RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART B
Neuropsychiatric Genetics

Glycogen Synthase Kinase 3 Beta Gene Structural Variants as Possible Risk Factors of Bipolar Depression

Zsolt Ronai,[1] Reka Kovacs-Nagy,[1] Eszter Szantai,[1] Zsuzsanna Elek,[1] Maria Sasvari-Szekely,[1] Gabor Faludi,[2] Judit Benkovits,[3] Janos M. Rethelyi,[3] and Anna Szekely[4]*

[1]Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary

[2]Department of Clinical and Theoretical Mental Health, Kutvolgyi Clinical Center, Semmelweis University, Budapest, Hungary

[3]Department of Psychiatry and Psychotherapy, Semmelweis University, Budapest, Hungary

[4]Institute of Psychology, Eotvos Lorand University, Budapest, Hungary

Manuscript Received: 28 May 2013; Manuscript Accepted: 10 January 2014

The glycogen synthase kinase 3B (GSK3B) is an important target protein of several antidepressants, such as lithium, a mood stabilizer. Recent studies associated structural variations of the GSK3B gene to bipolar disorder (BP), although replications were not conclusive. Here we present data on copy number variations (CNVs) of the GSK3B gene probing the 9th exon region in 846 individuals (414 controls, 172 patients with major depressive disorder (MDD) and 260 with BP). A significant accumulation (odds ratio: 5.5, $P = 0.00051$) of the amplified exon 9 region was found in patients (22 out of 432) compared to controls (4 of 414). Analyzing patient subgroups, GSK3B structural variants were found to be risk factors of BP particularly ($P = 0.00001$) with an odds ratio of 8.1 while no such effect was shown in the MDD group. The highest odds (19.7 ratio) for bipolar disorder was observed in females with the amplified exon 9 region. A more detailed analysis of the identified GSK3B CNV by a set of probes covering the GSK3B gene and the adjacent NR1I2 and C3orf15 genes showed that the amplified sequences contained 3′ (downstream) segments of the GSK3B and NR1I2 genes but none of them involved the C3orf15 gene. Therefore, the copy number variation of the GSK3B gene could be described as a complex set of structural variants involving partial duplications and deletions, simultaneously. In summary, here we confirmed significant association of the GSK3B CNV and bipolar disorder pointing out that the copy number and extension of the CNV varies among individuals.

Key words: copy number variation; structural variants; glycogen synthase kinase 3 beta; major depression; bipolar depression; GSK3B

How to Cite this Article:
Ronai Z, Kovacs-Nagy R, Szantai E, Elek Z, Sasvari-Szekely M, Faludi G, Benkovits J, Rethelyi JM, Szekely A. 2014. Glycogen synthase kinase 3 beta gene structural variants as possible risk factors of bipolar depression.
Am J Med Genet Part B 165B:217–222.

INTRODUCTION

Glycogen synthates kinase-3 is coded by two distinct genes (GSK3A and GSK3B). These ubiquitous serine/threonine protein kinases participate in various cellular pathways including neurodevelopment [Hur and Zhou, 2010]. While production of the coded proteins is constitutive, their activity is downregulated by phosphorylation during cell activation. Underphosphorylation or other changes in the regulation in GSK3B enzyme activity has been shown as risk factor of bipolar depression [Gould et al., 2004] and other psychiatric disorders, such as schizophrenia [Emamian et al., 2004] or Alzheimer disease [Van Wauwe and Haefner, 2003]. Lithium, a frequently used mood stabilizer in bipolar affective disorder, was shown to inhibit the enzyme coded by GSK3B gene both in vitro and in vivo [Klein and Melton, 1996]. Recent data support further evidences for the GSK3B protein as the target of lithium therapy in vivo and demonstrates that lithium disrupts the stabilizing effect of the GSK3B protein on the β-arrestin-2/Akt/PP2A complex [O'Brien et al., 2011]. In addition, the protein product of disrupted

Grant sponsor: NIH; Grant number: R03 TW007656; Grant sponsor: Fogarty International Research; Grant sponsor: Hungarian Scientific Research Funds; Grant numbers: OTKA K81466, OTKA, CK 80289, OTKA-PD 83876.

