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Functional Studies of HSPA5 Promoter Polymorphisms and Risk of Essential Tremor in Taiwan

Li-Ching Lee[1], Fen-Ju Hu[1], Yih-Ru Wu[2], Yu-Yao Lo[1], Ya-Yun Lin[1], Chiung-Mei Chen[2]*, Guey-Jen Lee-Chen[1]*

[1] Department of Life Science, National Taiwan Normal University
Taipei, Taiwan

[2] Department of Neurology, Chang Gung Memorial Hospital, Chang-Gung University College of Medicine
Taipei, Taiwan

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ABSTRACT

Endoplasmic reticulum (ER) stress induced by misfolded proteins and a malfunction of unfolded protein response (UPR) to ER stress have been implicated in neurodegenerative disease pathogenesis. Heat shock 70 kDa protein 5 (HSPA5) is one of the UPR chaperones reactive to ER stress to block the apoptotic process. Three polymorphisms in the HSPA5 promoter region, -415 G/A (rs391957), -370 C/T (rs17840761) and -180 del/G (rs3216733), may affect the gene expression. Using a reporter assay, we examined the functional implication of these three promoter polymorphisms. Reporter construct containing the polymorphic -415 A allele cloned into a luciferase reporter plasmid drove significantly lower transcriptional activity of HSPA5 compared with the common -415 G allele in human neuroblastoma SK-N-SH cells. The association of these polymorphisms with Taiwanese essential tremor (ET) was investigated using a case-control study. The genotype, allele or haplotype frequency distribution examined was not significantly different between the controls (n = 341) and the ET patients (n = 130). Our data suggest that while functionally, the HSPA5 -415 G/A polymorphism is unlikely to affect susceptibility to ET in Taiwanese subjects.

Key words: HSPA5/GRP78, promoter reporter assay, polymorphism, association study, essential tremor

Introduction

Endoplasmic reticulum (ER) stress is caused by disturbances in the structure and function of the ER with the accumulation of misfolded proteins and alterations in the calcium homeostasis. The unfolded protein response (UPR) serves to protect the ER and restores function by inducing chaperons, blocking translation and increasing protein folding in the ER (Lindholm *et al*., 2006). A malfunction of the ER stress response can result in neurodegenerative diseases such as Parkinson's, Alzheimer's and prion diseases (Yoshida, 2007). Among the UPR, HSPA5 (heat shock 70 kDa protein 5) also referred to as GRP78 (glucose-regulated protein, 78 kDa) or BiP (immunoglobulin heavy chain-binding protein), is an important chaperon involving in the folding and assembly of proteins in ER (Lee, 2005). Disrupted function of HSPA5 through defect in its co-chaperon SIL1 causes Marinesco-Sjogren syndrome characterized by cerebellar ataxia, progressive myopathy and cataracts (Senderek *et al*., 2005). Increased expression of HSPA5 can have neuroprotection from ER stress by enhancing UPR (Chen *et al*., 2000).

Essential tremor (ET), one of the most common neurological disorders, is characterized by postural tremor which worsens with movements (Thanvi *et al*., 2006). The estimated prevalence of ET worldwide ranges from 0.41 to 3.29% (Louis *et al*., 1998). The causes and mechanisms of ET remain poorly understood. Environmental agents and genetic factors are both believed to play an important role in ET. Linkage mapping efforts have found at least three loci for familial ET (*ETM1* on 3q13, *ETM2* on 2p24.1 and a locus on 6p23) (Gulcher *et al*., 1997; Higgins *et al*., 1997; Shatunov *et al*., 2006). More recently, a S9G variant in the dopamine D3 receptor gene, located

*Corresponding authors: Guey-Jen Lee-Chen; FAX: 886-2-29312904; E-mail: t43019@ntnu.edu.tw
Chiung-Mei Chen; FAX: 886-3-3285060; E-mail: cmchen@adm.cgmh.org.tw

in the *ETM1* locus, was found associated with risk of ET (Lucotte *et al*., 2006; Jeanneteau *et al*., 2006). An association between ET and an A265G variant in the HS1-binding protein 3 gene, located in the *ETM2* locus, has also been suggested as the cause of the disorder in American ET patients (Higgins *et al*., 2005, 2006). Additionally, association of the C677T and A1298C polymorphisms of methylenetetrahydrofolate reductase in patients with ET in Turkey were reported (Sazci *et al*., 2004).

