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ORIGINAL INVESTIGATION

A nonsynonymous SNP within *PCDH15* is associated with lipid traits in familial combined hyperlipidemia

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Abstract Familial combined hyperlipidemia (FCHL) is a common lipid disorder characterized by the presence of multiple lipoprotein phenotypes that increase the risk of premature coronary heart disease. In a previous study, we identified an intragenic microsatellite marker within the protocadherin 15 (*PCDH15*) gene to be associated with high triglycerides (TGs) in Finnish dyslipidemic families. In this study we analyzed all four known nonsynonymous SNPs within *PCDH15* in 1,268 individuals from Finnish and Dutch multigenerational families with FCHL. Association analyses of quantitative traits for SNPs were performed using the QTDT test. The nonsynonymous SNP rs10825269 resulted in a $P = 0.0006$ for the quantitative TG trait. Additional evidence for association was observed with the same SNP for apolipoprotein B levels (apo-B) ($P = 0.0001$) and total cholesterol (TC) levels ($P = 0.001$). None of the other three SNPs tested showed a significant association with any lipid-related trait. We investigated the expression of *PCDH15* in different human tissues and observed that *PCDH15* is expressed in several tissues including liver and pancreas. In addition, we measured the plasma lipid levels in mice with loss-of-function mutations in Pcdh15 ($Pcdh15^{av\text{-}Tg}$ and $Pcdh15^{av\text{-}3J}$) to investigate possible abnormalities in their lipid profile. We observed a significant difference in plasma TG and TC concentrations for the $Pcdh15^{av\text{-}3J}$ carriers when compared with the wild type ($P = 0.013$ and $P = 0.044$, respectively). Our study suggests that *PCDH15* is associated with lipid abnormalities.

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Introduction

Familial combined hyperlipidemia (FCHL) is a complex disease characterized by hypertriglyceridemia, hypercholesterolemia or both (Goldstein et al. 1973). In addition, high serum levels of apolipoprotein-B (apo-B) are often observed in FCHL affected individuals (Brunzell et al. 1983; Ayyobi et al. 2003). Several genome-wide scans have been performed to detect susceptibility loci for FCHL (Pajukanta et al. 1999; Aouizerat et al. 1999; Allayee et al. 2002). In a previous study, we identified an intragenic microsatellite marker (D10S546) within the protocadherin 15 (*PCDH15*) to be associated with high serum triglycerides (TGs) in Finnish dyslipidemic families (Lilja et al. 2004). Furthermore, *PCDH15* resides in a region on chromosome 10q11 that has been linked to lipid abnormalities in several studies (Pajukanta et al. 1999; Lilja et al. 2004; Huertas-Vazquez et al. 2005). *PCDH15* is a member of the

cadherin superfamily and encodes an integral membrane protein that mediates calcium-dependent cell–cell adhesion. Mutations in *PCDH15* have been associated with hearing-loss and visual-loss due to retinitis pigmentosa (Ahmed et al. 2001; Alagramam et al. 2001a). Several previous epidemiological studies have demonstrated a relationship between hearing loss and hyperlipidemia (Rosen et al. 1964; Rosen and Olin 1965; Evans et al. 2006; Chang et al. 2007). In this study, we investigated all known nonsynonymous SNPs within *PCDH15*, rs11004439, rs10825269, rs4935502 rs2135720, for association with the FCHL component traits, TGs, total cholesterol (TC) and apo-B in multigenerational Finnish and Dutch families with FCHL as well as the *PCDH15* expression pattern in different human tissues. In addition, we investigated the lipid profile in mice with two different loss-of-function mutations in Pcdh15.

Subjects and methods

Finnish FCHL families

A total of 60 Finnish FCHL families comprising 719 individuals were included in this study. The families were recruited in the Helsinki and Turku University Central Hospitals. The inclusion and exclusion criteria for FCHL have been described in detail previously (Pajukanta et al. 1999; Soro et al. 2002). All subjects gave their informed consent. The study design was approved by the ethics committees of the participating centers.

Dutch FCHL families

A total of 32 Dutch FCHL families comprising 549 individuals were included in this study. The families were recruited at the Lipid Clinic of the Utrecht Academic University Hospital, the Netherlands. The inclusion and exclusion criteria for FCHL have been described in detail previously (Allayee et al. 2002). All subjects provided written informed consent. The study design was approved by the ethics committee of the participating center.

