(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 July 2009 (16.07.2009)

PCT

(10) International Publication Number
WO 2009/086591 A1

(51) International Patent Classification:
G01N 33/68 (2006.01) *A61K 38/17* (2006.01)
A61P 25/00 (2006.01) *C07K 16/18* (2006.01)
C12N 15/12 (2006.01) *G01N 33/53* (2006.01)
A01K 67/00 (2006.01) *A61K 48/00* (2006.01)
C07K 14/435 (2006.01) *C12N 15/01* (2006.01)

(21) International Application Number:
PCT/AU2009/000008

(22) International Filing Date: 5 January 2009 (05.01.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/010,176 4 January 2008 (04.01.2008) US

(71) Applicant *(for all designated States except US)*: **MEDVET SCIENCE PTY LTD** [AU/AU]; IP & Commercialisation Office, Level 3 Imvs South Building, Frome Road, Adelaide, 5000 South Australia (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **DIBBENS Leanne Michelle** [AU/—]; 14 Trinity Street, College Park, 5069, South Australia (AU). **SCHEFFER, Ingrid Eileen** [AU/AU]; 3 Leura Grove, Hawthorn East, 3123, Victoria (AU). **BERKOVIC, Samuel Frank** [AU/AU]; 7 Polo Parade, Caulfield North, 3161, Victoria (AU). **MULLEY, John, Charles** [AU/AU]; 13 Dunkley Avenue, Firle, 5070, South Australia (AU). **GECZ, Jozef** [AU/AU]; 9 Garden Avenue, Burnside, 5066, South Australia (AU).

(74) Agent: **MADDERNS**; First Floor, 64 Hindmarsh Square, Adelaide, 5000, South Australia (AU).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(54) **Title:** DIAGNOSTIC AND THERAPEUTIC METHODS FOR EFMR (EPILEPSY AND MENTAL RETARDATION LIMITED TO FEMALES)

(57) **Abstract:** Methods and kits for the diagnosis of illnesses related to protocadherin 19 (PCDH 19) protein deficiency or altered PCDH 19 protein function, in particular EFMR (Epilepsy and Mental Retardation limited to Females) are provided, as well as methods and kits for the identification of a predisposition to such illnesses and methods of screening subjects to identify carriers of such illnesses and methods and kits for the therapeutic or prophylactic treatment of PCDH 19 deficiency or altered PCDH 19 protein function. Further, nucleotide and amino acid sequences corresponding to a complete *PCDH19* open reading frame (ORF), mutant sequences encoding non-functional *PCDH19* mRNA or altered *PCDH19* mRN A are described along with transformed cells and non-human transgenic animals comprising wild-type or mutant *PCDH19* ORF nucleotide sequences.

WO 2009/086591 A1

DIAGNOSTIC AND THERAPEUTIC METHODS FOR EFMR (EPILEPSY AND MENTAL RETARDATION LIMITED TO FEMALES)

FIELD OF THE INVENTION

The present invention relates to nucleotide and amino acid sequences corresponding to a complete protocadherin 19 (*PCDH19*) open reading frame (ORF), and mutant nucleotide sequences encoding non-functional or altered *PCDH19* mRNA or non-functional or altered PCDH19 protein which can result in illnesses related to PCDH19 protein deficiency or altered function in human subjects, in particular EFMR (Epilepsy and Mental Retardation limited to Females).

INCORPORATION BY REFERENCE

This patent application claims priority from:

- US 61/010176 titled "Therapeutic Compound" filed on 4 January 2008.

The entire content of this application is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Inherited diseases caused by mutations on the X chromosome are generally characterised by the affected status of carrier males and sparing of carrier females. EFMR (Epilepsy and Mental Retardation limited to Females) is a unique X-linked condition which, by contrast, spares carrier males and is expressed in females (Ryan SG *et al.*, 1997). EFMR is a rare condition characterised by seizure onset in early childhood (6-36 months) and cognitive impairment. The phenotype is restricted to females with males apparently spared, demonstrating normal cognitive function and absence of seizures.

Prior to the studies described herein, the cause of EFMR was unknown, with the presence of EFMR not previously attributed to any specific genetic factor. The studies described herein now identify the protocadherin 19 (*PCDH19*) gene as responsible for EFMR.

By the systematic re-sequencing of 737 X-linked genes, seven different mutations in the *PCDH19* gene were identified in seven unrelated families with EFMR. Five of these mutations result in the introduction of a premature termination codon resulting in non-functional *PCDH19* mRNA that is degraded by non-sense mediated decay (NMD) processes. The two other mutations have been determined to be missense mutations and are likely to affect adhesiveness of the PCDH19 protein through impaired calcium binding.

PCDH19 is the first cadherin to be implicated in epilepsy and mental retardation. The expression analysis described herein shows a role for PCDH19 in normal neuronal development. A mechanism of phenotype rescue that saves transmitting males (ie carrier males) from clinically expressing the disorder is suspected, through a related male-specific human gene, protocadherin 11Y (*PCDH11Y*) (Blanco P *et al.*, 2000). This mechanism is consistent with the remarkable mode of inheritance observed in EFMR.

The studies described herein have identified nucleotide and amino acid sequences corresponding to a complete *PCDH19* open reading frame (ORF) as well as mutant sequences encoding non-functional *PCDH19* mRNA or non-functional PCDH19 protein. These are shown to be related to illnesses associated with PCDH19 protein deficiency or altered function such as epilepsy and mental retardation, in particular EFMR. Further, male carriers of the PCDH19 deficient genotype have been shown to be rescued from the disease phenotype by the male-specific protocadherin PCDH11Y.

