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Testing the role of circadian genes in conferring risk for psychiatric disorders

Enda M Byrne[1], **Psychiatric Genomics Consortium Major Depressive Disorder Working Group**[*], **Andrew C Heath**[2], **Pamela AF Madden**[2], **Michele L Pergadia**[2], **Ian B Hickie**[3], **Grant W Montgomery**[4], **Nicholas G Martin**[4], and **Naomi R Wray**[1]

[1] The University of Queensland, Queensland Brain Institute, QLD 4072, Australia.

[2] Department of Psychiatry, Washington University School of Medicine, St.Louis, MO, USA

[3] Brain and Mind Research Institute, University of Sydney, Sydney, Australia

[4] Queensland Institute of Medical Research, Brisbane, Australia

Abstract

Disturbed sleep and disrupted circadian rhythms are a common feature of psychiatric disorders, and many groups have postulated an association between genetic variants in circadian clock genes and psychiatric disorders.

Using summary data from the association analyses of the Psychiatric Genomics Consortia (PGC) for schizophrenia, bipolar disorder and Major Depressive Disorder, we evaluated the evidence that common SNPs in genes encoding components of the molecular clock influence risk to psychiatric disorders. Initially, gene-based and SNP p-values were analysed for 21 core circadian genes. Subsequently, an expanded list of genes linked to control of circadian rhythms was analysed.

After correcting for multiple comparisons, none of the circadian genes were significantly associated with any of the three disorders. Several genes previously implicated in the etiology of psychiatric disorders harboured no SNPs significant at the nominal level of $p < 0.05$, and none of the the variants identified in candidate studies of clock genes that were included in the PGC datasets were significant after correction for multiple testing. There was no evidence of an enrichment of associations in genes linked to control of circadian rhythms in human cells.

Correspondence to: Enda Byrne, The University of Queensland, Queensland Brain Institute, Upland Road, St.Lucia, QLD 4072, Brisbane, Australia, e.byrne3@uq.edu.au.

*PGC contributors and affiliations are listed in the Supplementary Table III

Conflict of Interest:

Professor Ian B Hickie is supported by a National Health and Medical Research Council Australia Fellowship (No. 511921). He was a director of headspace: the national youth mental health foundation until January 2012. He is the executive director of the Brain and Mind Research Institute which operates two early-intervention youth services under contract to headspace. He is a member of the new Australian National Mental Health commission and was previously the CEO of beyondblue: the national depression initiative. He has led a range of community-based and pharmaceutical industry-supported depression awareness and education and training programs. He has led depression and other mental health research projects that have been supported by a variety of pharmaceutical partners. Current investigator-initiated studies are supported by Servier and Pfizer. He has received honoraria for his contributions to professional educational seminars related to depression, youth mental health and circadian-rhythms research"

Our results suggest that genes encoding components of the molecular clock are not good candidates for harbouring common variants that increase risk to bipolar disorder, schizophrenia or Major Depressive Disorder.

Introduction

Disruption in circadian rhythms and perceived changes in sleep continuity and architecture are common features of psychiatric disorders. Insomnia or hypersomnia are part of the diagnostic criteria for a major depressive episode, while reduced need for sleep is a prodromal marker for manic episodes in Bipolar Disorder patients. Disruptions of circadian rhythms are also seen in psychiatric disorder patients. The phase of prolactin secretion, a hormone with a diurnal secretion pattern that counteracts the effects of dopamine has been shown to be advanced in male depression sufferers [1]. Similar results have been found for plasma melatonin [2] and plasma cortisol in BPD[3]. Furthermore, BPD and schizophrenia (SCZ) have been shown to be associated with later bedtime and increased nocturnal activity [4].

