湖南大学学报（自然科学版）
Journal of Hunan University (Natural Sciences)

第 50 卷 第 12 期
2023 年 12 月

Available online at http://jonuns.com/index.php/journal/index

Vol. 50 No. 12
December 2023

Open Access Article

https://doi.org/10.55463/issn.1674-2974.50.12.10

Genetic Variants of Voltage-Gated Ca^{+2} Channel Genes, *cacna1c* and *cacnb2*, Conferring the Risk of Schizophrenia in Patients from Pakistan

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Received: September 22, 2023 / Revised: October 26, 2023 / Accepted: November 28, 2023 / Published: December 29, 2023

Abstract: Schizophrenia (SCZ) is a chronic mental disorder with a lifetime risk of approximately 1%. It is characterized by a profound disruption in cognition, behavior, and sensation with heritability estimated at up to 80%. It is a disorder with a complex etiology that involves multiple and heterogeneous genetic factors. The polygenic model of genetic susceptibility of psychiatric diseases explains that genetic variants of multiple genes determine the overall risk of disease because of their multiplicative effect. Two subunits of voltage-gated calcium channels, *cacna1c* and *cacnb2* genes, play important roles in balancing calcium in brain cells, and their single-nucleotide polymorphisms (SNPs) have been associated differently with schizophrenia risk in different ethnicities. Two SNPs (rs1006737 & 4765913) of *the cacna1c* gene and a SNP (rs12257556) of the *cacnb2* gene have been associated with schizophrenia. In this study, confirmed diagnosed schizophrenia patients and age-matched healthy control subjects were analyzed for the association of SNPs (rs1006737, 4765913 & rs12257556) with schizophrenia risk in Pakistani patients. Both SNPs of *the cacna1c* gene (rs1006737 and rs4765913) showed significant association with schizophrenia risk in this population. Moreover, two haplotypes (AT and GA) out of four for SNPs (rs1006737, rs4765913) of *cacna1c* further verified their significant association with the risk of schizophrenia. For *cacnb2* , no significant association with the risk of schizophrenia in the SNP (rs12257556) was observed. The results of this study offer compelling evidence of a strong link between *cacna1c* polymorphisms (individual variant & haplotypes) and schizophrenia risk in the Pakistani population.

Keywords: single-nucleotide polymorphisms, rs1006737, rs4765913, rs12257556.

使用人工智能技术的软件质量保证：巴基斯坦软件行业调查

摘要：软件产业以质量为基础，以市场为导向的软件产品至关重要。在软件行业中，质量保证（质量保证局）起着关键作用，也称为软件测试。测试策略可以包括几个需要考虑的

步骤，例如所有权、无脚本测试、文档和报告文学以及测试频率。测试为提议的软件产品提供软件质量并满足客户满意度。软件产品测试和交付比未经验证的软件一致性、预期性和非异常资源更合适。一些重要的软件测试类型是单元测试、集成测试、系统测试、健全性测试、冒烟测试、界面测试、回归测试和贝塔测试。人工智能方法将通过新的测试和回归测试方法，通过描述人工智能质量保证局工具的影响因子来提高应用程序的质量。它还将有助于开发高质量、不安全性较低且更安全的软件。人工智能可以帮助管理复杂的数字领域，例如动态分散的网络、监控、机器人和下一代数字服务（通常归为“下一代互联网”术语）。产出可以帮助促进基于人工智能的特定领域的工作，这些领域可能是实施现代数字平台智能活动所必需的，这些活动涉及涉及质量保证创新、外观和实施的密切相互关系。

关键词：单核苷酸多态性，rs1006737，rs4765913，rs12257556。

1. Introduction

Schizophrenia is a severe chronic mental disorder that may include delusions, false beliefs, unclear or confused thinking, agitation, social withdrawal, psychosis, and bizarre behavior [1]. People with schizophrenia cannot distinguish between the real and the unreal because they have an altered perception of reality. They think that others are harming, watching, cheating, spying on, poisoning, harassing, and plotting against them [2]. Symptoms of schizophrenia are categorized into three broad types: positive symptoms, negative symptoms, and cognitive symptoms [3]. The prevalence of schizophrenia is 1% worldwide and is equally common in men and women [4]. It has also been observed that schizophrenia is more severe in men than in women [5].

