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Differential Effects of the HESR/HEY Transcription Factor Family on Dopamine Transporter Reporter Gene Expression Via Variable Number of Tandem Repeats

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The 3′-untranslated region (UTR) of the human dopamine transporter (*DAT1*) gene contains a variable number of tandem repeats (VNTR) domain, which is thought to be associated with dopamine-related psychiatric disorders, personality, and behavior. However, the molecular and neuronal functions of polymorphisms within the VNTR domain are unknown. We previously identified the transcription factor HESR1 (HEY1) as a VNTR-binding protein. *Hesr1* knockout mice exhibit DAT up-regulation in the brain and low levels of spontaneous activity. Other members of the HESR (HEY) family, including HESR2 (HEY2) and 3 (HEYL), have similar DNA-binding domains. In this study, we analyzed the effects of HESR1, -2, and -3 on *DAT1* expression in human neuroblastoma SH-SY5Y cells using luciferase reporter assays. We found that the VNTR domain played an inhibitory role in *DAT1* reporter gene expression and that HESR1 and -2 inhibited expression via both the core promoter and the VNTR. The inhibitory effects of HESR family members on *DAT* reporter gene expression differed depending on the number of repeats in the VNTR domain. We also found that each Hesr was expressed in the dopaminergic neurons in the mouse midbrain. These results suggest that the HESR family is involved in *DAT* expression via the VNTR domain.

Key words: dopamine; genetic polymorphism; VNTR; luciferase reporter assay

The dopaminergic nervous system plays important regulatory roles in locomotion, cognition, reward, affection, and hormone release (Bannon et al., 2001; Jackson and Westlinddanielsson, 1994; Missale et al., 1998; Uhl, 2003). Thus, dopamine and its related genes are thought to be involved in neuropsychiatric disorders and behavioral traits. The human dopamine transporter (*DAT1*) gene is involved in many dopamine-related disorders. DAT levels are reduced in Parkinson's disease (PD) and elevated in attention deficit hyperactivity disorder (ADHD), Tourette's syndrome, and major depression (Madras et al., 1998; Muller-Vahl et al., 2000; Brunswick et al., 2003; Krause et al., 2003). In addition, several psychoactive drugs, including cocaine, amphetamine, and methylphenidate, are known to inhibit dopamine reuptake by DAT (Giros et al., 1991, 1992; Kilty et al., 1991; Shimada et al., 1991; Giros and Caron, 1993).

A functional genetic polymorphism has been described in the 3′-untranslated region (UTR) of exon 15 in *DAT1* (Michelhaugh et al., 2001). This 3′-UTR contains a 40-bp-long variable number of tandem repeats (VNTR) domain (Fig. 1; Vandenbergh et al., 1992; Michelhaugh et al., 2001). The polymorphism within this region is known to be associated with such neuropsychiatric disorders as ADHD, PD, alcoholism, and drug abuse (Cook et al., 1995; Ueno et al., 1999; Vandenbergh et al., 2000; Ueno, 2003; D'souza and Craig, 2008) and with modified gene expression depending on the genotype in vivo (Heinz et al., 2000; Jacobsen et al., 2000; Mill et al., 2002; D'souza and Craig, 2008) and in mammalian cell lines (Fuke et al., 2001, 2005; Inoue-Murayama et al., 2002; Miller and Madras, 2002; Greenwood and Kelsoe, 2003; Mill et al., 2005; VanNess et al., 2005; D'souza and Craig, 2008). It is expected that this region contains binding sites for interacting proteins, but, because these factors have not been described, the molecular and neuronal functions of the polymorphism are unknown (Michelhaugh et al., 2001).

We previously identified and characterized HESR1 (HEY1) as a *trans*-acting repressor of gene expression

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that acts through the 3′-UTR of *DAT1* via a yeast one-hybrid system (Fuke et al., 2005). In addition, we also showed that HESR1 binds directly to the region by electrophoretic mobility shift assay (EMSA) and represses the expression of the endogenous *DAT1* gene in the HEK293 cell line by RT-PCR assay (Fuke et al., 2006). In *Hes1* knockout (KO) mice, *DAT1* expression was enhanced, and the mice exhibited a reduction in spontaneous locomotor activity and exploration to novelty (Fuke et al., 2006). These findings suggest that the human *HESR1* gene and the polymorphisms could be related to many psychiatric disorders and behavioral traits. However, it is possible that other factors affect *DAT1* expression via the VNTR domain, insofar as more than one interacting factor is expected to bind this region (Michelhaugh et al., 2001). Conflicting results have been reported from studies using different cell lines, which may express different transcription factors (Fuke et al., 2001, 2005; Inoue-Murayama et al., 2002; Miller and Madras, 2002; Greenwood and Kelsoe, 2003; Mill et al., 2005; VanNess et al., 2005; D'souza and Craig, 2008). These results suggest that *DAT1* expression can be altered by cell-specific factors depending on the VNTR alleles present.

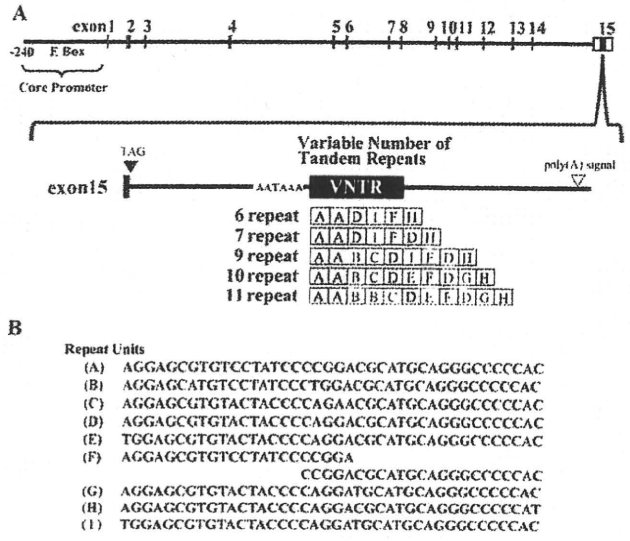

Fig. 1. Genomic structure of *DAT1* and allelic variants of the VNTR domain in exon 15. **A:** The coding region (black box), non-coding region (open box), VNTR domain, and constant parts of the repeat units (gray box) are shown. Exon 15 of *DAT1* contains a stop codon (solid arrowhead) and polyadenylation signal (open arrowhead). Upstream of the VNTR domain are six nucleotides (AATAAA) that resemble a polyadenylation signal. The allelic variants of the VNTR indicate the repeat unit type (A–I) for each allele. **B:** Nucleotide sequence of each unit of the VNTR polymorphism in the 3′-UTR of *DAT1*.

The Hesr family genes *Hesr1*, *-2*, and *-3* (*Hey1*, *Hey2*, and *HeyL*) were identified as the hairy/enhancer split-type basic helix-loop-helix (bHLH) genes. They have been shown to be direct transcriptional targets of the Notch signaling pathway, which is essential for neural development (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Henderson et al., 2001; Iso et al., 2001, 2003; Wang et al., 2002; Sakamoto et al., 2003). HESR family genes carry a bHLH domain essential for DNA binding as well as an Orange domain and YRPW motif, which mediate interaction with proteins and affect dimerization or recruitment of corepressors (Fischer and Gessler, 2007; Fig. 2). HESR proteins repress the expression of target genes by binding to E- or N-box bHLH-binding consensus sites (Nakagawa et al., 2000; Iso et al., 2001, 2003). The bHLH domain is highly conserved among HESR family members (Steidl et al., 2000), and Hesr1 and -2 repress gene expression via the same sequence (Kokubo et al., 2007). Thus, HESR1 along with HESR2 and -3 may be candidate factors regulating *DAT* expression via the VNTR. However, any roles of HESR2 and -3 in *DAT* gene expression have not yet been characterized.

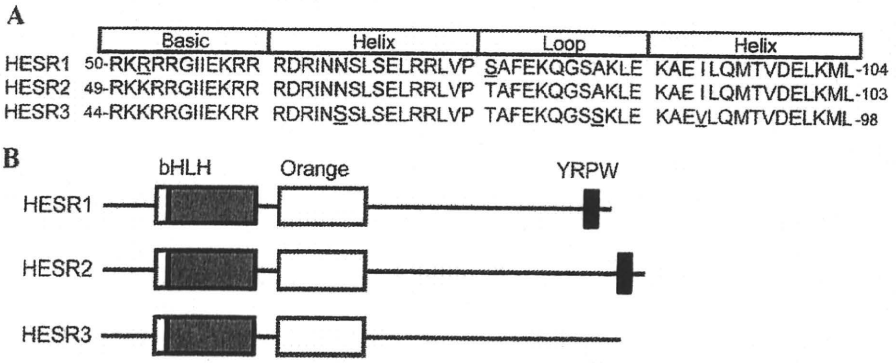

Fig. 2. Structure of HESR family members. **A:** Comparison of the primary sequences of the bHLH domain among human HESR family members (HESR1, 50–104 amino acids; HESR2, 49–103 amino acids; and HESR3, 44–98 amino acids). Those residues that differ among the family members are underscored. The primary sequences of the bHLH domain in the mouse Hesr family are identical to those in the human protein. **B:** Structure of the HESR family with the three major domains noted: bHLH domain, Orange domain, and YRPW motif.

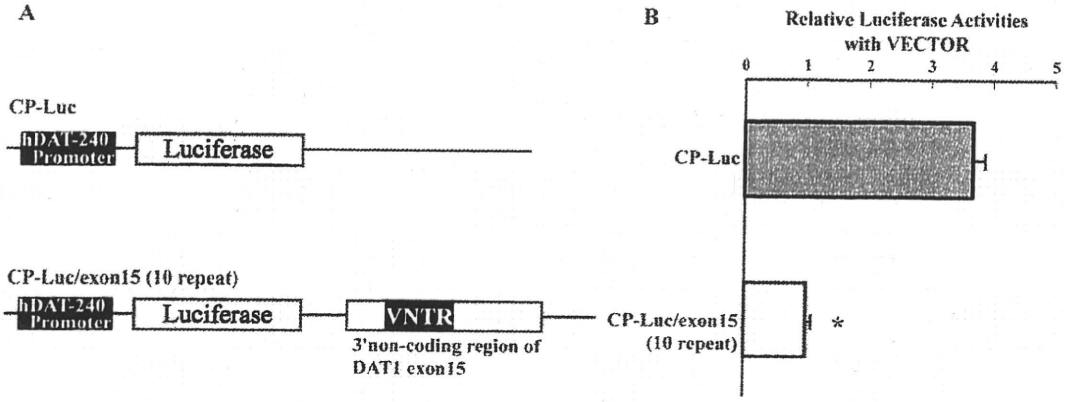

Fig. 3. Luciferase reporter vector and its activity in SY-SH5Y cells. **A:** Schematic diagram of the luciferase reporter vectors CP-Luc and CP-Luc/exon 15. CP-Luc contains only the *DAT* core promoter, whereas CP-Luc/exon 15 contains both the core promoter and the 3′-UTR with 10r, which is the most common allele. **B:** Negative regulation of gene expression through the 3′-UTR of *DAT* in SY-SH5Y cells. CP-Luc or CP-Luc/exon 15 and empty vector were transfected into the cells. Relative luciferase activity is expressed as the mean ± SEM. $*P < 0.0001$, Student's *t*-test.