Many of the components that regulate the circadian clock have been identified in model organisms and humans. Foremost among these are the *CLOCK* and *BMAL1* genes, which act as positive regulators of the genes *PER1*, *PER2* and *PER3*, as well as *CRY1* and *CRY2*. The PER genes in combination with CRY act to inhibit their own transcription, thus acting as negative regulators of the system. Other genes such as *GSK3B*, *TIMELESS*, *CSNK1D* and *CSNK1E* also play a role in control of the clock through post-translational modification of other components such as PER, that decrease the rate of degradation. For a comprehensive review of circadian genetics see [5]

The observation of circadian disruption in psychiatric patients, and the high heritability (h^2 ~0.8) of SCZ and BPD have led to the hypothesis that polymorphisms in genes encoding components of the circadian clock may influence susceptibility to psychiatric disorders. As a result, several candidate gene studies have been undertaken to test this hypothesis. One study that analysed 48 SNPs in 8 circadian genes for association with BPD and SCZ found associations of *BMAL1* and *TIMELESS* with BPD [6], while another also found evidence for *BMAL1* and *PER3* [7]. A more recent study that examined 209 SNPs in 19 circadian genes in a combined sample of Major Depressive Disorder (MDD) and BPD patients found suggestive evidence of association with a SNP in *CRY1*, one in *NPAS2* and one in *VIPR2* [8], while a variant in *CLOCK* and another in *VIP* were associated specifically in the BPD subgroup. Another study that examined SNPs in 21 circadian genes for association with BPD, SCZ and schizoaffective disorder found suggestive evidence for a SNP in *NPAS2* and another in *RORB* with all 3 disorders [9].

Almost all of these suggested associations have never been replicated and all of the studies were underpowered to detect variants of modest effect on disease risk. Large genome-wide association studies have demonstrated that common variants of large effect are unlikely to exist for many human traits [10], including psychiatric disorders [11-13]. Furthermore, many associations identified in candidate gene studies have not replicated in large-scale genome-wide association studies. The requirement for large sample sizes to detect common variants

of modest effects led to the formation of the Psychiatric Genomics Consortium (PGC, https://pgc.unc.edu/). The consortium combines information on psychiatric cases and controls from multiple sites around the world. This effort has facilitated the discovery of a number of genome-wide significant associations with BPD [14] and SCZ [15], with some associated with both disorders. A PGC study of MDD was also recently reported [13]. While no genome-wide significant associations were detected, this was still the largest GWAS undertaken for MDD (9240 cases and 9519 controls) and the evidence of association for the most highly ranked SNPs could be strengthened by demonstrating association with traits correlated with depression. The large sample size collected for the PGC analyses affords greater power for testing circadian genes for association with psychiatric disorders than previously possible.

While the PGC studies did not report genome-wide significant associations for any circadian genes, the BPD and SCZ results do provide some supporting evidence for the link between circadian disruption and psychiatric disorders. A SNP (rs1006737) in a gene that encodes a component of a calcium channel found in the brain, *CACNA1C*, was one of the first identified common variants influencing BPD risk [16]. An analysis of specific MDD symptoms associated with the BPD risk allele in an independent sample found that insomnia was the only symptom with evidence of significant association[17]. The hypothesis that *CACNA1C* may have a role in sleep disruption was supported by a GWAS for sleep latency[18], a key component of disturbed sleep.

Although the PGC studies did not report genome-wide significant associations for any circadian genes, it is well recognised that true positives are amongst associated SNPs not achieving association at the level of genome-wide significance [19]. The overall aim of our study was to determine if the PGC genome-wide association studies (GWAS) provide any evidence in support of a role of risk variants in circadian genes. Initially replication was attempted for previously reported associations between polymorphisms in circadian genes with BPD, SCZ and MDD using the results from PGC studies; to test whether there is evidence for association of other genes involved in the wider circadian clock with psychiatric disorders at both the SNP and gene-wide level.

Methods

Clock Gene Analysis

Summary statistics for the genome-wide association studies of SCZ, BPD and MDD are freely available from the PGC websites (https://pgc.unc.edu/Sharing.php#SharingOpp, http://www.broadinstitute.org/mpg/ricopili/). A total of 2,415,422 SNPs from the BPD analysis, 1,252,901 SNPs from the SCZ analysis and 1,253,093 SNPs from the MDD analysis were included in the present analysis. All SNPs were autosomal and the different numbers of SNPs for the different disorders reflects imputation to different reference panels, HapMap 2 for BPD and HapMap 3 for SCZ and MDD. Details on the quality-control steps applied to the datasets are given elsewhere[13-15] Total sample sizes were 9,394 cases and 12,462 controls for SCZ, 7,481 cases and 9,250 controls for BD, and 9,240 cases and 9,519 controls for MDD.

