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ORIGINAL ARTICLE

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Association of 14-3-3 ϵ gene haplotype with completed suicide in Japanese

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Abstract Genetic factors have been suggested to be involved in suicide. Although some genetic factors, such as serotonergic transduction, have been associated with suicide, the results are inconsistent. There is a possibility that various signaling anomalies are involved in the biological vulnerability to suicide. We carried out a genome-wide gene-expression study in the brains of suicide victims using DNA microarrays;14-3-3 ϵ, which is related to neurogenesis, was one of the genes upregulated in the brains of suicide victims in the microarray analysis. This was confirmed by Western blot analysis. To examine the possibility of the involvement of 14-3-3 ϵ in the pathogenesis of suicide, we investigated the association of the 14-3-3 ϵ gene and completed suicide. We used three high-frequency SNPs (rs1532976, rs3752826, and rs9393) and found a significant association of two alleles (rs1532976 and rs3752826) with completed suicide ($p < 0.05$). Moreover, the distribution of haplotype revealed a more significant difference between completed suicide and controls ($p = 0.0005$). This finding suggests that 14-3-3 ϵ is a potential suicide susceptibility gene and implies that dysregulation of neurogenesis may be involved in suicide.

Keywords Completed suicide victims · 14-3-3 ϵ (epsilon) · Postmortem brain · Microarray · Immunoreactivity · Single-nucleotide polymorphism

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Introduction

Genetic studies, such as family, twin, and adoption studies, have indicated an involvement of genetic factors in suicide (Roy et al. 1991; Wender et al. 1986). Neurobiological studies in suicidal behavior have demonstrated a low level of 5-hydroxyindoleacetic acid (5-HIAA) in cerebrospinal fluid (CSF) (Asberg 1997) and a blunted prolactin response to fenfluramine (Malone et al. 1996). These findings indicate that decreased serotonergic neurotransmission could be involved in suicide (Mann et al. 1999). Serotonergic genes, such as the genes for serotonin receptor1A (5-HT1A), 5-HT2A, 5-HT2C, tryptophan hydroxylase (TPH), and serotonin transporter (5-HTT), have been extensively examined in case-control association studies on suicide. Some of these studies have reported positive association with suicide, but most of them have shown no association (Anguelova et al. 2003; Bellivier et al. 2004).

Recent studies have shown a close relation between serotonin and neurogenesis (Gaspar et al. 2003; Gould 1999). In rats, chronic administration of serotonin reuptake inhibitor, an antidepressant, stimulated neurogenesis (Jacobs et al. 2000), and depletion of brain serotonin by a neurotoxin decreased newly generated neurons (Brezun and Daszuta, 1999). Thus, in addition to decreased serotonergic neurotransmission, the

dysfunction of neurogenesis may also underlie the etiology of suicide. In this study, we examined genome-wide gene expression in the brains of suicide victims using a DNA microarray to find candidate genes in suicide; 14-3-3 ϵ, which is related to neurogenesis, was found to be upregulated in the brains of suicide victims in the microarray analysis. To examine the possibility of the involvement of 14-3-3 ϵ in the pathogenesis of suicide, we investigated the association between the several single nucleotide polymorphisms (SNPs) of the 14-3-3 ϵ gene and completed suicide.

Materials and methods

Samples

Amygdala and peripheral blood samples used in this study were of Japanese descent. Amygdala samples of postmortem brains were obtained at autopsy. The subjects included 14 suicide victims (five men, nine women, mean age 43.9 years, SD 11.2) and 15 control subjects (six men, nine women, mean age 54.6 years, SD 13.8) who were without any history of neuropsychiatric disorders. Clinical characteristics and cause of death are shown in Table 1.

Peripheral blood was drawn from suicide completers and controls for genotyping study. The population consisted of 185 suicide completers (124 men, 61 women, mean age 47.7 years, SD 17.9) and 359 control subjects (153 men, 206 women, mean age 42.3 years, SD 16.3). The methods of suicide were hanging ($n = 91$), jumping from heights (60), drug overdose (8), drowning (8), several deep cuts (5), jumping in front of a vehicle (4), burning (3), gas poisoning (2), and other methods (4). Most (177) of the cases were classified as violent suicides according to the criteria previously proposed (Asberg et al. 1976). Accurate information about the clinical backgrounds of the suicide completers could not be obtained under the ethical code for this genetic study.