Biochemical analysis and SNP genotyping

Serum lipid parameters were measured as described earlier (Pajukanta et al. 1999; Soro et al. 2002; Allayee et al. 2002). We selected all nonsynonymous SNPs within the *PCDH15* gene, rs11004439, rs10825269, rs4935502 and rs2135720, for genotyping. The SNP primers were designed for PCR using the Primer3 program, and for detection, using the SNP Primer Design software (Pyrosequencing). Genotyping of the 1,268 Finnish and Dutch FCHL family members was performed with the Pyrosequencing technique on the automated PSQ HS96A platform. All SNPs had at least 92% genotype call rate. For quality control, we replicated 3.5% of the genotyped samples. The percentage agreement between samples was >99%. All SNPs were tested for a possible violation of Hardy–Weinberg equilibrium (HWE).

Statistical analysis

All of the association analyses were performed using quantitative lipid traits. The quantitative transmission disequilibrium test (QTDT) (Abecasis et al. 2000) implemented in the genetic analysis package SOLAR. QTDT was performed for each analyzed trait in the Finnish and Dutch families, both separately and in the combined dataset. We analyzed the quantitative TG, TC, and apo-B, traits, as they are the key component traits of FCHL. The residuals for these traits were adjusted by age and sex in the total sample, using the SPSS 12.0 program. The PedCheck program was used to assess the genotype data for pedigree inconsistencies (O'Connell and Weeks 1998). *P* values of less than <0.05 after Bonferroni correction for multiple testing were considered statistically significant. However, it is worth noting that the Bonferroni correction for the probability values obtained in these analyses can be considered conservative, because we investigated highly correlated lipid traits. Apo-A1 and HDL-C traits were analyzed as secondary traits for rs10825269 after establishing the significant associations with TGs and apo-B.

To analyze whether rs10825269 affects a combined trait of HDL-C and TGs, we utilized option 19 of Mendel software (Lange et al. 1976, 2001; Lange and Boehnke 1983). Mendel option 19 performs QTL association using a variance components model. We used a bivariate model consisting of an additive polygenic effect, a random environmental effect, and an additive SNP regression coefficient. Standardized residuals for HDL and Log(TG) were age and sex corrected, and proband ascertainment was corrected for within Mendel. A likelihood ratio test was performed using the formula: $\mathrm{LRT} = 2[\ln(L_{H1}) - \ln(L_{H2})]$, where L_{H1} = maximum likelihood of the bivariate model, and L_{H2} = maximum likelihood of the bivariate model without the additive SNP regression coefficient.

Simulation for functional change in coding nsSNPs

The PolyPhen software was used to investigate a possible impact of all nonsynonymous changes on the structure and function of the *PCDH15 in silico.*

Cross species comparisons

The cross species conservation of the nonsynonymous SNPs was evaluated using the UCSC genome browser.

RT-PCR analysis of *PCDH15* mRNA expression

The expression of *PCDH15* mRNA was analyzed using the human multiple tissue cDNA panel 1 (Clontech). Specific primers for the PCR amplification of *PCDH15* were 5′CCAGGACAAGCTATG TACTTCGAGTCCAAG-3′ (forward) and 5′-GACGAGTACATCGGCTTTGCCG CTCAGTC-3′ (reverse), amplifying a 396 bp fragment (Rouget-Quermalet et al. 2006). Amplification of specific DNA fragments was performed by adding 3 µl of cDNA from the Human Tissue panel I to a PCR mixture containing 0.2 mM dNTPs, 0.4 µM of each primer, 2 µl of 10× reaction buffer, 1.5 µM $MgCl_2$ and 0.2 µl of Taq DNA polymerase. PCR conditions were as follows: After initial denaturation for 10 min at 94°C, the reaction was subjected to 35 cycles of denaturation (30 s, 94°C), annealing (30 s, 61°C) and extension (1 min, 72°C). The amplified products were separated on a 1% agarose gel electrophoresis. G3PDH was used as a reference gene.