Schizophrenia is a heterogeneous and complex neuropsychiatric disorder, and both environmental and genetic factors are involved in its onset. Genome-wide association studies highlight schizophrenia as a highly polygenic disorder, and abnormal connections between different gene networks can also lead to schizophrenia [6]. Different pathways, including dopaminergic, glutamatergic, GABAergic, and calcium channels, have been causative of the disease. Moreover, it has been suggested that social environmental stressors have a stronger influence on genes involved in dopamine sensitization [7]-[8].

Recently, voltage-gated calcium channels have become major contributors to the pathogenesis of several psychotic disorders, including schizophrenia, and have been considered as potential therapeutic targets [9]. Voltage-gated calcium channels mediate depolarization-induced calcium entry into neurons. Because calcium channels are critical to brain function, their inappropriate expression or dysfunction results in a variety of neurological disorders, including schizophrenia [10]. The pleiotropic effects on the psychopathology of individuals with variation in calcium channel activity genes have been reported previously. The two subunits of voltage-gated calcium channels include *cacna1c* and *cacnb2,* which are involved in calcium balance in brain cells, and their single-nucleotide polymorphisms (SNPs) have been linked to schizophrenia [11].

The *cacna1c* gene spanning is an approximately 645-kb genomic region with 56 exons. It encodes the alpha-1C subunit of the L-type voltage-dependent calcium channel Cav1.2, which maintains a transient increase in membrane permeability for calcium-causing cell membrane depolarization, leading to activated intracellular gene transcription [12]-[13]. This gene transcription plays a major role in the development of dendrites, neuronal survival, memory formation, cognition, and behavior [14]-[15]. Recent studies have shown that *cacna1c* rs1006737 may have different effects on working memory in diseased patients of different populations, highlighting the need for further studies in different populations [16]. The *cacna1c* gene, which codes for subunit alpha-1C of the Cav1.2 voltage-dependent L-type calcium channel, has been consistently found to be a shared risk gene for several types of mental disorders in the Chinese Han population. It has been reported that *the cacna1c* SNP rs1006737 is associated with increased amygdala activity during emotional processing in different diagnostic groups of patients with bipolar disorder and schizophrenia [17].

Another gene, *cacnb2, also* manufactures part of the voltage-gated calcium channels. One of the most heavily implicated genes encodes a subunit of an auxiliary voltage-dependent L-type calcium channel that is mainly expressed in heart and brain tissue [18]. The cacnb2 subunit interacts with L-type calcium channel subunits, including *cacna1c*, to promote their trafficking to the plasma membrane, enhance their function, and modulate their expression by other molecules [19]. This subunit is essential for normal

surface expression, adequate trafficking, and functioning of voltage-gated calcium channels. By function, the calcium channel beta-2 subunit encoded by *cacnb2*, with the calcium channel alpha2/delta subunit, influences the kinetics and expression of CaV1.2. CaV1.2 is encoded by *cacna1c*, which is a well-established susceptibility gene for SCZ [11], [20]. The genetic variant rs12257556 of the *cacnb2* gene was one of the top hit regions in a GWAS of bipolar disorder I in the Chinese Han population [21]-[22]. Another study also highlighted *cacnb2* as a candidate gene and variants of *cacnb2*, including rs12257556, with schizophrenia risk [23].

In this study, the genetic associations of two genetic variants (rs1006737, rs4765913) of *the cacna1c* gene and a genetic variant, rs12257556, of *cacnb2, conferring the risk* of schizophrenia, were investigated in a case–control study in a Pakistani population. The identification of disease susceptibility loci may lead to a better understanding of the biological mechanism of schizophrenia, which will pave the way for better diagnostic, prevention, and treatment of schizophrenia.