The identification of the complete *PCDH19* ORF and the identification of mutations in the nucleotide sequence causing a disease state provide for methods for diagnosis of illnesses related to PCDH19 protein deficiency or altered PCDH19 protein function, methods for the identification of a predisposition to such illnesses, methods of screening to identify carriers of such illnesses methods, and agents for the therapeutic or prophylactic treatment of PCDH19 deficiency.

SUMMARY OF THE INVENTION

Thus, in a first aspect, the present invention provides a method of diagnosing an illness related to functional protocadherin 19 (PCDH19) protein deficiency or altered PCDH19 protein function, or assessing a predisposition to an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or screening to identify carriers of illnesses related to functional PCDH19 protein deficiency or altered PCDH19 protein function, wherein said method comprises the step of:

(i) detecting in a suitable biological sample from a subject, a loss of PCDH19 protein function or altered PCDH19 protein function.

In a second aspect, the invention provides a kit for diagnosing an illness related to functional protocadherin 19 (PCDH19) protein deficiency or altered PCDH19 protein function, or assessing a predisposition to an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or screening to identify carriers of illnesses related to functional PCDH19 protein deficiency or altered PCDH19 protein function, wherein said kit comprises one or more of the following: an antibody or fragment thereof which specifically binds to PCDH19 protein or polypeptide, or a fragment or variant thereof; and an oligonucleotide probe/primer molecule which specifically hybridises to a polynucleotide molecule encoding PCDH19 protein or polypeptide, or a fragment or variant thereof under high stringency conditions.

In a third aspect, the present invention provides for the use of: a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to a complete protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1, wherein said nucleotide sequence encodes a functional PCDH19 protein or polypeptide, or functional fragment or functional

variant thereof encoded by a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1; in the treatment of PCDH19 protein deficiency or altered PCDH19 protein function in a subject.

In a fourth aspect, the present invention provides a method for the therapeutic or prophylactic treatment of protocadherin 19 (PCDH19) protein deficiency or altered PCDH19 protein function in a subject, wherein said method comprises the step of:

(i) administering to said subject: a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1, wherein said nucleotide sequence encodes a functional PCDH19 protein or polypeptide, or a functional fragment or functional variant thereof; a functional PCDH19 protein or polypeptide, or functional fragment or functional variant thereof encoded by a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1; and/or an agent that compensates for the loss of PCDH19 protein function; optionally in combination with a pharmaceutically-acceptable carrier.

In a fifth aspect, the present invention provides an agent capable of treating a deficiency in functional protocadherin 19 (PCDH19) protein or altered PCDH19 protein function in a subject.

In a sixth aspect, the present invention provides a method for identifying an agent capable of treating a deficiency in functional protocadherin 19 (PCDH19) protein or altered PCDH19 protein function, wherein said method comprises the steps of;

(i) providing a cell or animal comprising a polynucleotide molecule comprising a mutant sequence of the *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1;

(ii) contacting a test agent with said cell or administering a test agent to said animal; and

(iii) comparing a response in said cell or animal with a control response.

In a seventh aspect, the present invention provides a kit for use in the method of the sixth aspect, wherein said kit comprises instructions for the operation of the method together with one or more containers and/or vessels containing one or more cell(s) or animal(s) comprising a polynucleotide molecule comprising a mutant sequence of the protocadherin 19 (*PCDH19*) ORF nucleotide sequence shown as SEQ ID NO: 1.

In an eighth aspect, the present invention provides a kit for identifying an agent capable of treating a deficiency in functional protocadherin 19 (PCDH19) protein or altered PCDH19 protein function, wherein said kit comprises;

(i) a cell or animal comprising a polynucleotide molecule comprising a mutant sequence of the *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1; and optionally,

(ii) a control cell or animal comprising a polynucleotide molecule comprising a wild-type form of the complete *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1, said wild-type form encoding a functional PCDH19 protein or polypeptide, or a functional fragment or functional variant thereof.

In a ninth aspect, the present invention provides an isolated protein or polypeptide comprising an amino acid sequence encoded by a nucleotide sequence showing at least 70% sequence identity to a complete protocadherin 19 (*PCDH19*) ORF nucleotide sequence according to SEQ ID NO: 1, or a functional fragment or variant thereof.

In a tenth aspect, the present invention provides an isolated polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to a complete protocadherin 19 (*PCDH19*) ORF nucleotide sequence according to SEQ ID NO: 1 or a complementary sequence thereto.

In an eleventh aspect, the present invention provides a cell transformed with the polynucleotide molecule of the tenth aspect.

In a twelfth aspect, the present invention provides a non-human animal comprising the polynucleotide molecule of the tenth aspect.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows pedigrees of the seven EFMR families assessed in the studies described herein. A specific mutation in the *PCDH19* gene, responsible for EFMR in each family, is indicated alongside the corresponding sequence chromatogram section showing the location of the mutation. Females presenting with EFMR are represented by filled circles and carrier males are represented by small circles within squares.

Figure 2 shows a schematic diagram of the PCDH19 protein with the signal peptide, extracellular cadherin (EC), transmembrane (TM) and cytoplasmic (CM) domains indicated. The relative locations of mutations found in the EFMR families are also shown.

