A polymorphism of the metabotropic glutamate receptor mGluR7 (*GRM7*) gene is associated with schizophrenia

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Abstract

Introduction: Glutamate dysfunction has been implicated in the pathophysiology of schizophrenia. The metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors. *GRM7*, the gene that encodes mGluR7, is expressed in many regions of the human central nervous system. The *GRM7* gene is located on human chromosome 3p26, which has been suggested by linkage analysis to contain a susceptibility locus for schizophrenia.

Methods: We screened for mutations in all exons, exon/intron junctions, and promoter regions of the *GRM7* gene in Japanese patients with schizophrenia and evaluated associations between the detected polymorphisms and schizophrenia. We examined the influence of one polymorphism associated with schizophrenia on the expression of *GRM7* by dual luciferase assay in transfected cells.

Results: Twenty-five polymorphisms/mutations were detected in *GRM7*. Case-control analysis revealed a potential association of a synonymous polymorphism (371T/C, rs3749380) in exon 1 with schizophrenia in our case-control study of 2293 Japanese patients with schizophrenia and 2382 Japanese control subjects (allelic $p = 0.009$). Dual luciferase assay revealed suppression of transcription activity by exon 1 containing this polymorphism and a statistically significant difference in the promoter activity between the T and C alleles.

Conclusions: Our results support the possible association of a *GRM7* gene polymorphism with genetic susceptibility to schizophrenia.

Introduction

Schizophrenia is a severe psychiatric disorder, which is equally prevalent in men and women and affects approximately one percent of the population worldwide. Several neurotransmitter systems and functional networks within the brain have been found to be affected in patients with schizophrenia. The glutamatergic neuronal dysfunction hypothesis is one of the main explanatory hypotheses (Carlsson et al., 1997). Glutamate is the primary excitatory neurotransmitter in brain. It is contained as a neurotransmitter in approximately 60% of brain neurons, including almost all cortical pyramidal neurons. Further, virtually 100% of brain neurons contain some type of glutamate receptor. Glutamate mediates its effects on the central nervous system via both ionotropic and metabotropic receptors. The metabotropic glutamate receptors (mGluRs), which are G protein-coupled receptors, are divided into 3 groups on the basis of sequence homology, putative signal transduction mechanisms, and pharmacologic properties (Nakanishi 1994; Pin and Duvoisin 1995). The mGluRs in group I are mGluR1 and mGluR5, those in group II are mGluR2 and mGluR3, and those in group III are mGluRs 4, 6, 7, and 8. Group II and group III mGluRs are linked to inhibition of the cyclic AMP cascade but differ in their agonist selectivities.

mGluR7 is the most highly conserved mGluR subtype across mammalian species (Flor et al., 1997). Makoff et al (1996) observed by in situ hybridization that *GRM7* is expressed in many areas of the human brain, especially the cerebral cortex, hippocampus, and cerebellum. mGluR7 is localized directly in the presynaptic zone of the synaptic cleft of glutamatergic synapses (Kinoshita et al 1998; Kosinski et al 1999), where it is thought to act as an autoreceptor that is activated by glutamate released from the presynaptic terminal during action potentials. Furthermore, mGluR7 is thought to be a key player in shaping synaptic responses at glutamatergic synapses as well as in regulating key aspects of inhibitory GABAergic transmission (Kinoshita et al 1998; Kosinski et al 1999).

mGluR7 has putative roles in anxiety, emotional responses, and spatial working memory (Callaerts-Vegh et al 2006; Cryan et al 2003; Mitsukawa et al 2006). Cognitive

dysfunction is estimated to occur in 75%–85% of patients with schizophrenia, often precedes the onset of other symptoms (Reichenberg et al 2006). Working memory is one of primary cognitive domains that are crucial for developing targets for the treatment of cognition in schizophrenia (Nuechterlein et al 2004).

mGluR7 ablation causes dysregulation of the hypothalamic-pituitary-adrenal axis and increases hippocampal BDNF protein levels (Mitsukawa et al 2005). Dysregulation of BDNF production or release is associated with neuropsychiatric disorders, such as schizophrenia (Kelley 2004; Harrison and Weinberger 2005). Association between the val66met polymorphism of the BDNF gene and hippocampal volume in human, particularly in patients with schizophrenia (Szeszko PR et al 2005).

