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Expression levels of *Protocadherin-α* transcripts are decreased by nonsense-mediated mRNA decay with frameshift mutations and by high DNA methylation in their promoter regions

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ABSTRACT

The mouse *protocadherin* (*Pcdh*) clusters, *Pcdh-α*, *-β*, and *-γ*, are located on chromosome 18. Many polymorphic variations are found in the *Pcdh-α* genes in wild-derived and laboratory mouse strains. In comparing the expression levels of *Pcdh-α* isoforms among several strains, we observed lower expression levels of *Pcdh-α9* in BLG2 and BFM/2, and of *Pcdh-α8* in C57BL/6 (B6) than in the other strains. For *Pcdh-α8*, high DNA methylation (72.7%) in the promoter region was found only in B6, whereas 36.4–44.3% methylation was seen in the other strains. On the other hand, the *Pcdh-α9* DNA-methylation levels were similar (23.6–36.3%) among the strains regardless of the difference in expression levels. Interestingly, however, the *Pcdh-α9* variable exon in both BLG2 and BFM/2 included a premature termination codon (PTC) generated by a nucleotide deletion or insertion. Treatment with emetine, a potent inhibitor of nonsense-mediated mRNA decay (NMD), increased the expression level of *Pcdh-α9* from the BLG2-*Pcdh-α* locus. These data indicate that the transcription levels of mature *Pcdh-α* mRNAs are decreased by the DNA-methylation state of the *Pcdh-α* promoter regions and by the NMD pathway during RNA maturation. And we correct some previous data on Sugino, H., Toyama, T., Taguchi, Y., Esumi, S., Miyazaki, M., Yagi, T., (2004) Negative and positive effects of an IAP-LTR on nearby Pcdaalpha gene expression in the central nervous system and neuroblastoma cell lines, Gene 337 91–103.

1. Introduction

The clustered protocadherin (*Pcdh*) genes, which encode a group of diverse cadherin-related transmembrane proteins, are expressed in the brain (Yagi, 2008). The clustered *Pcdh* genes are organized into three closely linked clusters, *Pcdh-α*, *-β*, and *-γ* (Wu and Maniatis, 1999). Single-cell RT-PCR analysis revealed differential and combinatorial expressions of the *Pcdh-α* and *-γ* isoforms in individual Purkinje cells (Esumi et al., 2005; Kaneko et al., 2006). One or two variable first exons are differentially expressed from each allele in single Purkinje cells, leading to the clustered *Pcdh* genes being suggested as candidates for the individualization of neurons in the vertebrate brain.

The *Pcdh-α* and *-γ* clusters are divided into variable and constant regions. In the variable region, multiple first exons are arrayed in tandem. In B6 mouse, *Pcdh-α* variable region contains 14 exons (*Pcdh-α1* – *-α12*, *-αC1* and *-αC2*) and *Pcdh-γ* variable region contains 22 exons (*Pcdh-γA1* – *-γA12*, *-γB1* – *-γB8*, *-γC3*, *-γC4* and *-γC5*). The constant region (CR) consists of three exons (CR1, CR2 and CR3) that encode a common cytoplasmic tail. Each *Pcdh-α* or *-γ* isoform is generated by splicing one of the multiple first exons to the constant exons. On the other hand, the *Pcdh-β* cluster is organized only into a variable region, with multiple single-exons arrayed in tandem. Upstream of each *Pcdh* variable exon is a specific promoter region, which contains a conserved sequence element (CSE). Using cell lines expressing different *Pcdh-α* isoforms, the methylation states of the CpG dinucleotides (CpGs) in the promoter and 5′ region of the variable exons were found to be different between the actively transcribed exons and the silent ones (Kawaguchi et al., 2008; Tasic et al., 2002). Moreover, the 5′ region of the *Pcdh-αC2* variable exon, which is actively transcribed, is extensively hypomethylated in vivo, suggesting that a status of DNA demethylation is important for the biallelic and active expression of each variable isoform (Kawaguchi et al., 2008). Many polymorphisms of the *Pcdh-α* gene cluster have been detected

Abbreviations: B6, C57BL/6; B6xBLG2, intercrossing between B6 and BLG2; CSE, conserved sequence element; CpG, CpG dinucleotide; CR, constant region exon; CTCF, CCCTC-binding factor; EC, extracellular cadherin; EJC, exon–junction complex; gDNA, genomic DNA; IAP, intracisternal A-particle; NMD, nonsense-mediated mRNA decay; P0 or P2, postnatal day 0 or 2; Pcdh, protocadherin; PTC, premature termination codon.