2. Methods/Materials

2.1. Sample Collection

Blood samples of 60 confirmed diagnosed schizophrenia patients from the Institute of Mental Health, Lahore and 60 age-matched healthy control were collected in k3 EDTA vials. These samples were processed for molecular analysis. Informed consent according to the Declaration of Helsinki was obtained from patients or their relatives, as well as healthy subjects who were involved in the study. The COMSATS Ethics Committee approved the study.

2.2. DNA Extraction

DNA of patients and healthy controls was collected from fresh blood samples using either a DNA extraction kit (K0512-Thermo Scientific Genomic DNA Purification kit; Thermo Fisher Scientific, Waltham, MA, USA). DNA quality and quantity were determined using agarose gel electrophoresis, as described below.

2.3. Primers Designing

Allele- specific amplification primers for allelic variants of *cacna1c* and *cacnb2* were designed by using Primer 3 software. Two allele-specific primers and one universal primer were designed for each marker. The strategy for designing allele-specific primers was followed [24]. The sequences of primers and their annealing temperatures are given in table S1 of the Supplementary Material.

2.4. PCR Amplification

PCR was performed in a 25µl reaction volume containing genomic DNA (10 ng), oligonucleotide primers 0.4ρM each, 1X PCR Buffer, dNTPs 200 µM, 2 mM MgCl, and 1U Taq Polymerase. The following PCR cycling conditions were performed: 5 minutes at 95°C for 1 cycle, 32 cycles at 95°C for 30 seconds, with varied annealing temperatures given in table 1 for 30 seconds and 72°C for 30 seconds followed by 1 cycle at 72°C for 5 minutes.

2.5. Gel Electrophoresis

Amplified SNP products were electrophoresed on 2% agarose gel stained with ethidium bromide (EtBr) and visualized on a UV transilluminator. The allelic variants of *cacna1c* and *cacnb2* were genotyped using the gel-based genotyping method described in the literature.

2.6. Sanger Sequencing

Allele-specific amplification of selected SNPs was validated by Sanger sequencing. Purified PCR products of the selected samples were used for this purpose. Sequencing of the purified products using universal primers was performed using the Big Dye Sequencing Kit according to the manufacturer's instructions (Applied Biosystems).

2.7. Statistical Analysis

For estimating the association of genetic variants of *cacna1c* and *cacnb2* with schizophrenia, chi-square tests along with odds ratios (OR) and 95% confidence intervals (CI) were calculated. A p-value of <0.05 was considered statistically significant. Fisher's exact test was performed to determine the association of haplotypes with the risk of schizophrenia. All statistical analyses were performed using the SHEsis online software [25].

3. Results

This study was conducted on 120 subjects to determine the association of two SNPs (rs1006737, rs4765913) of the *cacna1c* gene and a SNP, rs12257556, of the cacnb2 gene with schizophrenia in the Pakistani population. Allele- specific PCR was performed to amplify the targeted region of genes. To determine the possible variants of each genetic marker and confirm their amplified fragment sizes, the amplified PCR products of the three genetic variants were run on an agarose gel (Figure 1). For further validation, Sanger sequencing of selected subjects was performed, and the results were in 100 % concordance with the allele-specific primer-based amplification method (Figure 2). Genotypes of all subjects are given in the supplementary material (Tables S2 and S3).

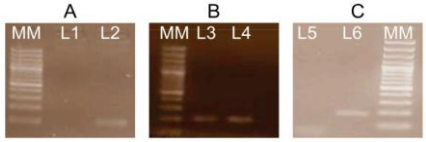

Fig. 1 Allele-specific amplification of allelic variants of genes (*cacna1c* gene & *cacnb2)* for the selected SNPs and their

electrophoresis (2% agarose): A. rs4765913 (103bp), B. rs1006737 (203bp), C. rs12257556 (182bp). Different lanes were labeled as follows: MM - molecular marker (Thermo Fisher Scientific™ O'GeneRuler™ 100 bp Plus DNA Ladder), L1 - no amplification of the T variant of rs4765913 (due to homozygosity), L2 - a variant of rs4765913 (homozygous), L3 - a variant of rs1006737, L4 - G variant of rs1006737 (heterozygous), L5 - no amplification of A variant of rs12257556 (due to homozygosity), G variant of rs12257556 (homozygous) (Developed by the authors)