In the present study, we examined the *GRM7* gene as a candidate for schizophrenia.

Materials and Methods

Subjects

All subjects were of Japanese descent and were recruited from the main island of Japan. Patients with schizophrenia were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Control subjects were mentally healthy, unrelated subjects with no self-reported family history of mental illness within second-degree relatives. We sequenced the 5' region, exons and exon-intron boundaries of the *GRM7* gene in 32 patients (mutation screening patients) with schizophrenia (mean age ± SD, 46.5 ± 10.9 years; 17 men and 15 women) to identify polymorphisms. We then genotyped 576 patients including the mutation screening patients (mean age ± SD, 46.6 ± 14.8 years; 322 men and 254 women) and 576 control subjects (mean age ± SD, 46.8 ± 12.9 years; 268 men and 308 women) (1st association population) with Predesigned TaqMan single nucleotide polymorphism (SNP) genotyping assays. When Predesigned TaqMan SNP genotyping assays were not available, we performed direct sequencing of DNAs from 96 patients including the mutation screening patients (mean age ± SD, 50.3 ± 13.1 years; 55 men and 40 women) and

96 control subjects (mean age ± SD, 53.6 ± 9.1 years; 42 men and 54 women). For SNPs for which an association with schizophrenia was suggested in the first association population, we performed genotyping in an independent sample of 1817 patients (mean age ± SD, 45.5 ± 14.1 years; 962 men and 855 women) and 1728 control subjects (mean age ± SD, 46.2 ± 13.6 years; 958 men and 770 women) (confirmation population). The present study was approved by the ethics committees of the University of Tsukuba and participating institutes. All participants provided written informed consent.

DNA isolation and genotyping

DNAs were extracted from peripheral lymphocytes by standard phenol-chloroform extraction. The genomic structure of *GRM7* was determined from the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgGateway) and the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). To screen for polymorphisms, we performed direct sequencing of genomic DNAs with a Big Dye Terminator Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All exons, exon-intron junctions, and 1.6 kb of the 5' flanking region of *GRM7* were amplified from the genomic DNAs of 24 randomly selected patients. The sequences of primers and conditions used for amplification for the mutation screening are available upon request. We genotyped polymorphisms with the TaqMan SNP Genotyping Assay (Applied Biosystems) and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells with the SV Total RNA Isolation System (Promega, Madison, WI, USA). cDNA was synthesized from RNA with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primer. Expression of *GRM7* was quantified by real-time quantitative RT-PCR with the TaqMan Gene Expression Assay and ABI PRISM

7900HT Sequence Detection System (Applied Biosystems) per the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems (Assays-on-Demand Assay ID: Hs00179051_m1). *GAPDH* was used as an internal control. Data were collected and analyzed with Sequence Detector Software (SDS) version 2.1 (Applied Biosystems) and the standard curve method.

Luciferase reporter assay

To assay promoter activity of the 5'-flanking region and exon 1 of the *GRM7* gene, 9 fragments of the 5' region were cloned into the pGL3-Basic plasmid with and without a Simian virus 40 enhancer sequence (Promega, Madison, WI, USA). The day before transfection, NH-12 cells (Japanese Collection of Research Bioresources Gene Bank, http://genebank.nibio.go.jp/gbank/index_e.html) were plated at 1 X 10^5 cells per well in a 24-well plate and grown in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA). One microgram of each test plasmid was transiently cotransfected into the cells with 0.1 μg of pRL-TK plasmid (an internal standard reporter) (Promega) with Lipofectamine 2000 (Invitrogen) per manufacturer's protocol. After 48 h, the dual-luciferase assay was performed with a PicaGene Dual SeaPansy Kit (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions.

