD5 1481            | 4p16.1       | rs1967551              | C         | T                           | 3'-UTR   | 0.65                  |
| Tyrosine hydroxylase<br>(TH)                              | TH 241 –243                           | TH 2066              | 11p15.5      | rs6356                 | G         | A                           | V81M     | 0.61                  |
|   | TH 614                                | TH 3891              | 11p15.5      | rs # n.a.              | T         | C                           | L205P    | 0.96                  |
| 5-hydroxytryptamine<br>(serotonin) receptor<br>1A (HTR1A) | HTR1A –1018                           | HTR1A –1019          | 5q11.2–q13   | rs6295                 | C         | G                           | 5'-UTR   | 0.43                  |
|   | HTR1A –480                            | HTR1A –480           | 5q11.2–q13   | rs # n.a.              | A         | del                         | 5'-UTR   | 0.91                  |
| 5-hydroxytryptamine                                       | HTR1B                                 | HTR1B –1089          | 6q13         | rs1778258              | T         | C                           | 5'-UTR   | 0.24                  |

Table 1 (cont.)

| Gene name<br>(abbreviation)  | Gene and SNP                  | Position<br>from ATG | Location      | db SNP rs # | Allele 1  | Allele 2 | Function | Allele 1<br>frequency |
|--|-------------------------------|----------------------|---------------|-------------|-----------|----------|----------|-----------------------|
| (serotonin) receptor<br>1B (HTR1B)   | HTR1B                         | HTR1B -700           | 6q13          | rs1228814   | C         | A        | 5'-UTR   | 0.55                  |
|  | HTR1B -511                    | HTR1B -511           | 6q13          | rs130056    | G         | T        | 5'-UTR   | 0.995                 |
|  | HTR1B -161                    | HTR1B -161           | 6q13          | rs130058    | A         | T        | 5'-UTR   | 0.78                  |
|  | HTR1B 129                     | HTR1B 129            | 6q13          | rs6298      | C         | T        | S43S     | 0.74                  |
|  | HTR1B 276                     | HTR1B 276            | 6q13          | rs130059    | G         | A        | A92A     | 0.96                  |
|  | HTR1B 371                     | HTR1B 371            | 6q13          | rs130060    | T         | G        | F124C    | 0.99                  |
|  | HTR1B 705                     | HTR1B 705            | 6q13          | rs130062    | C         | T        | A235A    | 0.80                  |
|  | HTR1B 861                     | HTR1B 861            | 6q13          | rs6296      | G         | C        | V287V    | 0.74                  |
|  | HTR1B                         | HTR1B 1180           | 6q13          | rs6297      | G         | A        | 3'-UTR   | 0.23                  |
| 5-hydroxytryptamine<br>(serotonin) receptor<br>2A (HTR2A)  | HTR2A -1438                   | HTR2A -1437          | 13q14-q21     | rs6311      | A         | G        | 5'-UTR   | 0.42                  |
|  | HTR2A 73                      | HTR2A 74             | 13q14-q21     | rs1805055   | C         | A        | T25N     | 0.98                  |
|  | HTR2A 102                     | HTR2A 102            | 13q14-q21     | rs6313      | T         | C        | S34S     | 0.37                  |
|  | HTR2A 1354                    | HTR2A 61008          | 13q14-q21     | rs6314      | C         | T        | H452Y    | 0.94                  |
| 5-hydroxytryptamine<br>(serotonin) receptor<br>2C (HTR2C)  | HTR2C 68                      | HTR2C 4390           | Xq24          | rs6318      | G         | C        | C23S     | 0.83                  |
|  | HTR2C 2831                    | HTR2C 181359         | Xq24          | rs1801412   | T         | G        | 3'-UTR   | n.a.                  |
| 5-hydroxytryptamine<br>(serotonin) receptor<br>3A (HTR3A)  | HTR3A 1302                    | HTR3A -507           | 11q23.1-q23.2 | rs1150226   | T         | C        | 5'-UTR   | 0.31                  |
|  | HTR3A 1596                    | HT3A 14378           | 11q23.1-q23.2 | rs1176713   | G         | A        | L459L    | 0.26                  |
