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Association of Aggression With a Novel MicroRNA Binding Site Polymorphism in the Wolframin Gene

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Rare mutations in the WFS1 gene lead to Wolfram syndrome, a severe multisystem disorder with progressive neurodegeneration and diabetes mellitus causing life-threatening complications and premature death. Only a few association studies using small clinical samples tested the possible effects of common WFS1 gene variants on mood disorders and suicide, the non-clinical spectrum has not been studied yet. Self-report data on Aggression, Impulsiveness, Anxiety, and Depression were collected from a large (N = 801) non-psychiatric sample. Single nucleotide polymorphisms (SNPs) were selected to provide an adequate coverage of the entire WFS1 gene, as well as to include putative microRNA binding site polymorphisms. Molecular analysis of the assumed microRNA binding site variant was performed by an in vitro reporter-gene assay of the cloned 3′ untranslated region with coexpression of miR-668. Among the 17 WFS1 SNPs, only the rs1046322, a putative microRNA (miR-668) binding site polymorphism showed significant association with psychological dimensions after correction for multiple testing: those with the homozygous form of the minor allele reported higher aggression on the Buss–Perry Aggression Questionnaire ($P = 0.0005$). Functional effect of the same SNP was also demonstrated in a luciferase reporter system: the minor A allele showed lower repression compared to the major G allele, if co-expressed with miR-668. To our knowledge, this is the first report describing a microRNA binding site polymorphism of the WFS1 gene and its association with human aggression based on a large, non-clinical sample. © 2013 Wiley Periodicals, Inc.

Key words: aggression; microRNA; WFS1; rs1046322; SNP

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INTRODUCTION

Wolfram syndrome (WS) is an autosomal recessive multisystem disorder with progressive neurodegeneration and diabetes mellitus [Rigoli et al., 2011], also known as DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy and deafness). Although the clinical course of WS is highly variable, the prognosis is poor as patients have an impaired quality of life and usually die in their thirties from central respiratory failure as a result of brainstem atrophy [Barrett et al., 1995]. Psychiatric manifestations are common in WS patients, including severe depression, psychosis, as well as impulsive verbal and physical aggression [Swift et al., 1990].

The mutated gene, WFS1 was identified in 1998 on chromosome 4p16.1 spanning 33.4 kb [Inoue et al., 1998]; it has eight exons but the first exon is non-coding. The WFS1 gene encodes a transmembrane protein (wolframin) of the endoplasmatic reticulum (ER), predominantly expressed in the brain and in the pancreatic β cells, but detected nearly in all tissues. Although the molecular function of wolframin is not fully understood to date, current results suggest that wolframin has a critical role in the regulation of ER stress [Fonseca et al., 2005, 2010]. ER homeostasis is based on a sensitive balance of protein synthesis and protein folding capacity of the ER. Accumulation of unfolded proteins induces ER stress, triggering the unfolded protein response (UPR). At the same time it is of vital importance to avoid UPR hyperactivation, which could trigger apoptotic pathways and lead to cell death. According to recent data, wolframin might participate in down regulation of ER stress and thus, in the reestablishment of ER homeostasis [Oslowski and

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Urano, 2011]. Further findings pointed out another possible role of wolframin in controlling ER calcium homeostasis [Takei et al., 2006]. A recent study demonstrated that wolframin is a calcium-calmodulin binding protein, and three mutations found in WS patients completely abolish this function [Yurimoto et al., 2009]. Taken together the findings above about the molecular function of wolframin, this protein is expected to have a vital role in ER homeostasis of excretory cells, such as the insulin producing pancreatic β cells or the neurons producing synaptosomes for neurotransmission.

Studies on the WFS1 knockout mouse provided further evidence for the significant role of wolframin in the brain and in the pancreas. WFS1 deficient mice had a dramatic decrease in the mass of the pancreatic islets and a serious impairment of glucose homeostasis [Ishihara et al., 2004]. Results from behavioral studies of the WFS1 knockout mouse suggested that it could be used as an animal model of mood disorder [Kato et al., 2008], impaired behavioral stress adaptation [Luuk et al., 2009] and post-traumatic stress disorder [Kesner et al., 2009].

Although more than 200 different mutations causing WS have been identified (for a recent database see: [Lesperance, 2010]), phenotype–genotype relationships are still not clear. Similarly, little is known about the clinical and molecular effects of common variants. Several single nucleotide polymorphisms (SNPs) have been shown as possible risk factors for type 2 diabetes mellitus, such as the rs10010131 in intron 4 and rs1046320 in the 3′ untranslated region (UTR) [Fawcett et al., 2010]. Other studies raised the possibility of association between several WFS1 SNPs and neuropsychiatric disorders, such as mood disorders [Kawamoto et al., 2004; Koido et al., 2005] and impulsive suicide [Lemonde et al., 2003; Must et al., 2009]. Although the level of significance did not survive corrections for multiple testing in most studies, the possible link between WFS1 genetic variants and psychiatric disorders is strongly supported by the fact that carriers of various WFS1 mutations are 26-fold more likely to be hospitalized in psychiatric institutions than non-carriers [Swift and Swift, 2000].