Statistics

Deviation from predicted Hardy-Weinberg frequencies was examined by chi-square test. Individual allelic associations were examined by Fisher's exact test. Genotypic associations were examined by Armitage's Trend Test for the reasons discussed by Devlin and Roeder (Devlin and Roeder 1999). A significant association was defined when the given p value for allelic or genotypic tests was less than 5% (uncorrected $p < 0.05$) and the same association was confirmed in an independent population with $p < 0.05$. Linkage

disequilibrium (LD) between polymorphisms and haplotype block structures was evaluated with Haploview software version 3.11 (Barrett et al 2005). Haplotype blocks were generated with the default algorithm taken from Gabriel et al (Gabriel et al 2002). Haplotypic associations with disorders were examined with Haploview software, which performs association tests on the set of blocks selected by obtaining counts for case control association tests by summing the fractional likelihoods of each individual for each haplotype by the EM algorithm.

Results

Twenty-five polymorphisms/mutations were detected in the exons, exon-intron junctions, and 5'-flanking region of the *GRM7* gene (Figure 1). Genotyping was carried out for all detected polymorphisms except rare variants with allele frequencies < 0.05 and polymorphisms in LD with each other ($r^2 = 1$). Among these SNPs, 1724A/G (rs34373930), 1938C/T (rs7614915), IVS8+49T/A (T/A at the position of 49 base-pair starting from the G of the donor site of intron 8), and 2345C/T (rs1485175) were in complete LD ($r^2 = 1$), and IVS8+114T/C and 2384G/A (rs1485174) were also in complete LD ($r^2 = 1$) in 24 screening samples. Therefore, we genotyped the 2345C/T and 2384G/A polymorphisms as representative SNPs. The IVS9+97C/T, 2912T/C, and 3292(A)3-4 (rs3840229) SNPs were genotyped in 96 patients and 96 controls by direct sequencing, and the other 12 SNPs were genotyped in 576 patients and 575 control subjects by TaqMan SNP Genotyping Assay (Applied Biosystems). The genotypic distributions of these 15 SNPs did not deviate significantly from Hardy-Weinberg equilibrium ($p > 0.05$). A synonymous polymorphism (371T/C) in exon 1 of *GRM7* showed a potential association for schizophrenia (allelic uncorrected $p = 0.04$) (Table 1). We did not detect a significant association with schizophrenia of any of the other 14 SNPs, including the Tyr433Phe polymorphism (allelic uncorrected $p = 0.33$; genotypic uncorrected $p = 0.63$), which was previously reported to be associated

(Bolonna et al 2001). The haplotype blocks consisted of IVS1-76T/C, IVS1-67A/G, and IVS1-23G/A and of 2345C/T and 2384A/G. No significant haplotypic association was suggested for these 2 haplotype blocks ($p > 0.05$). We confirmed the association of the 371T/C polymorphism with schizophrenia in an independent population of 1717 patients and 1807 control subjects and confirmed the association (allelic $p = 0.03$, one-sided) (Table 2). The allelic p value of the association in the total population of 2293 patients with schizophrenia and 2382 control subjects was 0.009 (Table 2). According to the HapMap database (http://www.hapmap.org/index.html), the 371T/C polymorphism (rs3749380) was not in the LD block and was not in LD with other SNPs within 80 kb ($r^2 < 0.7$). Weak LD was observed with rs458351 ($D' = 0.89$ and $r^2 = 0.69$), which is 30 kb upstream of rs3749380.

Expression of *GRM7* mRNA was assessed by RT-PCR in 7 different human cell lines (IMR-32, NH-12, TN-2, NB-1, SCCH-26, A172, and T98G). *GRM7* was expressed in NH-12 and SCCH-26 cells (data not shown). Because expression in NH-12 cells was higher than in SCCH-26 cells, we used NH-12 cells, a human cell line derived from neuroblastoma, for luciferase assays. Dual luciferase assay revealed that the strongest promoter activity for *GRM7* was contained in the 1-kb fragment upstream of the ATG site of exon 1. However, a promoter construct containing the sequence from the ATG to the end of exon 1 showed significantly lower activity, indicating that the 371T/C polymorphism is in a regulatory region. When an SV40 enhancer was added downstream of the luciferase gene, the 371C allele showed significantly higher promoter activity than the 371T allele (Figure 2).