Figure 3 shows the partial alignment of the human *PCDH19* with orthologues of *PCDH19* from other species and other human protocadherins. The high conservation of residues affected by two missense mutations, V441E (top panel) and N557K (bottom panel) are indicated by rectangular boxes. The calcium ion-binding acidic residues are also indicated by a bracket against both alignments.

Figure 4 shows Northern blot (Clontech Laboratories, Inc., Mountain View, CA, United States of America) analyses of *PCDH19* and *PCDH11X/Y* in various human brains tissues. The position of the ~9.8 kb *PCDH19* transcript is indicated by an asterisk, while the position of the smaller ~9.5 kb *PCDH11X/Y* mRNAs is shown by an arrowhead. The brackets indicate either non-specific binding of the *PCDH19* probe or *PCDH19* degradation products.

Figure 5 shows a section of a nucleotide sequence chromatogram from an EFMR affected female, indicating the detection of a mutation, 253C>T, in genomic DNA (gDNA) (top panel), the absence of the mutant sequence in fibroblast cDNA (middle panel) and the presence of both the mutant and wild-type cDNA after the treatment of fibroblasts with cyclohexamide (bottom panel). The position of the mutation is boxed.

Figure 6 shows the expression of PCDH19 in murine central nervous system (CNS) at 15.5 days postcoital (a-f) and postnatal day 2 (g-l). (a, b) are adjacent sections stained with Haematoxylin and Eosin and processed for PCDH19 *in situ*, respectively. (c, d, e) are higher magnification images of the boxed regions in b. Arrowheads in c indicate PCDH19 expressing cells within the cortex; the asterisk in e highlights the dorsolateral wall of the lateral ventricle. (g, h) are adjacent sections stained with Haematoxylin and Eosin and processed for PCDH19 *in situ,* respectively. (i) is a posterior brain section (to h) highlighting PCDH19 expression. (j, k, l) are higher magnification images of the boxed regions in (g, h, respectively). Cx/P, cortical plate; Hn, Hippocampal neuroepithelium; lv, lateral ventricle; Th, thalamus; Hy, hypothalamus; icf, intercerebral fissure; Ob, olfactory bulbs; Ne, nasal epithelium. Magnification bars (a, b, f-i) represent 200 μM, bars in (c-e; and j-l) represent 50 μM.

Figure 7 shows a diagrammatic representation of the expected mechanism underlying the inheritance of EFMR whereby PCDH11Y functionally rescues *PCDH19* mutations in transmitting males.

Figure 8 shows expression profiles of PCDH19 (top panel), PCDH11Y (middle panel) and PCDH11X (bottom panel) in various regions of adult human brain tissues.

DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to methods and kits for the diagnosis of illnesses related to PCDH19 protein deficiency or altered PCDH19 protein function, methods and kits for the identification of a predisposition to such illnesses, methods of screening subjects to identify carriers of such illnesses,

and methods and kits for the therapeutic or prophylactic treatment of PCDH19 deficiency or altered PCDH19 protein function. The present invention also relates to nucleotide and amino acid sequences corresponding to a complete *PCDH19* ORF, mutant sequences encoding non-functional *PCDH19* mRNA (eg which may be degraded by nonsense mediated mRNA degradation (NMD) processes) or altered *PCDH19* mRNA, or non-functional PCDH19 protein or altered PCDH19 protein causative of illnesses related to PCDH19 protein deficiency or altered function in human subjects, and transformed cells and transgenic non-human animals comprising wild-type or mutant *PCDH19* ORF nucleotide sequences.

Thus, in a first aspect, the present invention provides a method of diagnosing an illness related to functional protocadherin 19 (PCDH19) protein deficiency or altered PCDH19 protein function, or assessing a predisposition to an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or screening to identify carriers of illnesses related to functional PCDH19 protein deficiency or altered PCDH19 protein function, wherein said method comprises the step of:

(i) detecting in a suitable biological sample from a subject, a loss of PCDH19 protein function or altered PCDH19 protein function.

Illnesses resulting from PCDH19 deficiency or altered PCDH19 protein function include epilepsy and/or mental retardation, in particular EFMR. Thus, preferably, the method of the first aspect provides a method of diagnosing EFMR, assessing a predisposition to EFMR or screening carriers of EFMR, in particular, male carriers of EFMR. In particular, preferred methods include prenatal diagnosis or screeing of EFMR.

The detection of a loss of PCDH19 protein function or altered PCDH19 protein function can be used, in the case of a subject for which EFMR has not previously been diagnosed, either on its own or in combination with other tests, to diagnose EFMR in the subject. For a subject in which EFMR has not previously been diagnosed and who is not showing any signs of ill health due to EFMR, the detection of a loss of PCDH19 protein function or altered PCDH19 protein function, can be used in an assessment of a predisposition to EFMR or carrier status of EFMR.

The detection of a loss of PCDH19 protein function or altered PCDH19 protein function can involve one or more of detecting a mutant sequence in a *PCDH19* ORF of the subject which encodes non-functional or altered *PCDH19* mRNA or non-functional or altered PCDH19 protein (eg by genotyping the subject) or causes reduced expression of PCDH19 protein (eg mutations of the *PCDH19* gene expression control sequences). Conveniently, this may be achieved by amplifying the *PCDH19* nucleotide sequences (or a target region thereof) within a suitable biological sample and, thereafter sequencing the amplication product. Preferably, the detection of a loss of PCDH19 protein function involves detecting a mutant sequence in the extracellular (EC) domain-encoding region of a *PCDH19* ORF of the subject such as, for example, a mutant sequence causing an amino acid substitution within or adjacent to a calcium ion-

binding site (eg within 20 amino acids of a calcium ion-binding site) such that calcium ion binding is impaired, or a mutant sequence comprising a premature termination codon (PTC).