In this study, we sought to clarify whether HESR2 and -3, as well as HESR1, also affect *DAT1* expression in human neuroblastoma cells via the 3′-UTR, including the VNTR region. Luciferase reporter constructs were made containing the endogenous *DAT1* core promoter and the VNTR with 6, 7, 9, 10, or 11 repeats, which is consistent with the sequences reported previously (Fuke et al., 2001). The differential effects on VNTR among HESR1, -2, and -3 were also characterized. Furthermore, we conducted immunohistochemistry for Hesrs and tyrosine hydroxylase (TH), a marker of dopaminergic neurons, in mouse midbrains to analyze localization of Hesr proteins for the investigation of HESR family function in the brain dopamine systems, because localization of HESR proteins in the adult brain has not been reported, although dopaminergic neurons localize in the specific brain regions (Bjorklund and Dunnett, 2007).

MATERIALS AND METHODS

General Procedure

First, to characterize the role of the 3′-UTR in *DAT* expression, a CP-Luc or CP-Luc/exon15 (10r) reporter vector and empty vector (control) were expressed in cells. Ten repeats is the most common allele in the population (Fuke et al., 2005; D'souza and Craig, 2008). CP-Luc contained only the *DAT* core promoter, whereas CP-Luc/exon15 contained both the core promoter and the 3′-UTR (Fig. 3). Next, the empty vector or each HESR was cotransfected with CP-Luc or CP-Luc/exon15 (10r). Finally, to compare the VNTR alleles and HESRs, each repeat (6, 7, 9, 10, or 11r) of CP-Luc/exon15 and the HESR or vector were cotransfected. The relative luciferase activity was standardized to that of the vector. In the experiment examining the repeat effect, the relative luciferase activity was standardized to that of 10r in each group. In addition, the localization of Hesrs in the mouse midbrain was detected by immunohistochemistry.

Cloning and Construction

Two kinds of luciferase reporter vectors were prepared: CP-Luc and CP-Luc/exon 15 (see Fig. 3A). CP-Luc contained the human *DAT* core promoter (−240 to +2; Fig. 1A; Kouzmenko et al., 1997; Sacchetti et al., 1999) cloned from the DAT1-8317 plasmid (Sacchetti et al., 1999), a gift from Dr. Michael J. Bannon (Wayne State University, School of Medicine, Detroit, MI), upstream of the firefly luciferase site in the modified pGL3 vector (Promega, Madison, WI). CP-Luc/exon 15 contained the human *DAT* core promoter and 3′-UTR region including the VNTR domain downstream of the luciferase site in CP-Luc. There are five kinds of VNTR alleles (6, 7, 9, 10, and 11r), which is consistent with the sequence (Fig. 1B) reported in our previous study (Fuke et al., 2001). These reporter vectors are the same as the constructs used in our previous study (Fuke et al., 2005); schematic structures of the reporter vectors are shown in Figure 3A. Additional information on these constructs is described in our previous work (Fuke et al., 2005).

All of the HESR family expression vectors were made by cloning the cDNA into myc-pcDNA modified from pcDNA 3.1+ (Invitrogen, Carlsbad, CA), an expression vector for mammalian cell lines. The Myc-tag is located upstream of the multicloning site. Myc-pcDNA was also used as a control vector (the vector described in Figs. 3–6). Myc-pcDNA and Myc-HESR1 (human) are the same as the constructs described in our previous study (Fuke et al., 2005). Myc-Hesr1 (mouse) was a gift from Dr. Hiroki Kokubo (Division of Mammalian Development, National Institute of Genetics, Mishima, Japan). Human HESR2 and -3 cDNAs were amplified from a fetal brain cDNA library (Clontech, Palo Alto, CA) by PCR with oligonucleotide primers and the high-fidelity DNA polymerase PrimeStar (TaKaRa, Shiga, Japan). Mouse Hesr2 and -3 cDNAs were amplified from cloned cDNAs in pBluescript (gifts from Dr. Hiroki Kokubo) by PCR with oligonucleotide primers and PrimeStar (TaKaRa). Each oligonucleotide primer was designed to

contain a restriction site (italicized). The primer sequences are as follows: human HESR2, Fw (XhoI) 5′-TTA*CTCGAG*AT GAAGCGCCC-3′ and Rv (ApaI) 5′-TTAGGGCCCT TAAAAAGCTCCAAC-3′; human HESR3, Fw (XhoI) 5′-TTA*CTCGAG*ATGAAGCGACCC-3′ and Rv (XhoI) 5′-TTA*CTCGAG*TCAGAAAGCCCC-3′; mouse Hesr2, Fw (XhoI) 5′-TTA*CTCGAG*ATGAAGCGCCCT-3′ and Rv (ApaI) 5′-ATA*GGGCCC*TTAAAAGCTGGCTCC-3′; and mouse Hesr3, Fw (XhoI) 5′-TTA*CTCGAG*ATGAA GCGGCC-3′ and Rv (ApaI) 5′-TTA*GGGCCC*TCAGAAA GCC-3′. These amplified fragments were cloned into T-Vectors (Promega p-GEM T Easy Kit) and sequenced by the dideoxy chain termination method with CEQ DTCS and CEQ8000 (Beckman Coulter, Brea, CA). Finally, the fragments were digested with each restriction enzyme and subcloned into myc-pcDNA.

Cell Culture and Transient Transfection: Luciferase Reporter Assay

The methods used for culture, transfection, cell harvesting, and luciferase activity measurements followed the standard methods of the Dual-Luciferase Reporter Assay System (Promega). SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2. SH-SY5Y cells were plated into 24-well plates and cultured until they grew to 80% confluence before transfection. The cells were transfected with 1 μg total plasmid using Lipofectamine 2000 reagent (Invitrogen). The firefly luciferase reporter gene (0.5 μg) and each HESR or vector (0.5 μg) were coexpressed in the cells. Plasmid pRL (Promega) containing the sea pansy luciferase gene was cotransfected (20 ng) as an internal control to normalize the transfection efficiency in all experiments. After 48 hr, the cells were harvested and stored at −80°C. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega). The firefly and sea pansy luciferase activity was measured using a Centro LB 960 (Berthold, Bad Wildbad, Germany) for 10 sec after a 2-sec delay, and then the value of each sample was calculated as light units of firefly luciferase per that of sea pansy. Each HESR group and its controls were measured at the same time on a Centro LB 960.

Animals

Adult (9-week-old) male C57BL6/J mice (CLEA Japan, Tokyo, Japan) were kept under a controlled temperature (23–25°C) and photoperiod (LD 14:10, lights off at 22:00 hr). Food and water were available freely. All experiments were conducted according to the Regulations for Animal Experimentation at the University of Tokyo (Tokyo, Japan).

Tissue Preparation

The mice were deeply anesthetized using sodium pentobarbital (50 mg/kg body weight) and then perfused intracardially with 0.05 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in 0.05 M phosphate buffer (PB). The brains were removed and postfixed with the same fixative for 2 hr and immersed in 30% sucrose in 0.05 M PB for several days at 4°C. Serial coronal brain sections (20 μm) including the midbrain ventral tegmental area (VTA) and substantia nigra (SN; −3.04 to −3.49 to the bregma) were made with a cryostat and collected according to a brain map (Franklin and Paxinos, 2008). Five animals were used in this experiment.

Immunohistochemistry

Immunoperoxidase staining. Free-floating sections of the midbrain were incubated with 0.6% H_2O_2 in 10 mM PBS for 30 min at room temperature (RT) before and after rinsing with 10 mM PBS. Next, the sections were incubated with 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA), 0.4% Triton X-100, and 10 mM PBS for 1 hr at RT and then with a primary rabbit antibody against Hesr1 (working dilution 1:500; Chemicon, Temecula, CA), Hesr2 (1:1,000; Chemicon), or Hesr3 (1:500; Chemicon) containing 5% NGS, 0.4% Triton X-100, and 10 mM PBS for 3 nights at 4°C. After washing with 10 mM PBS, the sections were reacted with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) in 5% NGS, 0.4% Triton X-100, and 10 mM PBS overnight at 4°C. The sections were rinsed with 10 mM PBS three times and then incubated in avidin-biotin-peroxidase complex (ABC) solution (Vectastain Elite ABC Kit; Vector Laboratories). Next, the sections were reacted with 0.05% 3,3′-diaminobenzidine (DAB) in 0.01% H_2O_2 and 100 mM Tris-HCl to visualize Hesr1-, -2-, or -3-immunoreactive (-ir) cells.

For single staining of the TH-ir (a marker of dopaminergic neurons) cells, similar steps were taken, except for the steps involving incubation with the primary or secondary antibody and visualization. The sections were incubated with a mouse anti-TH antibody (1:10,000; Chemicon) overnight at 4°C. The sections were then reacted with biotinylated goat anti-mouse IgG (1:200, Vector Laboratories) in 5% NGS, 0.4% Triton X-100, and 10 mM PBS overnight at 4°C after washing with 10 mM PBS three times. The sections were rinsed with 10 mM PBS three times and then incubated in ABC solution (Vector Laboratories). Next, the sections were reacted with 0.02% DAB solution to visualize TH-ir cells.

For double labeling, the same staining steps as for TH were performed after staining for each Hesr. Instead of DAB, a Vector SG Kit was used for visualization of TH after the ABC reaction. Immunostained sections mounted on slides were dehydrated through a graded ethanol series, cleared with xylene, and then coverslipped with an embedding agent.