Discussion

In the present study, we examined associations between polymorphisms in the *GRM7* gene and schizophrenia. Weak association was found for a synonymous SNP (371T/C) in exon 1 in the first association population, and this association was replicated in the confirmation population. The T allele, which is associated with schizophrenia, has lower promoter activity than the C allele. On the basis of this finding, we hypothesized that lower

expression of mGluR7 may increase risk of developing schizophrenia, though studies of the expression of GRM7 in brains of patients with schizophrenia have not been reported.

mGluR7 was the first group III mGluR found to be enriched presynaptically at active zones of hippocampal pyramidal cells (Shigemoto et al 1996). The low affinity of mGluR7 for glutamate suggests that mGluR7 might act as a “low-pass filter” that suppresses release of glutamate only when action potentials arriving at a high frequency produce massive glutamate release. The interaction with PICK1 (protein interacting with PRKCA 1) is crucial for the clustering of mGluR7 at presynaptic release sites (Boudin et al 2000). *PICK1* is reported to be associated with schizophrenia (Dev and Henley 2006; Fujii et al 2006).

In the present study, we found an association between a functional SNP, 371T/C, in the gene encoding mGluR7 and schizophrenia. Recently, a genome-wide association study of 2000 individuals with bipolar disorder and 3000 control subjects found a strong association of SNP marker rs1485171 (p = 9.7 X 10^{-5}) in *GRM7* with bipolar disorder (Consortium 2007; Consortium. 2007). Therefore, genetic variations in the GRM7 gene may be involved in both schizophrenia and bipolar disorder.

An association between Tyr433Phe polymorphism of *GRM7* and schizophrenia was reported (Bolonna et al 2001). However, we failed to detect the association (allele, p = 0.33; genotype, p = 0.63). Recently, a copy number variation of the *GRM7* locus has been reported in patients with schizophrenia (Wilson et al 2006). In the present study, we did not observe significant deviation from Hardy-Weinberg expectancy of the genotypic distributions of SNPs, indicating that copy number variations at the SNP examined in the present study are not common and are unlikely.

In the present study, the association of 371T/C with schizophrenia was observed in two independent case control populations. However, its weak association (OR = 1.12) requires replication studies in large sample populations of more than 2000 cases and 2000 control subjects with a power greater than 0.8. We believe that *GRM7* is an interesting target worth such studies for schizophrenia and other psychiatric disorders.

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FIGURE LEGENDS

Figure 1. Schematic representation of the *GRM7* gene and relevant mutations/polymorphisms (A), linkage disequilibrium plot of the Japanese population from HapMap database (B), and between SNPs genotyped in this study shown in Table 1 (C). Figures in rhombus are D' between SNPs and filled rhombus without figures indicates D' = 1 in (C).

Figure 2. Luciferase assays.

(A) Schematic representation of the *GRM7* gene and reporter gene constructs.

(B) Transcriptional activity of various constructs of the 5' region and exon 1 of the human *GRM7* gene in NH-12 cells. The region indicated by fine lines were not included in the constructs. Cotransfections were performed with pRL-TK (*Renilla* luciferase) to normalize transfection efficiency. Luciferase activity was assayed 48 h after transfection. Relative ratio of expression is shown as relative to that of pGL3-Basic, which was set at 1. The mean ±SEM was calculated from triplicate assays.

(C) Effect of the 371C/T polymorphism on *GRM7* promoter activity. This assay was performed with pGL3-enhancer vector, because the constructs that contain *GRM7* exon 1 show extremely low luciferase activity.