The detection of a loss of PCDH19 protein function or altered PCDH19 protein function may also be indirectly achieved by conducting, for example, assays for *functional* PCDH19 protein or polypeptide. Assays for detecting functional PCDH19 protein or polypeptide, preferably comprise the use of an antibody or fragment thereof that is capable of specifically binding with PCDH19 protein or polypeptide, or a functional fragment or functional variant thereof, to determine the relative amount of the protein or polypeptide that is present in a suitable biological sample taken from the subject. This can involve the use of any of the methods well known to persons skilled in the art (eg standard ELISA-based methods or *in situ* immunofluorescence using tissue section samples). As such, the relative amount of functional *PCDH19* protein or polypeptide can be determined by quantitatively detecting the protein or polypeptide with a specific antibody or fragment thereof (ie a primary antibody) which is either directly conjugated to a detectable label or is otherwise detected via a secondary antibody or fragment thereof directly conjugated to a detectable label. Suitable detectable labels include chromophores, fluorophores (eg fluorescein or FITC), radiolabels (eg ^{125}I), and enzymes such as horseradish peroxidase. These labels can be used in methods and systems as are well known to persons skilled in the art, which provide for the automation or partial automation of the step of detecting the functional *PCDH19* protein or polypeptide (eg by a microplate reader or use of a flow cytometer). Generally, the relative amount of functional PCDH19 protein will be determined by comparison against the amount, or range of amounts, present in "normal samples" (eg samples from equivalent biological samples taken from normal subject(s)).

Functional PCDH19 protein or polypeptide may be characterised as being encoded by a nucleotide sequence showing at least 70% sequence identity, preferably at least 85% sequence identity, and, more preferably, at least 95% sequence identity to a complete *PCDH19* open reading frame (ORF) nucleotide sequence according to:

```
atggagtcgc tcctgctgcc ggtgctgctg ctgctggcca tactgtggac gcaggctgcc     60
gccctcatta atctcaagta ctcggtagaa gaggagcagc gcgccgggac ggtgattgcc    120
aacgtggcca aagacgcgcg agaggcgggc ttcgcgctgg acccccggca ggcttcagcc    180
tttcgcgtgg tgtccaactc ggctccacac ctagtggaca tcaatcccag ctctggcctg    240
ctggtcacca agcagaagat tgaccgtgat ctgctgtgcc gccagagccc caagtgcatc    300
atctcgctcg aggtcatgtc cagctcaatg gaaatctgcg tgataaaggt ggagatcaag    360
gacctgaacg acaatgcgcc cagtttcccg gcagcacaga tcgagctgga gatctcggag    420
gcagccagcc ctggcacgcg catcccgctg gacagcgctt acgatccaga ctcaggaagc    480
tttggcgtgc agacttacga gctcacgccc aacgagctgt tcggcctgga gatcaagacg    540
cgcggcgacg gctcccgctt tgccgaactc gtggtggaaa agagcctgga ccgcgagacg    600
cagtcgcact acagcttccg aatcactgcg ctagacggtg gcgacccgcc gcgcctgggc    660
accgttggcc ttagtatcaa ggtgaccgac tccaatgaca acaacccggt gtttagcgag    720
tccacctacg cggtgagcgt gccagaaaac tcgcctccca acacacccgt catccgcctc    780
aacgccagcg atccagacga gggcaccaac ggccaggtgg tctactcctt ctatggctac    840
gtcaacgacc gcacgcgcga gctctttcag atcgacccgc acagtggcct ggtcactgtc    900
actggcgctt tagactacga agaggggcac gtgtacgaac tggacgtgca ggctaaggac    960
ttggggccca attccatccc ggcacactgc aaggtcaccg tcagcgtgct ggacaccaat   1020
gacaatccgc cggtcatcaa cctgctgtca gtcaacagtg agcttgtgga ggtcagcgag   1080
```

```
agcgcccccc cgggctacgt gatcgccttg gtgcgggtgt ctgatcgcga ctcaggcctc   1140
aatggacgtg tgcagtgccg tttgctgggc aatgtgccct ttcgactgca ggaatatgag   1200
agcttctcca ctattctggt ggacggacgg ctggaccgcg agcagcacga ccaatacaac   1260
ctcacaattc aggcacgcga cggcggcgtg cccatgctgc agagtgccaa gtcctttacc   1320
gtgctcatca ctgacgaaaa tgacaaccac ccgcactttt ccaagcccta ctaccaggtc   1380
attgtgcagg agaacaacac gcctggcgcc tatctgctct ctgtgtctgc tcgcgacccc   1440
gacctgggtc tcaacggcag tgtctcctac cagatcgtgc cgtcgcaggt gcgggacatg   1500
cctgtcttca cctatgtctc catcaatccc aactcaggcg acatctacgc gctgcgatcc   1560
tttaaccacg agcagaccaa ggcgttcgaa ttcaaggtgc tggccaagga cggcggcctt   1620
ccctcactgc aaagcaacgc tacggtgcgg gtcatcatcc tcgacgtcaa cgacaacacc   1680
ccggtcatca cagccccacc tctgattaac ggcactgccg aggtctacat accccgcaac   1740
tctggcatag gctacctggt gactgttgtc aaggcagaag actacgatga gggcgaaaat   1800
ggccgagtca cctacgacat gaccgagggc gaccgcggct tctttgaaat agaccaggtc   1860
aatggcgaag tcagaaccac ccgcaccttc ggggagagct ccaagtcctc ctatgagctt   1920
atcgtggtgg ctcacgacca cggcaagaca tctctctctg cctctgctct cgtcctaatc   1980
tacttgtccc ctgctctcga tgcccaagag tcaatgggct ctgtgaactt gtccttgatt   2040
ttcattattg ccctgggctc cattgcgggc atcctctttg taactatgat cttcgtggca   2100
atcaagtgca agcgagacaa caaagagatc cggacctaca actgcagtaa ttgtttaacc   2160
atcacttgtc tcctcggctg ttttataaaa ggacaaaaca gcaagtgtct gcattgcatc   2220
tcggtttctc ccattagcga ggagcaagac aaaaagacag aggagaaagt gagcctaagg   2280
ggaaagagaa ttgctgagta ctcctatggg catcaaaaga aatcaagcaa gaagaaaaaa   2340
atcagtaaga atgacatccg cctggtaccc cgggatgtgg aggagacaga caagatgaac   2400
gttgtcagtt gctcttccct gacctcctcc ctcaactatt ttgactacca ccagcagacg   2460
ctgcccctgg gctgccgccg ctctgagagc actttcctga atgtggagaa ccagaatacc   2520
cgcaacacca gtgctaacca catctaccat cactctttca acagccaggg gccccagcag   2580
cctgacctga ttatcaacgg tgtgcctctg cctgagactg aaaactattc ttttgactcc   2640
aactacgtga atagccgagc ccatttaatc aagagcagct ccaccttcaa ggacttagag   2700
ggcaacagcc tgaaggatag tggacatgag gagagtgacc aaactgacag tgagcatgat   2760
gtccagcgga gcctgtattg tgatactgct gtcaacgatg tgctgaacac cagtgtgacc   2820
tccatgggat ctcagatgcc tgatcatgat cagaatgaag gatttcattg ccgggaagaa   2880
tgccggattc ttggccactc tgacaggtgc tggatgcccc ggaaccccat gcccatccgt   2940
tccaagtccc ctgagcatgt gaggaacatc atcgcgctgt ctattgaagc tactgctgct   3000
gatgtcgagg cttatgacga ctgcggcccc accaaacgga ctttcgcaac ctttgggaaa   3060
gatgtcagcg accacccggc tgaggagagg cctaccctga aaggcaagag gactgtcgat   3120
gtgaccatct gcagccccaa ggtcaacagc gttatccggg aggcaggcaa tggctgtgag   3180
gcgattagcc ctgtcacctc ccccctccac ctcaagagct ctctgcccac caagccttcc   3240
gtgtcttaca ccattgccct ggctccccca gcccgtgatc tggagcagta tgtcaacaat   3300
gtcaacaatg gccctactcg tccctctgaa gctgagcccc gtggagctga tagcgagaaa   3360
gtcatgcatg aggtcagccc cattctgaag gaaggtcgca acaaagagtc ccctggtgtg   3420
aagcgtctga aggatatcgt tctctaa                                       3447
```