Fluorescence immunohistochemistry for Hesrs and TH. Free-floating sections were rinsed with 10 mM PBS. The sections were then incubated with 5% normal donkey serum (NDS; Jackson Immunoresearch, West Grove, PA), 0.4% Triton X-100, and 10 mM PBS for 1 hr at RT and then with the primary rabbit antibody against Hesr1 (1:500; Chemicon), Hesr2 (1:1,000; Chemicon), or Hesr3 (1:500; Chemicon) and the primary mouse antibody for TH (1:10,000; Chemicon) in 5% NDS, 0.4% Triton X-100, and 10 mM PBS for 2 nights at 4°C. After washing with 10 mM PBS, the sections were reacted with donkey anti-rabbit IgG (1:200; Jackson Immunoresearch; Cy3) and donkey anti-mouse IgG (1:200; Jackson Immunoresearch; Cy2) in 5% NDS, 0.4% Triton X-100, and 10 mM PBS overnight at

4°C. Next, the sections were rinsed with 10 mM PBS before and after incubating with Hoechst 33342 (1:1,000; Dojindo, Tokyo, Japan) for 15 min. The immunostained sections were mounted on slides and then coverslipped. Images were captured with a digital CCD camera (DP70; Olympus, Tokyo, Japan) and the microscope software manager DP (Olympus) and analyzed in Photoshop CS4 (Adobe, San Jose, CA).

Statistical Analysis

Statistical analysis was performed in JMP 8.0 (SAS Institute). All values are reported as the mean ± SEM. Each value was standardized to that of CP-Luc/exon15 (10r) (Fig. 3), vector (Figs. 4, 5), or 10r (Fig. 6) in each group. Student's *t*-test was performed to detect statistical significance between two experimental objects. Tukey-Kramer's honestly significant difference (HSD) test was used as a post hoc test after one-way ANOVA. Tukey-Kramer's HSD test is a standard method of JMP to find significant difference among data after ANOVA. In addition, two-way ANOVA was conducted to detect interactive effects between the number of VNTR repeats and HESRs. Differences were considered significant at $P < 0.05$.

RESULTS

Lusciferase Activity of CP-Luc and CP-Luc/Exon15 (10r) in SH-SY5Y Cells

To determine the effect of the VNTR domain on *DAT1* expression, we made luciferase reporter constructs (Fig. 3). The relative luciferase activity of CP-Luc was 3.68 ± 0.15, whereas that of CP-Luc/exon 15 (10r) was 1.00 ± 0.03. A significant difference was noted between CP-Luc and CP-Luc/exon 15 (10r) ($P < 0.0001$, n = 8; Fig. 3). These results indicate that the 3′-UTR including the VNTR domain strongly inhibited luciferase expression in this reporter assay.

Effects of Human HESR and Mouse Hesr Family on Lusciferase Activity of CP-Luc

We next examined the effect of HESR family members on expression of the CP-Luc plasmid (Fig. 4A).

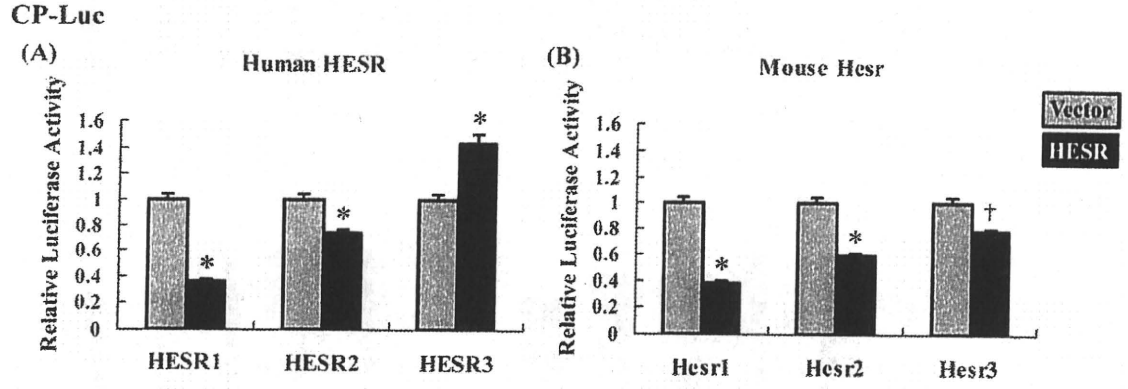

Fig. 4. Effects of the HESR family on the luciferase activity of CP-Luc. Cells were transfected with the CP-Luc reporter construct and human (**A**) and mouse (**B**) Hesrs or with empty vector. The values represent the mean ± SEM. of relative luciferase activity of the reporter construct in each group. *$P < 0.0001$ vs. vector, †$P < 0.0003$ vs. vector, Student's *t*-test.

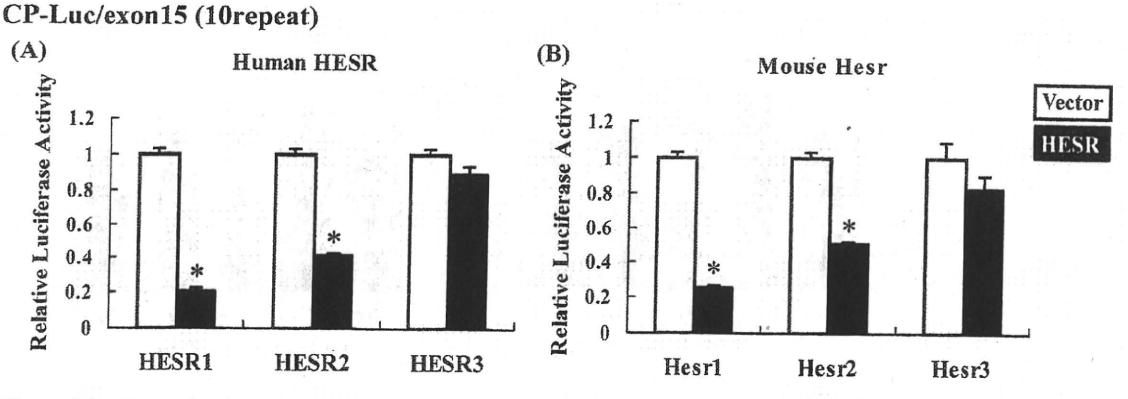

Fig. 5. Effects of the HESR family on the luciferase activity of CP-Luc/exon 15 (10r). Cells were transfected with CP-Luc/exon 15 (10r) and human (**A**) and mouse (**B**) Hesrs or with empty vector. The values represent the mean ± SEM. of relative luciferase activity of the reporter construct in each group. *$P < 0.0001$ vs. vector, Student's *t*-test.

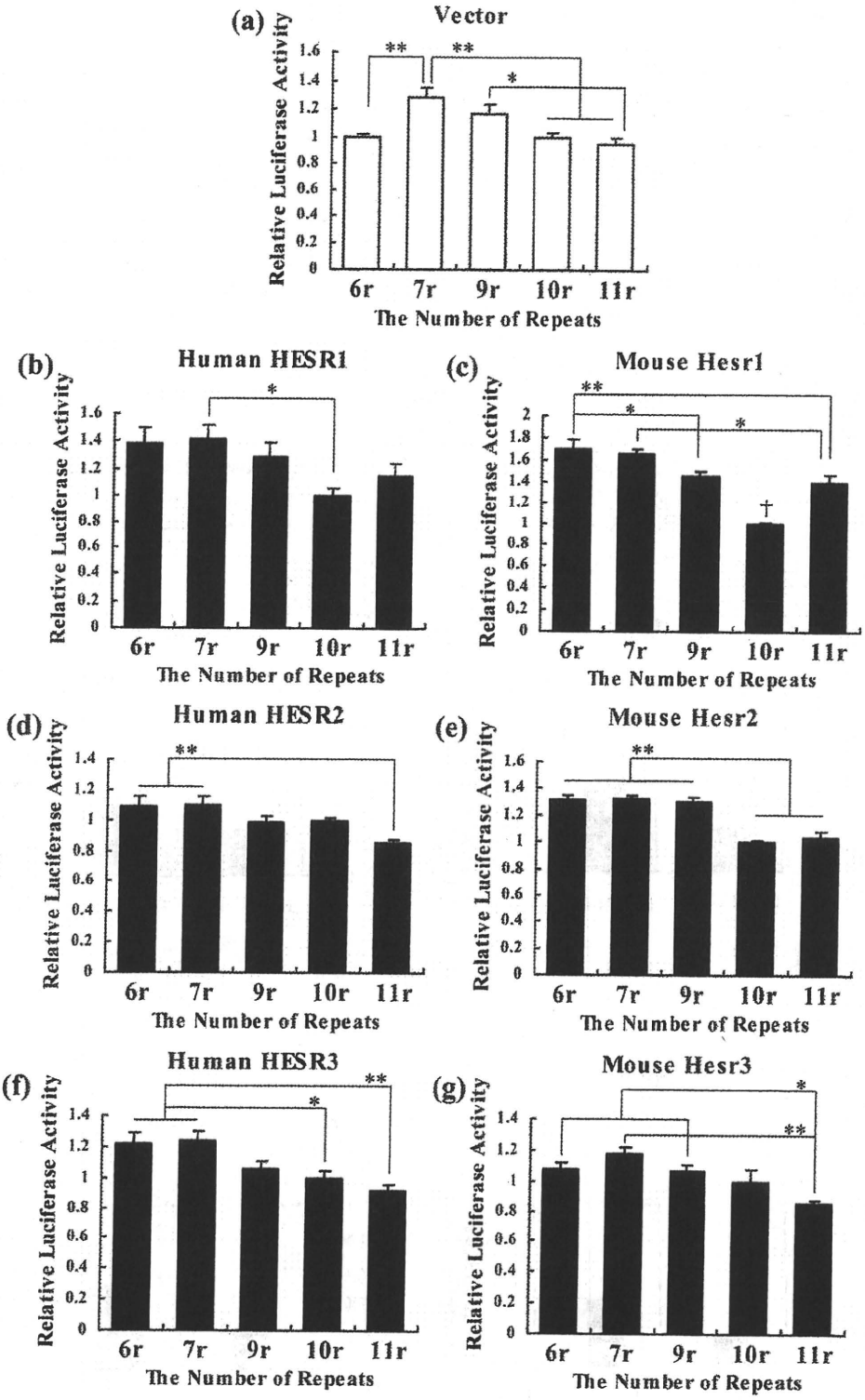

Fig. 6. Differential effects of the HESR family on the VNTR alleles. Cells were transfected with CP-Luc/exon 15 containing the indicated number of repeats and human (**b,d,f**) or mouse Hesrs (**c,e,g**) or with empty vector (**a**). Relative luciferase activity is expressed as the mean ± SEM. of relative luciferase activity of the reporter construct in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $\dagger P < 0.01$ vs. the other repeats, Tukey-Kramer's HSD test after one-way ANOVA.