Promoter polymorphism may affect gene expression. HSPA5 promoter polymorphisms may affect the individual variability of ER stress response and has been reported to be a risk factor for bipolar disorder in a Japanese population (Kakiuchi *et al*., 2005). Therefore, HSPA5 promoter polymorphisms may potentially confer a genetic risk factor to ET. We examined whether the HSPA5 single nucleotide polymorphisms (SNPs) (-415, -370 and -180) and haplotypes affect gene expression and predispose to the risk of developing ET in Taiwan.

Materials and Methods

Subjects

A total of 130 unrelated subjects (49.2% women, 50.8% men), diagnosed with ET by neurological specialist C. M. Chen and Y. R. Wu, were recruited from the neurology clinics of Chang Gung Medical Center. Diagnosis of ET was based on the Tremor Investigational Group criteria for definite or probable essential tremor (Findley and Koller, 1995). To the extent possible, other types of tremor with a recognizable cause, such as tremor caused by hyperthyroidism, medical intoxication, drug withdrawal, and chronic alcoholism were excluded. The mean age of ET at time of examination was 63.8 ± 13.5 years, ranging between 34 and 89 years. A group of 341 normal control individuals without neurodegenerative diseases including any kind of tremor were recruited from the same ethnic community. Control subjects (49.6% women, 50.4% men) had mean age at examination of 62.5 ± 11.4 years, ranging between 39 and 94 years. All examinations were performed after obtaining informed consent from patients and control individuals.

Genotyping

DNA was extracted from leukyocytes by using the standard protocols. The polymorphisms of -415 G/A (rs391957) and -370 C/T (rs17840761) were determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (Table 1) (Fig. 1A). To genotype -180 del/G (rs3216733), the length of the amplified products was determined by electrophoresis in a linear polyacrylamide gel on an automated MegaBACE Analyzer (Molecular Dynamics, Division of Amersham Pharmacia Biotech) (Fig. 1B). In addition, aliquots of the amplified products were mixed with an equal volume of 95% formamide buffer and subjected to single strand conformation polymorphism (SSCP) analysis using GeneGel Excel gels as recommended by the manufacturer (Pharmacia Biotech) (Fig. 1C). Alleles del and G were confirmed by DNA sequencing.

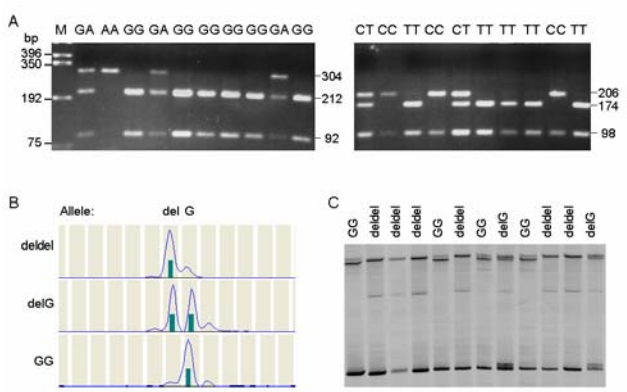

Figure 1. (**A**) RFLP analysis of *HSPA5* -415 G/A (*Xmn*I 212, 92 / 304) and -370 C/T (*Bst*YI 206 / 174, 32) polymorphisms. Lane M (*Hin*fI digest of pGEM4 DNA) was used for size markers. (**B**) Electrophoresis of fluorescenced -180 del/G PCR products in linear polyacrylamide gels on an automated sequencer. (**C**) Electrophoresis of denatured -180 del/G PCR products for SSCP analysis. The template genotypes are marked on the top (**A** and **C**) or left (**B**) of panel.