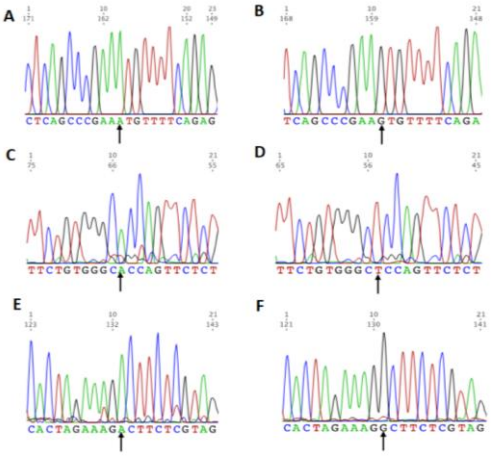

Fig. 2 Sequencing chromatograms of A – *cacna1c* rs10067737 (variant A), B – *cacna1c* rs10067737 (variant G), C – *cacna1c* rs4765913 (variant A), D – *cacna1c* rs4765913 (variant T), E – *cacnb2* rs12257556 (Variant A), F – *cacnb2* rs12257556 (Variant G). *Cacna1c*, calcium voltage-gated channel subunit alpha1; C, cacnb2, calcium voltage-gated channel auxiliary subunit alpha2 (Developed by the authors)

To determine the association of all three SNPs with schizophrenia, the chi-square test was applied. The genetic variant, rs1006737, *cacna1c* gene was significantly associated with schizophrenia in the Pakistani population, with an odds ratio of 2.103679 and 95% confidence interval (CI) ranging 1.256251~3.522755 with *p*-value (0.000227). Another genetic variant, rs4765913, of *cacna1c* also showed a highly significant association (OR=0.435626, CI= 0.258022~0.735481) with schizophrenia risk in our patients (*p*-value=5.03e-005) (Table 1). However, the SNP, rs12257556, of the *cacnb2* gene did not show any association with schizophrenia in the Pakistani population (Table 1).

Table 1 Association of genetic variants of *cacna1c* (rs4765913, rs1006737) and *cacnb2* (rs12257556) with schizophrenia risk in the Pakistani population (Developed by the authors)

Gene	Marker	Odds Ratio	95% CI	Fisher's *p* value	Pearson's *p* value
Cacna1c	rs1006737	2.103679	[1.256251~3.522755]	0.000227	0.000224*
	rs4765913	0.435626	[0.258022~0.735481]	5.03e-005	4.94e-005*
Cacnb2	rs12257556	1.472255	[0.851510~2.545518]	0.332887	0.332806

Haplotype analysis was also conducted to further ensure the epistatic interactions of these SNPs using the SHEsis online software. The combined effect (haplotype) of the two SNPs of *the cacna1c* gene (rs1006737, rs4765913) identified 4 haplotypes. Two of these haplotypes (AT and GA) with frequencies higher than 0.03 were significantly associated (*p*-value of 0.003438 & 0.000227, respectively) with schizophrenia in the Pakistani population (Table 2).

Table 2 Association of haplotypes of both genetic variants (rs1006737 & rs4765913) of *cacna1c* with schizophrenia risk in the Pakistani population (Developed by the authors)

Gene	Marker	Haplotype	Case Frequency	Control Frequency	*p*-value	OR (95%CI)
Cacna1c	rs1006737	A A	20.44(0.170)	19.39(0.162)	0.856036	1.065 [0.539~2.103]
	rs4765913	A T*	47.56(0.396)	26.61(0.222)	0.003438	2.305 [1.310~4.055]
		G A*	18.56(0.155)	43.61(0.363)	0.000227	0.321 [0.173~0.595]
		G T	33.44(0.279)	30.39(0.253)	0.656366	1.139 [0.642~2.020]