With the expression of human HESR family members, the luciferase activity of CP-Luc relative to the vector control (1.00 ± 0.04) was as follows: HESR1, 0.36 ± 0.01; HESR2, 0.74 ± 0.02; and HESR3, 1.44 ± 0.06. The activity of CP-Luc with HESR1 or 2 was significantly lower than that with the vector control ($P < 0.0001$, n = 8 in each group), whereas that with HESR3 was higher than the control ($P < 0.0001$, n = 8 in each group).

With the expression of mouse Hesr family members (Fig. 4B), the luciferase activity of CP-Luc relative to the vector control (1.00 ± 0.04) was as follows: Hesr1, 0.39 ± 0.01; Hesr2, 0.60 ± 0.01; and Hesr3, 0.79 ± 0.01. The activity of CP-Luc with Hesr1 ($P < 0.0001$), -2 ($P < 0.0001$), or -3 ($P < 0.0003$) was significantly lower than with the vector (n = 8 in each group). These results indicate that only human HESR3 stimulated the expression of the CP-Luc construct containing the human *DAT1* core promoter, whereas expression of the other family members inhibited expression.

Effects of Human HESR and Mouse Hesr Family on Lusciferase Activity of CP-Luc/Exon 15 (10r)

To investigate the role of the VNTR domain, we cotransfected HESR genes with CP-Luc/exon 15 (10r). With the expression of human HESR family members (Fig. 5A), the luciferase activity of the CP-Luc/exon 15 (10r) relative to the vector control (1.00 ± 0.03) was as follows: HESR1, 0.21 ± 0.01; HESR2, 0.42 ± 0.01; and HESR3, 0.90 ± 0.04. These results illustrate that the luciferase activity of CP-Luc/exon 15 (10r) with HESR1 or 2 was significantly lower than with the vector ($P < 0.0001$, n = 8 in each group), although no significant decrease was observed with HESR3 ($P = 0.07$, n = 8).

The same trend was observed for mouse Hesr (Fig. 5B). With the expression of mouse Hesr family proteins, the luciferase activity of CP-Luc/exon 15 (10r) with each Hesr relative to the vector control (1.00 ± 0.07) was as follows: Hesr1, 0.25 ± 0.01; Hesr2, 0.51 ± 0.01; and Hesr3, 0.83 ± 0.09. The level of luciferase activity with Hesr1 or -2 was significantly lower than that with the vector ($P < 0.0001$, n = 8 in each group), whereas no significant decrease in activity was detected with Hesr3 ($P = 0.16$, n = 7).

Comparison of Effects of HESR Family and VNTR on Lusciferase Activity of CP-Luc/Exon15 (nr)

We investigated the effect of human and mouse HESR family members on the VNTR domain by first evaluating the effect of the repeat number. Luciferase expression from the CP-Luc/exon 15 construct is shown in Figure 6a. The following levels of activity were calculated: 6r, 0.99 ± 0.02; 7r, 1.28 ± 0.06; 9r, 1.16 ± 0.07; 10r, 1.00 ± 0.03; and 11r, 0.95 ± 0.04. One-way ANOVA indicated that there was a significant effect of the number of repeats ($F_{4,35} = 8.0167$, $P < 0.0001$). Post hoc analysis indicated that the luciferase activity level associated with 7r was significantly higher than that with 6, 10, or 11r ($P < 0.01$) and that the level of activity associated with 9r was significantly higher than that associated with 11r ($P < 0.05$; n = 8 in each group; Fig. 6a).

The effect of human HESR1 on the VNTR repeat number is shown in Figure 6b. With the expression of HESR1, CP-Luc/exon 15 activity (6r, 1.38 ± 0.11; 7r, 1.41 ± 0.11; 9r, 1.29 ± 0.11; 10r, 1.00 ± 0.06; and 11r, 1.14 ± 0.10) was compared with that for the vector alone (6r, 4.69 ± 0.11; 7r, 6.06 ± 0.28; 9r, 5.48 ± 0.35; 10r, 4.72 ± 0.16; and 11r, 4.50 ± 0.19). One-way ANOVA indicated a significant effect of the number of repeats ($F_{4,34} = 3.006$, $P < 0.032$). Post hoc analysis indicated that the luciferase activity associated with 7r was significantly higher than that associated with 10r (n = 7 for 11r, n = 8 in the other groups; Fig. 6b). In addition, two-way ANOVA indicated that there was a significant interaction between the number of repeats with HESR1 (or vector) ($F_{4,69} = 5.04$, $P < 0.002$).

We also investigated the effects of mouse Hesr1 (Fig. 6c). With the expression of Hesr1, CP-Luc/exon 15 activity (6r, 1.70 ± 0.08; 7r, 1.65 ± 0.05; 9r, 1.46 ± 0.04; 10r, 1.00 ± 0.02; and 11r, 1.41 ± 0.06) was compared with the control vector (6r, 3.90 ± 0.09; 7r, 5.04 ± 0.23; 9r, 4.56 ± 0.29; 10r, 3.92 ± 0.13; and 11r, 3.74 ± 0.16). As shown for human HERS1, one-way ANOVA indicated that there was a significant effect of the number of repeats ($F_{4,35} = 25.7587$, $P < 0.0001$). Post hoc analysis indicated that the level of luciferase activity associated with 6r was significantly higher than that associated with 9r ($P < 0.05$) or 11r ($P < 0.01$), and that the level for 7r was also higher than that for 11r (0.05). Furthermore, the lowest level of activity was observed for 10r ($P < 0.01$, n = 8 in each group; Fig. 6c). In addition, two-way ANOVA indicated that there was a significant interaction between the number of repeats with Hesr1 or the control vector ($F_{4,70} = 6.34$, $P < 0.002$).

We also investigated the effects of human HESR2 and mouse Hesr2. With the expression of human HERS2, CP-Luc/exon 15 activity (6r, 1.08 ± 0.07; 7r, 1.10 ± 0.06; 9r, 0.99 ± 0.04; 10r, 1.00 ± 0.02; and 11r, 0.85 ± 0.02) was again compared with the control vector (6r, 2.36 ± 0.05; 7r, 3.05 ± 0.14; 9r, 2.76 ± 0.17; 10r, 2.36 ± 0.08; and 11r, 2.26 ± 0.10) (Fig. 6d). One-way ANOVA indicated that there was a significant effect of the number of repeats ($F_{4,35} = 4.6640$, $P < 0.004$). Post hoc analysis indicated that the level of luciferase activity associated with 6 or 7r was significantly higher than that associated with 11r ($P < 0.01$, n = 8 in each group; Fig. 6d). In addition, two-way ANOVA indicated that there was a significant interaction between the number of repeats with HESR2 (or the control vector) ($F_{4,70} = 5.29$, $P < 0.0009$).

With the expression of mouse Hesr2 (Fig. 6e), CP-Luc/exon15 activity (6r, 1.32 ± 0.02; 7r, 1.32 ± 0.03; 9r, 1.30 ± 0.03; 10r, 1.00 ± 0.01; and 11r, 1.04 ± 0.04) was again compared with the control vector (6r, 1.94 ± 0.04; 7r, 2.51 ± 0.11; 9r, 2.26 ± 0.14; 10r,

1.95 ± 0.07; and 11r, 1.86 ± 0.08). One-way ANOVA indicated that there was a significant effect of the number of repeats ($F_{4,35} = 31.6769$, $P < 0.0001$). Post hoc analysis indicated that the level of luciferase activity for 6, 7, or 9r was significantly higher than that for 10 or 11r ($P < 0.01$, n = 8 in each group; Fig. 6e). In addition, two-way ANOVA indicated that there was a significant interaction between the number of repeats with Hesr2 (or the control vector; $F4, 70 = 4.19$, $P < 0.0045$).

Human HESR3 and mouse Hesr3 were also investigated. With human HESR3 expression, CP-Luc/exon 15 activity (6r, 1.22 ± 0.07; 7r, 1.24 ± 0.06; 9r, 1.06 ± 0.05; 10r, 1.00 ± 0.04; and 11r, 0.92 ± 0.04) was compared with the control vector (6r, 1.11 ± 0.03; 7r, 1.43 ± 0.07; 9r, 1.29 ± 0.08; 10r, 1.12 ± 0.04; and 11r, 1.06 ± 0.05). One-way ANOVA again indicated a significant effect of the number of repeats ($F_{4,35} = 7.0211$, $P < 0.0003$). Post hoc analysis indicated that the level of luciferase activity using 6r or 7r was significantly higher than that using 10 ($P < 0.05$) or 11r ($P < 0.01$; n = 8 in each group; Fig. 6f). In addition, two-way ANOVA indicated that there was a significant interaction between the number of repeats with HESR3 or control vector ($F_{4,70} = 3.14$, $P < 0.02$).

With the expression of mouse Hesr3, CP-Luc/exon 15 activity (6r, 1.08 ± 0.04; 7r, 1.18 ± 0.04; 9r, 1.07 ± 0.04; 10r, 1.00 ± 0.08; and 11r, 0.85 ± 0.03) was again compared with that of the control vector (6r, 1.15 ± 0.07; 7r, 1.44 ± 0.07; 9r, 1.28 ± 0.05; 10r, 1.20 ± 0.10; and 11r, 1.07 ± 0.05). One-way ANOVA indicated that there was a significant effect of the number of repeats ($F_{4,30} = 6.4123$, $P < 0.0007$). Post hoc analysis indicated that the level of luciferase activity for 11r was significantly lower than that for 6 ($P < 0.05$), 7 ($P < 0.01$), and 9r ($P < 0.05$; n = 7 in each group; Fig. 6g). In addition, two-way ANOVA indicated no significant interaction between the number of repeats with Hesr3 or the control vector ($F_{4,65} = 0.67$, $P = 0.62$). The value of luciferase expression from the each CP-Luc/exon 15 construct (6, 7, 9, 10, and 11r) with empty vector compared in Figure 6b–g was measured from same samples as used in Figure 6a.