Table 1. Genotypic and allelic distributions of polymorphisms in the *GRM7* gene

Polymorphism	Population	n	Genotype count (frequency)						*p*	Allele count (frequency)				*p*
371T/C (rs3749380)			CC		TC		TT			C		T		
	Patients	576	241	(0.42)	256	(0.44)	79	(0.14)		738	(0.64)	414	(0.36)	
	Controls	575	274	(0.48)	236	(0.41)	65	(0.11)	0.04	784	(0.68)	366	(0.32)	0.04
IVS1-76T/C (rs3749450)			CC		TC		TT			C		T		
	Patients	568	10	(0.02)	148	(0.26)	410	(0.72)		168	(0.15)	968	(0.85)	
	Controls	561	18	(0.03)	129	(0.23)	414	(0.74)	0.95	165	(0.15)	957	(0.85)	0.96
IVS1-67A/G (rs3749449)			GG		AG		AA			G		A		
	Patients	570	8	(0.01)	95	(0.17)	467	(0.82)		111	(0.10)	1029	(0.90)	
	Controls	567	7	(0.01)	89	(0.16)	471	(0.83)	0.60	103	(0.09)	1031	(0.91)	0.59
IVS1-23G/A (rs3749448)			AA		GA		GG			A		G		
	Patients	569	26	(0.05)	181	(0.32)	362	(0.64)		233	(0.20)	905	(0.80)	
	Controls	566	30	(0.05)	178	(0.31)	358	(0.63)	0.75	238	(0.21)	894	(0.79)	0.75
IVS4-35C/T (rs712774)			CC		CT		TT			C		T		
	Patients	571	121	(0.21)	260	(0.46)	190	(0.33)		502	(0.44)	640	(0.56)	
	Controls	559	117	(0.21)	268	(0.48)	174	(0.31)	0.66	502	(0.45)	616	(0.55)	0.65
1447T/A (rs2229902, Phe433Tyr)			AA		AT		TT			A		T		
	Patients	575	488	(0.85)	81	(0.14)	6	(0.01)		1057	(0.92)	93	(0.08)	
	Controls	569	484	(0.85)	82	(0.14)	3	(0.01)	0.76	1050	(0.92)	88	(0.08)	0.75
2345C/T (rs1485175)			CC		CT		TT			C		T		
	Patients	569	120	(0.21)	275	(0.48)	174	(0.31)		515	(0.45)	623	(0.55)	
	Controls	562	118	(0.21)	282	(0.50)	162	(0.29)	0.69	518	(0.46)	606	(0.54)	0.69
2384A/G (rs1485174)			AA		GA		GG			A		G		
	Patients	569	18	(0.03)	150	(0.26)	401	(0.70)		186	(0.16)	952	(0.84)	
	Controls	561	24	(0.04)	162	(0.29)	375	(0.67)	0.15	210	(0.19)	912	(0.81)	0.14
IVS8-123T/C (rs162802)			CC		TC		TT			C		T		
	Patients	569	3	(0.01)	91	(0.16)	475	(0.83)		97	(0.09)	1041	(0.91)	
	Controls	566	9	(0.02)	88	(0.16)	469	(0.83)	0.49	106	(0.09)	1026	(0.91)	0.48
IVS9+15G/A (rs2280739)			AA		GA		GG			A		G		
	Patients	570	4	(0.01)	84	(0.15)	482	(0.85)		92	(0.08)	1048	(0.92)	
	Controls	564	3	(0.01)	86	(0.15)	475	(0.84)	0.94	92	(0.08)	1036	(0.92)	0.94
IVS9e+97C/T			CC		CT		TT			C		T		
	Patients	96	75	(0.78)	20	(0.21)	1	(0.01)		170	(0.89)	22	(0.11)	
	Controls	95	78	(0.82)	16	(0.17)	1	(0.01)	0.53	172	(0.91)	18	(0.09)	0.62
IVS9e+131C/T (rs162777)			CC		CT		TT			C		T		
	Patients	570	22	(0.04)	186	(0.33)	362	(0.64)		230	(0.20)	910	(0.80)	
	Controls	566	23	(0.04)	178	(0.31)	365	(0.64)	0.82	224	(0.20)	908	(0.80)	0.82
2912T/C			TT		TC		CC			T		C		
	Patients	96	78	(0.81)	18	(0.19)	0	(0.00)		174	(0.91)	18	(0.09)	
	Controls	95	83	(0.87)	12	(0.13)	0	(0.00)	0.32	178	(0.94)	12	(0.06)	0.34
3175C/T (rs9826579)			CC		CT		TT			C		T		
	Patients	574	25	(0.04)	203	(0.35)	346	(0.60)		253	(0.22)	895	(0.78)	

	Controls	567	23	(0.04)	189	(0.33)	355	(0.63)	0.44	235	(0.21)	899	(0.79)	0.44
3292(A)3-4 (rs3840229)			33		34		44			3		4		
	Patients	96	80	(0.83)	16	(0.17)	0	(0.00)		176	(0.92)	16	(0.08)	
	Controls	95	78	(0.82)	17	(0.18)	0	(0.00)	0.85	173	(0.91)	17	(0.09)	0.86

Genotypic *p* was calculated by Armitage's Trend Test and allelic *p* was calculated by Fisher's exact test.