Animals

All the mice serum samples were collected at the Department of Otolaryngology-Head and Neck Surgery, Case Western Reserve University, University Hospitals-Case Medical Center. All mice used in this study were maintained on regular mouse diet (6% fat IsoPro 3000 from Purina that contains 6% fat).The mice were fasted for 12 h, beginning one hour after the start of their light cycle. At the conclusion of the fast, the blood was collected from each mouse using a retro-orbital bleed. A total of 41 mice serums were collected for the FVB/N genetic background $Pcdh15^{av\text{-}Tg}$ ($n = 13$ mutants: 3 males and 10 females; and $n = 8$ controls: 4 males and 4 females), and for the C57BL/6J genetic background $Pcdh15^{av\text{-}3J}$ ($n = 9$ mutants: 5 males, 4 females; and $n = 11$ controls: 4 males, 7 females). Mutant mice were generated as described previously (Alagramam et al. 2001b). All animal experimental protocols were approved by Institutional Animal Care and Use Committee, Case Western Reserve University.

Mice serum lipid measurement

All mice were fed a normal diet for 100 days and lipid concentrations were determined. TC and TGs were determined as described previously (Castellani et al. 2004). Each lipid determination was measured in triplicate. The statistical analysis to evaluate differences in the mice lipid measurements was determined by using the unpaired, two tailed Student's *t* test. Sex was included as a covariate in these analyses. Values of $P \leq 0.05$ were considered to be significant.

Results

The mean lipid values of the 92 Dutch and Finnish FCHL families included in this study are shown in Table 1. All nonsynonymous SNPs within *PCDH15* were genotyped in these 92 Finnish and Dutch FCHL families. Genotype distributions for the four investigated SNPs in both populations were consistent with the Hardy–Weinberg equilibrium in nonrelated groups of family members ($P > 0.05$). Of the four nonsynonymous SNPs investigated, SNP rs10825269 showed significant evidence for association for the different quantitative lipid traits, TGs ($P = 0.001$), apo-B ($P = 0.002$) and TC ($P = 0.04$) in the Finns, and for the quantitative apo-B trait in the Dutch ($P = 0.04$) for the same allele (C). No significant association signals were observed with the other three SNPs rs11004439, rs4935502 and rs2135720 ($P > 0.05$). None of the investigated SNPs were in linkage disequilibrium with each other. Next we performed a combined data analysis of both the Finnish and Dutch families with FCHL, and observed a significant increase of statistical significance for all investigated quantitative lipid traits (uncorrected $P = 0.001$–0.0001, Bonferroni corrected $P = 0.02$–0.002). Association results for the combined study sample for SNP rs10825269 are presented in Table 2. We also investigated the nonsynonymous SNP rs10825269 within PCDH15 for associations with quantitative Apo-A1 and HDL-C levels in the Finnish and Dutch FCHL families. No evidence for association was observed for these traits ($P > 0.05$). The frequency of the minor allele of the SNP rs10825269 in both populations was 10%, which is in a good agreement with the allele frequency reported by the International HapMap project in the CEPH samples (http://www.hapmap.org).

The chromosomal region on 10q11, where PCDH15 is located, was also implicated for a combined trait of HDL-C and TGs in our previous study (Lilja et al. 2004). Therefore, we investigated whether rs10825269 affects the combined trait of HDL-C and TGs in the Dutch and Finnish families with FCHL. For this analysis, we utilized option

Table 1 Mean lipid values of the 92 FCHL families included in the study

	Finnish FCHL	Dutch FCHL
No. of families	60	32
No. of subjects (M/F)	719 (356/363)	549 (273/276)
TG, mg/dl (mean ± SD)	316.4 ± 151.0	315.0 ± 201.7
TC, mg/dl (mean ± SD)	298.8 ± 41.3	301.7 ± 61.2
Apo-B, mg/dl (mean ± SD)	146.7 ± 31.1	141.8 ± 24.6
HDL-C, mg/dl (mean ± SD)	40.9 ± 13.1	39.4 ± 12.8

M/F male/female, *TG* triglycerides, *TC* total cholesterol, *Apo-B* apolipoprotein-B, *HDL-C* HDL cholesterol

Table 2 Quantitative family-based association analysis of lipid phenotypes with SNP rs10825269 in the Finnish and Dutch FCHL families using the QTDT program