*Correspondence to:
Anna Szekely, Associate professor, Institute of Psychology, Eotvos Lorand University, Izabella u. 46, Budapest 1064, Hungary.
E-mail: szekely.anna@ppk.elte.hu

Article first published online in Wiley Online Library (wileyonlinelibrary.com): 21 February 2014
DOI 10.1002/ajmg.b.32223

in schizophrenia 1 (DISC1) gene, having allelic variants with established genetic risk factor of psychiatric disorders, was shown to inhibit the GSK3B enzyme activity through direct physical interaction [Mao et al., 2009]. These functional data suggest pathological consequences of abnormal GSK3B levels.

A significant source of individual genetic variability has been recently shown to originate from structural variations, termed also as copy number variations [CNVs, Iafrate et al., 2004; Sebat et al., 2004]. These chromosomal changes involve amplification or deletion of DNA segments with lengths from a few hundred to several million basepairs. There is a growing body of evidence supporting the role of structural variation in psychiatric disorders [Stankiewicz and Lupski, 2010], as well as in other common diseases [Fanciulli et al., 2010].

A real-time PCR method was previously developed and validated in our laboratory for detection of copy number variations of complement C4A and C4B candidate genes [Szilagyi et al., 2006] and applied for an association study of cardiovascular disease mortality [Arason et al., 2007; Blasko et al., 2008]. Moreover, we also demonstrated recently that the β-arestin gene dosage associated with the risk of pancreatitis [Tiszlavicz et al., 2010].

Expansion of the GSK3B structural variant was shown to involve nearby genes as well [Iafrate et al., 2004], namely NR1I2 (pregnane X receptor isoform 2) and C3orf15 (expressed exclusively in the testis). An association of increased copy number of this region was found in patients with BP using a probe complementary to the NR1I2 gene [Lachman et al., 2007], however this was not replicated by probing the exon 10 region within the GSK3B gene [Saus et al., 2010]. Probing the 9th exon region of the GSK3B gene here we replicated the association between GSK3B CNV and BP previously described by Lachman et al. using another probe. Moreover, fine mapping of this region in the present study revealed a wide variety of unusual structural variants, involving partial or complete amplification of GSK3B gene, as well as amplification of the downstream sequences combined with a deletion of the upstream region of the same gene.

MATERIALS AND METHODS

Ethics Statement

The study protocol was approved by the Hungarian Research Ethics Committee with signed informed consent from all participants after explanation of the purpose and the design of the study.

Subjects

Four hundred thirty-two patients with a current major depressive episode were recruited at the Department of Clinical and Theoretical Mental Health of Kutvolgyi Clinical Center and at the Department of Psychiatry and Psychotherapy, Semmelweis University. All patients were characterized by mood disorders according to the DSM-IV criteria. Diagnoses of MDD and BPD were established independently by two trained psychiatrists. Patients with any neurological or DSM-IV Axis I psychiatric diagnosis other than depression were excluded. The 414 control subjects were volunteers recruited at the Institute of Psychology, Eotvos Lorand University. Controls subjects had no past or current psychiatric history according to their self-report. Most important clinical and demographic characteristics of subsamples are summarized in Table I. Sex ratio did not differ significantly in patient or BP or MDD subgroups as compared to that of controls. Age of the control group, however, was significantly lower than that of the patient groups.