Table 2. Association of the 371T/C polymorphism (rs3749380) in the *GRM7* gene with schizophrenia

Polymorphism		Genotype count (frequency)				Allele count (frequency)		
population	n	CC	CT	TT	*p*	C	T	*p*
Screening population								
Patients	576	241 (0.42)	256 (0.44)	79 (0.14)		738 (0.64)	414 (0.36)	
Controls	575	274 (0.48)	236 (0.41)	65 (0.11)	0.04	784 (0.68)	366 (0.32)	0.04
Confirmatory population		CC	CT	TT		C	T	
Patients	1717	715 (0.42)	771 (0.45)	231 (0.13)		2201 (0.64)	1233 (0.36)	
Controls	1807	799 (0.44)	794 (0.44)	214 (0.12)	0.07	2392 (0.66)	1222 (0.34)	0.03
Total		CC	CT	TT		C	T	
Patients	2293	956 (0.42)	1027 (0.45)	310 (0.14)		2939 (0.64)	1647 (0.36)	
Controls	2382	1073 (0.45)	1030 (0.43)	279 (0.12)	0.01	3176 (0.67)	1588 (0.33)	0.009*

Genotypic *p* was calculated by Armitage's Trend Test and allelic *p* was calculated by Fisher's exact test.

*Odd ratio = 1.12, 95% confidence interval = 1.03-1.22

A)

-1889(CAA)$_{4-5}$
-1006G/C$_{(rs17046184)}$
-445C$_{7-8}$
IVS1+781(TAACTGC) I/D$_{(rs34216931)}$
IVS1+820 T I/D
371C/T
N74N
(rs3749380)
IVS1-76T/C$_{(rs3749450)}$
IVS1-67A/G$_{(rs3749449)}$
IVS1-23G/A$_{(rs3749448)}$
IVS4-35C/T$_{(rs712774)}$
IVS5-20(TG)$_{13-17}$
1447A/T
Y433F$_{(rs2229902)}$
1938C/T
L597L
(rs7614915)
2345C/T
Y732Y
(rs1485176)
2384G/A
G745G$_{(rs1485174)}$
IVS8+49T/A$_{(rs1485173)}$
IVS8+114T/C$_{(rs1485172)}$
1724A/G
L525L
(rs34373930)
IVS8-123T/C
(rs162802)
IVS9+15G/A
(rs2280739)
IVS9e+97C/T
IVS9e+131C/T$_{(rs162777)}$
3175C/T$_{(rs9826579)}$
3061T/C
3292(T)$_{3-4}$
(rs3840229)

1 2 3 4 5 6 7 8 9 9a 9b 9c 9d 9e 10

↑ATG

CpG Island

880 Kb

B)

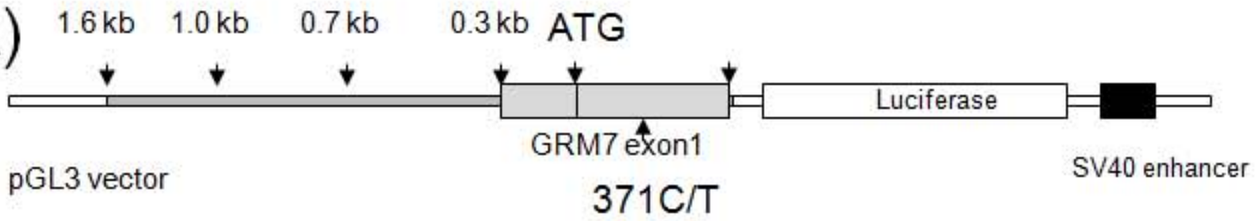
C)

Block 1 (0 kb)
Block 2 (0 kb)
Block 3 (0 kb)
Block 4 (0 kb)