Localization of Hesr Family Members in Mouse Midbrain

We next analyzed the localization of Hesr1, -2, and -3 by immunoperoxidase staining. Hesr1-, Hesr2-, and Hesr3-ir cells were observed in both dopaminergic and nondopaminergic regions in the mouse midbrain (Fig. 7a). Immunoreactivity against Hesr1 and -2 was observed mainly in the nucleus, whereas Hesr3 was detected in the cytoplasm (Fig. 7b). However, in the nondopaminergic regions, immunoreactivity against Hesr1 and -2 was detected in the nucleus and cytoplasm (Fig. 7c). We also analyzed the localization of Hesr family members in the TH-ir cells using double-fluorescence immunostaining. Hesr1-, Hesr2-, and Hesr3-TH-ir cells were observed in both the SN and the VTA (Fig. 8).

DISCUSSION

Roles of the 3′-UTR Including VNTR Domain for *DAT* Expression

The relative luciferase activity of CP-Luc was significantly higher than that of CP-Luc/exon 15 (10r) in SH-SY5Y cells (Fig. 3). This suggests that the 3′-UTR plays an inhibitory role in *DAT1* expression. It is possible that endogenous factors affect *DAT1* expression via the 3′-UTR; in fact, it is predicted that more than one factor binds to this region (Michelhaugh et al., 2001). Thus, we investigated the HESR family as novel candidate regulatory factors modulating *DAT1* expression.

Effects of HESR Family on the *DAT* Core Promoter

Human HESR1 and -2 or mouse Hesr1, -2, and -3 significantly decreased the relative luciferase activity level of CP-Luc containing the *DAT1* core promoter, whereas human HESR3 increased CP-Luc activity (Fig. 4). These results were unexpected, insofar as HESR1 has been identified as a 3′-UTR-binding protein. This suggests that the HESR family generally down-regulates *DAT* expression through the core promoter region. In particular, HESR1 showed strong inhibitory effects in this region, with a 36% decrease in luciferase activity with human HESR1 expression and a 39% decrease with mouse Hesr1. In fact, the core promoter region has an E-box known to be bHLH-binding consensus sites. Moreover, it has been reported that mouse *Hesr2* does not contain an E- or N-box and is repressed by Hesr proteins (Nakagawa et al., 2000). Therefore, it is likely that HESR family members recognize a binding site different from that recognized by other bHLH family members. In addition, a functional single nucleotide polymorphism (SNP; −67 A/T) in the promoter was reported to be associated with personality traits, ADHD, and bipolar disorder (Greenwood and Kelsoe, 2003; Ohadi et al., 2006, 2007; Shibuya et al., 2009). This SNP may be a point of interaction with HESR family members.

Human HESR3 was the only HESR family member that significantly increased luciferase activity of CP-Luc containing the *DAT1* core promoter. We previously reported that HESR1 containing the Leu94Met SNP in the second helix of the bHLH domain lacked inhibitory activity (Fuke et al., 2005). It was also demonstrated that this SNP dramatically transforms HESR1 from an androgen receptor corepressor to an activator (Villaronga et al., 2010). HESR3 is the only HESR with a single-amino-acid substitution adjacent to the Leu in HESR1 and -2 located in the second helix of the bHLH domain (Fig. 2). Thus, the second helix in HESR family members may be critical in the modulation of gene expression.

Effects of HESR Family on the 3′-UTR of DAT

As shown in Figure 5, human and mouse HESR1 and -2 significantly inhibited the luciferase activity of CP-Luc/exon 15 (10r). Taken together with the results shown in Figure 4, this result indicates that HESR1 and

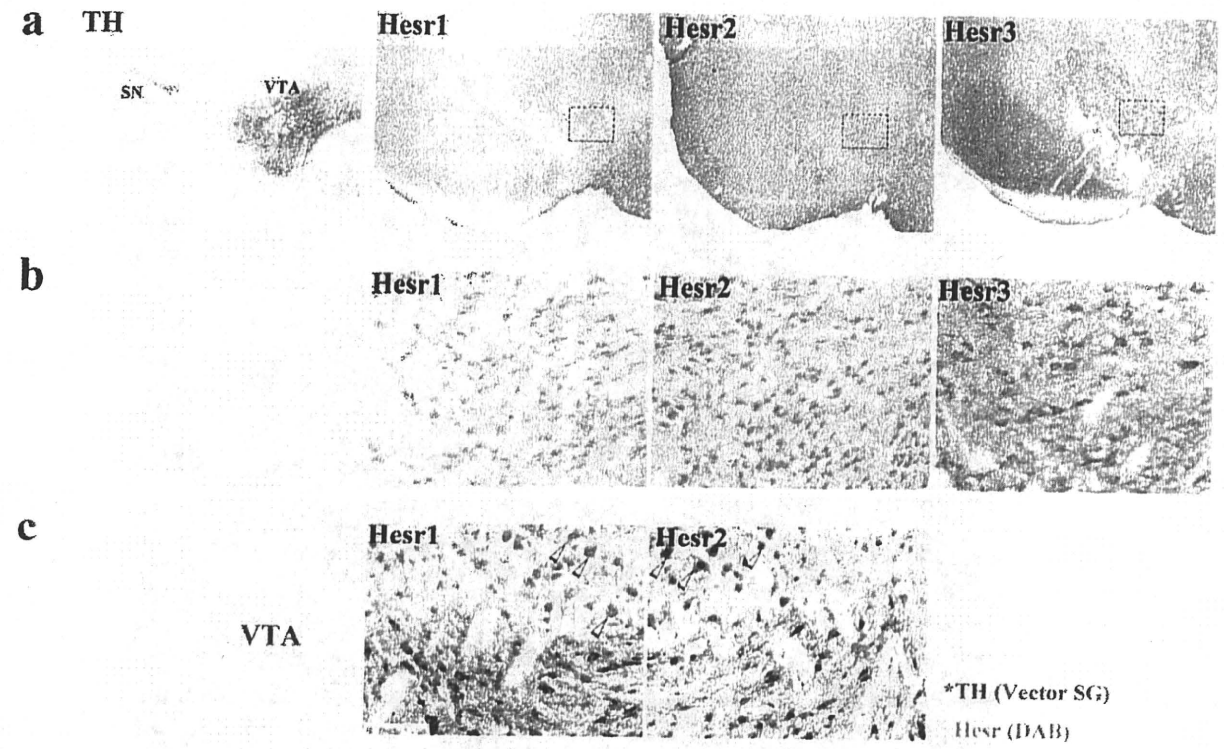

Fig. 7. Distribution of the Hesr family in mouse midbrain. Photomicrographs showing immunoperoxidase staining visualized by DAB (brown) or Vector SG (blue/gray). **a:** Localization of tyrosine hydroxylase (TH) or each Hesr family member in mouse midbrain, including the substantia nigra (SN) and ventral tegmental area (VTA). **b:** High-magnification images of the boxed areas in a. Hesr1 and -2 are localized primarily in the nucleus, whereas Hesr3 is located predominantly in the cytoplasm. **c:** Photomicrographs of the VTA and surrounding area depicting dual labeling for TH (Vector SG) and Hesr1 or 2 (DAB). Arrowheads indicate cells in which Hesr1 or -2 immunoreactivity was observed in both the nucleus and the cytoplasm. Scale bars = 500 μm in a; 100 μm in b,c.

-2 inhibit *DAT1* expression through both the core promoter and the 3′-UTR. HESR1 and -2 decreased the relative level of luciferase activity to less than 25% (HESR1) and 50% (HESR2). This degree of decrease is relatively high compared with that seen with the CP-Luc. Thus, HESR1 and -2 may have a stronger inhibitory effect on *DAT1* expression in the presence of the 3′-UTR.

We previously showed that HESR1 bound the 3′-UTR of *DAT1* directly by electromobility shift assays (Fuke et al., 2006). Because the bHLH domain is highly conserved (Steidl et al., 2000), we investigated the effect of other HESR family members to determine whether they affect gene expression by interacting with this same region. It has been proposed that more than one factor binds to this region and that the 3′-UTR, including the VNTR domain, modulates gene expression (Michelhaugh et al., 2001). Functional VNTR polymorphisms also exist in the serotonin transporter (*SERT*) gene located in intron 2, and two transcription factors, Y box-binding protein 1 (YB-1) and CCTC-binding factor (CTCF), were found to be responsible for the modulation of VNTR function (Klenova et al., 2004). This suggests that the VNTR domain functions as a modulator of gene expression (Nakamura et al., 1998) with other binding proteins.

Comparison of *DAT* Expression With the VNTR: Differential Effects of HESR Family on Each VNTR Allele

The number of repeats in the VNTR domain significantly affected the level of luciferase activity of CP-Luc/exon 15 in SH-SY5Y cells (Fig. 6a). This suggests that the VNTR domain in the *DAT* 3′-UTR is the functional sequence for *DAT* expression and is supported by in vivo neuroimaging involving SPECT (Heinz et al., 2000; Jacobsen et al., 2000; Martinez et al., 2001) and ex vivo RT-PCR analysis (Mill et al., 2002; Brookes et al., 2007). When the HESR family was transiently transfected, the number of repeats in the VNTR domain significantly affected the luciferase activity of CP-Luc/exon 15 (Fig. 6b–g). In addition, interactive effects between the number of repeats and the HESR or control vector were detected for all groups except mouse Hesr3. This suggests that the HESR