Brain and peripheral blood samples of the suicide victims were obtained from the Division of Legal Medicine, Department of Environmental Health and Safety, Faculty of Medical Sciences, Kobe University

Table 1 Autopsy and clinical data

	Gender[a]	Age	PMI[b] (h)	Cause of death
Suicide				
S-1	F	26	Unknown	Jumping
S-2	F	28	30	Drug overdose
S-3	F	32	9	Burning
S-4	F	33	29	Hypothermia (by starvation)
S-5	F	40	24	Drowning
S-6*[e]	M	41	9	Cutting
S-7	F	47	16	Hanging
S-8	F	47	16	Drug overdose
S-9	M	48	14	Gas poisoning
S-10*	M	48	5	Hanging
S-11*	M	50	28	Cutting
S-12	F	52	24	Drowning
S-13*	M	57	22	Drowning
S-14	F	65	10	Drowning
	Mean ± SD	43.9 ± 11.2[c]	18.2 ± 8.5[d]	
Control				
C-1	M	28	Unknown	Traffic accident
C-2	M	35	17	Traffic accident
C-3	M	38	Unknown	Asthma
C-4	F	44	17	Aplastic anemia
C-5	M	49	5	Myocardial infarction
C-6	F	49	28	Homicidal cardiac wound
C-7*	M	56	12	Myocardial infarction
C-8	F	57	2	Myocardial infarction
C-9*	M	59	9	Pulmonary tuberculosis
C-10	F	62	8	Myocardial infarction
C-11	F	63	Unknown	Myocardial infarction
C-12	F	66	22	Cardiomyopathy
C-13	F	67	Unknown	Cardiomyopathy
C-14	F	71	8	Suffocation (choke on food)
C-15	F	75	Unknown	Suffocation (choke on food)
	Mean ± SD	54.6 ± 13.8	12.8 ± 8.1	

[a] *M* male, *F* female
[b] Postmortem interval
[c] Significant change in age between two groups ($p = 0.029$, Mann-Whitney *U* test)
[d] No significant change in PMI between two groups ($p = 0.13$, Mann-Whitney *U* test)
[e] *Samples used in microarray analysis

Graduate School of Medicine. The suicide was assessed by medicolegal autopsy. Brain samples of control subjects were obtained from the Department of Anatomy, Kobe University Graduate School of Medicine. The consent of the relatives was obtained after the purpose and procedures of the study were fully explained. Peripheral blood samples of control subjects were recruited from the general population of Kobe city area, Japan, with consent after the purpose and procedures of the study were fully explained. All were healthy and had not manifested psychiatric problems in brief interviews by psychiatrists. This study was approved by the Ethical Committee for Genetic Studies of Kobe University Graduate School of Medicine.

Microarray procedure

Total RNA was extracted from 0.1 g of frozen tissue using ISOGEN (Nippon Gene Tokyo, Japan). The purity of total RNA was evaluated by the OD260/OD280 ratio, and its integrity was evaluated by denaturing agarose gel electrophoresis. We also used amygdala mRNA (Clontech Palo Alto, CA, USA) that was pooled from 70 male and female Caucasians ages 17–76 who had died from trauma. Microarray analysis was performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Twenty micrograms of total RNA from five suicide victims and two controls, which was of good quality, was used to synthesize cDNA. Additionally, 1 μg of mRNA obtained from Clontech was used to synthesize cDNA. These samples were used to generate biotinylated complementary RNA (cRNA). cRNA was fragmented and first applied to the Test2 Chip (Affymetrix) to assess the sample quality and then applied to the HU95A chip (Affymetrix), which contained probes for about 12,000 genes. The hybridization signal on the chip was scanned with a GeneArray scanner (Hewlett-Packard, Palo Alto, CA, USA) and was processed by GeneSuite software (Affymetrix).

Immunoreactivity

Western blot analysis was performed, as previously described (Ono et al. 2002). Anti-14-3-3 ϵ polyclonal antibody was obtained from Santa Cruz (Santa Cruz, CA, USA). A 10-μg protein/lane for 14-3-3 ϵ was fractionated in a 10% sodium dodecyl sulfate/ polyacrylamide gel (SDS-PAGE) by electrophoresis. Measurements were made in duplicate. Data were calculated as percentages of the mean values in the control subjects.