Trait	Major/minor allele	Minor allele frequency	QTDT[a]	QTDT[b]
TG	**C**/T	0.10	0.0006	0.01
Apo-B	**C**/T	0.10	0.0001	0.002
TC	**C**/T	0.10	0.001	0.02

The risk allele is indicated in bold

TG triglycerides, *TC* total cholesterol, *apo-B* apolipoprotein-B

[a] Uncorrected *P* values (The Bonferroni correction for the probability values obtained in these analyses can be considered conservative, because we investigate highly correlated lipid traits)

[b] *P* values obtained after Bonferroni correction for 24 test (4 SNPs, 3 traits, 2 different populations)

19 of Mendel software (Lange et al. 1976, 2001; Lange and Boehnke 1983) (see Subjects and methods section). We observed that rs10825269 does not significantly alter this combined trait ($P = 0.08$).

The nonsynonymous changes of rs10825269, rs11004439 and rs2135720 were predicted to be benign by the PolyPhen software (PSIC score difference: 0.057, 1.023 and 1.034, respectively). The SNP rs4935720, 166 bp away from SNP rs10825269, was predicted to be possibly damaging (PSIC score 1.7). We also examined the sequence conservation across species of the nonsynonymous variants within *PCDH15*. The cross-species conservations of these nonsynonymous SNPs are shown in Fig. 1.

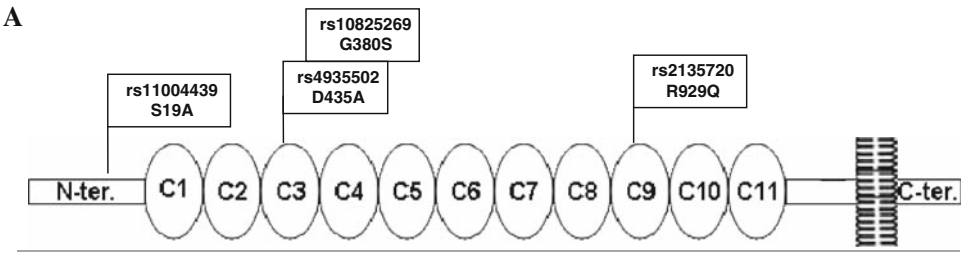
Next, we investigated the tissue distribution of *PCDH15* in different human tissues using a commercial human multiple tissue cDNA panel of eight different tissues. We observed that *PCDH15* was expressed in brain, heart, kidney, liver, lung and pancreas. Figure 2 shows the expression patterns of *PCDH15* in eight human adult tissues.

To investigate possible alterations in the lipid profiles of the Pcdh15 mouse mutants, we measured the lipid levels of two mouse mutants homozygous for different loss of function mutation in Pcdh15 ($Pcdh15^{av\text{-}Tg}$ and $Pcdh15^{av\text{-}3J}$) (Fig. 3a). We observed a significant decrease in plasma TG and TC concentrations between the $Pcdh15^{av\text{-}3J}$ homozygotes and age-match wild type siblings ($P = 0.013$ and $P = 0.044$, respectively) (Fig. 3b). No statistically significant differences were observed between the $Pcdh15^{av\text{-}Tg}$ homozygotes and controls for any lipid trait (data not shown).

Discussion

Results from our study suggest that the common allele of SNP rs10825269 within *PCDH15* is associated with TG, apo-B and TC levels in FCHL. This SNP resides in the same exon as the microsatellite D10S546 that was previously associated with high TGs in the Finnish families with FCHL (Lilja et al. 2004). The functional role of the amino acid substitution G380S in the lipid metabolism is unknown. This amino acid substitution is located in the extracellular domain and resides in a highly conserved residue. Although the amino acid change was predicted to be benign using the Polyphen software (Ramensky and Bork 2002), nonsynonymous SNPs in the coding region of a gene could affect the structure and function of the protein. It

A



B

Variant	hs	pt	rm	mm	rt	cf	dn	la	ec
rs11004439	S	S	S	S	S	A	S	N	S
rs10825269	G	S	S	S	S	S	S	S	S
rs4935502	D	D	D	D	D	D	D	D	D
rs2135720	R	R	R	R	R	R	R	R	R