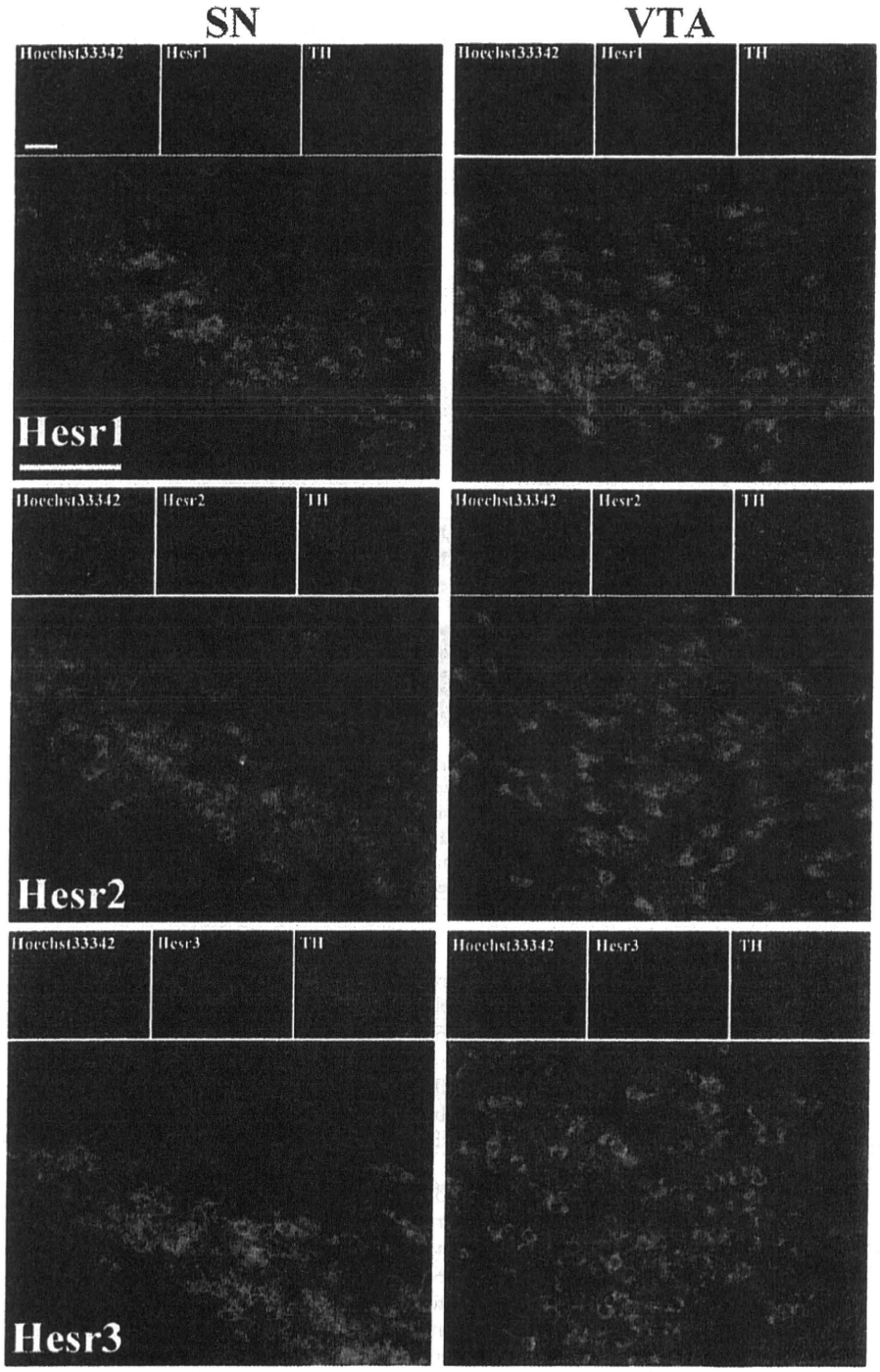

Fig. 8. Colocalization of Hesr family proteins with dopaminergic neurons. Fluorescence immunohistochemical analysis of the Hesr family protein and tyrosine hydroxylase (TH) distribution in the substantia nigra (SN) and ventral tegmental area (VTA). Hesr, magenta (Cy3); TH, green (Cy2); Hoechst 33342, blue. Scale bars = 100 μm.

family, or at least HESR1 and -2, differentially alter *DAT1* expression depending on the VNTR allele. This supports our idea that cell-specific factors regulate *DAT1* expression in a VNTR allele-specific manner and may explain the discrepancies among previous studies (D'souza and Craig, 2008).

Functional Considerations

We first reported the functional significance of the *DAT* VNTR sequence at the molecular level using luciferase reporter assays (Fuke et al., 2001). After our report, other groups reported interesting but conflicting in vitro results (Fuke et al., 2001, 2005; Inoue-Murayama et al., 2002; Miller and Madras, 2002; Greenwood and Kelsoe, 2003; VanNess et al., 2005; Mill et al., 2005; D'souza and Craig, 2008). One explanation for this is that different promoters and cell lines were used in each study. In fact, our previous study (Fuke et al., 2005) revealed that the inhibitory effect of the *DAT* 3′-UTR in CP-Luc/exon15 was not detected in HEK293 cells, but we could detected it in SH-SY5Y, Neuro2A, and COS-7 cell lines. Every cell line might not express all the transcription factors necessary for regulation of the *DAT1* gene, as D'souza and Craig (2008) speculated. Thus, the identification of the regulating factor for *DAT1* was necessary. We previously identified HESR1 as a 3′-UTR-binding protein using a yeast one-hybrid system (Fuke et al., 2005) and showed that HESR1 inhibited the luciferase activity of CP-Luc/exon 15 in SY-SY5Y, Neuro2A, COS-7, and also HEK293 cells. This indicates that HESR1 may regulate *DAT1* expression.

We also reported the increased expression of *DAT* in the brains of *Hesr1* KO mice. These mice also showed a decrease in spontaneous locomotor activity and a reduction in exploration of novelty (Fuke et al., 2006). This is consistent with our previous and present results, insofar as HESR1 is thought to be a *DAT*-inhibitory factor. In addition, the expression of several dopamine receptor genes (*DRD1*, *DRD2*, *DRD4*, and *DRD5*), the main targets of synaptic dopamine responsiveness, were enhanced in *Hesr1* KO mice. These phenomena are in contrast to what is seen in *DAT* KO mice. Mice lacking *DAT* show decreased intraneural dopamine storage and spontaneous hyperlocomotion following the down-regulation of several dopamine-related genes, including those encoding dopamine receptors D1 and D2 (Giros et al., 1996; Caine, 1998; Jaber et al., 1999; Fauchey et al., 2000; Gainetdinov et al., 2002). This result highlights the importance of Hesr1 in the dopaminergic system in vivo and suggests that further investigation of the in vivo functions of Hesr2 and -3 is needed.

The *Hesr1/2* double mutation is known to be embryonic lethal as a result of cardiac and vascular dysplasia, and both genes have been shown to be involved in neural development (Sakamoto et al., 2003; Fischer et al., 2004; Kokubo et al., 2005). However, whereas *Hesr2* KO mice exhibited heart dysfunction (Kokubo et al., 2004), *Hesr1* KO mice exhibited no obvious morphological or anatomical phenotype (Fischer et al., 2004; Kokubo et al., 2005). On the other hand, interactions between *Hesr1* and transforming growth factor-β (TGF-β) or bone morphogenetic protein (BMP) signaling (Dahlqvist et al., 2003; Takizawa et al., 2003; Zavadil et al., 2004), which functions in the differentiation and maintenance of the dopaminergic nervous system, have been reported (Stull et al., 2001; Farkas et al., 2003; Sanchez-Capelo et al., 2003). *HESR1* may play important roles in the dopaminergic nervous system and the regulation of *DAT*. *HESR* family genes encode a bHLH domain that is essential for DNA binding, an Orange domain and YRPW motif. HESR proteins dimerize with other bHLH proteins via the bHLH domain and bind to corepressors via the YRPW motif (Fischer and Gessler, 2007). Additional investigations are needed to characterize these interacting proteins. In the present study, the results obtained for HESR3 sometimes differed from those obtained for HESR1 and -2. One possible reason for this is the lack of a YRPW motif in HESR3 (Fig. 2).

Our findings may lead to novel therapies for DAT-related disorders. In the case of SERT, YB-1 and CTCF act as regulators of SERT (Klenova et al., 2004) and are targeted by lithium chloride, a mood stabilizer that modifies CTCF and YB-1 expression (Roberts et al., 2007).

DAT gene is expressed exclusively in the dopaminergic neurons (Uhl, 2003). If Hesr family genes are not expressed in the dopaminergic neurons, Hesrs cannot inhibit *DAT* expression via 3′-UTR of DAT. However, as shown in Figures 7 and 8, each Hesr was expressed in dopaminergic neurons throughout the SN and VTA. This suggests that the HESR family influences *DAT* expression in vivo, as observed in our present culture studies. At the same time, these data suggest that HESR family members are involved in mesostriatal and mesocorticolimbic dopamine systems underlying motor control, emotion, and cognition (Bjorklund and Dunnett, 2007).

In a study of prostate cancer, HESR1 was reported to be an androgen receptor-interacting factor (Belandia et al., 2005). It has also been shown that HESR1 is excluded from the nucleus in most human prostate cancers, raising the possibility that abnormal HESR1 subcellular distribution plays a role in the aberrant hormonal responses observed in prostate cancer. In the present study, as shown in Figure 7c, Hesr1 and -2 were localized not only to the nucleus but also to the cytoplasm of nondopaminergic cells in the brain. Thus, the cellular localization of HESR family members may be important for some physiological functions or pathological conditions and warrants further study.

Although it seems clear that the VNTR domain plays a role in regulating *DAT* expression in vitro, there are discrepancies in the proposed differential effects of the various alleles. In vivo studies using transgenic mice with *DAT-9r* or *10r* knock-ins may facilitate the characterization of the mechanisms underlying *DAT* transcriptional regulation. The mouse Hesr data presented here will serve as a molecular basis for generating these animals.

In conclusion, we have demonstrated the differential expression of a luciferase reporter vector containing the *DAT1* 3′-UTR and VNTR domain as well as the differential inhibitory effects of HESR family members on *DAT1* expression via the VNTR domain. Given these findings, additional behavioral and psychiatric studies of personality should be conducted.

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Case report

Distal lipid storage myopathy due to *PNPLA2* mutation

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Abstract

Distal myopathy is a group of heterogeneous disorders affecting predominantly distal muscles usually appearing from young to late adulthood with very rare cardiac complications. We report a 27-year-old man characterized clinically by distal myopathy and dilated cardiomyopathy, pathologically by lipid storage, and genetically by a *PNPLA2* mutation. The patient developed weakness in his lower legs and fingers at age 20 years. Physical examination at age 27 years revealed muscle weakness and atrophy predominantly in lower legs and hands, and severe dilated cardiomyopathy. The patient had a homozygous four-base duplication (c.475_478dupCTCC) in exon 4 of *PNPLA2*.

Keywords: Distal myopathy; Lipid storage myopathy; Neutral lipid storage disease with myopathy; *PNPLA2*

1. Introduction

Lipid storage myopathy (LSM) is a pathologically defined entity with accumulation of triglycerides in the muscle fiber. Six causative genes for only four diseases have been identified: *SLC22A5* for primary carnitine deficiency (PCD); *ETFA*, *ETFB*, and *ETFDH* for multiple acyl-CoA dehydrogenase deficiency (MADD); *ABHD5* for neutral lipid storage disease with ichthyosis or Chanarin–Dorfman syndrome; and *PNPLA2* for neutral lipid storage disease with myopathy (NLSDM) [1–3].