Initially, we examined the evidence for association in the 21 circadian genes listed in Mansour et al [9], by performing a lookup of the single SNP test statistics in the PGC studies. We specifically focussed on variants previously reported to be associated with any of the three disorders in previous candidate studies, to test whether the evidence for association remains in the large sample size of the PGC. In cases where previously reported SNPs had not been genotyped or imputed in the PGC studies, we tried to identify a SNP in high linkage disequilibrium (LD) with the variant of interest.

Previous candidate gene studies have in many cases only examined a fraction of the common variants in the coding and non-coding regions of the clock genes. We evaluated the evidence for association at all available individual SNPs across each of the genes, including SNPs up to 50kb upstream or downstream of the genes of interest. We downloaded allele frequency and linkage disequilibrium data from the HapMap Project Phase III (Release III) for each of the core clock genes and tested how many of the SNPs with Minor Allele Frequency of greater than or equal to 5% were directly included in the PGC study or were represented by a proxy SNP ($r^2 >= 0.8$). On average 82% of the common variation in the clock genes were represented in the PGC study (range: 68-100).

A gene-based test of association was also carried out using the freely-available software VEGAS [20]. This test uses summary statistics from genome-wide association studies and performs multivariate gene-based simulation to account for the number of SNPs in a gene and the linkage disequilibrium between them to compute an empirical p-value for each gene based on the p-values for the individual SNPs. SNPs were considered to be part of a gene if they were located within 50kb of the start or stop site of the gene, to include regulatory regions. The output from VEGAS also provides the most significantly associated SNP from each gene.

Subsequently, we examined the evidence for association with genes previously identified as potential modulators of the clock in an siRNA screen for modulators of circadian period and amplitude in human cells [21]. An initial list of 343 of potential clock modulators first identified by Zhang et al[21] was extracted. 19 of those genes are located on the X chromosome and were excluded as genes on the sex chromosomes were not included in the PGC results for all disorders. In addition, a further 2 genes identified in Zhang et al were not included in the gene-based test owing to their not being any SNPs in the PGC results that mapped in those genes. A total of 322 genes circadian genes remained. We tested whether circadian genes were more significantly associated at the gene level than would be expected by chance as follows:

Firstly, all genes (n = 17,802) were ranked based on their gene-based p-value. Then, the ranks of the circadian modifier genes (n = 323) were extracted and the mean of the ranks calculated, in addition to the total number of SNPs across all of the genes. The mean of the ranks of the circadian genes was then compared to sets of 343 randomly selected genes. In each iteration, 343 genes were sampled from the list of all genes at random and the mean was calculated. As there was a small but significant negative correlation between a gene's rank and the number of SNPs in the gene ($r^2 = -0.01$, $p = 0.02$), a random gene set was discarded if the total number of SNPs in the set of random genes was not within 25 SNPs of

the total number of SNPs in the circadian genes. This process was repeated until 1,000 sets of 343 randomly selected genes had been drawn and the mean calculated for each set. The p-value of the set of circadian genes was calculated as the proportion of the 1,000 replicates that had a lower mean than the circadian genes. This process was repeated for all three psychiatric disorders.

Results

Core circadian genes list (n=21)

Table I shows the results of the gene-based test for the 21 circadian genes. More details (most associated SNP, percentile rank in gene-based test, percentile rank for most associated SNP) are provided in Supplementary Table I. These results show that while there are several circadian genes that harbor SNPs with nominally significant p-values, there are no associations beyond what would be expected after controlling for the number of SNPs in each gene. The gene-based test not only controls for the number of SNPs, but also combines evidence from multiple SNPs after controlling for the linkage disequilibrium between them. Hence, if any of the circadian genes contain many independent common variants each of small effect, this would likely be identified by the gene-based test, although this can depend on the genetic architecture of the gene. However, we see that there is no significant evidence of multiple effects in a gene for any of the PGC GWAS studies from the gene-based test.

In the SCZ analysis, no circadian genes had a nominally significant p-value, indicating there is little evidence for a role for common variants in these genes for influencing risk to SCZ. It is particularly noteworthy that *CLOCK* and *NPAS2*, core components of the molecular clock and for which unconfirmed associations with mood disorders have been found, harbor no SNPs with a nominally significant p-value (minimum 0.07). The results from the gene-based test indicate that *DBP1*, *AANAT* and *CSNK1E* are the best candidates among the circadian genes to influence SCZ risk. Five of the twenty-one genes have a SNP with a p-value less than 0.01 for association with SCZ, however none remain significant after correcting for multiple testing of SNPs within those genes (Supplementary Table I).