(SEQ ID NO: 1).

Most preferably, functional PCDH19 protein or polypeptide is characterised by comprising an amino acid sequence according to:

```
MESLLLPVLLLLAILWTQAAALINLKYSVEEEQRAGTVIANVAKDAREAGFALDPRQASA   60
FRVVSNSAPHLVDINPSSGLLVTKQKIDRDLLCRQSPKCIISLEVMSSSMEICVIKVEIK  120
DLNDNAPSFPAAQIELEISEAASPGTRIPLDSAYDPDSGSFGVQTYELTPNELFGLEIKT  180
RGDGSRFAELVVEKSLDRETQSHYSFRITALDGGDPPRLGTVGLSIKVTDSNDNNPVFSE  240
STYAVSVPENSPPNTPVIRLNASDPDEGTNGQVVYSFYGYVNDRTRELFQIDPHSGLVTV  300
TGALDYEEGHVYELDVQAKDLGPNSIPAHCKVTVSVLDTNDNPPVINLLSVNSELVEVSE  360
SAPPGYVIALVRVSDRDSGLNGRVQCRLLGNVPFRLQEYESFSTILVDGRLDREQHDQYN  420
LTIQARDGGVPMLQSAKSFTVLITDENDNHPHFSKPYYQVIVQENNTPGAYLLSVSARDP  480
DLGLNGSVSYQIVPSQVRDMPVFTYVSINPNSGDIYALRSFNHEQTKAFEFKVLAKDGGL  540
```