Identification of 14-3-3 ϵ gene polymorphisms

DNA was extracted from whole blood with the sodium iodide method using a DNA Extractor WB kit (Wako Chemicals, Tokyo, Japan). We checked the SNP database (http://snpper.chip.org/bio/snpper-enter) and focused on the three common (frequency $>10\%$ in a Japanese population) polymorphisms that cover the 14-3-3 ϵ gene: T-5424C (5′upstream, rs1532976), C38340A (intron, rs3752826), and T55012C (3′UTR, rs9393). Genomic DNA was amplified using the following primer sets: For T-5424C, the forward primer was 5′-gcctcttcctactcctacgtaactgct and the reverse primer was 5′-ggacggactgagtgagttctt; for C38340A, the mismatch forward primer was 5′-tagcccaaccacctttgcat and the reverse primer was 5′-ggcgagtccaaggttttcta; for T55012C, the forward primer was 5′-ccgtgccagatgtggcaagat and the mismatch reverse primer was 5′-cctctctcttagatgcttgcagca. Target sequences were amplified in a polymerase chain reaction (PCR) with a Gene Amp PCR System 9700 (ABI, Foster City, CA, USA). The PCR products were then digested with each enzyme, followed by electrophoresis on a 4% agarose gel. The DNA was visualized by ethidium bromide staining and UV transillumination. The PCR product of T-5424C was digested with *Mnl*I at 37°C overnight, and the T allele was cut into 381-bp and 13-bp fragments while the C allele was cut into 207-bp, 174-bp, and 13-bp fragments. The PCR product of C38340A was digested with *Bsm*I at 65°C for 4 h, and the C allele was cut into 166-bp and 16-bp fragments while the A allele was not cut and kept at 182 bp. The PCR product of T55012C was digested with *Alw*NI at 37°C overnight, and the C allele was cut into 71-bp and 22-bp fragments whereas that for the T allele was left uncut. Restriction enzymes *Mnl*I, *Bsm*I and *Alw*NI were obtained from Boehringer Mannheim (Mannheim, Germany).

Statistical significance

Student's t test was used to estimate the significance of differences between the two groups. Comparisons of the genotype or allele frequencies between groups were performed with a χ^2 test. The level of significance was set at $p=0.05$. Haplotype analysis (estimation of maximum-likelihood haplotype frequencies and differences in haplotypic distribution between suicide completers and control groups) was carried out using Alrequin software (http://anthropologie.unige.ch/arlequin/methods.html). The haplotype counts were estimated based on EM estimates of haplotype frequency. The permutation test was conducted to confirm differences in haplotypic distribution using SNPAlyze software (DYNACOM, Yokohama, Japan, http://www.dynacom.co.jp).

Results

Microarray data analysis

Gene expression data generated by the microarray were normalized by dividing each expression value by the

Table 2 Result of microarray analysis

Accession	Gene	Signal of gene expression							Fold change against Clontech	Fold change against controls
		S6[a]	S10	S11	S13	Clontech	C7	C9		
Upregulated										
U54778	14-3-3 epsilon	1404	1582	1652	1169	1110	529	372	1.3	3.3
J03473	Poly (ADP-ribose) synthetase	2228	2111	2159	2032	1889	832	951	1.1	2.4
D50916	KIAA0126	1181	1121	1014	1116	438	380	430	2.6	2.8
Z11793	Selenoprotein P	2608	2085	1050	1299	762	240	363	3.1	7.8
M38690	CD9 antigen	1226	716	568	575	537	78	148	1.8	8.6
AL050050	DKFZp566D133	680	514	526	496	395	177	213	1.5	3.1
S70154	Cytosolic acetoacetyl-coenzyme A thiolase	195	168	168	179	161	77	81	1.1	2.3
L10379	Clone CTG-B45d	268	264	283	259	227	89	98	1.2	2.8
U96750	Putative tumor supressor NOEY2	79	189	122	105	54	8	29	2.5	7.4
Downregulated										
	Cytochrome P450, subfamily IIc, alt. splice Form 2	54	54	45	29	93	121	125	0.6	0.4
AL080169	DKFZp434C171	450	394	448	382	498	1184	955	0.8	0.4

[a]Sample number in Table 1

median gene expression value. The genes of interest were genes whose expressions were (1) all elevated or depressed with respect to the expression in the Clontech sample, and (2) over twofold greater or less than that in the controls. Of approximately 12,000 genes examined, 11 satisfied both criteria (Table 2). Nine were upregulated in the brains of suicide victims with respect to both controls, and two of them were downregulated with respect to both controls. Of these genes, we selected the 14-3-3 ϵ gene for further analysis because of its relation to neurogenesis.