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among wild-derived and laboratory mouse strains (Taguchi et al., 2005) and in humans (Miki et al., 2005; Noonan et al., 2004). Here we examined the transcriptional regulation of *Pcdh-α* isoforms by analyzing their expression levels in wild-derived and laboratory mouse strains, using strain-specific polymorphisms.

In the present study, we found lower mRNA expression of *Pcdh-α9* in the BLG2 and BFM/2 mouse strains than in other strains. In both of the low-expression strains, there is an aberrant stop codon in the *Pcdh-α9* variable exon caused by a nucleotide deletion or insertion that results in a frame-shift. Treatment with an inhibitor of nonsense-mediated mRNA decay (NMD) significantly increased the expression level of *Pcdh-α9* from the BLG2-*Pcdh-α* locus. Thus, the lower mRNA expression of *Pcdh-α9* in BLG2 and BFM/2 was related to NMD. We also analyzed the DNA-methylation states of *Pcdh-α4*, *α8*, *α9*, and *α10* in the cerebral cortex of BLG2, BFM/2, JF1, and B6 mice, and correct previous data for strains BLG2 and B6 (Sugino et al., 2004). Our present results demonstrated that the transcription levels of mature *Pcdh-α* mRNAs are decreased by the DNA-methylation state of the *Pcdh-α* promoter regions and by the NMD pathway during RNA maturation.

2. Materials and methods

2.1. Animals

C57BL/6J (B6) and CBA/JNcrlj (CBA) mice were purchased from Charles River Japan. Wild mouse strains JF1, BLG2, and BFM/2 were obtained from the National Institute for Genetics (Mishima, Shizuoka, Japan). B6xBLG2 F1 hybrid offspring were obtained by intercrossing mice of the laboratory strain B6 with the wild-derived strain BLG2. The mice were used at the age of 2 months (adult males), postnatal day 2 (P2, both gender), and postnatal day 0 (P0, both gender). All the experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the Science Council of Japan and were approved by the Animal Experiment Committee of Osaka University.

2.2. Mice harboring the BLG2-Pcdh-α locus under a B6 genetic background

Mice harboring the BLG2-*Pcdh-α* locus on a B6 genetic background were generated by backcrossing the BLG2 with the B6 mouse. The progeny obtained by the intercrossing of mice that were backcrossed for 7 generations were used for experiments. The $B6^{\alpha BLG2/\alpha BLG2}$ mice were genotyped by checking for the presence of the BLG2-*Pcdh-α* locus by PCR followed by restriction enzyme digestion. The regions amplified by PCR were both upstream (*Pcdh-α1* 1st exon) and downstream (*Pcdh-α* CR3 exon) of the mouse *Pcdh-α* locus. The primers used to amplify the upstream region were as follows: upstream, a1+1061F (CATCCTTGTCTTTGCCCATC) and v01-8 (CCACAGCTCAAATTTGGACT); downstream, CP3+2.1F (GCTATCCG-GAATATCAGC) and CP3-8 (CCATTGCTTTCACACCACCA). The restriction enzymes used were *Mbo*I for the upstream sequence and *Dra*I for the downstream sequence. Both enzymes digested only BLG2-derived sequences.