PNPLA2 encodes an adipose triglyceride lipase; mutations in this gene were recently reported in three patients who presented with LSM and variable cardiac involvement [1]. Here, we report a Japanese patient with a *PNPLA2* mutation presenting with distal myopathy and severe dilated cardiomyopathy and showing numerous rimmed vacuoles on muscle pathology.

2. Case report

A 27-year-old man had slowly progressive muscle weakness. Despite being a slow runner since childhood, he belonged to a mountaineering club and had no difficultly climbing mountains. At 20 years, he noticed difficulty climbing down the stairs, and gradually developed distal dominant muscle weakness and atrophy. Family history was non-contributory.

Upon consultation with us at 27 years, he had marked muscle weakness and atrophy in the extremities predominantly in the lower legs (Fig. 1A) and fingers (Fig. 1B). Examination of the muscle strength showed 3–4/5 asymmetric weakness over the deltoid, biceps brachii, extensor digitorum, gastrocnemius, and tibialis anterior. Grasping power was 12 kg on right and 10 kg on left (normal

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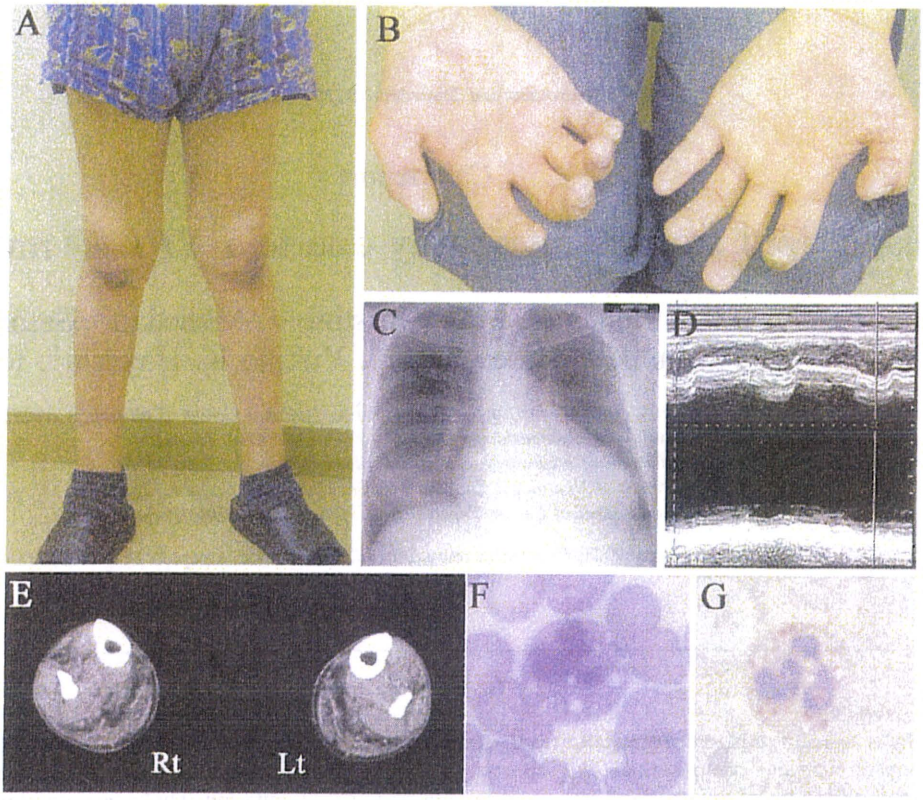

Fig. 1. The patient had distal muscle atrophy especially in the lower legs (A) and thenar muscles (B). Chest X-ray showed cardiomegaly with cardiothoratic ratio of 63% (normal cardiothoratic ratio <50%) (C). Echocardiogram showed left ventricular enlargement with decreased ejection fraction of 18% (normal >60%) (D). Calf muscles were involved relatively sparing tibialis anterior on CT (E). Note many vacuoles of leukocyte by Wright–Giemsa (F), which are positively stained by oil red O (G).

values = 43–56 kg). Deep tendon reflexes were absent. No skin abnormality was seen. Chest X-ray revealed cardiomegaly (Fig. 1C). Echocardiogram showed left ventricular enlargement with decreased left ventricular ejection fraction of 18% (normal >60%), left ventricular end-diastolic dimension of 78 mm, left ventricular end-systolic dimension of 70 mm, interventricular septum thickness of 8 mm and posterior wall thickness of 8 mm (Fig. 1D). ECG showed negative Q wave in lead I, negative P wave in V_1 and occasional ventricular extra-systoles. EMG showed myopathic changes. His respiratory function was normal. Serum creatine kinase was elevated (412–1697 IU/L; normal value <170). Serum cholesterol, TG, LDL-cholesterol and glucose were within normal ranges. In leukocytes, Jordans anomaly [4], multiple tiny vacuoles due to lipid accumulation, was seen (Fig. 1F and G). Muscle CT showed decreased densities in both soleus, both gastrocnemius, and right tibialis anterior muscles (Fig. 1E).

Muscle biopsy from the left biceps brachii muscle revealed marked variation in fiber size. Numerous lipid droplets were seen in virtually all type one fibers (Fig. 2A). In addition, rimmed vacuoles were observed in scattered fibers (Fig. 2B). Dystrophin, caveolin-3, and dysferlin immunohistochemistry were normal. On electron microscopy, markedly increased lipid droplets were seen between myofibrils where mitochondria appeared pyknotic (Fig. 3A). Numerous autophagic vacuoles were also observed (Fig. 3B). Total and free muscle carnitine levels were 13.2 and 3.9 nmol/mg non-collagen protein, respectively (reference: total, 15.7 ± 2.8; free, 12.9 ± 3.7).

We sequenced all exons and the flanking intronic regions of all six known causative genes for LSM in genomic DNA. In the patient, we identified a homozygous four-base duplication (c.475_478dupCTCC) in exon 4 of *PNPLA2* (Gene ID: 57104), predicted to result in a premature stop codon at amino acid position 178. Heterozygous c.475_478dupCTCC mutation was confirmed in both healthy parents. We did not find any sequence variant in other candidate genes, including *GNE* gene.

3. Discussion

The patient presented has been followed up with a tentative diagnosis of distal myopathy. In fact, one patient in the first report of *PNPLA2* mutations had distal dominant muscle weakness although the other two had proximal muscle involvement [1]. Therefore, distal myopathy may not be uncommon in LSM associated with *PNPLA2* mutations.

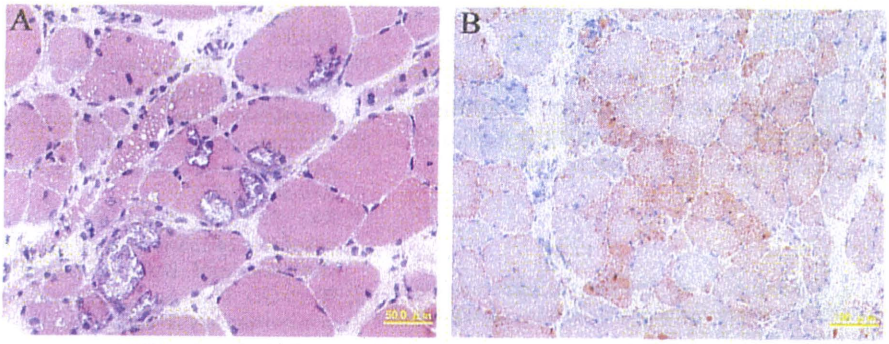

Fig. 2. In addition to variation in fiber size, numerous small vacuoles and rimmed vacuoles were seen with H&E staining (A). Numerous lipid droplets were seen with oil red O (B).

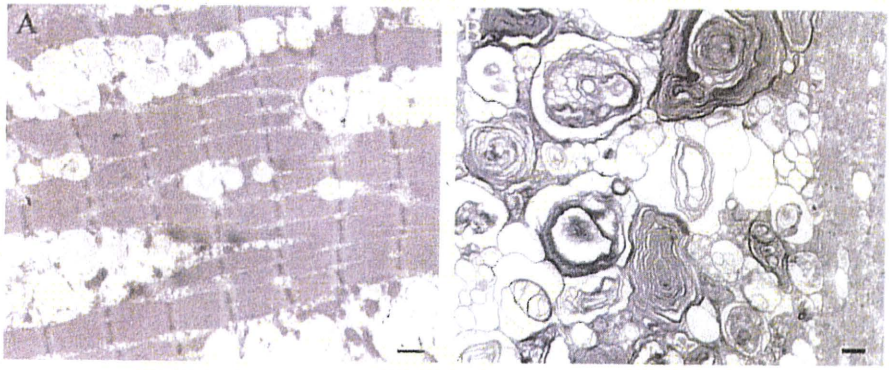

Fig. 3. Onelectron microscopy, markedly increased lipid droplets were seen intermyofibrillar spaces in most of fibers (A). In areas with the rimmed vacuoles, the lipid droplets were not actively scavenged by autophagosome (K). Bar = 1 μm.

Miyoshi myopathy and distal myopathy with rimmed vacuoles are the two most common distal myopathies in Japan, but these were excluded by immunohistochemistry for dysferlin and sequence analysis of *GNE* gene; moreover, finger muscle atrophy and weakness are not usually seen in these distal myopathies. There is a peculiar distal myopathy due to caveolin-3 gene mutation that selectively affected small muscles in hands and feet [5]. However, caveolin-3 immunohistochemistry was normal (data not shown).

Rimmed vacuoles can also be seen in myofibrillar myopathy and inclusion body myopathy with Paget's disease of bone and frontotemporal dementia (IBMPFD) [6,7]. Myofibrillar myopathy is pathologically characterized by disorganization of myofibrillar alignment and protein aggregations, such as cytoplasmic body and spheroid body, which were absent in our patient. IBMPFD is caused by mutations in the gene encoding valosin-containing protein and is clinically characterized by variable extent of dementia and polyostotic skeletal disorganization. IBMPFD is unlikely as our patient had neither intellectual deficit nor bone abnormality although Kimonis et al. recently postulated that IBMPFD is underdiagnosed and reported that 86% of patients had muscle disease while frontotemporal dementia and Paget disease of bone was diagnosed in 27% and 57%, respectively [8]. On top of it, lipid droplets are not a feature of any of the above-mentioned disorders.