PSLQSNATVRVIILDVNDNTPVITAPPLINGTAEVYIPRNSGIGYLVTVVKAEDYDEGEN 600
GRVTYDMTEGDRGFFEIDQVNGEVRTTRTFGESSKSSYELIVVAHDHGKTSLSASALVLI 660
YLSPALDAQESMGSVNLSLIFIIALGSIAGILFVTMIFVAIKCKRDNKEIRTYNCSNCLT 720
ITCLLGCFIKGQNSKCLHCISVSPISEEQDKKTEEKVSLRGKRIAEYSYGHQKKSSKKKK 780
ISKNDIRLVPRDVEETDKMNVVSCSSLTSSLNYFDYHQQTLPLGCRRSESTFLNVENQNT 840
RNTSANHIYHHSFNSQGPQQPDLIINGVPLPETENYSFDSNYVNSRAHLIKSSSTFKDLE 900
GNSLKDSGHEESDQTDSEHDVQRSLYCDTAVNDVLNTSVTSMGSQMPDHDQNEGFHCREE 960
CRILGHSDRCWMPRNPMPIRSKSPEHVRNIIALSIEATAADVEAYDDCGPTKRTFATFGK 1020
DVSDHPAEERPTLKGKRTVDVTICSPKVNSVIREAGNGCEAISPVTSPLHLKSSLPTKPS 1080
VSYTIALAPPARDLEQYVNNVNNGPTRPSEAEPRGADSEKVMHEVSPILKEGRNKESPGV 1140
KRLKDIVL 1148

(SEQ ID NO: 2).

On the other hand, assays for detecting loss of PCDH19 protein function may comprise the use of an antibody or fragment thereof that is capable of distinguishing between, for example, a wild-type PCDH19 protein or polypeptide and a non-functional variant thereof. This may or may not result in the identification of the particular form of the PCDH19 protein or polypeptide that is present in the biological sample taken from the subject.

Otherwise, assays for detecting loss of PCDH19 protein function may comprise determining the relative amount of messenger RNA (mRNA) encoding functional PCDH19 protein or polypeptide in a suitable biological sample taken from the subject. The relative amount of mRNA encoding the protein or polypeptide may be determined by any of the methods well known to persons skilled in the art including Northern blot (by comparison to reference samples) and PCR-based mRNA quantification methods (eg using RT-PCR with primers conjugated to a detectable label). Generally, the relative amount of mRNA encoding the protein or polypeptide will be determined by comparison against the amount, or range of amounts, present in "normal samples" (eg samples from equivalent biological samples taken from normal subject(s)).

Most preferably, the detection of a loss of PCDH19 protein function comprises detecting a mutant sequence encoding a non-functional variant of the PCDH19 amino acid sequence shown as SEQ ID NO: 2. Said mutant sequence may comprise any one or more of the nucleotide changes, relative to the nucleotide sequence shown as SEQ ID NO: 1, as follows: 1322 T>A, 253 C>T, 2012 C>G, 2030_2031insT, 1671 C>G, 357delC and 1091_1092insC. Methods for the detection of such nucleotide changes may comprise the step of detecting any hybridisation of a suitable oligonucleotide probe/primer molecule under high stringency conditions to the mutant sequence present in a suitable biological sample. High stringency conditions are well known to persons skilled in the art, and are typically characterised by high temperature (ie high annealing temperature) and low ionic strength (ie low salt concentration, especially of $MgCl_2$, KCl and NaCl). Thus, high stringency conditions may vary according to the

circumstances of the hybridisation (ie for probe hybridisation, PCR amplification, etc). For the purposes of the present invention, the term "high stringency conditions" is to be understood as referring to such conditions applicable to probe hybridisation (eg conditions which: (1) employ low ionic strength and high temperature for washing, for example, 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 50°C; (2) employ, during hybridisation, a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5X SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS and 10% dextran sulfate at 42°C in 0.2X SSC (30 mM NaCl, 3 mM sodium citrate) and 0.1% SDS). An oligonucleotide molecule useful in the detection of a mutant sequence according to the present invention may be suitable for use as, for example, a probe or primer sequence. Typically, the oligonucleotide molecule will consist of 10 to 50 nucleotides and, more preferably, about 15 to 30 nucleotides. Preferably, the oligonucleotide molecule is derived from the nucleotide sequence shown as SEQ ID NO: 1 or a complementary sequence thereto, or the nucleotide sequence as shown as SEQ ID NO: 1 but incorporating one or more of the nucleotide changes mentioned above (ie 1322 T>A, 253 C>T, 2012 C>G, 2030_2031insT, 1671 C>G, 357delC and 1091_1092insC) or a complementary sequence thereto.

For the step of detecting a loss of PCDH19 protein function or altered PCDH19 protein function, a suitable biological sample taken from the subject may be selected from, for example, tissue biopsies and fixed sections (eg formalin fixed or paraffin embedded) or fixed cell samples prepared therefrom, including epithelial samples, smear samples, blood samples, faecal samples, urine samples or buccal samples. The sample may be pre-treated by, for example, filtration, separation or extraction methods to partly or completely purify or isolate cells, proteins, polypeptides, polynucleotide molecules, oligonucleotide molecules or fragments thereof or fractions containing these components.

In a second aspect, the invention provides a kit for diagnosing an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or assessing a predisposition to an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or screening to identify carriers of illnesses related to functional PCDH19 protein deficiency or altered PCDH19 protein function, wherein said kit comprises one or more of the following: an antibody or fragment thereof which specifically binds to PCDH19 protein or polypeptide, or a fragment or variant thereof; and an oligonucleotide probe/primer molecule which specifically hybridises to a polynucleotide molecule encoding PCDH19 protein or polypeptide, or a fragment or variant thereof under high stringency conditions.