The evidence for a role for common risk variants in circadian genes is slightly stronger for BPD, with 2 genes – *BHLHB3* and *DBP1* - showing nominal significance for the gene-based test and 9 genes harbouring a SNP with a p-value < 0.01 (Supplementary Table I). The most significant single SNP association with BPD was with rs4132063 (p = 0.0006), a SNP located upstream of *CRY2*. This SNP remains significant after correcting the nominal significance threshold for the number of SNPs tested in the gene. It is notable that there are no SNPs in or near the *TIMELESS* gene that show evidence of association with risk to BPD, and similarly, the *CLOCK* gene harbours only one SNP that just reaches the nominal significance threshold (p =0.042), indicating that these genes are not strong candidates for influencing BPD risk in the population.

The *NR1D1* gene was the only circadian gene to show nominal significance with major depression (p =0.03). When ranking genes based on the most significant SNP, the strongest signal was rs9353523 in the *CNR1* gene (p=0.001). This gene ranked in the 95th percentile when ranking genes based on their most significant p-value.

When analysing the results from all genes in the gene-based test, 12,964 genes out of a total of 17,803 (72.8%) that were tested had at least one SNP within 50kb of the gene with a p-value less than 0.05, and 6,175 (34.7%) had a SNP with a p-value less than 0.01. Therefore, a gene having at least one SNP with nominal significance should not be considered strong evidence for a role for variants in that gene in influencing disease risk.

To compare the evidence from the most significant circadian genes to all of the other genes in the genome, we ranked all genes by their gene-based p-value and the p-value of their most significant SNP. None of the circadian genes rank in the top 5% of all genes when considering either the gene-based p-value or the p-value of the most significant SNP in the gene.

We also investigated whether any of the individual variants that showed evidence of association in candidate gene studies of BPD[6; 9; 22-25], MDD[22; 24; 26], and SCZ[6; 9] also show evidence in the PGC analyses (Supplementary Table II). It is noteworthy that none of the candidate SNPs were nominally significant with the same disorder in the PGC studies. Furthermore, the well-studied CLOCK3111T/C (rs1801260) polymorphism did not show evidence of association with any of the three disorders. There is therefore little evidence for a role for candidate SNPs in clock genes in affective disorders or schizophrenia.

Expanded circadian genes list (n=323)

The search for evidence for a role for circadian genes was expanded to include genes that have been linked to control of circadian clock in human cells. There was no overall evidence for an enrichment of associations at the gene level among the list of clock modifiers (p = 0.47 for BPD, p = 0.49 for SCZ and p = 0.51 for MDD). The circadian genes that ranked in the top 5 percentile of all genes based on the gene-based association test are listed in Table II. Full details are listed in Supplementary Table III.

The most significant evidence was found for *HIST1H1B* with SCZ (gene-based p = 0.00001). This gene is located in a cluster of histone genes on chromosome 6 and signals of association have been detected in this region in several studies. Owing to the linkage disequilibrium in this region, many genes show evidence of association. The genes *BTN2A2* and *BTNL2* are also located in this region and all 3 genes affect the periodicity of gene expression in cells when knocked down and hence represent strong candidates for functional follow-up for a role in schizophrenia.

The most significantly associated circadian gene with BPD is the *TUBA1B* gene on chromosome 12 (p = 0.000002). This gene encodes a subunit of the tubulin complex. The gene *CNNM4* on chromosome 2 is located downstream of the genome-wide significant SNP (rs6746896, BPD $p = 2.45 \times 10^{-9}$) and ranks second among the extended circadian gene list for association with BPD. This gene is thought to play a role in ion transport and mutations therein are associated with Jalili Syndrome, which is characterised by cone-rod dystrophy and amelogenesis imperfecta[27].

The most significant gene in the MDD analysis was *FHIT*, which encodes Fragile Histidine Triad. It is notable that this gene shows evidence of association with MDD because a

polymorphism in the *FHIT* gene was among the top hits in a previous association study of daytime sleepiness [28; 29]. McCarthy et al note that this gene is associated with ADHD and schizophrenia in addition to having altered expression in the mouse brain in response to lithium[30]. Multiple sources of evidence therefore suggest *FHIT* as a strong candidate for association with psychiatric disorders.