Assay of Copy Number Variations (CNVs)

DNA was isolated from buccal cells using the DNA-purification kit obtained from Gentra (Minneapolis, MN). Concentration and quality of isolated DNA was assessed by UV absorption at 230, 260, and 280 nm, as well as by gel electrophoresis. The average yield was 4 (range: 2–10) μg DNA/sample. Primers and probes for GSK3B exon 9, NR1I2 exon 9 and for RNase P control and conditions of TaqMan assays for measuring copy number were as described earlier [Szantai et al., 2009]. Primers and probes for C3orf15 exon 18 and GSK3B exon 5 have been obtained from Applied Biosystems (Grand Island, NY) (Hs02571551_s1 and Hs02558733_s1, respectively). NR1I2 5′ untranslated region (−179 to −80) was measured by forward primer (5′ TTGTGAGCCAGAAGGGATTTG 3′), reverse primer (5′ GGCATGACTCCAGCTCAGGTA 3′) and probe (5′ FAM–TACGTATGTTACCCATAACC–MGB 3′), while GSK3B promoter region (−415 to −266) was assessed by forward primer (5′ GGCCACTGTTGCCATTGTC 3′), reverse primer (5′ AGCCGATCAGCCTGAGAAAC 3′) and probe (5′ VIC–CCAGCCAGCCACCGA–MGB 3′). All probes were labeled with FAM or VIC reporter dyes at their 5′ end and a minor groove binding nonfluorescent quencher (MGB) at their 3′ end. Relative quantification of the assessed region was based on RNAse P control applied in the same sample, as described earlier [Szantai et al., 2009]. The gene region was considered as amplified (>2 copy number) or deleted (<2 copy number) if the average values of three parallel measurements were higher than 2.5, or lower than 1.5, respectively, in at least two, separate experiments.

RESULTS

As a first step, copy number variation was assessed by real time PCR using specific probe and primers for exon 9 of GSK3B and RNase P control as described earlier [Szantai et al., 2009]. Amplified (copy number > 2) exon 9 region was detectable in all subgroups (MDD: 3 of 172; BP: 19 of 260, control; 4 of 414, see Table II). There was a significant difference in occurrence of amplification among patients (MDD + BP) and controls according to the Pearson chi-square test ($P = 0.00051$). This association was significant across male ($P = 0.05$) and female ($P = 0.002$) subpopulations. The effect was more pronounced in the female subgroup (13 of

TABLE I. Clinical and Demographic Data of the Studied Samples

	Control (N = 414)	MDD (N = 172)	BD (N = 260)
Gender (M/F)	32.6%/67.4%	25.6%/74.4%	30.4%/69.6%
Mean age (years) ±SD	25.3 ± 8.3	49.0 ± 11.9	45.2 ± 11.7
Age (min–max)	18–71	19–70	19–70

TABLE II. Amplification of the GSK3B Exon 9 Region

	Wild type (n = 2)		Amplified (n > 2)				
	N	%	N	%		OR	*P*-value
Controls (C)	410	99.0	4	1.00			
Males	132	97.8	3	2.20			
Females	278	99.6	1	0.40			
Patients (P)	410	94.9	22	5.10	C vs. P	**5.5**	**0.00051**
Males	114	92.7	9	7.30	C vs. P in males	3.5	0.05
Females	296	95.8	13	4.20	C vs. P in females	12.2	0.002
MDD	169	98.3	3	1.70	C vs. MDD	1.8	0.43
BP	241	92.7	19	7.30	C vs. BP	**8.1**	**0.00001**
Males	72	91.1	7	8.90	C vs. P in males	4.3	0.03
Females	169	93.4	12	6.60	C vs. P in females	19.7	0.00007

N, number of individuals; n, copy number; OR, odd ratio; C, controls; P, patients (MDD + BP); MDD, major depressive disorder; BP, bipolar disorder.

296; OR = 12.2). Analyses of patient subgroups revealed that the accumulation of amplified exon 9 region was a significant ($P = 0.00001$) risk factor of bipolar disorder with an odds ratio of 8.1 (2.7–27 at 95% confidence interval), while its accumulation in major depression was not significant ($P = 0.43$). Analyses of gender subgroups within bipolar patients showed similar results; the odds ratio for bipolar disorder in females with the amplified exon 9 region was 19.7.