Immnunoquantification of 14-3-3 ϵ

To confirm the microarray result, we compared the protein expression levels of 14-3-3 ϵ in amygdala using immunoreactivity on suicide victims and controls (Fig. 1). The anti-14-3-3 ϵ immunoreactive bands had an apparent molecular weight of 30 kDa, as previously reported (Chen et al. 2003). A 14-3-3 ϵ immunoreactivity in the amygdala of suicide victims (mean ± SE 136.5 ± 11.3) was significantly higher ($p = 0.029$) than that in the controls (100 ± 5.2). In the control samples, the immunoreactivity levels of 14-3-3 ϵ were not correlated with age ($r = -0.14$, $p = 0.63$). There was no gender difference in the control samples (men 100.7 ± 0.63, women 99.6 ± 0.80, $p = 0.92$).

Genotyping analysis

Table 3 shows the genotype distribution and allele frequencies of the T-5424C, C38340A, and T55012C

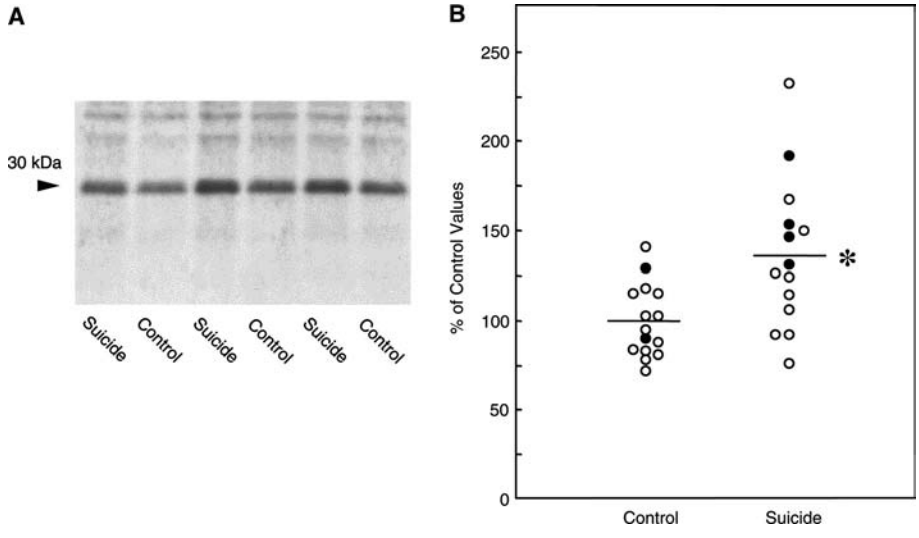

Fig. 1 Representative immunoblots of 14-3-3 ϵ (**a**) and changes in amygdala of suicide victims and controls (**b**). **a** *Each lane* was loaded with 10 μg protein obtained from the amygdala. *C* control subject, *S* suicide victim. **b** The results are expressed as a percentage of the control values. * $p <$ 0.05, Student's *t* test. *Closed circles* show the samples that were used in the microarray analysis

Table 3 Distribution of 14-3-3 ϵ gene polymorphisms in suicide completers and controls

Polymorphism		Suicide completers ($n=185$) (frequency)		Controls ($n=359$) (frequency)
T-5424C				
Genotype	A/A	32 (0.17)		82 (0.23)
	A/G	86 (0.47)		179 (0.50)
	G/G	67 (0.36)		98 (0.27)
	p value		0.072	
Allele	A	150 (0.41)		343 (0.48)
	G	220 (0.59)		375 (0.52)
	p value		0.023	[a]
C38340A				
Genotype	A/A	27 (0.14)		72 (0.20)
	A/C	90 (0.49)		182 (0.51)
	C/C	68 (0.37)		105 (0.29)
	p value		0.118	
Allele	A	144 (0.39)		326 (0.45)
	C	226 (0.61)		392 (0.55)
	p value		0.041	[b]
T55012C				
Genotype	C/C	13 (0.07)		29 (0.08)
	C/T	72 (0.39)		159 (0.44)
	T/T	100 (0.54)		171 (0.48)
	p value		0.365	
Allele	C	98 (0.26)		217 (0.30)
	T	272 (0.74)		501 (0.70)
	p value		0.198	