4. Discussion

The *cacna1c* gene encodes 1C subunit (Cav 1.2) of the L-type voltage-dependent calcium channel. Calcium influx through such channels is coupled to signaling pathways that stimulate the expression of genes essential for neuronal survival and plasticity [26] and rare mutations in *cacna1c* are known to cause cognitive abnormalities such as schizophrenia [27]. *Cacna1c* mutations are involved in various psychiatric disorders including depression [28], bipolar disorder [29], and schizophrenia [30]-[31]. *Cacnb2* also manufactures part of voltage-gated calcium channels. One of the most heavily implicated genes; it encodes an auxiliary voltage-dependent L-type calcium-channel subunit that is mainly expressed in brain tissue [19], [32]. This subunit is essential for normal surface expression, adequate trafficking, and functioning of voltage-gated calcium channels [19]. Rare mutations in *the cacnb2* gene have been linked to five major disorders, including autism spectrum disorder, attention deficit hyperactivity disorder, bipolar disorder, major depressive disorder, and schizophrenia [33].

The current study, conducted on Pakistani population, represents a significant association of *cacna1c* gene SNPs, rs1006737 (*p*-value=0.000224, OR=2.103679, 95% CI=1.256251~3.522755), and rs4765913 (*p*-value=5.03e-005, OR=0.435626, CI= 0.258022~0.735481) with risk of schizophrenia. Our results were consistent with those of a previous study conducted on the Chinese Han population. In a case-

control study with 312 schizophrenia patients and 305 control subjects, the authors represented the correlation of cognitive function of schizophrenia patients with the A allele of variant rs1006737 of *the cacna1c* gene. Carriers of the A allele had a significant reduction in delayed memory [34]. It has been reported in a GWAS that the risk SNP (rs1006737) for bipolar disorder was also a conferred risk for schizophrenia and early recurrent major depressive disorder [35]. Another study was conducted to examine the replicative effect of *cacna1c* single-nucleotide polymorphisms as a risk factor in three different populations (Jewish, European, Exploratory African American). The authors concluded that variants of SNP (rs1006737) played a significant role in the pathogenesis of schizophrenia [36]. The association of the *cacna1c* gene (SNP; rs1006737) with the susceptibility of schizophrenia in a meta-analysis was reported in [37]. According to [38], rs1006737 was associated with schizophrenia (p=0.015, OR=1.16) in a European population. As reported in [39], the SNP rs1006737 ((p_{allele}= 0.0014, $p_{genotype}$= 0.006, OR= 1.384, 95% CI= 1.134–1.690) is significantly associated with schizophrenia as a risk allele in the Han Chinese population.

The genetic variant rs4765913 of the *cacna1c* gene is also significantly associated with various psychiatric disorders, including schizophrenia [28], bipolar disorder [40], and sleep latency [41]. In this study, we also found a significant association of rs4765913 (p value = 5.03e-005, OR = 0.435626, CI = 0.258022~0.735481) with schizophrenia in the studied subjects. A combined study conducted by the Psychiatric GWAS Consortium working on bipolar disorder and the Psychotic Genome Consortium showed that combined samples of bipolar disorder and schizophrenia had a stronger association with rs4765913 than only with schizophrenia patients [28]. A study provided evidence that schizophrenia and bipolar disorder partly share a common genetic cause [42]. Two different GWAS conducted on European cohorts determined rs4765913 to be a risk factor for autism spectrum disorder [43]-[44].