According to our best knowledge, WFS1 polymorphisms have not been studied yet in relation to either aggression or mood characteristics within the non-clinical spectrum. Here, we present an association study of WFS1 SNPs and four psychological dimensions: Aggression, Impulsiveness, Anxiety, and Depression, using self-report data from a large, non-psychiatric sample. Study of these phenotypes was based on the main psychiatric symptoms of WS patients (depression and aggression), as well as on the results of the few psychogenetic association studies related to mood disorders and impulsive suicide. In addition, we characterized the molecular function of rs1046322 which had a significant effect on the measured phenotypes.

MATERIALS AND METHODS

Sample

Caucasian (Hungarian) participants of the study signed written informed consent, provided buccal samples and filled out questionnaires. They were recruited at different higher education sites on a voluntary basis. The study protocol was approved by the Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB). Valid DNA and questionnaire data were available from 801 subjects (46.2% males and 53.8% females). Mean age was 21.3 years (±3.3) within the age range of 18–35 years.

Phenotypic Measures

All subjects completed a panel of self-report measures relevant to the study of Aggression, Impulsiveness, Anxiety, and Depression. The Buss–Perry Aggression Questionnaire [Buss and Perry, 1992] includes 29 items, rated from 1 (extremely uncharacteristic of me) to 5 (extremely characteristic of me). The aggression construct was measured with items loading on four distinct factors: Verbal Aggression (five items), Anger (seven items), Hostility (eight items), and Physical Aggression (nine items). The total score for aggression was the sum of ratings for all the items, thus possible total scores range from 29 to 145. The 11th version of the Barratt Impulsiveness Scale was designed to assess general impulsiveness using 30 items scored from 1 to 4 (BIS-11; [Patton et al., 1995]). In the present study, the total score for impulsiveness was used, with values ranging from 30 to 120. The English version of the questionnaires measuring aggression and impulsiveness were translated using the "forward–backward" procedure and were pilot tested prior to the present study to enhance translation clarity and applicability. Anxiety and depression was measured by the Hungarian version of the Hospital Anxiety and Depression Scale (HADS; [Muszbek et al., 2006]). Items are scored from 0 to 3 based on the selected response category, possible total scores for both Anxiety (seven items) and Depression (seven items) range from 0 to 21.

To assess the internal consistency, Cronbach's alpha values were calculated for each phenotype construct in the present sample, and were found satisfactory (Aggression Total Score: 0.90; Impulsiveness Total Score: 0.82; Anxiety Scale: 0.77; Depression Scale: 0.74). Internal consistency measures of the four aggression factors were also sufficient (Verbal Aggression: 0.64; Anger: 0.84; Hostility: 0.80; Physical Aggression: 0.84).

Marker Selection

Common (higher than 5% minor allele frequency [MAF]) polymorphic loci were selected from dbSNP database using HapMap information about linkage disequilibrium (LD) to provide adequate coverage of the entire WFS1 gene. LD blocks were defined by the "Four Gamete Rule" using Haplowiev 4.2. At least two SNPs have been investigated in each block taking the chromosomal localization also into consideration. Our goal was to generate a balanced "physical coverage" so that no segments longer than 6 kb remained uncovered. Priority was given to those SNPs which were referred in various association studies in connection with diabetes mellitus and psychiatric disorders. SNPs, theoretically influencing microRNA binding, were searched in the polymiRTS and Patrocles databases.

DNA and Genotyping

Genomic DNA was extracted from buccal swabs by Gentra purification kit (Minneapolis, MN) according to manufacturer's instructions and quantified by fluorometry employing an intercalation

assay (AccuBlue Broad Range dsDNA Quantitation Kit, Biotium, Hayward, CA). Typical DNA concentration was about 50 ng/μl (range: 15–200 ng/μl).