2.3. Conventional RT-PCR

Total RNA was prepared from mouse cerebral cortex using Trizol reagent (Invitrogen, Carlsbad, CA). Next, 2.5 μg of the total RNA was treated with DNase I (Takara, Japan) and then reverse transcribed by Superscript III reverse transcriptase (Invitrogen) using random primers, according to the manufacturer's protocol, in a 40-μl reaction volume. The PCR of each *Pcdh-α* isoform was performed using 0.4 μl of complementary DNA as a template, 2 μl of 10x Ex-taq PCR buffer, 2 μl of 2.5 mM each dNTP mix, 2 μl of RT-PCR primer set, 0.1 μl of Ex-taq HS polymerase (Takara), and up to 20 μl of water. The PCR conditions were 1 cycle of 3 min at 95°C and then 35 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C. The primer sets and sequences are shown in Supplementary Table S1. The band intensities in Fig. 6b were examined by using NIH Image software.

2.4. Quantitative RT-PCR

Pcdh-α mRNA and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA were quantified by a real-time fluorescence detection method, as described previously (Kawaguchi et al., 2008). The cDNA was prepared as described above. PCR amplification was carried out with the gene-specific primers shown in Supplementary Table 1 using the 7900HT Sequence Detection System and SYBR-Green, according to the manufacturer's instructions (Applied Biosystems). To determine a suitable gene for normalization, we compared the expression levels of two housekeeping genes, *Gapdh* and *β-actin*. In our pilot study, the expression level of *Gapdh* was not influenced by emetine treatment, while that of *β-actin* was slightly decreased. Therefore, we used *Gapdh* for normalization in this study. Statistical significance was calculated by Student's *t*-test.

2.5. Genomic DNA preparation and bisulfite genomic sequencing

Genomic DNA (gDNA) was prepared from mouse cerebral cortex by standard techniques. Bisulfite genomic sequencing was performed according to our previous report (Kawaguchi et al., 2008). gDNA was treated with sodium bisulfite, and the promoter regions of *Pcdh-α4*, *-α8*, *-α9,* and *-α10* were amplified by PCR. The primers used for DNA amplification are shown in Supplementary Table 2. The primers used for analyzing *Pcdh-α8*, *-α9,* and *-α10* were newly designed to improve the amplification efficiency. The reliability of the newly designed primers was confirmed by analyzing whether the methylation patterns of these promoter regions in two mouse cell lines; neuroblastoma C1300 and melanoma M3 (obtained from the Imperial Cancer Research Fund Laboratories, Clar Hall, UK) were the same as reported previously (Kawaguchi et al., 2008) and consistent with the results of DNA methylation-sensitive Southern blotting (for *Pcdh-α8,* Supplementary Fig. 2; for *Pcdh-α9* and *-α10*, data not shown). To obtain the methylation profile from the acquired data, we used the web-based tool, "QUMA" (http://quma.cdb.riken.jp/) (Kumaki et al., 2008).

2.6. Methylation-sensitive Southern blotting for the Pcdh-α8 locus

Genomic DNA was digested by *Sac*I and *Apa*I, then by *Msp*I or the CpG methylation-sensitive isoschizomer *Hpa*II. The probe used in Southern blots to recognize the upstream region of *Pcdh-α8* was amplified by KOD-plus (Toyobo, Japan) PCR with the following primers: forward, GAGCTCTCCCTAAACCCTGA; reverse, ACGAATAAGG-CAGCCGAGAG. The template for PCR was the *Mus musculus* genomic BAC clone RP23-303I8. The amplified PCR products were cloned into pCR-Blunt vectors (Invitrogen) and cut out from the vector. For labeling, the High Prime Labeling Kit for fluorescein (Roche Diagnostics, Mannheim, Germany) was used. The probe fragments were recognized by an anti-fluorescein alkaline phosphatase-conjugated antibody (GE Healthcare UK Ltd.: diluted 100 000 times) and detected by the CDP-Star detection reagent (GE Healthcare UK Ltd.).

2.7. Emetine treatment

Emetine (Sigma E2375) was dissolved in saline. The emetine or vehicle solution was injected into the right lateral ventricle using a glass micropipette. The dose of injected emetine was 10 mg/kg body weight for an adult mouse and 5 mg/kg for a newborn infant. Six hours after the injection, all the mice were still living. The mice