[a] $\chi^2=5.15$ df = 1
[b] $\chi^2=4.19$ df = 1

Table 4 Haplotype frequencies of the 14-3-3ϵ gene in completed suicides and controls

Haplotype			Estimated haplotype frequency	
T-5424C	C38340A	T55012C	Suicide ($n=185$)	Control ($n=359$)
A	C	A	0.0027	0.0014
A	C	G	0.0000	0.0014
A	T	A	0.3595	0.4415
A	T	G	0.0216	0.0084
C	C	A	0.0108	0.0153
C	C	G	0.2514	0.2841
C	T	A	0.0324	0.0195
C	T	G	0.3216	0.2284
				p value: 0.0005

polymorphisms of the 14-3-3 ϵ gene. All genotype distributions in the control group were in Hardy-Weinberg equilibrium and very similar to those in other Japanese populations (Hirakawa et al. 2002; Haga et al. 2002). The allele distributions at positions T-5424C and C38340A in suicide completers were significantly different from those in controls ($p=0.023$ and 0.041, respectively). Moreover, the difference in the distributions of the three haplotypes between the two groups was even more significant ($p=0.0005$) (Table 4) (Fig. 2). This association was confirmed by the permutation test ($p=0.002$ for 1,000 permutations). Similar results were observed ($p=0.005$ by Alrequin software and $p=0.011$ for 1000 permutations) between suicide completers and age-matched and gender-matched controls (124 men, 61 women, mean age 48.5 years, SD 15.6).

Discussion

We carried out the genetic study depending on the expression change of 14-3-3 ϵ in the amygdala of suicide victims. We found a significant difference of the 14-3-3 ϵ gene haplotype on completed suicides. This result suggests 14-3-3 ϵ as a potential suicide-susceptibility gene. We did not employ finer mapping techniques by using a denser collection of markers around the 14-3-3 ϵ locus. Therefore, we cannot exclude the possibility that the observed association signal originates from genes in the vicinity of the 14-3-3 ϵ locus. However, the altered expression of 14-3-3 ϵ indicates its involvement in suicide. This finding implies that the observed association originates from the 14-3-3 ϵ gene.

A 14-3-3 ϵ is a subtype of the 14-3-3 protein family. Until now, seven mammalian 14-3-3 subtypes have been found (β, γ, ϵ, η, ζ, σ, and θ) (Berg et al. 2003). The 14-3-3 proteins have their highest tissue concentrations in the brain and function as adapter proteins in multiple cellular processes, such as the mitogen-activated protein (MAP) kinase cascade, apoptosis signaling, and TPH activation (Berg et al. 2003; Ichimura et al. 1995). Although subtype-specific roles are not clear, several genetic studies have reported the involvement of 14-3-3 subtypes in the pathogenesis of mental disorder (Jia et al. 2004; Toyo-oka et al. 1999; Wong et al. 2003).

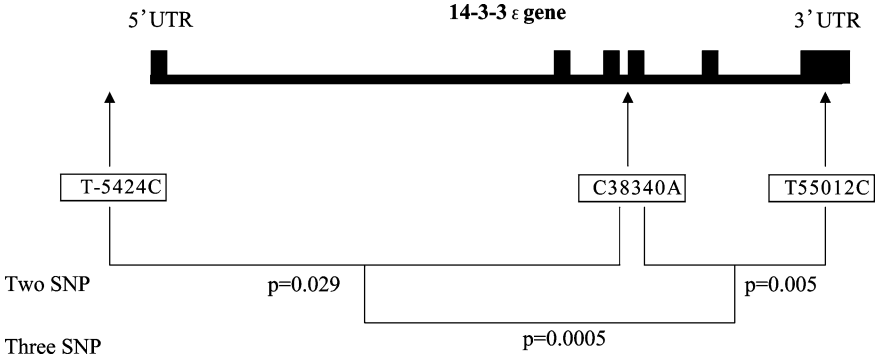

Fig. 2 Schema of 14-3-3 ϵ gene and haplotype results of 14-3-3 ϵ gene polymorphisms in suicide completers and controls. *Arrow* shows the locations of SNPs. The *p* values represent each haplotype result of two SNPs and three SNPs. Haplotype analysis was carried out using Alrequin software