Discussion

Genes known to play a role in the circadian clock have long been considered candidate genes for psychiatric disorders, and variants therein have been tested in a number of association studies. The results of our analyses which are based on those from the Psychiatric Genomics Consortium, the largest association studies performed for any psychiatric disorders, indicate that common variants in circadian genes are unlikely to play a significant role in the etiology of psychiatric disorders. When ranking all genes based on the most significant SNP that is located within or near them, from the 21 core circadian genes only *CNR1* ranked in the top 5% of for any of the 3 disorders under analysis (95^{th} percentile with MDD, Supplementary Table 1). Furthermore, the results of the gene-based test indicate that none of the circadian genes carry multiple independent common variants that are associated with increased disease risk.

One other previous study has investigated the role of clock genes in conferring risk to mood disorders [30]. McCarthy et al identified an increase in associations in clock genes in BPD relative to random lists of similar numbers of genes. The analysis performed in this study differs in several ways. Firstly, their study used results from several GWAS studies and analysed the evidence for each one separately. Therefore, if a variant was associated in one study for a particular disorder, but showed no evidence of association in another, the variant would be counted as having an association. In contrast, our study analysed data from large meta-analyses of multiple datasets that combined the information across studies to give a single measure of association. In this way, if a SNP was associated at $p < 10^{-3}$ in one study, but not in any others, it is not considered to be associated in our analysis. But if a SNP that is associated at $p < 10^{-3}$ in the PGC meta-analysis, it suggests that there is evidence for association across studies. Secondly, our study utilised the latest publicly available results from the PGC, and hence the power to detect true associations is higher than in the McCarthy et al study. Their study lists a number of GWAS studies that were not included in their study, but the majority are included as part of the PGC meta-analyses. Lastly, McCarthy et al consider only associated SNPs and correct for gene length. This does not correct for the number of SNPs in a gene or for the linkage disequilibrium between SNPs in a gene, both of which are accounted for in the gene-based test. These two parameters can vary from gene to gene, and hence their inclusion in the association test should provide a more accurate correction for multiple testing in a given gene.

Despite the lack of associations with clock genes in our study, there are clear links between disturbed sleep, circadian timing and mood and thought disorders. We investigated whether other genes that have been linked to altered circadian rhythms show strong evidence of association with mood disorders. Several candidate genes emerged from this analysis including *HIST1H1B* SCZ, *CNNM4* with BPD, and *FHIT* with MDD. However, few other

genes linked to the circadian clock were identified as associated with SCZ, BPD or MDD. This supports the finding that candidate genes for SCZ show no more evidence than would be expected by chance in a large meta-analysis of SCZ genome-wide association studies [31].

There is a wealth of biological and epidemiological evidence linking disruption of the circadian clock and psychiatric disorders. Furthermore, a significant proportion of the overall risk for these disorders is due to genetic risk factors. The lack of evidence for association with common clock variants support the hypothesis that disruption of the circadian system may be consequence rather than a cause of the illness. The results also support the hypothesis that common variants in genes that are not part of the clock act to disrupt the clock through some as yet unknown mechanism. The results of the PGC GWAS studies, combined with analyses that examine the overall contribution of all common SNPs to psychiatric disorders suggest that the effects of common variants in any given gene will be small. However, genes such as CACNA1C and ODZ1 clearly harbour variants with stronger risk than other genes, and functional testing of the effects of robustly associated common variants on the clock will likely prove more fruitful for our understanding of circadian disruption in psychiatric disorders than further studies of common clock variants. Moreover, our results suggest that it will be more beneficial to our understanding of the role of genetic variation in mental health for smaller studies to focus on trying to replicate variants that approach genome-wide significance in the PGC analyses than to focus on the core circadian genes.

The circadian genetic system has been highly conserved over evolutionary time and a disruption of the clock can have serious consequences. It is therefore likely that variants that cause even a slight disruption of the system would be subject to strong negative selection. Such variants are more likely to be rare in the population. Hence, analysis of the effects of rare variants in clock genes may prove more fruitful in the future. It is worth noting that the sample sizes required to reliably demonstrate association between rare variants and complex diseases will likely be even bigger than those required for GWAS. Just as the results from small candidate gene studies of common SNPs have for the most part not replicated in larger studies, candidate studies of rare variants in the clock gene should be interpreted with caution.