For further characterization of the structural variants, a set of TaqMan probes and primers (labeled as A–F in Fig. 1) were designed for positions located in GSK3B and in the two adjacent genes (C3orf15/AAT-1 and NR1I2). Please, note that the orientation of NR1I2 and GSK3B genes are opposite to each other. All the newly designed assay systems were validated for applicability of DNA dosage assays by testing the linear dependence of C_T values on the logarithm of DNA concentration from a serial dilution of the same sample (see Supplementary Fig S1). Copy number variation of the measured regions was defined as described in the Method section: if the average of the measured copy number in at least two separate experiments testing three parallel samples in each were higher than 2.5 or lower than 1.5, the measured region of the sample was accepted as amplified or deleted, respectively.

Surprisingly, the detected amplicons were different from the published GSK3B CNV variants, and showed a considerable variety aligned as variation_HU1, HU2, and HU3 as shown on Figure 1. The C3orf15/AAT-1 gene (position A) and the NR1I2 5′ upstream region (position B) were found to be excluded from the repeating units in all of the studied samples. On the other hand, the 3′ regions of both the NR1I2 and GSK3B genes (positions C and D) seemed to be an obligatory component of the identified repeating units. Complete amplification of GSK3B gene (probes C, D, E, and F) was shown only in two patients (one BP and one MDD). Partially amplified GSK3B gene region (probes C, D, E, but not F) was found in seven patients (5 BP and 2 MDD). In addition, the amplification of downstream sequences was combined with the deletion of upstream region of GSK3B in 17 samples as measured by probes E and F and labeled as variation_HU3. Occurrence of GSK3B CNV variation_HU3 was significantly ($P = 0.00115$) higher in BP patients (13 of 260) than in controls (4 of 414), while no such variant was found in our MDD (N = 172) group.

The estimated copy numbers in three different positions of the GSK3B gene showed a large individual variation (see: Supplementary Table SI). As the amplification-based methods are not sensitive enough for exact quantification, we grouped the individually determined copy numbers into two classes (lower than 5 amplicons and higher than 5 amplicons). There were two samples with completely amplified GSK3B genes (1 BP and 1 MDD patient) both of them possessing three copies of exon 9–exon 5 and the 5′ upstream region of the GSK3B gene (variation_HU1). The high (higher than 5) copy number of exon 9–exon 5 region was found in BP patients only, and was either combined

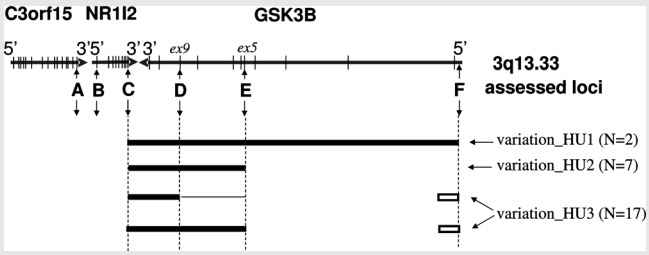

FIG. 1. Characterization of structural variants in the GSKB gene region. The name of the three genes in the assessed region, their orientation and the positions of their exons (vertical lines) are labeled on the top of the figure. Position of the applied probes are indicated by arrows and capital letters A–F (A: C3orf15 exon 18; B:NR1I2 5′ non coding region −179 to −80; C: NR1I2 exon 9; D: GSK3B exon 9; E: GSK3B exon 5; F: GSK3B 5′ non coding region −415 to −266). Main types of the structural variants determined by real time PCR (see Methods) are labeled as variation_HU1, HU2, and HU3. N: number of individuals possessing the labeled variant from the total of 846 participants. Filled lines: amplified region; solid lines: normal (2) copy number; empty lines: deletion.

(variation_HU3) or not (variation_HU2) with deletion at the other end of the gene.