| Solute carrier family<br>6 (neurotransmitter<br>transporter,<br>serotonin), member 4<br>(SLC6A4) | SLC6A4                        | SLC6A4 18784         | 17q11.1-q12   | rs6352      | A         | C        | K605N    | 0.96                  |
|  | SLC6A4                        | SLC6A4 10647         | 17q11.1-q12   | rs6353      | G         | A        | T439T    | 0.92                  |
|  | SLC6A4                        | SLC6A4 167           | 17q11.1-q12   | rs6355      | G         | C        | G56A     | 0.77                  |
| Tryptophan<br>hydroxylase 1<br>(tryptophan 5-<br>monooxygenase)<br>(TPH1)                        | TPH1 218                      | TPH1 14494           | 11p15.3-p14   | rs1800532   | A         | C        | intron   | 0.29                  |
|  | TPH1 779                      | TPH1 15055           | 11p15.3-p14   | rs1799913   | A         | C        | intron   | 0.27                  |
| Opioid receptor, mu 1<br>(OPRM1)   | OPRM1 31                      | OPRM1 50665          | 6q24-q25      | rs # n.a.   | G         | A        | intron   | 0.92                  |
|  | OPRM1 118                     | OPRM1 118            | 6q24-q25      | rs1799971   | A         | G        | N40D     | 0.78                  |
|  | OPRM1 440                     | OPRM1 50431          | 6q24-q25      | rs # n.a.   | C         | G        | S147C    | 0.84                  |
|  | OPRM1 691                     | OPRM1 51325          | 6q24-q25      | rs2075572   | C         | G        | intron   | 0.54                  |
|  | OPRD1 80                      | OPRD1 80             | 1p36.1-p34.3  | rs1042114   | T         | G        | C27F     | 0.91                  |
| Opioid receptor,<br>delta 1 (OPRD1)  | OPRD1 921                     | OPRD1 50702          | 1p36.1-p34.3  | rs2234918   | T         | C        | G307G    | 0.63                  |
| Opioid receptor,<br>kappa 1 (OPRK1)  | OPRK1 36                      | OPRK1 36             | 8q11.2        | rs1051660   | G         | T        | P12P     | 0.84                  |
|  | OPRK1                         | OPRK1 10807          | 8q11.2        | rs1365097   | A         | G        | intron   | 0.69                  |
|  | OPRK1                         | OPRK1 10915          | 8q11.2        | rs1365098   | G         | T        | intron   | 0.66                  |
|  | OPRK1                         | OPRK1 11220          | 8q11.2        | rs997917    | A         | G        | intron   | 0.54                  |
|  | OPRK1 459                     | OPRK1 16128          | 8q11.2        | rs7815824   | C         | T        | S153S    | 0.90                  |
|  | OPRK1 843                     | OPRK1 21441          | 8q11.2        | rs702764    | A         | G        | A281A    | 0.72                  |
|  | OPRK1 846                     | OPRK1 21444          | 8q11.2        | rs # n.a.   | C         | T        | V282V    | 0.97                  |
|  | Proopiomelanocortin<br>(POMC) | POMC 18              | POMC 18       | 2p23.3      | rs8192605 | C        | T        | C6C                   |
| POMC 282   | POMC 3170                     | 2p23.3               | rs # n.a.     | C           | T         | S94S     | 0.92     |                       |
| POMC 313   | POMC 3201                     | 2p23.3               | rs # n.a.     | G           | T         | E105Stop | 0.96     |                       |
| POMC 346   | POMC 3234                     | 2p23.3               | rs # n.a.     | C           | T         | L116L    | 0.98     |                       |
| POMC 585   | POMC 3473                     | 2p23.3               | rs2071345     | C           | T         | A195A    | 0.94     |                       |
| POMC 866   | POMC 3755                     | 2p23.3               | rs1042571     | C           | T         | 3'-UTR   | 0.85     |                       |
| Proenkephalin<br>(PENK)  | PENK 28                       | PENK -588            | 8q23-q24      | rs2609999   | C         | A        | 5'-UTR   | 0.57                  |
|  | PENK 808                      | PENK 4686            | 8q23-q24      | rs3839874   | C         | del      | 3'-UTR   | 0.67                  |

db SNP rs # – accession number of SNP in NCBI dbSNP database; allele frequency is based on controls of this study. rs # n.a. – SNP is not listed in NCBI dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>).