Genotyping was performed by an Open Array real-time PCR platform of Applied Biosystems (Foster City, CA) based on the 5′ nuclease assay with pre-designed and validated primers and allele-specific, fluorescent (TaqMan) probes immobilized to a solid surface. Approximately 100 ng (range: 30–150 ng) DNA was applied for each SNP subarray. The master mix, containing each dNTP and the AmpliTaq Gold DNA-polymerase, was supplied by the manufacturer. The thermocycle was carried out according to the manufacturer's instruction. Allele specific FAM and VIC fluorescent intensities were measured after the PCR amplification, genotypes were called using the TaqMan Genotyper v1.2 software. Call rates for SNPs on the OpenArray system was 88.87% in average (range: 69.37–96.00%). For quality control, three SNPs (rs9457, rs1046320, and rs1046322) were genotyped additionally on a conventional 96-well-plate system with the commercially available TaqMan assay kits on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Re-genotyping proved the reliability of the high-throughput OpenArray platform, as 99.1% agreement was observed comparing the two approaches, moreover increased the call rate of genotypes at these three loci. The genotype and allele frequencies obtained as a combination of data determined by the two genotyping platforms for the 801 individuals with valid data of questionnaires were used for association analysis between genotypes and phenotypic measures. Genotype frequency data for each SNP are given in Supplementary Table I, MAFs are shown in Table I. The measured genotype frequencies corresponded to the Hardy–Weinberg equilibrium for all SNPs (for *P* values see: Supplementary Table I). Call rates for each SNP were calculated as the sum of the genotypes given in Supplementary Table I divided by the total number of participants with valid phenotypic data (801).

Luciferase Constructs of the WFS1 3′ UTR

The entire 3′ UTR region of the human WFS1 gene was amplified by PCR of a sample with known rs1046322 (GG) genotype using the 5′ TCG GCG **GAG CTC** GGA TGG TCC GCC ACG AGG AGC 3′ forward and 5′ AAA GGA **AAG CTT** GCG CTG CAG GTT CCA CCA GAG G 3′ reverse primers. Bold letters indicate the *Sac*I and *Hin*dIII recognition sites in the forward and reverse primer, respectively. PCR product was purified and cloned between the *Sac*I and *Hin*dIII sites downstream to the firefly luciferase gene in pMIR-Report vector (Life Technologies, Grand Island, NY) by standard protocols (pMIR-G). Construct with WFS1 3′ UTR possessing the rs1046322 A allele (designated pMIR-A) was generated by quick change site directed mutagenesis of the cloned pMIR-G, both constructs were confirmed by direct sequencing. A control pMIR construct (pMIR-c) was also created by cloning a DNA-fragment with identical length, however, without the binding site of miR-668 downstream to the luciferase gene.

Cell Transfection and Luciferase Assay

The Human Embryonic Kidney 293 (HEK293) cell line was cultured in DMEM medium (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland) at 37°C in an atmosphere of 5% CO_2. HEK293 cells were transferred into 24-well plate and incubated for 24 hr before transfection. Subsequently, 0.05 g of the luciferase reporter construct were cotransfected with 0.2 g β-galactosidase vector (Ambion), and 5 pmol miR-668 using Lipofectamine 2000 (Invitrogen). Cells were collected 48 hr after transfection and extracted by three consecutive freeze–thaw cycles followed by a centrifugation. Supernatants were used for luciferase assay. Luminescence for luciferase as well as optical density for β-galactosidase were measured by Varioskan Flash Multimode Reader, luciferase activity data were normalized by β-galactosidase activities. All experiments

TABLE I. Main Characteristics of the Selected WFS1 SNPs

dbSNP number	Genomic position	Inter-SNP distance (bp)	Type	MAF
rs10002743	6276581		Intron 2	0.191
rs6824720	6278647	2,066	Intron 2	0.159
rs752854	6281961	3,314	Intron 3	0.310
rs4689393	6287241	5,280	Intron 3	0.435
rs10010131	6292915	5,674	Intron 4	0.391
rs13147655	6293474	559	Intron 6	0.384
rs4467645	6294305	831	Intron 7	0.394
rs13128674	6294517	212	Intron 7	0.409
rs6446482	6295693	1,176	Intron 7	0.398
rs4689395	6295985	292	Intron 7	0.359
rs28716718	6301910	5,925	Intron 8	0.053
rs1801208	6302889	979	Exon 8 (p.R456H)	0.050
rs734312	6303354	465	Exon 8 (p.H611R)	0.462
rs1046316	6304087	733	Exon 8 (p.S855S)	0.341
rs1046320	6304344	257	3′ UTR	0.403
rs1046322	6304448	104	3′ UTR	0.099
rs9457	6304799	351	3′ UTR	0.433

were carried out in triplicates. Statistical analysis of normalized reporter data was performed using one-way ANOVA followed by Tukey–Kramer test (GraphPad InStat 3.05 GraphPad Software, Inc., San Diego, CA).

Statistical Analysis

Statistical analyses were carried out using SPSS 19.0 for Windows. Allele and genotype frequency distributions were tested by Chi-square analyses. Independent-Samples t-test was applied for assessment of gender differences on self-report scales; their correlation with age was also calculated. Genetic associations were tested by one-way analyses of covariance (ANCOVA) assuming a bi-allelic inheritance model and the recessivity of the minor allele. Sex and age were used as covariates, since both were possible confounds. Significant ($P < 0.001$) gender differences were found in aggression and anxiety (males exposed higher aggression and lower anxiety scores). Impulsiveness and depression scores showed no significant sex differences. A significant weak negative correlation was found between age and aggression $r = -0.073$ ($P = 0.04$). Other measured scales did not correlate with age. In association analyses nominal level of significance was corrected to $P < 0.0007$ based on the Bonferroni correction for multiple testing: $P = 0.05$ was divided by 68, the number of analyses performed (4 phenotypes $\times$ 17 SNPs). Lewontin's D′ as well as R^2 values of LD were determined using HaploView 4.2 [Barrett et al., 2005].