DISCUSSION

A growing body of evidence demonstrates the contribution of CNVs to the genetic background of multifactoral psychiatric disorders, such as schizophrenia, BD and MDD [for a review see: Lachman, 2008]. One of possible approaches is the identification of rare, patient-specific CNVs by the analysis of large families, such as the discovery of the rare chromosomal translocation disrupting DISC1, a schizophrenia candidate gene [St Clair et al., 1990]. Structural variants, however, were detected in control populations as well. This fact initiated the adaptation of a widely used strategy in genetic association studies, the case-control setup comparing the frequencies of CNVs related to selected candidate genes. One of the first reports focused on copy number variations of the CHRFAM7A gene, obtaining a modest association ($P = 0.04$) between the psychosis phenotype and decreased copy number of the CHRFAM7A gene [Flomen et al., 2006]. The first genome wide analysis identified a total of 35 CNVs (22 gains and 13 losses) in 30 schizophrenic patients [Moon et al., 2006]. Most of the studies, however, provided contradictory results, as structural variants of specific genes seemed to be rare and often could not be assessed properly with the analyzed sample size.

The increased copy number of GSK3B gene locus among patients with BP was first demonstrated by Lachman et al. [2007] in a combined sample of US and Czech patients and controls. In this study the NR1I2 gene region was probed and a single deletion was found in the combined control sample (N = 275), while 4 deletions (less than 2%) and 6 duplications (less than 3%) were shown among patients (N = 225). Subsequent results of GSK3B structural variants using a probe for the exon 10 region of the GSK3B gene showed a relatively high (3–6%) occurrence of amplification in both the patient and control groups with no significant difference among 243 patients with MDD, 173 BP and 356 controls [Saus et al., 2010]. Here, we report a significant association ($P = 0.00051$) of increased copy number of GSK3B 9 exon region among MDD and BP patients (N = 432) compared to the control group (N = 414). Analyzing the patient subgroups, we found a significant increase in the occurrence of amplification among BP patients (7.3%) compared to the control group (1.7%) with an odds ratio of 8.1 and a *P*-value of 0.00001, replicating the association first described by Lachman's group. It should be noted, however, that the probe applied by Lachman et al. was located at the 5′ end of the NR1I2 gene, between our probes B and C. In the present article, however, amplification was detected only by probe C but not by probe B. On the other hand, we did not obtain any significant difference in CNV frequency of the MDD patient group compared to the control group in accordance with the results of Saus et al. [2010] concerning the MDD group. Here, we demonstrated, however, a significant association between GSK3B CNV and BP, which is in contrast with the results of Saus et al. It should be noted, that Saus et al. applied a probe located in exon 10th of GSK3B. This region was found to be an obligatory component of the repeating units identified here by probes C and D (see Fig. 1), thus, the different findings cannot be explained by differences in probe position. Interestingly, when the case-control analyses were stratified by sex, the highest odds (19.7 ratio) for bipolar disorder was observed in females with the amplified exon 9 region. These results are in line with previous results Szczepankiewicz et al. [2006], who found that the T-50C polymorphism of GSK3B gene was associated with bipolar disorder in females.

Our further analysis revealed that in most of the cases it is a segment rather than the complete GSK3B gene which is involved in the structural variation. Interestingly, the 3′ untranslated regions of both the NR1I2 and GSK3B (adjacent genes located in opposite directions) seemed to be an obligatory component of the amplified downstream region as measured by probe C (NR1I2 exon 9) and probe D (GSK3B exon 9, see Fig. 1 and Supplementary Table SI). Although sensitivity of amplification based methods is not high enough for determination of the exact copy numbers, a higher than 5 copy number of the GSK3B downstream region including exon 9 and exon 5 was clearly demonstrated in several BP samples.

Another novel finding of this study was that the amplification of the GSK3B upstream sequence was often combined with the

TABLE III. Structural Variants of the GSK3B Gene Region on the 3q13.33 Chromosome