a gene involved in the pathogenesis of emotional disorders, especially anxiety and panic disorders (Bowen et al., 1998; Geraciotti et al., 1989; Hattori et al., 2002; Kennedy et al., 1999b). Opioid peptides are also implicated in the development of emotional disorders (Alda et al., 2000; Peckys and Hurd, 2001). As proopiomelanocortin (POMC) is a precursor for adrenocorticotropin hormone (ACTH) and patients with mood disorders have disturbances in the hypothalamic–pituitary–adrenal (HPA) system, POMC is a good target for association studies (Galard et al., 2002). 5-HT and DA are monoamines which are frequently studied in respect to mood disorders (Nutt, 2002; Pania and Gessab, 2002). Genes of the above described neurotransmitters and their receptors were chosen for genotyping. Altogether we analysed 91 polymorphisms located in 21 candidate genes (detailed information about the studied polymorphisms is available in Table 1). SNP analysis was performed by arrayed primer extension (APEX) technology. APEX is a genotyping and resequencing technology that combines Sanger dideoxy sequencing with the parallelization and high-throughput potential of microarray format (Tõnisson et al., 2002). APEX technology is suitable for SNP analysis allowing the screening of hundreds of SNPs in one sample.

## Methods

### *Subjects and psychiatric assessment*

Unrelated patients ( $n=177$ ; 39 male, 138 female; age range 18–73 yr; mean age 40.3 yr) with MDD were recruited in the study along with healthy control individuals ( $n=160$ ; 49 male, 111 female; age range 18–71 yr; mean age 37.7 yr) from the Estonian population. Diagnoses of patients were substantiated by psychiatric interview and verified by Mini International Neuropsychiatric Interview (M.I.N.I. 5.0.0) based on DSM-IV (Sheehan et al., 1998). The case group consisted of patients with only MDD ( $n=69$ ) and MDD patients with comorbid anxiety disorders [panic disorder, generalized anxiety disorder (GAD), obsessive–compulsive disorder (OCD), social phobia] ( $n=108$ ). Controls were evaluated using M.I.N.I. to exclude those with psychiatric morbidity, and with a family history interview to exclude those with a known history of major psychiatric disorders in first-degree relatives. Patients were recruited among consecutive outpatients and in-patients at the Clinic of Psychiatry of Tartu University Clinics and controls were recruited by newspaper advertisement in Tartu, Estonia. The study was conducted in accordance with

the principles of the Declaration of Helsinki. The study protocol was approved by the Ethics Review Committee on Human Research of the University of Tartu. Each subject provided written informed consent.

### *Template preparation and genotyping*

Standard high-salt extraction method was used to isolate genomic DNA from 9 ml venous blood samples. Two different PCR programs were used to amplify the genomic regions containing the whole set of studied 91 polymorphisms with 64 individual PCR reactions. In program 1 amplification reactions consisted of an initial 5 min denaturation at 95 °C, followed by 34 cycles of: 95 °C for 30 s, 55 °C for 40 s, 72 °C for 40 s. The final extension step was 72 °C for 6 min. Program 2 contained temperature decrements of 1 °C per cycle in annealing step for first 10 cycles. Samples were processed in a PTC-200 thermal cycler (MJ Research Inc., Watertown, MA, USA). Primer sequences and PCR conditions used for amplification are available upon request.

A 20% fraction of the dTTP in the amplification mixture was substituted by dUTP, allowing later fragmentation of PCR products with uracil-*N*-glycosylase. Pooled amplification products were concentrated and purified, followed by fragmentation and functional inactivation of the unincorporated dNTPs as described in Tõnisson et al. (2002). Production of oligonucleotide microchips and APEX reactions were performed as described earlier (Tõnisson et al., 2002). Slides were imaged with Genorama Quattroimager detector (Asper Biotech Ltd, Tartu, Estonia) and polymorphisms were identified by Genorama™ 4.1 genotyping software (Asper Biotech Ltd) by using signal patterns from a wild-type DNA sequence as the reference.

### *Selection of SNPs*

By choosing missense SNPs for genotyping, we reasoned that at least some of them are probably causative mutations affecting function of the encoded protein associated with the underlying phenotype. We included common synonymous SNPs in our study under the assumption that silent SNPs, being in linkage disequilibrium (LD) with unknown functional polymorphism, can reveal an association with the actual disease-causing SNP(s). SNPs in regulatory sequences are thought to have the potential to control the level of gene expression, therefore, in some genes polymorphisms in 5' or 3' untranslated regions and intronic SNPs were included.