Limitations

While there is little evidence to suggest a role for common variants in clock genes in increasing risk to psychiatric disorders, this does not preclude a role for multi-SNP haplotypes or rare variants in these genes. Our study was unable to assess potential epistatic effects between variants in clock genes or to test other for other non-additive effects as we only used summary statistics from the GWAS studies. Moreover, our use of most associated SNP or whole gene association represent only two out of many possible genetic architectures of causal variants within clock genes. For example, a set of nominally associated markers in the first exon of a gene may not generate a significant gene-based test in a long gene. Choosing the optimal genetic architecture for each gene is not possible without independent knowledge of gene function. Lastly, our study does not address the

potential role of clock gene variants in other aspects of the disorders, such as age-at-onset [32] and response to treatment [33], for which associations have previously been found.

Conclusions

Despite the large number of candidate gene studies that have identified polymorphisms associated with mood and other psychiatric disorders, our study shows that the risk conferred by common variants in core components of the molecular clock is likely to be small relative to common variants in other genes. Several other genes that show evidence for a role in control of circadian gene expression show stronger evidence of association and represent statistically stronger candidates for follow-up studies of the role of circadian genes in psychiatric disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table I

Association p-values for gene-based tests of the 21 core circadian genes

Gene	Chr	Length(kb)	SCZ	BPD	MDD
AANAT	17	2.55	0.17	0.71	0.24
ARNTL	11	109.49	0.53	0.26	0.51
ARNTL2	12	87.48	0.42	0.36	0.95
BHLHB3	12	4.89	0.60	0.03	0.74
CLOCK	4	114.34	0.98	0.29	0.54
CNR1	6	5.47	0.76	0.11	0.12
CRY2	11	36.13	0.21	0.07	0.68
CSNK1D	17	29.33	0.38	0.60	0.58
CSNK1E	22	27.39	0.20	0.83	0.80
CSNK2A1	20	61.15	0.67	0.20	0.80
DBP1	19	6.82	0.13	0.04	0.58
EGR3	8	5.64	0.77	0.10	0.63
NFIL3	9	14.82	0.41	0.19	0.16
NPAS2	2	176.68	0.87	0.47	0.39
NR1D1	17	7.93	0.64	0.31	0.03
PER1	17	11.97	0.40	0.15	0.61
PER2	2	44.53	0.52	0.45	0.67
PER3	1	60.48	0.24	0.14	0.64
RORB	9	189.87	0.21	0.37	0.66
TIMELESS	12	32.26	0.33	0.27	0.70
TIPIN	15	20.05	0.88	0.06	0.36

Table II

Genes from the expanded circadian genes list that have gene-based association in the top 5% of all genes

Disorder	Gene (Chr/percentile)
SCZ	HIST1H1B (6/99), BTNL2 (6/99), BTN2A2 (6/999), RRP12 (10/98), HEATR5B (2/98), RBX1 (22/98), PLEKHJ1 (19/98), SLC23A1 (20/97), MKL1 (22/97), DDX56 (7/97), SFN (1/97), SPR (2/96), CENPM (22/95), CNNM4 (2/95), NPC1L1 (7/95)
BPD	TUBA1B (12/99), CNNM4 (2/99), LYG1 (2/99), HIST1H1B (6/99), CCDC87 (11/99), CSE1L (20/99), OR2W1 (6/98), BTN2A2 (6/98), RRP12 (10/98), GIPC2 (1/98), KCNG2 (18/98), CKLF (16/98), MKL1 (22/98), SERPINC1 (1/97), STX4 (16/97), UBAP2 (9/96), TNK2 (3/96), BMP4 (14/96), SRPRB (3/95), BOLA3 (2/95)
MDD	FHIT (3/99), SART3 (12/99), UBA3 (3/99), C1orf85 (1/99), CNNM4 (2/99), SUV420H1 (11/99), OPN5 (6/99), TUBA1B (12/99), RCVRN (17/99), BTNL2 (6/99), GPR124 (8/98), MAP7D1 (1/98), LYG1 (2/98), KCNT2 (1/97), CNOT2 (12/97), FRAP1 (1/97), INTS2 (17/97), CDC73 (1/97), GPR137 (11/96), GPR158 (10/95), PTK2 (2/95), TBCB (19/95)