RESULTS

Higher Aggression in AA Homozygotes of WFS1 rs1046322

Table I summarizes the most important information for the 17 studied WFS1 SNPs. The average distance between the selected SNPs was 1,764 bp. Most of them are intronic, but three SNPs in the longest (8th) exon and three SNPs in the 3′ UTR region were also investigated. MAF was calculated from the genotype distribution of each polymorphism (see Supplementary Table I). As our aim was to study common allelic variants, we selected SNPs with MAF ≥ 0.05.

Figure 1 shows the pairwise LD estimates between the studied SNPs of the WFS1 gene region. R^2 (Fig. 1A) as well as D′ values (Fig. 1B) were assessed, the most apparent differences between the two measures could be observed regarding rs28716718 and rs1801208 polymorphisms. R^2 values suggested practically no LD between these two SNPs and the other sites, whereas D′ showed a high level of LD in this region. This finding can be demonstrated in that special form of LD, which can be characterized by the lack of exactly one haplotype that is one allele is freely combined with both alleles of the adjacent site; however, the other allele is always present together with one of the two variants of the neighboring polymorphism. The LD structure of WFS1 gene could be characterized by two (D′, block definition according to confidence intervals) or three (R^2, block definition according to Four Gamete Rule) haploblocks as labeled in Figure 1. All SNPs were used for the subsequent association analysis.

Individual scores for Aggression, Impulsiveness, Anxiety, and Depression were derived from self-report questionnaire data. Mean values are listed for all three genotypes in Table II. Based on the applied recessive model of the minor allele we compared mean values of minor homozygotes (mm) to those carrying the major allele (MM and Mm) for each polymorphism. Results of these one-way ANCOVAs are depicted in Table II, sex and age were used as covariates.

The WFS1 rs1046322 showed significant association with Aggression ($F[1,797] = 12.241$, $P = 0.0005$, $\eta^2 = 0.015$, power = 0.938), Impulsiveness ($F[1,791] = 6.362$, $P = 0.012$, $\eta^2 = 0.008$, power = 0.712), Anxiety ($F[1,797] = 7.801$, $P = 0.005$, $\eta^2 = 0.010$, power = 0.797), and Depression ($F[1,797] = 5.074$, $P = 0.025$, $\eta^2 = 0.006$, power = 0.614). These scales are highly related, correlation values reported below are all positive with a P value lower than 0.0001 Aggression showed a strong correlation with Impulsiveness ($r = 0.43$) and Anxiety ($r = 0.41$) and a somewhat weaker correlation with Depression ($r = 0.37$). Impulsiveness was related to both Anxiety ($r = 0.27$) and Depression ($r = 0.25$). Correlation was the highest between anxiety and depression ($r = 0.57$). Participants with the homozygous form of the rs1046322 minor allele (mm) reported significantly higher aggression, they were also more impulsive, more anxious and more depressed than those with genotypes including the major allele (see total scores for each self-report scale in Table II).

Other WFS1 SNPs did not show significant association with Aggression, Impulsiveness, or Anxiety scores. Depression was associated with two other WFS1 SNPs from the first haploblock: rs10002743 ($F[1,678] = 6.777$, $P = 0.009$, $\eta^2 = 0.01$, power = 0.739) and rs6824720 ($F[1,673] = 6.789$, $P = 0.009$, $\eta^2 = 0.01$, power = 0.740). These SNPs are in strong LD with each other, but not with the WFS1 rs1046322 polymorphism (see Fig. 1).

Due to performing multiple comparisons, the accepted level of significance was corrected to rule out false positive results. From the nominally significant associations (labeled by bold in Table II) only effect of the WFS1 rs1046322 on Aggression remained significant after the stringent Bonferroni correction (corrected P value was 0.0007). As highlighted in Table II, participants with the AA genotype (homozygous for the minor allele) showed the highest aggression (mean total score = 85.8), whereas subjects with the AG and GG genotypes scored notably lower (their mean total score was 66.3 and 66.4, respectively). It is important to note that only 9 participants out of 801 carried this rare variant (see Supplementary Table II for genotype distribution). To test consistency of this genetic effect we calculated mean aggression raw scores for each factor, and compared the average Verbal Aggression, Anger, Hostility and Physical Aggression values for the WFS1 rs1046322 genotypes. Figure 2 shows that aggression level of those with the AA variant are notably higher in all four dimensions as compared to aggression level of carriers of the major allele (AG and GG genotypes). This result indicates that the applied genetic model fits data from the different dimensions of aggression as well as the main construct.