In our patient, free carnitine was low in the muscle while total amount was normal. Two patients in the first report of *PNPLA2* mutations showed normal serum carnitine levels [1]. However, muscle carnitine levels were not measured in these patients. Further studies are necessary to determine a relationship between NLSDM and carnitine levels.

The increased amount of lipid droplets in muscle fibers led us to make a diagnosis of LSM. In PCD and MADD, lipid droplets are seen next to mitochondria that are structurally normal. In contrast, mitochondria are pyknotic in our case. Furthermore, autophagic vacuoles have never been reported in other LSM. These observations suggest a possibility that NLSDM may have a myodegenerative process different from other LSM.

We have 47 muscle biopsies diagnosed as LSM collected from 1978–2006. Interestingly, all other 46 patients had proximal dominant muscle weakness except for the present case, suggesting a possibility that distal muscle involvement may be unique to *PNPLA2* mutations although further studies are necessary to draw any conclusion.

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Case report

Rigid spine syndrome caused by a novel mutation in four-and-a-half LIM domain 1 gene (*FHL1*)

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ABSTRACT

Four-and-a-half LIM domain 1 gene (*FHL1*) has recently been identified as the causative gene for reducing body myopathy (RBM), X-linked scapuloperoneal myopathy (SPM) and X-linked myopathy with postural muscle atrophy (XMPMA). Rigid spine is a common clinical feature of the three diseases. We searched for *FHL1* mutations in eighteen patients clinically diagnosed as rigid spine syndrome (RSS). We identified one RSS patient with *FHL1* mutation. Reducing bodies were observed in few fibers of the patient's muscle sample. Amount of FHL1 protein was decreased on immunoblotting. In conclusion, *FHL1* can be one of the causative genes for RSS.

1. Introduction

FHL1, four-and-a-half LIM domain 1 is a 32 kDa protein which is highly expressed in skeletal muscle with intermediate expression in the heart [1]. LIM domains are a cysteine-rich double zinc finger protein-binding motif denoted by the sequence (CX2–CX17–19HX2C)X2(CX2CX16–20CX2(H/D/C)) and mediate interactions with transcription factors and cytoskeletal proteins. LIM domain proteins play critical roles in tissue differentiation and cytoskeletal integrity, respectively. FHL1 was implicated in many cellular functions; (1) α5β1-integrin-dependent myocyte elongation [2], (2) regulation of myosin filament formation and sarcomere assembly by binding to myosin-binding protein C [3], and (3) modulation of Notch signalling pathway through interaction of FHL1C (one of the splicing isoforms of FHL1) with transcription factor RBP-J and RING1 [4].

Recently, mutations in *FHL1* have been identified in patients with RBM [5], SPM [6] and XMPMA [7]. We have also identified mutations in *FHL1* in all RBM patients we reported previously, and confirmed that *FHL1* is the causative gene for RBM (unpublished data). Clinical picture of RBM patients varies from congenital lethal form to benign childhood and adult forms. However, four out of the six RBM families reported to date show rigid spine [5,8]. In addition rigid spine was reported in SPM families [9] and was also seen in the British and Italian–American families reported as XMPMA [7]. This finding suggests that rigid spine is a common clinical feature of patients with *FHL1* mutations.

Here we found a patient with rigid spine syndrome (RSS) harboring a mutation in *FHL1* among 18 patients clinically diagnosed as RSS.

2. Case report

The patient is a 16-year-old male who was a good runner during his childhood. He was first noted to have scoliosis on a routine medical examination when he was 13 years old. Gradually, his walking and running speed became slower, and hip muscle atrophy was noted. Two years late he started experiencing difficulty in bending his body and difficulty in neck flexion. He could not stand on one foot. By the age of 16 years, bilateral hip and thigh muscle atrophy was prominent. On examination, he showed muscle weakness and atrophy in the sternomastoid, trapezius, paravertebral, pelvic girdle and proximal lower limb muscles. Winging of scapula and Gowers' sign were observed. Funnel chest and joint contractures in neck, spine, hip and ankle joints were seen. He walked slouchingly and his left leg was slightly lagged and outward rotated. Serum creatine kinase level was mildly elevated and respiratory functions were mildly impaired. His elder brother showed mild scoliosis but not rigid spine or muscle weakness. His father had IRBBB while his mother was healthy.

Genomic DNA was isolated from peripheral lymphocytes using a standard technique after obtaining informed consent. Seven sets of primers were used to amplify genomic fragments of *FHL1*. All

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exons and their flanking intronic regions of *FHL1* were directly sequenced using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems). We identified a hemizygous in-frame nine base-pair (bp) deletion mutation at c.451–459delGTGACTTGC (p.151–153delVTC) of *FHL1* in this patient. A total 250 controls and the other 17 RSS patients did not carry the mutation in *FHL1*. Genetic analysis of other family members including the elder brother was not allowed.

Biopsied muscle specimen was frozen in isopentane cooled in liquid nitrogen. Serial 10 μm cryostat sections were stained with haematoxylin and eosin (HE), modified Gomori trichrome (mGt) and a battery of histochemical methods. Menadione–nitroblue tetrazolium (NBT) staining in the absence of the substrate α-glycerophosphate was also performed to detect reducing bodies (RBs). Histological analyses of muscle showed marked variation in fiber size and fibers with rimmed vacuoles. Only a limited number of fibers contained RBs. These abnormal fibers detected were localized in focal areas of the muscle specimen (Fig. 1A and B).

Immunohistochemical analysis revealed diffusely increased FHL1 staining in some muscle fibers. The strong FHL1 staining was observed in both types of fibers as seen in serial sections stained by slow type of myosin heavy chain (MHC-slow) (Fig. 1C and D). Protein amount of FHL1 by immunoblotting analysis was significantly reduced in the patient muscle when compared to normal control after normalization to actin amount (Fig. 2).

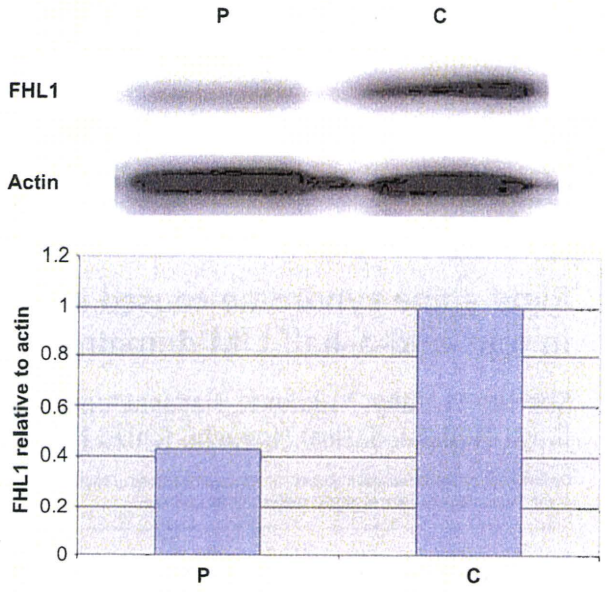

Fig. 2. Immunoblotting analysis of FHL1. Amount of FHL1 in biopsied muscle from the RSS patient show significant reduction compared to actin.

3. Discussion

The term *rigid spine syndrome* was first proposed by Dubowitz to highlight the essential clinical problem seen in myopathy with prominent spinal rigidity [10]. Nevertheless, spinal rigidity is not a specific finding as it is a characteristic feature in Emery–Dreifuss muscular dystrophy, Bethlem myopathy, and in selenoprotein related myopathies. In addition it has also been reported in other congenital myopathies and muscular dystrophies. Patients with *FHL1* mutations also show spinal rigidity [5,7,9].

Here we identified a RSS patient with a novel mutation in *FHL1*. The mutation affects a cysteine residue in the second LIM domain of FHL1 similar to all mutations causing RBM [5].

The most important feature to differentiate RSS from other muscular diseases associated with spinal rigidity is the limitation of flexion of the cervical and dorsolumbar spine in absence of

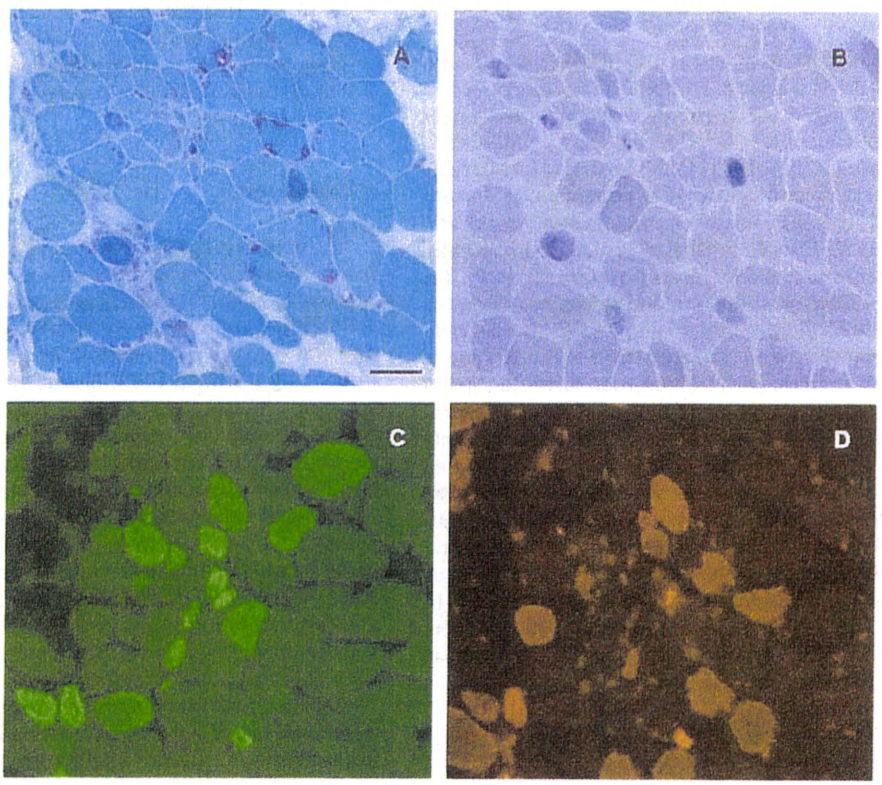

Fig. 1. Muscle pathology. (A) Intracytoplasmic inclusions and rimmed vacuoles are seen on mGT staining. (B) Reducing bodies are positive on melanodine–NBT staining. (C) Diffuse strong immunoreactivity to FHL1 is seen in both MyHC-slow positive and negative fibers (D). Bar = 20 μm.