In this study, the association of the genetic variant rs12257556 of *cacnb2* was also verified in Pakistani schizophrenia patients. No statistically significant association between the rs12257556 SNP of the *cacnb2* gene and the risk of schizophrenia was observed in the subjects included in this study. Although it has been reported that the genetic variant, rs12257556, of *cacnb2* has shown the strongest functional consequences with respect to schizophrenia risk [23], our results are contradictory to those of this study. The sample size could be a limitation of our study, and further association studies of the variant rs12257556 of *cacnb2* are needed to validate its involvement in schizophrenia. Of note, *cacnb2*, L-type calcium channels regulate vascular endothelial growth factor (VEGF) expression [45]. Recent studies have reported that VEGF is involved in neurogenesis, neuronal patterning, and neuroprotection [46]. VEGF has been reported to be involved in neurodevelopmental disorders, including schizophrenia [47]. Particularly, VEGF plays a role in blood– brain barrier disruption, increased extracellular volume, and frontal lobe degeneration in major psychiatric disorders [48]. It has also been reported that VEGF levels inversely correlate with the severity of cognitive impairment, which results in a critical endophenotype of schizophrenia [49]. Hence, *the Cacnb2–Vegf* axis may play a major role in the development of schizophrenia.

5. Conclusion

In conclusion, both SNPs of *the cacna1c* gene (rs1006737 and rs4765913) have shown a significant association with schizophrenia risk in this population. Two haplotypes (AT and GA) out of four for SNPs (rs1006737, rs4765913) of *cacna1c* further verified their significant association with the risk of schizophrenia. In this study sample size analyzed for schizophrenia patients, the genetic variant (rs12257556) of *the cacnb2* gene did not show significant association with the risk of schizophrenia in this population. Our results suggest that there is a strong link between *cacna1c* polymorphisms (both at individual variant and haplotype level) and schizophrenia risk in the Pakistani population.

Acknowledgment

We are thankful to the Punjab Institute of Mental Health (PIMH), Lahore, Punjab, Pakistan for facilitation in blood sample collection from patients.

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Supplementary Material

Table S1 Allele specific primer sequences for *cacn1ac* and *cacnb2* alleles and their annealing temperatures (Developed by the authors)

Allele Name	Name of Primer	Sequences of primers	Annealing Temperature (ºC)
Cacn1ac rs1006737	A Forward	5’-ATTCCATCTCAGCCCGAAA-3’	56
	G Forward	5’-ATTCCATCTCAGCCCGAAG-3’	56
	Universal Reverse	5’-GACCAGCAATCAGTCACACT-3’	56
Cacn1ac rs4765913	A Forward	5’-GTTCTTTCATTCTGTGGGCA -3’	56
	T Forward	5’-GTTCTTTCATTCTGTGGGCT-3’	56
	Universal Reverse	5’-CATTGGTTCTTCTGATGTGC-3’	56
Cacnb2 rs12257556	T Reverse	5’-TCAGTACATCTACGAGAAGT-3’	54
	C Reverse	5’-TCAGTACATCTACGAGAAGC -3’	54
	Universal Forward	5’-TTCTACCCACTGTTAAGCAC-3’	54

Table S2 List of genotypes for three genetic markers in schizophrenia patient samples (Developed by the authors)