Molecular-Functional Study of rs1046322

The rs1046322 SNP which had a significant effect on aggression, impulsiveness, and depression is located in the 3′ UTR of the WFS1 gene (see Table I) and does not fall into any of the identified haploblocks (see Fig. 1). This SNP was selected as a putative

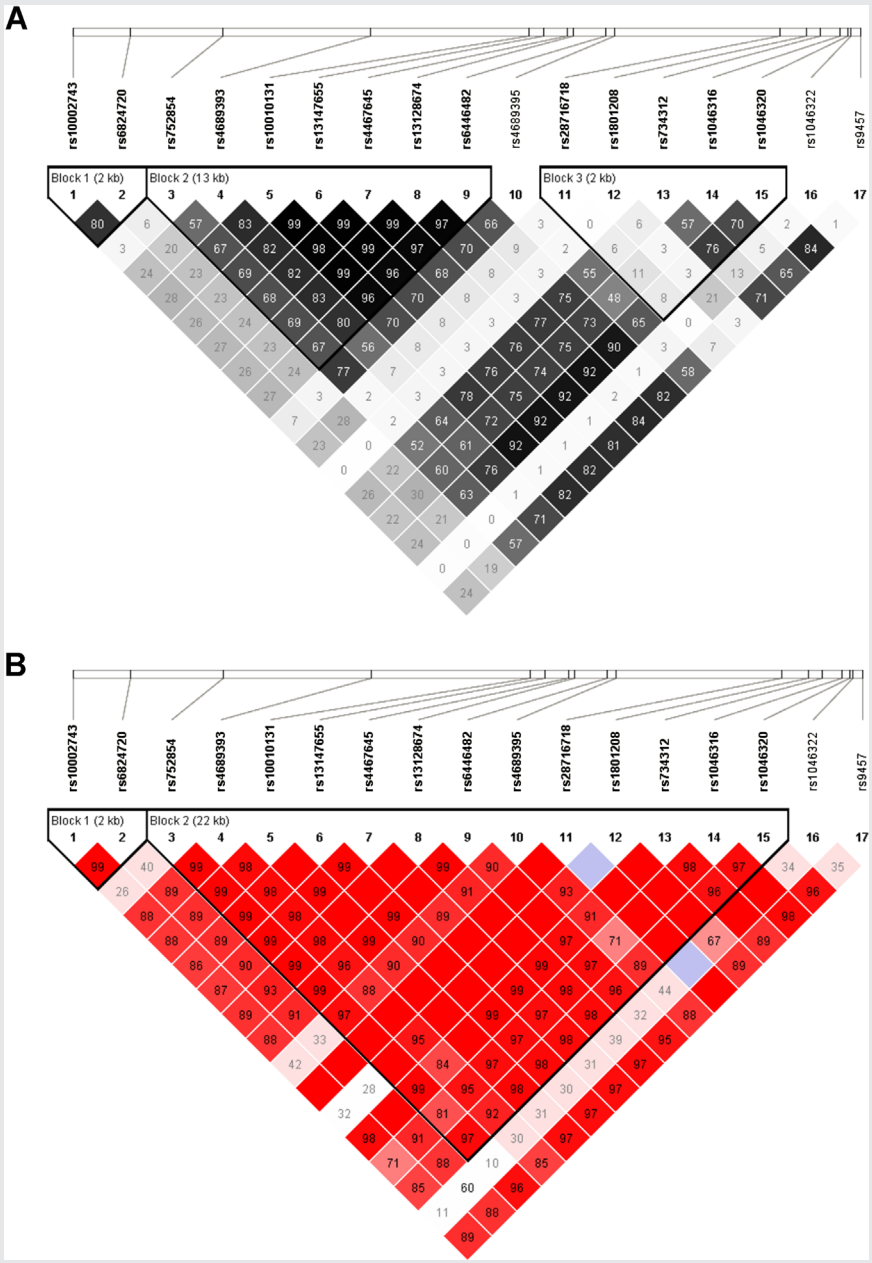

FIG. 1. Linkage disequilibrium plots of the WFS1 SNPs. A: R^2 (%) measure of linkage disequilibrium between any pair of SNPs, as determined by HaploView, version 4.2. The gray-scale spectrum indicates pairwise R^2 values ranging from black ($R^2 = 1$) to white ($R^2 = 0$). B: Pairwise LD values expressed by D′ (HaploView v4.2). Red squares indicate 100% LD.