**Table 2.** Results of association analysis of 91 polymorphisms in major depressive disorder

| SNP   | Allele |   | Gene  | Allelic P<br>MDD | Allele 2 frequencies |          |
|-------|--------|---|-------|------------------|----------------------|----------|
|       | 1      | 2 |       |                  | MDD                  | Controls |
| 246   | G      | A | CCKAR | 0.006            | 0.09                 | 0.03     |
| -2102 | C      | A | DRD1  | 0.008            | 0.02                 | 0.07     |
| -7054 | C      | A | DRD2  | 0.03             | 0.14                 | 0.08     |
| 68    | G      | C | HTR2C | 0.02             | 0.10                 | 0.17     |

SNP, single-nucleotide polymorphism; MDD, major depressive disorder.

### Statistical analysis

Association analysis statistics was performed using GENEPOP Version 3.3 software (Raymond and Rousset, 1995). *p* values for allelic and genotypic association were calculated using Fisher's exact test. The significance level for all statistical tests was 0.05. Haplotype analysis was performed using the maximum-likelihood method for estimating simultaneously haplotype frequencies and haplotype-phenotype association as described in Tregouet et al. (2002). Pairwise LD was estimated by a log-linear model and the extent of disequilibrium was expressed in terms of standardized *D'* characteristic. Bonferroni correction was used after association and haplotype analysis to adjust for multiple testing.

### Results

We genotyped 91 polymorphisms (87 SNPs and 4 insertions/deletions) in 21 candidate genes in 177 unrelated MDD patients and 160 healthy controls. In our screening set, genetic variations in altogether four genes displayed association with MDD. Data for statistically significant SNPs are presented in Table 2. Namely, SNPs 246G/A in CCKAR, -2102C/A in DRD1, -7054C/A in DRD2, and 68G/C (rs6318) in HTR2C genes were associated with MDD. In the case of CCKAR and DRD2 markers an excess of minor alleles in the affected group was found. In contrast, the minor alleles of DRD1 and HTR2C markers were more frequent in control subjects. After Bonferroni correction, none of the described marker-disease associations remained statistically significant. There was no deviation from Hardy-Weinberg equilibrium expectations at any of the genotyped loci. A gender comparison between females (*n*=139) and males (*n*=38) of the MDD sample did not show any significant differences

with regard to alleles and/or genotypes. Our data indicate that the relationship between unipolar affective disorder and analysed loci appear to be independent of sex. Haplotype analysis was performed according to particular pairwise LD pattern for each gene (cases + controls, *n*=337). Only genes that were genotyped for two or more SNPs and showing the presence of LD in both affected and control groups, and having preliminary evidence of marker-disease association were included in haplotype analysis. It was also possible to investigate the effect of each SNP on different haplotypic background using the inference method. The odds ratio for MDD was estimated according to the haplotypic background conferred by other polymorphisms. Haplotype analysis revealed CCKAR haplotypes to be associated with MDD and altogether six haplotypes (HT) were found (Table 3). Reference haplotypes combined with the major alleles at each locus, which taken together with another common haplotype constituted almost 90% of all alleles. Both haplotypes were almost equally represented in cases and control subjects. Other haplotypes were rare. Haplotype 3 (GAGT) was significantly over-represented in the affected group, reflecting a higher frequency of the rare 246A allele in cases by comparison to the reference haplotype (GGGT). This haplotype (GAGT) was associated with a higher risk for MDD (OR 7.42, 95% CI 2.13-25.85, *p*=0.002) compared to the reference haplotype (GGGT). This haplotype effect also remained significant after Bonferroni correction (*p*=0.04 after Bonferroni's adjustment). We detected a significant individual SNP effect (OR 7.40, *p*=0.002) for 246G/A in a haplotype context HT1 (GGGT) vs. HT3 (GAGT). The test of a global CCKAR haplotypic association with MDD was significant in the population studied ( $\chi^2 = 17.60$ , d.f. = 5, *p*=0.004).

Taken together, results of haplotype analysis confirmed our findings from the association study. Haplotype analysis revealed that CCKAR haplotype (GAGT) formed by SNPs at positions -128G/T (rs1800908), 246G/A, 608G/A (rs1800856), and 1266T/C (rs1800857) is a possible susceptibility haplotype for MDD.