Such kits may comprise, for example, instructions for the operation of the method and, optionally, for thereafter diagnosing an illness related to functional PCDH19 protein deficiency or altered PCDH19

protein function, or assessing a predisposition to an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or identifying carriers of illnesses related to functional PCDH19 protein deficiency or altered PCDH19 protein function, together with one or more containers or vessels containing said antibody or fragment thereof and/or said oligonucleotide probe/primer molecule.

Preferably, said antibody or fragment thereof will bind to a protein or polypeptide comprising an amino acid sequence showing at least 70% sequence identity to the PCDH19 amino acid sequence according to SEQ ID NO: 2. Further, preferably, said oligonucleotide probe/primer molecule will hybridise to a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1.

In a third aspect, the present invention provides for the use of: a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1, wherein said nucleotide sequence encodes a functional PCDH19 protein or polypeptide, or a functional fragment or functional variant thereof; or a functional PCDH19 protein or polypeptide, or functional fragment or functional variant thereof encoded by a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1; in the treatment of PCDH19 protein deficiency or altered PCDH19 protein function in a subject.

Preferably, the said nucleotide sequence shows at least 85% sequence identity, and, more preferably, at least 95% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1.

Most preferably, the said functional PCDH19 protein or polypeptide comprises an amino acid sequence according to SEQ ID NO: 2.

For the sake of clarity, percentage levels of nucleotide sequence identity and amino acid sequence identity referred to herein are to be understood as meaning the "match percentage" calculated by the EMBL-EBI Multiple Alignment Using Fast Fourier Transform (MAFFT) tool using the Blosum 62 matrix (http://www.ebi.ac.uk/mafft/) and standard default settings.

The term "functional fragment" as used herein is to be understood as referring to a fragment which exhibits biological activity that is substantially equivalent to a protein or polypeptide comprising the complete PCDH19 amino acid sequence shown as SEQ ID NO: 2.

The term "variant" as used herein in relation to an amino acid sequence, is to be understood as referring to a protein or polypeptide, or fragment thereof, comprising an amino acid sequence showing a high level of

sequence identity to the corresponding complete (or part thereof as the case may be) of the amino acid sequence shown as SEQ ID NO: 2, but which includes one or more variations in the sequence which do not result in any significant alteration of the biological activity of its derivative protein or polypeptide (ie a protein or polypeptide comprising the complete PCDH19 amino acid sequence shown as SEQ ID NO: 2) or which otherwise results in enhanced or reduced biological activity (eg variants may include one or more amino acid substitutions, additions or deletions, or may include the addition or deletion of a sequence of amino acids, which enhances or reduces biological activity). A variant with enhanced or reduced biological activity can therefore be regarded as a "functional variant", whereas a variant which has no or minimal biological activity can be regarded as a "non-functional variant". Variations that do not result in any significant alteration of the biological activity may include conservative amino acid substitutions. Exemplary conservative amino acid substitutions are provided in Table 1 below. Particular conservative amino acids envisaged are: G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkylamino acids.

Table 1 Exemplary conservative amino acid substitutions

	Conservative Substitutions
Ala	Val*, Leu, Ile
Arg	Lys*, Gln, Asn
Asn	Gln*, His, Lys, Arg, Asp
Asp	Glu*, Asn
Cys	Ser
Gln	Asn*, His, Lys,
Glu	Asp*, γ-carboxyglutamic acid (Gla)
Gly	Pro
His	Asn, Gln, Lys, Arg*
Ile	Leu*, Val, Met, Ala, Phe, norleucine (Nle)
Leu	Nle, Ile*, Val, Met, Ala, Phe
Lys	Arg*, Gln, Asn, ornithine (Orn)
Met	Leu*, Ile, Phe, Nle
Phe	Leu*, Val, Ile, Ala
Pro	Gly*, hydroxyproline (Hyp), Ser, Thr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe*, Thr, Ser
Val	Ile, Leu*, Met, Phe, Ala, Nle

*indicates preferred conservative substitutions

In a fourth aspect, the present invention provides a method for the therapeutic or prophylactic treatment of protocadherin 19 (PCDH19) protein deficiency or altered PCDH19 protein function in a subject, wherein said method comprises the step of:

(i) administering to said subject: a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1, wherein said nucleotide sequence encodes a functional PCDH19 protein or polypeptide, or a functional fragment or functional variant thereof; a functional PCDH19 protein or polypeptide, or functional fragment or functional variant thereof encoded by a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1; and/or an agent that compensates for the loss of PCDH19 protein function; optionally in combination with a pharmaceutically-acceptable carrier.

Preferably, the method comprises administering a functional PCDH19 protein or polypeptide comprising an amino acid sequence according to SEQ ID NO: 2, or a functional fragment or functional variant thereof.

Alternatively or additionally, the method comprises administering an agent that compensates for the loss of PCDH19 function in the subject. Preferably, such an agent is a protein or polypeptide that compensates for PCDH19 function, such as another protocadherin/cadherin protein, or a functional fragment or functional variant thereof. An example of a preferred compensatory agent is PCDH11Y.

It is envisaged that the method of the fourth aspect may include the administration of whole cells or recombinant delivery vehicles (eg viral vectors). Suitable polynucleotide molecule-delivery vectors include those suitable for the chromosomal integration of the polynucleotide molecule of the present invention (eg retroviral vectors), or simply for the non-integrated expression of the polynucleotide molecule (eg plasmid vectors). Alternatively, the administration of a polynucleotide molecule encoding a PCDH19 protein or polypeptide, or functional fragment or functional variant thereof, may involve any of the methods and/or agents for the delivery of "naked DNA" to cells well known to persons skilled in the art (eg liposomes, lipoplexes, polyplexes, gold microparticles, and conjugation to mannose and the like).