Statistical analysis

Genotype and allele frequencies for each polymorphic site were calculated, and the differences between patients and controls were tested by the χ^2 test of association and the Fisher's exact test where appropriate. The χ^2 test was used to test Hardy-Weinberg equilibrium for each polymorphic site. The SNPSpD method (Nyholt, 2004) was used to generate an adjusted significance threshold for correction of multiple SNP testing. The experiment-wide significance threshold of

Table 1. Specific PCR conditions and detection for genotyping.

Polymorphism	Anneal (℃) / $MgCl_2$ (mM)	Enzyme* (fragment bp)
-415 G/A and -370 C/T		
F: TCAGAGACTGGATGGAAGCTGG	56 / 1.0	*Xmn*I: GAANNNNTTC (212, 92 / 304)
R: TGGCTGCTATTCGTTTCTAACG		*Bst*YI: RGATCY (206, 98 / 174, 98, 32)
-180 del/G		
F: hex-CGGGGTCAGAAGTCGCAGGAGAGAT	63 / 1.0	(212 / 213)
R: CGTTGGAGGCCGTTCATTGG		

*The underlines in the enzyme recognition site indicate the polymorphic site.

0.031 was required to keep the type I error rate at 5%. Measures of pairwise linkage disequilibrium (LD) between SNPs, including Lewontin's standardized disequilibrium coefficients (D'), the squared pairwise correlations (Δ^2), and eigenvalues (λs) were computed with the LDMAX software-part of the GOLD Command Line Tools package (Abecasis and Cookson, 2000). PHASE version 2.1 was used to reconstruct the HSPA5 gene haplotypes (Stephens *et al*., 2001; Stephens and Donnelly, 2003). The HSPA5 pairwise haplotype frequencies were computed and Chi-square tested for significance. One-way analysis of variance was used to test between-group differences in age of disease onset. Odds ratios (ORs) with 95% confidence intervals (95% CI) were calculated to test association between genotype/allele/haplotype and disease.

Promoter functional assay

The HSPA5 promoter fragment (-606 ~ +15, where +1 represents the first nucleotide to be transcribed) containing G-T-del, G-C-del and A-C-G haplotypes (-415, -370 and -180 sites) were cloned into the pGEM-T Easy vector (Promega) and sequenced. In addition, site-directed mutagenesis was performed to generate G-C-G haplotype for comparison. The cloned promoter fragments were placed upstream of the firefly luciferase reporter gene at the *Eco*RI site added between the *Hin*dIII/*Xho*I sites of the pGL3-basic vector (Promega). The orientation of the insert in the HSPA5-pGL3 construct was confirmed by restriction analysis. Human neuroblastoma SK-N-SH and embryonic kidney HEK-293 cells cultivated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum were plated into 24-well dishes (2×10^5/well), grown for 20 h, and transfected by the lipofection method (GibcoBRL). The test plasmid (0.95 μg) and internal *Renilla* luciferase control plasmid (phRL-TK, 0.05 μg) were co-transfected into cells. The cells were grown for 48 h. Cell lysate were prepared and the activity of each promoter was directly measured by the ratio of the firefly luciferase level to the *Renilla* luciferase level using a dual luciferase assay system (Promega). For each reporter construct, four independent transfection experiments each performed in quartet were performed. An allele-specific difference in luciferase activity was tested using the two-tailed Student's *t* test.

Results

The genotype distributions in ET and controls did not deviate significantly from Hardy-Weinberg equilibrium for all three polymorphisms examined (data not shown). The modified SNPSpD method was employed for correction of multiple SNP testing. SNPSpD output of three λs was shown in Table 2. As described by Cheverud (2001), high correlation among variables leads to high λs. In this case, the first λ (1.63) is less than 2 (the number of variables in the correlation matrix), suggesting that not all variables are completely correlated. The magnitude of pair-wise LD was quantified by the metrics D' and Δ^2. The D' coefficient of -415 G/A and -180 del/G was equal to 1 (D' = 1.0), strongly

Table 2. Pairwise linkage disequilibrium measures* for *HSPA5* SNPs.