microRNA binding site polymorphism (miRSNP). According to the PolymiRTS database, the minor allele (A) generates a mismatch in the target site of the seed region of miR-668. To explore whether the rs1046322 polymorphism alters the miR-668 binding efficiency under experimental conditions, a firefly luciferase reporter vector containing the 3′ UTR region of the WFS1 gene (pMIR-G) was constructed, and its minor allelic variant was generated by site directed mutagenesis (pMIR-A). The control construct (pMIR-c) possessed an independent sequence with the same length, lacking any complementary region with miR-668. HEK293 cells were cotransfected with miR-668 and with pMIR-G, pMIR-A or pMIR-c, respectively, transfection efficiency was determined by β-galactosidase reporter vector. The average values of the relative luciferase activities are shown in Figure 3. Expression level was decreased by miR-668 in the presence of both allelic variants (pMIR-A and pMIR-G) compared to the control (pMIR-c). The wild type 3′ UTR variant (pMIR-G) inhibited the luciferase expression upto 28% of the control, while pMIR-A resulted only in a 47%

TABLE II. Association of WFS1 Genotypes With Self-Report Measures

	Aggression (Buss–Perry)				Impulsivity (Barratt)				Anxiety (HADS)				Depression (HADS)			
WFS1 SNP	(MM)	(Mm)	(mm)	P	(MM)	(Mm)	(mm)	P	(MM)	(Mm)	(mm)	P	(MM)	(Mm)	(mm)	P
rs10002743	66.2	68.1	66.1	0.8711	58.3	59.8	61.1	0.2133	5.7	5.7	6.5	0.3196	2.5	2.7	3.8	0.0094
rs6824720	66.4	67.1	64.1	0.5821	58.8	59.7	61.6	0.2611	5.7	5.6	6.7	0.3235	2.5	2.7	4.1	0.0094
rs752854	67.5	66.5	66.8	0.8727	58.6	58.8	57.9	0.5744	5.7	5.5	5.3	0.6456	2.7	2.6	2.6	0.9468
rs4689393	66.2	67.5	66.6	0.9975	58.0	59.4	59.4	0.5180	5.5	5.7	5.6	0.9226	2.4	2.7	2.7	0.6312
rs10010131	66.8	66.2	64.8	0.3542	58.1	59.8	58.5	0.6086	5.8	5.9	5.4	0.2070	2.6	2.5	2.7	0.7347
rs13147655	66.6	67.4	66.1	0.7131	57.9	59.4	58.7	0.9993	5.7	5.7	5.5	0.4438	2.6	2.7	2.8	0.7246
rs4467645	66.5	67.2	66.2	0.7985	58.3	59.4	58.9	0.9656	5.6	5.8	5.6	0.6563	2.6	2.6	2.7	0.6657
rs13128674	66.9	67.4	67.2	0.8759	58.1	58.8	59.0	0.6345	5.5	5.6	5.6	0.8510	2.7	2.7	2.9	0.6256
rs6446482	66.3	67.3	67.0	0.8364	57.8	59.4	59.1	0.6555	5.5	5.7	5.6	0.8530	2.5	2.6	2.8	0.4933
rs4689395	66.6	66.6	67.0	0.8654	58.6	59.2	59.0	0.9261	5.7	5.7	5.5	0.7063	2.6	2.6	2.7	0.9011
rs28716718	66.5	66.8	72.2	0.4960	58.8	59.9	57.0	0.6773	5.6	5.7	7.0	0.5337	2.6	2.8	3.8	0.3753
rs1801208	66.6	68.5	72.5	0.5980	59.0	58.0	59.0	0.938	5.6	5.9	7.8	0.1544	2.6	2.7	2.8	0.9045
rs734312	66.0	67.4	66.5	0.9215	58.7	58.8	58.9	0.8644	5.6	5.7	5.7	0.9185	2.6	2.6	2.8	0.4407
rs1046316	67.3	67.5	66.4	0.8790	58.8	58.2	59.7	0.3845	5.5	5.6	5.6	0.9628	2.7	2.7	2.8	0.8360
rs1046320	66.9	66.5	66.3	0.8793	58.1	59.3	58.4	0.6323	5.7	5.8	5.3	0.1697	2.7	2.7	2.5	0.5612
rs1046322	66.4	66.3	85.8	**0.0005***	58.6	59.1	66.9	**0.0119**	5.6	5.8	8.8	**0.0053**	2.6	2.7	4.7	**0.0246**
rs9457	66.3	67.3	65.8	0.5462	57.8	59.2	58.2	0.6403	5.4	5.8	5.5	0.6373	2.6	2.8	2.7	0.9249

Mean values of the total score are presented for each genotype. *Significant with Bonferroni correction.

silencing effect. ANOVA followed by post hoc analysis showed a statistically significant ($P < 0.05$) difference between the luciferase reporter activity of the pMIR-G and pMIR-A constructs (ANOVA $P = 0.0005$). Co-transfection of a control miRNA lacking a binding site in the WFS1 3′ UTR did not result in the decrease of relative luciferase activity.