Fig. 1 Nonsynonymous sequence variants in *PCDH15*. **a** All nonsynonymous sequence variants within *PCDH15*. *N-ter* N-terminus of the amino acid sequence, *C1* cadherin domain 1, *C2* cadherin domain 2, etc.; *C-ter* C-terminus of the amino acid sequence. **b** Sequence conservation across species of nonsynonymous variants in *PCDH15*. The alignment includes Human (*hs*), chimpanzee (*pt*), rhesus monkey (*rm*), mouse (*mm*), rat (*rt*), dog (*cf*), armadillo (*dn*), elephant (*la*), horse (*ec*). *S* serine, *A* alanine, *G* glycine, *D* aspartic acid, *R* Arginine, *Q* Glutamine, *N* aligning has one or more unalignable bases in the gap region

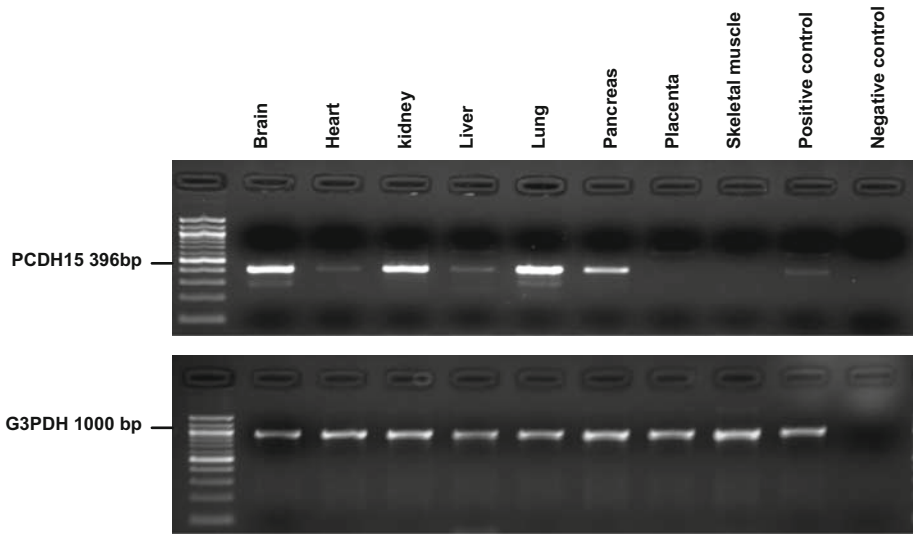


Fig. 2 Expression patterns of *PCDH15* in eight human adult tissues (*upper panel*). G3PDH was used as a positive control (*lower panel*)