		D'		
		-415 G/A	-370 C/T	-180 del/G
Δ^2	-415 G/A	***1.63***	1.00	1.00
	-370 C/T	0.39	***0.37***	1.00
	-180 del/G	1.00	0.39	-

*Lewontin's standardized disequilibrium coefficients (D') are given above the diagonal and the squared pairwise correlations (Δ^2) are given below the diagonal; the eigenvalues (λs) associated with the LD correlation matrix are given along the diagonal (***bold, italic***).

suggesting that there has been no recombination in the region over time, and a very strong LD was observed between -415 G/A and -180 del/G sites ($\Delta^2 = 1.0$). SNP -415 G/A and SNP -180 del/G were completely linked in sampled patients and controls as all individuals tested had identical polymorphic genotypes.

The genotype and allele frequency distributions of the polymorphisms in ET patients and controls are displayed in Table 3. Neither the genotypic ($P = 0.438$) nor the allelic frequencies ($P = 0.705$) of -415 G/A (-180 del/G) polymorphism were statistically different between the ET and the control groups. The genotypic and the allelic frequencies of -370 C/T polymorphism were also similar between the ET patients and the controls ($P = 0.530$ for genotype; $P = 0.865$ for allele). For ET patients, the mean ages at time of examination for the -415 G/A (-180 del/G) genotype-carrying groups (in years) were 66.8 ± 13.2 for AA, 62.0 ± 13.4 for GA and 64.9 ± 13.5 for GG. The mean ages at time of examination for the -370 C/T genotype-carrying groups (in years) were 65.3 ± 12.8 for CC, 63.6 ± 14.1 for CT and 62.0 ± 12.4 for TT. The ages at time of examination were not significantly different when comparing the three -415 G/A (-180 del/G) genotype groups ($P = 0.373$) and when comparing the three -370 C/T genotype groups ($P = 0.630$).

Pairwise haplotype analysis of the three polymorphisms showed that none of the haplotype frequencies was different between the controls and the ET groups ($P = 0.925$) (Table 3). ORs of the at-risk genotype were calculated by comparing each value to the common genotype, allele or haplotype (Table 3). None of the genotypes, alleles or halotypes of the *HSPA5* gene polymorphisms confers an increased risk to ET ($P = 0.259 \sim 0.932$).

To test the effect of a polymorphic sequence on gene expression, fragments containing the haplotypes G-T-del, G-C-del, A-C-G and G-C-G (-415, -370 and -180 sites) were fused to firefly luciferase reporter construct and transient transfection experiments were carried out to assess the promoter activity by measuring the ratio of firefly luciferase activity to *Renilla* luciferase activity in SK-N-SH and HEK-293 cells. As shown in Fig. 2, the -370 C did not appreciably affect the promoter activity compared with the -370 T (G-C-del vs. G-T-del) in both SK-N-SH (111%, $P = 0.294$) and HEK-293 cells (75%, $P = 0.055$). This is also true for the -180 G compared to the -180 del (G-C-G vs.G-C-del) (103%, $P = 0.811$ in SK-N-SH; 89%, $P = 0.576$ in HEK-293). However, compared with the -415 G (A-C-G vs. G-C-G), the -415 A drove significantly lower (59%, $P = 0.011$) luciferase activity in SK-N-SH cells but not in HEK-293 cells (87%, $P = 0.570$). When haplotype

Table 3. Genotype, allele and haplotype distributions and association analysis.