DISCUSSION

Loss of function mutation of WFS1 gene leads to serious neurodegeneration and early death, suggesting a central role of this protein in the function of the nervous system. Studies on the common WFS1 polymorphisms to date focused primarily on major

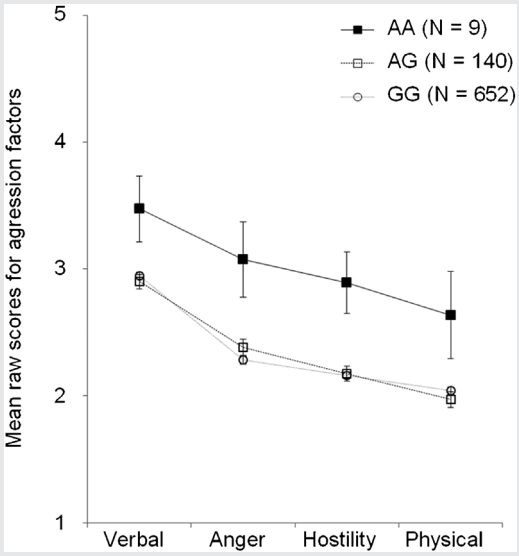

FIG. 2. The effect of the rs1046322 WFS1 SNP in the Buss–Perry Aggression Scale. Mean raw score values with standard errors of mean for the four aggression factors are shown.

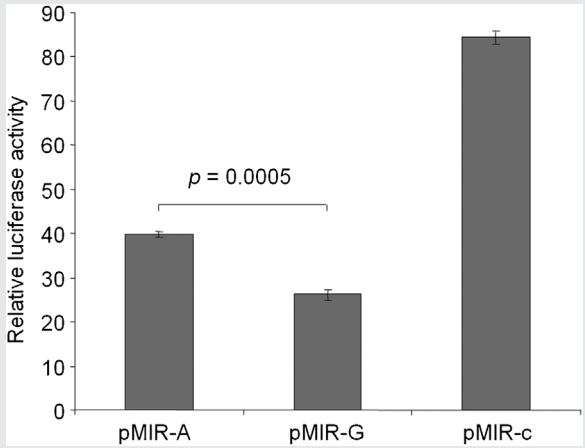

FIG. 3. The silencing effect of the rs1046322. HEK293 cells were cotransfected with miR-668 and with pMIR-G, pMIR-A or pMIR-c construct, respectively. Relative luciferase activities (mean ± SD) from three independent experiments are shown. ANOVA showed a statistically significant difference between the luciferase activity of pMIR-A and pMIR-G constructs ($P = 0.0005$).

depression, bipolar depression and suicidality. Kawamoto et al. [2004] examined association of mood disorders and certain haplotypes of WFS1. The p.H611R (rs734312) missense exon polymorphism of the WFS1 gene has been linked to mood disorders [Koido et al., 2005] in different populations [Zalsman et al., 2009]. Suicidal behavior is often a symptom of an underlying psychological disorder, especially mood disorders. Sequeira et al. [2003] found higher frequency of the WFS1 611R/611R genotype in a small sample of completed suicide victims in the general population. This result, however, has not been confirmed by Zalsman and coworkers [Zalsman et al., 2009]. A recent study of 4p chromosomal region identified various risk factors of completed suicide in males in several genes including the p.H611R (rs734312) of WFS1, but these results were not significant after the stringent corrections for multiple testing. Interestingly, the risk allele was A in this recent study in opposite to the results of Sequeira et al. [2003] where the G allele emerged as the risk factor of completed suicide.

Based on the above only a handful of association studies investigated the effect of certain WFS1 gene variants on mood disorders and suicide using clinical samples. Results are rather contradictory, which may be a consequence of the small sample size. It is also possible that the most widely investigated WFS1 p.H611R (rs734312) polymorphism is only a marker, being in LD with another functional polymorphism of the WFS1 gene, such as the miRSNP studied here, which has not been assessed previously.

Recent investigations suggested that miRNAs are important regulators of protein synthesis [Bartel, 2004]. There is a short (6–8 bp) complementary region between the miRNA binding site of the target mRNA and the "seed sequence" of miRNA. An SNP, which influences the microRNA binding (miRSNP), probably has a significant effect on the rate of target protein synthesis. Therefore, miRSNPs represent a novel class of functional polymorphisms, playing an important role in the genetics and pharmacogenetics of complex diseases [Bertino et al., 2007]. For example, the miRSNP of dihydrofolate reductase gene was associated with resistance to methotrexate, an anticancer drug [Mishra et al., 2007]. There are also a few studies emphasizing the role of the miRSNPs in neuropsychiatric disorders, such as Parkinson's disease and Tourette syndrome [Sethupathy and Collins, 2008]. The rs686 of the DRD1gene, previously associated with nicotine dependence, was shown to function as miRSNP [Huang and Li, 2009]. Recently, it was demonstrated [Jensen et al., 2009] that the rs13212041 in the serotonin receptor 1B gene, which was associated to aggression among college students, disrupts a sequence critical for a miR-96 binding. An additional evidence for this miRSNP was also provided by the same group showing an association of a haplotype involving rs13212041 with self-reported Anger and Hostility in young men [Conner et al., 2010].