### Discussion

Clinical as well as molecular genetic studies indicate that MDD is a polygenic disorder. Many genes, each of minor individual contribution, are likely to be involved in the development of affective disorders. In our screening set of 91 polymorphisms in 21 candidate genes, variations in four genes displayed an association with MDD. Polymorphisms in CCKAR

**Table 3.** Estimated haplotype (HT) frequencies and HT effects in the CCKAR gene

| HT | Single-nucleotide polymorphism |        |        |         | Haplotype frequency |          | Haplotypic OR (95% CI) | <i>p</i> |
|----|--------------------------------|--------|--------|---------|---------------------|----------|------------------------|----------|
|    | –128G/T                        | 246G/A | 608G/A | 1266T/C | Controls            | Patients |                        |          |
| 1  | G                              | G      | G      | T       | 67.5                | 65.6     | *                      |          |
| 2  | G                              | G      | G      | C       | 21.6                | 20.0     | 0.905 (0.611–1.338)    | 0.625    |
| 3  | G                              | A      | G      | T       | 1.2                 | 7.5      | 7.418 (2.129–25.85)    | 0.002*   |
| 4  | T                              | G      | G      | T       | 3.8                 | 2.6      | 0.517 (0.203–1.320)    | 0.168    |
| 5  | G                              | G      | A      | T       | 2.4                 | 1.3      | 0.588 (0.137–2.523)    | 0.475    |
| 6  | G                              | A      | G      | C       | 2.4                 | 1.3      | 0.588 (0.137–2.523)    | 0.475    |

\*  $p=0.04$  after Bonferroni's adjustment.

(246G/A), DRD1 (–2102C/A), DRD2 (–7054C/A), and HTR2C (68G/C, rs6318) genes were associated with MDD phenotypes.

Pharmacological studies have suggested that MDD is associated with impairment of brain monoaminergic transmission (Nemeroff, 2002). The role of 5-HT in the pathology of mood disorders is based mainly on the efficacy of selective 5-HT reuptake inhibitors in the treatment of MDD. DA has also been implicated in the pathophysiology of mood disorders and hypoactivity of the mesolimbic DA pathway may be related to depressive symptoms. Thus, genes that control the brain 5-HT and DA pathways seem to be good candidates for mediating genetic susceptibility to MDD.

Association of CCKAR gene polymorphism with MDD was further confirmed by haplotype analysis, where the GAGT haplotype carrying the risk for MDD (OR 7.418,  $p=0.002$ ) was established. CCKAR polymorphisms have been shown to be involved in schizophrenia and auditory hallucinations (Wang et al., 2002; Wei and Hemmings, 1999), and also in panic disorder (Miyasaka et al., 2004). Preclinical studies suggest that CCKAR directly regulates the release of DA in the nucleus accumbens and amygdala (Hamilton and Freeman, 1995). Therefore, CCKAR is implicated in the regulation of emotional behaviour and motivation. Supportive evidence of CCKAR gene involvement in mood disorders is also related to its genomic localization (4p15.1–p15.2). This locus is close to the 4p16 region which has been repeatedly shown to be related to bipolar disorder (Kennedy et al., 1999a). In our previous study we found that polymorphisms in the wolframin (WFS1) gene, also located in the 4p16 region, are possibly related to an increased risk for mood disorders (Koido et al., 2004). This study sample was partially the same as in the present study. Als and colleagues found that markers in the 4p15 region appeared to be associated with schizophrenia and

schizophrenia combined with bipolar disorder, and also supportive evidence for schizophrenia and bipolar disorder being associated with the 4p16 region (Als et al., 2004). Therefore, the 4p15–p16 region seems to be a good candidate risk locus for psychiatric disorders.

Results of this study provide further evidence for the involvement of genes related to monoaminergic and peptidergic neurotransmission in the regulation of mood disorders. However, we cannot exclude a hypothesis describing polymorphisms as being in LD with other functionally significant polymorphisms, which could actually be involved in mood disorders. It has been shown that missense SNP itself probably does not cause disease but it is in strong LD with non-functional SNP which may actually contribute to the susceptibility for disease (Handoko et al., 2004). This warrants studying not only functional polymorphisms but also untranslated SNPs.

Due to the limited size of our sample this study should be considered an exploratory in nature. A multi-stage approach is recommended to distinguish false-positive discoveries from real associations (Hirschhorn and Daly, 2005). As many association studies produce unreplicable results due to false-positive findings induced by multiple testing, it is suggested that first, many markers should be typed for a subset of individuals. Afterwards the most promising markers can be evaluated on a larger sample (van den Oord and Sullivan, 2003). Therefore, replication studies with larger and independent samples are needed.

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### Statement of Interest

A. Metspalu is a scientific advisor and member of the Council of Asper Ltd.

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