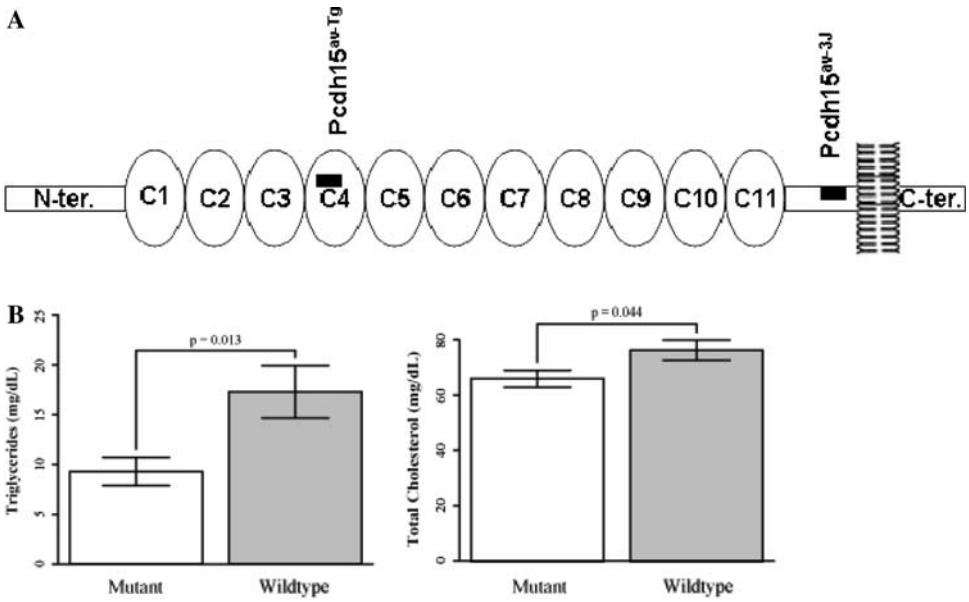


Fig. 3 **a** Mouse loss-of function mutations in Pcdh15 investigated in this study. The *solid rectangle* indicates protein truncation due to premature stop mutations in the mutant mouse. *N-ter* N-terminus of the amino acid sequence, *C1* cadherin domain 1, *C2* cadherin domain 2, etc.; *C-ter* C-terminus of the amino acid sequence. **b** Levels of TGs and total cholesterol in the Pcdh15 mouse mutant in the loss-of-function allele $Pcdh15^{av\text{-}3J}$, when compared to sibling controls. Groups of mice were as follows: 9 mutant and 11 control mice (C57BL/6J genetic background). TG and total cholesterol levels are expressed in mg/dl

also remains possible that SNP rs10825269 is in linkage disequilibrium with another functional DNA variant at this locus.

PCDH15 is a member of the cadherin superfamily of calcium-dependent cell–cell adhesion molecules. *PCDH15* plays an essential role in the maintenance of normal retinal and cochlear function, and mutations in *PCDH15* have been associated with nonsyndromic (DFNB23) (Ahmed et al. 2003) and syndromic hearing loss (the Usher syndrome type 1F, USH1F) (Ahmed et al. 2001; Alagramam et al. 2001a). Although *PCDH15* has not been directly related with lipid abnormalities, previous biochemical analysis suggested that USH1F patients have decreased levels of long-chain polyunsaturated fatty acids in plasma (Maude et al. 1998). In addition, previous epidemiological studies have linked hearing loss to lipid abnormalities, showing that hyperlipidemia and atherosclerosis can induce alteration in cochlear function (Rosen et al. 1964; Rosen and Olin 1965; Evans et al. 2006; Chang et al. 2007). The biological role of protocadherins in lipid abnormalities is unclear. The large size and diversity of members of the protocadherin family suggest the participation of these

proteins in a wide variety of biological processes. Previous studies of the Usher syndrome and visual abnormalities have shown that *PCDH15* is expressed in several tissues including retina, brain, cerebellum, kidney, cochlea and liver (Alagramam et al. 2001a; Rouget-Quermalet et al. 2006). In this study, the expression pattern of *PCDH15* in human was consistent with the pattern previously observed in mice (Alagramam et al. 2001b; Rouget-Quermalet et al. 2006). Importantly, we also demonstrate that *PCDH15* is expressed in human pancreas. Further investigation is necessary to confirm the role of PCDH15 in lipid abnormalities.

To the best of our knowledge, this is the first time that lipid traits have been investigated in the Pcdh15 mouse mutant. Although additional studies are necessary to confirm our findings, these observations suggest a possible alteration in the lipid profile of the Pcdh15 mouse mutant due to the $Pcdh15^{av\text{-}3J}$ loss-of-function mutation. No statistically significant differences were observed in the $Pcdh15^{av\text{-}Tg}$ loss-of-function mutation. The observed results suggest differences in the genetic background between the FVB/N and C57BL/6J strains. This suggestion is indirectly supported by a previous study demonstrating that the FVB/N strain is susceptible to diet induce-atherosclerosis whereas the C57BL/6J strain is resistant (Hoover-Plow et al. 2006). A given phenotype could be obvious in one inbred genetic background but it could be suppressed in another genetic background (potential genetic modifier effect; Nadeau 2001).

Genome-wide association analyses in unrelated individuals have identified several loci associated with lipid abnormalities. However, the variants identified so far explain a small fraction of the disease risk, suggesting that many genes implicated in the lipid metabolism still remain undiscovered. We have previously identified several genes associated with FCHL using family-based studies and replicated our results in different cohorts (Pajukanta et al. 2004; Huertas-Vazquez et al. 2005, 2008; Weissglas-Volkov et al. 2006; Lee et al. 2008; Plaisier et al. 2009). Family-based studies are more robust to population stratification and families ascertained for the disease of interest provide a powerful tool for association studies.

In conclusion, we have identified a nonsynonymous variant in *PCDH15* associated with TG, apo-B and TC levels in multigenerational Caucasian FCHL families. Replication in additional FCHL study samples and sequencing of *PCDH15* are warranted to further explore the effects of *PCDH15* in FCHL.

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