Schizophrenia	**CACNA1C**		**CACNB2**		**CACNB2**	
Sample No.	**rs1006737**		**rs4765913**		**rs12257556**	
	Allele A	**Allele G**	**Allele A**	**Allele T**	**Allele A**	**Allele G**
C01	A	G	T	T	A	A
C02	A	A	T	T	A	A
C03	A	G	T	T	A	G
C04	G	G	T	T	G	G
C05	G	G	A	A	A	G
C06	A	G	A	A	G	G
C07	A	G	A	A	G	G
C08	A	G	A	A	A	A
C09	G	G	T	T	A	G
C10	A	G	T	T	A	G
C11	A	A	T	T	A	A
C12	A	G	T	T	A	A
C13	A	G	T	T	A	A
C14	G	G	A	A	A	G
C15	G	G	A	A	A	A
C16	A	A	T	T	A	A
C17	A	A	T	T	A	G
C18	G	G	T	T	A	A
C19	A	G	A	A	A	G
C20	A	G	A	A	A	G
C21	A	A	A	A	A	G
C22	A	A	A	A	A	G
C23	A	A	T	T	A	G
C24	A	G	T	T	A	G
C25	A	G	T	T	A	A
C26	G	G	T	T	A	G
C27	A	G	A	A	A	G
C28	G	G	A	A	G	G
C29	A	G	A	A	A	A
C30	A	G	T	T	A	A
C31	A	A	T	T	A	G
C32	A	A	A	A	A	A
C33	A	A	A	A	G	G
C34	G	G	T	T	A	G
C35	G	G	T	T	A	A
C36	A	G	A	T	A	A
C37	A	A	T	T	A	G
C38	A	A	T	T	A	G
C39	A	A	T	T	A	G
C40	A	A	T	T	A	A
C41	A	A	T	T	G	G
C42	G	G	T	T	A	A
C43	A	G	A	A	A	G
C44	A	G	A	A	A	A
C45	G	G	T	T	A	G
C46	G	G	T	T	A	G
C47	G	G	A	T	A	G
C48	G	G	T	T	A	G
C49	A	A	A	T	A	G
C50	A	A	T	T	A	G
C51	A	A	T	T	A	G
C52	A	A	A	T	A	A
C53	A	A	T	T	A	A
C54	A	A	T	T	A	A
C55	A	A	T	T	A	A
C56	A	A	A	T	A	G
C57	G	G	T	T	A	G
C58	A	A	T	T	A	G
C59	G	G	T	T	A	G
C60	A	A	T	T	A	A

Table S3 List of genotypes for three genetic markers in healthy controls (Developed by the authors)

Controls	**CACNA1C**					
	rs1006737		**rs4765913**		**rs12257556**	
Sample No.	**Allele A**	**Allele G**	**Allele A**	**Allele T**	**Allele A**	**Allele G**
N01	G	G	T	T	A	A
N02	G	G	T	T	A	A
N03	G	G	T	T	A	G
N04	A	G	T	T	A	G
N05	G	G	T	T	A	G
N06	A	G	T	T	A	G
N07	A	G	A	T	A	A
N08	A	G	T	T	G	G
N09	A	G	T	T	G	G
N10	A	A	T	T	G	G
N11	A	A	A	T	A	A
N12	G	G	T	T	A	A
N13	G	G	A	A	A	A
N14	G	G	A	T	A	G
N15	G	G	A	T	A	G
N16	A	G	A	A	A	G
N17	A	G	A	T	A	G
N18	A	G	A	A	A	G
N19	A	G	A	A	A	A
N20	A	G	A	T	A	A
N21	A	G	T	T	A	A
N22	A	G	A	A	A	G
N23	G	G	A	A	A	A
N24	G	G	A	A	A	A
N25	A	G	T	T	A	G
N26	A	A	T	T	A	G
N27	G	G	T	T	A	A
N28	G	G	A	A	A	A
N29	A	A	T	T	A	A
N30	A	G	T	T	A	A
N31	G	G	A	A	A	A
N32	G	G	A	A	A	A
N33	G	G	A	A	A	G
N34	G	G	A	A	A	G
N35	G	G	T	T	A	G
N36	A	G	A	A	A	G
N37	A	G	A	A	A	G
N38	A	G	A	T	A	G
N39	A	G	A	A	A	A
N40	A	A	A	T	A	A
N41	A	G	A	T	A	A
N42	A	G	A	A	A	G
N43	A	G	A	T	A	A
N44	A	A	A	T	A	G
N45	A	G	A	T	A	G
N46	A	G	A	T	A	A
N47	A	G	A	A	A	A
N48	A	G	A	A	A	A
N49	A	G	A	T	A	A
N50	A	G	A	T	A	G
N51	A	G	A	T	A	A
N52	A	G	A	T	A	G
N53	G	G	A	A	A	G
N54	G	G	A	T	A	G
N55	G	G	A	T	A	A
N56	A	G	A	T	A	G
N57	A	G	A	A	A	A
N58	A	G	A	T	A	G
N59	A	G	A	T	A	A
N60	A	G	A	T	A	A