Searching in various databases we found two putative miRSNPs (rs9457, rs1046322) in the WFS1 gene. According to our best knowledge, these common variants have not been tested previously in psychogenetic or diabetes mellitus related association studies. We found that one of these putative miRSNPs, the rs1046322 was associated with aggression and related dimensions. Our in silico studies indicated that the A allele weakens the binding of miR-668. We also provided experimental evidence about the effect of this SNP on miR-668 binding: coexpressing the cloned WFS1 3′ UTR possessing A or G alleles with miR-668 resulted in changes of luciferase reporter activity. These results suggest that the rs1046322 SNP has an important molecular function in the regulation of WFS1 gene expression and should be included into further association studies of the WFS1 gene. We hypothesize that the negative regulation of wolframin protein synthesis is less pronounced in the presence of the minor allele of the rs1046322 leading to an overexpression of wolframin and an imbalance in ER stress regulation. Limitation of our hypotheses is the lack of in vitro or in vivo studies that would support the role of miR-668 in the regulation of the wolframin protein synthesis.

Studies to date did not investigate association of WFS1 polymorphisms with impulsivity, aggression or mood characteristics within the non-clinical spectrum. According to the psychiatric literature, these dimensions are interrelated [Apter et al., 1990]. Results of the correlation analysis from our present study also showed that Aggression was in a strong correlation with Impulsiveness ($r = 0.43$, $P < 0.0001$) and Anxiety ($r = 0.41$, $P < 0.0001$), although they were assessed with different tools and in populations of different type and size.

The aim of the present study was to detect possible genetic effects of a comprehensive set of WFS1 polymorphisms, involving putative miRSNPs, on psychological dimensions using self-report data from 801 non-related, healthy Caucasians. Our set of 17 WFS1 SNPs included the p.H611R (rs734312) polymorphism that was previously associated with mood disorders; however, we did not find any association with impulsivity, aggression or mood characteristics in our large, non-clinical sample. Results from our association analyses revealed a novel risk factor of the impulsive-aggressive tendencies within the non-clinical range: The WFS1 rs1046322 polymorphism, which was identified here as a miRSNP, showed significant association with all the measured scales: Aggression ($P = 0.0005$), Impulsiveness ($P = 0.0119$), Anxiety ($P = 0.0053$), and Depression ($P = 0.0246$). Subjects homozygous for the minor allele (AA) showed higher aggression, higher impulsivity, higher anxiety and higher depression as compared to those without the rare risk genotype. It is important to note, that when correcting the level of significance to rule out false positive results of multiple comparisons only the effect of rs1046322 on Aggression remained significant. Although application of the stringent Bonferroni correction is an important principle in current association studies, we would like to emphasize that most of our nominally significant associations emerged between the WFS1 rs1046322 and the other three dimensions (Impulsiveness, Anxiety, and Depression) interrelated with Aggression suggesting a significant and complex effect of this miRSNP on the behavior.

Dimensions of Aggression, Impulsivity, Anxiety, and Depression are correlated and are linked to certain psychopathologies but also cause disturbances in the emotions, decision making and self-regulation within the non-clinical spectrum. Association studies highlight the role of various genetic and environmental predisposing factors in the polygenic model of these behaviors and acknowledge the importance of understanding interaction effects between these components through the study of psychiatric patients as well as the non-clinical population [Courtet et al., 2011]. To our knowledge, this is the first association study of a validated miRSNP

in the WFS1 gene. This functional polymorphism was shown as a genetic factor of individual variations in aggression.

Limitation of the present study is the applied self-report method for portraying such a complex dimension as aggression; however, this tool was sufficient for large-scale assessment. We tested 801 subjects, but there were only nine subjects (five males and four females) homozygous with the risk allele (AA) of the miRSNP (rs1046322) of the WFS1 gene, although the call rate for this SNP was 100% as a result of double genotyping (see Supplementary Table I). Recessivity of the risk allele might be explained by the identified molecular effect of the rs1046322 minor A allele. If microRNA serves as a fine tuning of gene expression, the ER balance could be disturbed only in the presence of both risk alleles. These subjects showed substantially higher levels of aggression (mean value was 86($\pm$22) as compared to the overall aggression average: 67 ($\pm$16). Due to the small number of subjects with the risk genotype replication of these findings would be important in an independent non-clinical population. Investigation of this novel functional polymorphism in clinical samples with mood disorders and suicide would also be essential.

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