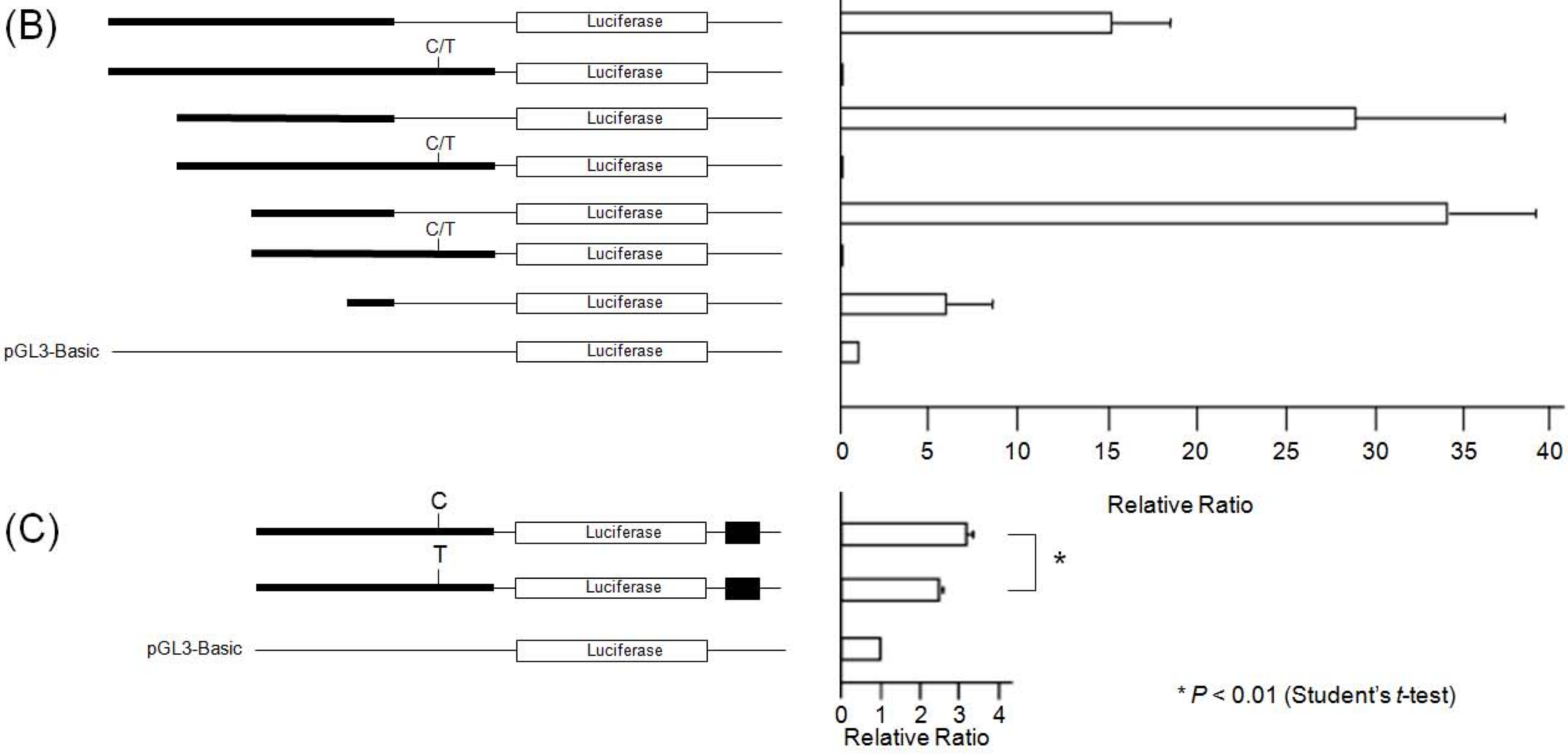
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

28 57 1 85 6 6 13 90
24 53 4 11 9 12 30 9 65 2 80 23 56
2 43 4 13 7 41 68 71 88 44

(A)



(B)



(C)

* $P < 0.01$ (Student's *t*-test)

Figure 2