	ET		Control		Odds ratio[b]	*P*-value
	No.	(%)	No.	(%)	(95% CI)	
Genotype/allele						
-415 G/A (-180 del/G)[a]						
GG (deldel)	59	(45.4)	170	(49.9)	1.00	
GA (delG)	59	(45.4)	133	(39.0)	1.28 (0.83-1.96)	0.259
AA (GG)	12	(9.2)	38	(11.1)	0.91 (0.43-1.81)	0.795
G (del)	177	(68.1)	473	(69.4)	1.00	
A (G)	83	(31.9)	209	(30.6)	1.06 (0.78-1.44)	0.705
-370 C/T						
CC	33	(25.4)	94	(27.6)	1.00	
CT	74	(56.9)	175	(51.3)	1.20 (0.75-1.96)	0.448
TT	23	(17.7)	72	(21.1)	0.91 (0.49-1.68)	0.763
C	140	(53.8)	363	(53.2)	1.00	
T	120	(46.2)	319	(46.8)	0.98 (0.73-1.30)	0.865
Haplotype (-415/-370/-180)						
G-T-del	120	(46.2)	319	(46.8)	1.00	
G-C-del	57	(21.9)	154	(22.6)	0.98 (0.68-1.42)	0.932
A-C-G	83	(31.9)	209	(30.6)	1.06 (0.76-1.47)	0.747

[a]For -415 G/A and -180 del/G, all individuals tested had identical polymorphic genotypes; [b]Odds ratios for minor common genotypes, alleles or haplotypes were calculated by comparing each value to the major common genotype, allele or haplotype.

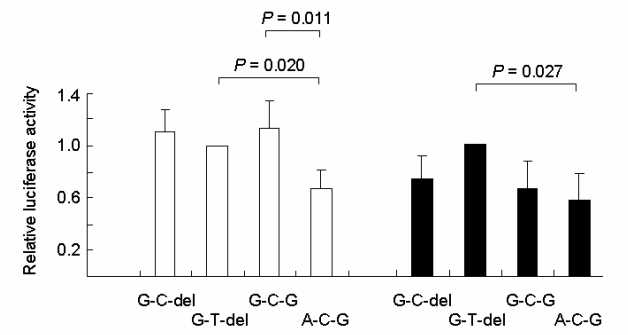

Figure 2. Luciferase reporter assay. Transient expression of luciferase enzymatic activity driven by the HSPA5 5'-flanking promoter fragments in SK-N-SH (open bars) and HEK-293 (filled bars) cells. Each value is the mean ± SD of four independent experiments, each performed in quartet.

A-C-G was compared with haplotype G-T-del directly, significantly lower luciferase activity was observed in both SK-N-SH (67%, P = 0.020) and HEK-293 (58%, P = 0.027) cells.

Discussion

Neuronal death can be induced by ER stress in Alzheimer's disease (Katayama *et al*., 2004) and ER chaperones can protect against the disease by inhibiting the production of amyloid-β peptide (Hoshino *et al*., 2007). Similarly, loss of parkin function and mutation of α-synuclein can increase ER stress and then lead to neuronal loss in Parkinson's disease, if UPR is not adequately activated (Imai and Takahashi, 2004; Smith *et al*., 2005). Among UPR, HSPA5 is one of the chaperons to protect cell from pro-apoptotic pathways (Lee, 2005). Recently, polymorphisms and haplotypes in HSPA5 promoter was found to affect the basal activity of HSPA5 promoter and haplotypes carrying high activity of promoter were found to be a risk factor for bipolar disorder in a Japanese population (Kakiuchi *et al*., 2005). Although how the haplotypes carrying high promoter activity increase the disease risk is not clear, the biological function makes HSPA5 a good candidate for the risk study of neurodegenerative disease. Given that multi-factors involving both genetics and environment are implicated in the ET pathogenesis, we examined if the HSPA5 polymorphisms influence risk of ET. However, our study did not demonstrate an association between the HSPA5 polymorphisms and haplotypes with ET in our Taiwanese population.