Suicide mostly occurs under psychosocial stress conditions. Stressful experiences have been shown to inhibit granule cell production in the dentate gyrus of rodents and primates (Gould et al. 1997, 1998; Tanapat et al. 1998). In addition, lithium, which is thought to be efficacious in preventing suicide (Baldessarini et al. 2001; Goodwin et al. 2003; Muller-Oerlinghausen et al. 2002), has been found to stimulate neurogenesis in the dentate gyrus of rodents (Chen et al. 2000; Kim et al. 2004). Clozapine, which is an atypical antipsychotic that has been suggested to have some efficacy in preventing suicide (Meltzer 2001; Meltzer et al. 2003), was also reported to stimulate neurogenesis in the hippocampus of rats (Halim et al. 2004). These lines of evidence support the relation of neurogenesis with suicide.

Recently, 14-3-3 ϵ was found to have a role in neuronal migration, which is one part of neurogenesis (Toyo-oka et al. 2003). The amygdala is a region critical to emotional regulation and one of the areas in which newly generated neurons are found in primates (Bernier et al. 2002). Additionally, several reports have demonstrated an abnormal serotonergic neurotransmission and intracellular signal transduction in the amygdala of suicide victims (Cheetham et al. 1989; Hrdina et al. 1993; Young et al. 2004). In view of the functional relation of 14-3-3 ϵ to both neurogenesis and TPH activity, the increased 14-3-3 ϵ in suicide victims seems to be a compensatory reaction against inhibited neurogenesis under stress condition and/or decreased serotonergic neurotransmission. The significant association of the 14-3-3 ϵ gene haplotype on completed suicide indicates the genetic involvement of 14-3-3 ϵ in suicide. There are two possibilities about the relation of the genetic involvement with the increased 14-3-3 ϵ protein in suicide. First, the genetic alteration of the 14-3-3 ϵ gene may affect the expression of the 14-3-3 ϵ gene. Second, the genetic alteration may result in the dysfunction of 14-3-3 ϵ protein and could induce the increase of impaired 14-3-3 ϵ proteins in suicide victims.

We found the associations of allele distribution at two SNPs in the 14-3-3 ϵ gene. However, genotype distributions were not significant, and observed significances of allele distribution disappeared after Bonferroni correction. These findings indicate that the associations of each SNPs with suicide are marginal. Therefore, we cannot exclude the possibility that these associations are due to a type I error. Since three SNPs are located on -5424 at 5′UTR, intron, and 3′UTR, it is unlikely that they alter the function of 14-3-3 ϵ. Observed weak associations of allele distribution may represent the association of adjacent functional polymorphisms. Further studies are needed to find other polymorphisms, which have strong association with suicide in the 14-3-3 ϵ gene.

This study has some limitations. Psychiatric disorders, such as mood disorders and schizophrenia, are known to show high suicide risk. Our results might reflect, in part, the pathogenesis of these disorders. However, we were unable to obtain clinical backgrounds of the suicide victims because of the ethical code for this genetic study. To exclude the possibility that our results reflect the pathogenesis of psychiatric disorders, association studies in a specific mental disorder are needed. The information about psychotropic medications in suicide victims was also not available. Actually, psychotropic medications may affect the gene expression of 14-3-3 ϵ. However, Iwamoto et al. (2004) reported that the gene expression of 14-3-3 ϵ was not significantly affected by psychotropic medications in bipolar disorder. Although the information about the mood state and the psychiatric diagnose in suicide victims is not available, it seems that the expression change of 14-3-3 ϵ in this study was not due to psychotropic medications. Next, the gene expression in the controls showed very low compared with that in the Clontech sample and the suicide victims. Although we used the RNA samples, which were of adequate RNA quality (28S/18S ratio >1.5), the integrity of RNA in the controls may be worse than that in the suicide victims and the Clontech sample.

Our initial analysis of the 14-3-3 ϵ gene has detected a significant association between it and completed suicide. This finding indicates the possibility that the 14-3-3 ϵ gene contributes to the biological susceptibility to suicide. Further studies are needed, with samples from a greater number of suicides, to verify this study.

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