Sample	Assigned term	Amplification	Deletion	Affected region	Reference
Control, N = 39	Variation_0035	1 (control)	1 (control)	C3orf15 3′ region, NR1I2 complete gene, GSK3B 3′ region	Iafrate et al. [2004]
Control, N = 36	Variation_6205	0	1 (control)	NR1I2 5′region	Mills et al. [2006]
	Variation_12326	0	1 (control)	NR1I2 5′region	Mills et al. [2006]
Control, N = 1 (JD Watson)	Variation_39343	0	1 (control)	NR1I2 1 intron	Wheeler et al. [2008]
Control, N = 1 (Nigerian man)	Variation_98419	0	1 (control)	GSK3B 7 intron	McKernan et al. [2009]
Control (N = 414); MDD (N = 172); BP (N = 260)	Variation_HU1	2 (1MDD, 1BD)	0	GSK3B complete gene	Present study
	Variation_HU2	7 (2MDD, 5 BD)	0	NR1I2 3′ region, GSK3B 3′ region	Present study
	Variation_HU3	17[a] (4 controls, 13 BP)		*Amplification*: NR1I2 3′ region and GSK3B 3′ region; *deletion*: GSK3B 5′ region	Present study

[a]Amplification and deletion detected in the same sample.

deletion at the 5′ promoter region as detected by probe F. From the technical point of view the coexistence of amplification and deletion in the same gene points out a possible source of contradiction in replication studies using single probes. On the other hand it was shown recently, that GSK3B gene expression is regulated at the microRNA level [Suh et al., 2012]. Based on this one might hypothesize that an overrepresentation of the 3′ region might result in a decreased effect of microRNA downregulation, which in turn could compensate the deleted upstream regulatory elements.

Our detailed analysis regarding extension of amplicons lead to the unexpected conclusion that the repeating units identified here are different from the previously found structural variants of GSK3B gene region (see: Table III). Copy number variation in the GSK3B gene region was originally described in an array-based comparative genomic hybridization (aCGH) using BAC (bacterial artificial chromosome) clones [Iafrate et al., 2004]. The CNV breakpoints in this study were defined as the size of the entire BAC clone, resulting in a copy number variant with all the three genes in this region (C3orf15, NR1I2, and GSK3B). Several smaller variations involving the NR1I2 but not the GSKB gene has also been reported later [Mills et al., 2006; Wheeler et al., 2008; McKernan et al., 2009]. It is important to note that all these variants were described in control individuals. Here, we identified three novel variants assigned as variation_HU1, variation_HU2 and variation_HU3. All structural variants possessed the 3′ segment of both the NR1I2 and GSK3B genes, but none of the variants contained the C3orf15 gene. There were only two samples (one patient with BP and another with MDD) where the complete GSK3B gene was amplified (variation_HU1). Segmental amplification of GSK3B gene at its downstream sequences was characteristic in BP patients. Moreover, the large majority of the samples with 3′ amplification were combined with a deletion at the 5′ end of GSK3B gene which might originate from different events on the homologous chromosome pair. Unfortunately, relatives of these participants were not available to clarify this question.

Results presented here are in line with the concept that copy number variants might play an important role in the genetic background of common diseases, and serves further evidence for the role of GSK3B in the development of mood disorders. Further molecular studies are needed, however, to clarify the molecular mechanism of the unusual CNV variants of the GSK3B gene detected here.

A possible limitation of our studies could be the relatively low sample size (N = 846), however, power calculations using the PGA software package [Menashe et al., 2008] indicate that size of our sample was sufficient. In addition, we cannot exclude the possibility of false positive association as a consequence of population stratification, as no genomic control was applied. Another limitation of the study is the significantly lower age of the controls compared to the patient sample. Therefore, further replication of association between bipolar disorder and GSK3B CNV variants is of vital importance. Moreover, the extension of the amplicons were estimated with a probe set but should be reinforced with sequencing. Determination of the precise CNV breakpoints in the different samples could also be of great interest for further studies aiming to explore these structural variants in different populations. The novelty of our analysis is that the fine mapping of the CNVs in the GSK3B region revealed several novel forms of structural variants including high copy number in the downstream region combined with a deletion in the upstream region of GSK3B. Further studies testing effect of these structural variants on the expression level of GSK3B gene would be of great importance.

ACKNOWLEDGMENT

This work was supported by the NIH R03 TW007656, Fogarty International Research grant to Maria Sasvari-Szekely and the following Hungarian Scientific Research Funds: OTKA K81466, OTKA CK 80289, and OTKA-PD 83876.

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