The HSPA5 promoter contains three ER stress response elements (ERSEs) consisting of a tripartite structure CCAATN$_9$CCACG, with N being 9-bp GC-rich region (Yoshida *et al*., 1998). Transcription factors CBF/NF-Y, YY1 and TFII-I bind to the CCAAT, CCACG and GC-rich N$_9$ region, respectively, to activate the ERSE (Li *et al*., 1997; Marcus *et al*., 1997; Parker *et al*., 2001). The three SNPs examined located not within but upstream of ERSE, and did not alter ER stress response (Kakiuchi *et al*., 2005). Among the three SNPs examined, the promoter activity of polymorphic -415 A was significantly lower than that of the common -415 G in SK-N-SH cells (Fig. 2). The -415 G/A lies within the regulatory element (AGAYAAGATAA) involved in the binding of ecotropic viral integration site 1 (EVI-1) (http://mbs.cbrc.jp/research/db/TFSEARCH.html). Binding of EVI-1 to -450 A may interfere with the transcriptional activity of some factors controlling the HSPA5 level expression in SK-N-SH cells. Alternatively, the -415 G/A may affect the binding of unknown transcription factors to alter the HSPA5 expression.

We have examined the limitations and advantages of our study. Although our study cohort includes 130 unrelated ET subjects and 341 normal controls, which may still be unable to detect an uncommon disease locus with a small effect. Secondly, environmental agent exposure and its interaction with HSPA5 promoter were not assessed, and these factors may be relevant in the context of this particular gene. However, our study have also two strengths: (1) laboratory genotyping was performed blind to case-control status, minimizing experimental bias and genotyping was rechecked if there is ambiguity; (2) all ET patients and control subjects had a similar ethnic background, limiting the possible confounding effect of population stratification.

As locus heterogeneity for the disease pathogenesis in different genetic backgrounds has been indicated in ET (Ma *et al*., 2006), our negative results do not exclude an association between HSPA5 and ET in other ethnic populations. While our results do not exclude a possible association of other genetic variants within the HSPA5 gene in ET, we conclude that the HSPA5 -415 G/A, -370 C/T and -180 del/G polymorphisms are unlikely to play a major role in ET in our population.

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HSPA5 啟動子多型性的功能性分析及其與台灣原發性顫抖症的相關性研究

李麗卿[1] 胡雱茹[1] 吳逸如[2] 羅于堯[1] 林雅芸[1] 陳瓊美[2]* 李桂楨[1]*

[1]國立臺灣師範大學生命科學系

[2]長庚醫院神經內科

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摘　要

蛋白質不正常摺疊引起的內質網壓力(ER stress)及不正常蛋白質摺疊反應(unfolded protein response；UPR)的機能失常，和神經退化性疾病的致病機制相關。70 kDa 熱休克蛋白 5 (HSPA5)為一 UPR 伴隨蛋白，可反應內質網壓力、抑制細胞凋零。HSPA5 基因的啟動子多型性-415 G/A (rs391957)、-370 C/T (rs17840761)及-180 del/G (rs3216733)可能影響其表現量。利用啟動子報告基因試驗，我們檢測了這三個多型性所暗示的功能。在神經癌 SK-N-SH 細胞中，帶有-415 A 多型性啟動子的螢光酵素報告質體表現的轉錄活性顯著低於帶有-415 G 者。利用個案-正常對照研究，我們分析了 HSPA5 基因啟動子多型性與台灣原發性顫抖症的相關性。在正常人(n = 341)及原發性顫抖症患者(n = 130)間，所分析的各多型性點，其基因型、等位基因與單套型頻率皆無顯著差異。我們的實驗數據顯示，HSPA5 -415 G/A 多型性雖然影響其功能，但和台灣原發性顫抖症的風險無關。

關鍵詞：HSPA5/GRP78、啟動子報告基因試驗、多型性、相關性研究、原發性顫抖症

*通信作者：李桂楨（Guey-Jen Lee-Chen）；FAX：886-2-29312904；E-mail：t43019@ntnu.edu.tw

陳瓊美（Chiung-Mei Chen）；FAX：886-3-3285060；E-mail：cmchen@adm.cgmh.org.tw