In a fifth aspect, the present invention provides an agent capable of treating a deficiency in functional protocadherin (PCDH19) protein or altered PCDH19 protein function in a subject.

Preferred agents according to the fifth aspect include: a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity, preferably at least 85% sequence identity, and, more preferably, at least 95% sequence identity to the complete *PCDH19* ORF nucleotide sequence according

to SEQ ID NO: 1; or a functional PCDH19 protein or polypeptide, or functional fragment or functional variant thereof encoded by a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity, preferably at least 85% sequence identity, and, more preferably, at least 95% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1. Most preferably, the agent is a polynucleotide molecule comprising a nucleotide sequence encoding a PCDH19 protein or polypeptide comprising the amino acid sequence shown as SEQ ID NO: 2; or a functional PCDH19 protein or polypeptide comprising the amino acid sequence shown as SEQ ID NO: 2.

Further preferred agents include an isolated or recombinantly expressed PCDH11Y protein or polypeptide, or a functional fragment or functional variant thereof. Homologues, analogues, orthologues or mimetics of PCDH19 or PCDH11Y may also be suitable and, indeed, these may possess further desirable characteristics for use as therapeutic agents, for example *in vivo* stability, safety and toxicity, pharmaceutical acceptability and the like. The selection of preferred homologues, analogues, orthologues or mimetics of PCDH19 or PCDH11Y according to desirable characteristics may be readily determined by methods well known to persons skilled in the art.

Particularly preferred agents according to the fifth aspect are agents that are capable of providing treatment to EFMR or prophylactic treatment to subjects predisposed to EFMR.

Agents of the fifth aspect may be administered with a pharmaceutically acceptable carrier, and/or formulated into any suitable pharmaceutical/veterinary composition or dosage form (eg compositions for oral, buccal, nasal, intramuscular and intravenous administration). Typically, such a composition or dosage form will be administered to the subject in an amount which is effective to treat EFMR or provide prophylaxis to a subject predisposed to EFMR, and may therefore be provided at between about 0.01 and about 100 μg/kg body weight per day of the agent, and more preferably, at between 0.05 and 25 μg/kg body weight per day of the agent. A suitable composition may be intended for single daily administration, multiple daily administration, or controlled or sustained release, as needed to achieve the most effective result.

In a sixth aspect, the present invention provides a method for identifying an agent capable of treating a deficiency in functional protocadherin 19 (PCDH19) protein or altered PCDH19 protein function, wherein said method comprises the steps of;

(i) providing a cell or animal comprising a polynucleotide molecule comprising a mutant sequence of the *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1;

(ii) contacting a test agent with said cell or administering a test agent to said animal; and

(iii) comparing a response in said cell or animal with a control response.

The method of the sixth aspect may identify agents capable of providing a treatment of illness caused by a deficiency in functional *PCDH19* protein or altered PCDH19 protein function, or which may be capable of providing a prophylactic treatment of functional *PCDH19* protein deficiency or altered PCDH19 protein function.

The control response referred to in step (iii) of the method may include a baseline response detected in said cell or animal without exposure to the test agent or, alternatively, the control response may be a response following exposure to the test agent in cells or animals comprising a normal or wild-type complete *PCDH19* ORF nucleotide sequence.

The test agent may be selected from known and novel compounds, complexes and other substances which may, for example, be sourced from private or publicly accessible agent libraries (eg the Queensland Compound Library (Griffith University, Nathan, QLD, Australia) and the Molecular Libraries Small Molecule Repository (NIH Molecular Libraries, Bethesda, MD, United States of America). The test agent may therefore comprise a protein, polypeptide or peptide (eg a recombinantly expressed PCDH19 or PCDH11Y protein or polypeptide, or a functional fragment or functional variant thereof), or a mimetic thereof (including so-called peptoids and retro-inverso peptides), but more preferably comprises a small organic molecule and especially one which complies or substantially complies with Lipinski's Rule of Five for "druglikeness" (Lipinski, CA *et al.*, 2001). The test agent may also be selected on the basis of structural analysis of known or novel compounds or may otherwise be designed following the further structural analysis of PCDH19 or PCDH11Y binding sites, particularly calcium ion binding sites.

The method of the sixth aspect may be adapted for high-throughput screening of large numbers of test agents.

The step of comparing a response in said cell or animal with a control response may be conducted using one or more standard binding assay formats (eg ELISA-based or competition-based assays). Preferably, the test agent will be labelled with a readily detectable label (eg a fluorochrome or radioisotope) to allow detection of binding to, for example, a calcium channel receptor. A change in activity may be observed in such assays by using standard methods including spectrophotometric, fluorimetric, calorimetric or chemiluminescent means preferably providing for the automation or partial automation of the detecting step (eg by a microplate reader or use of a flow cytometer).

Preferred steps for comparing a response in an animal with a control animal (ie comprising a normal or wild-type complete *PCDH19* ORF nucleotide sequence) involve the identification of a disease state in said animal, in particular, the analysis of neurological indicators of illness.

In a seventh aspect, the present invention provides a kit for use in the method of the sixth aspect, wherein said kit comprises instructions for the operation of the method together with one or more containers and/or vessels containing one or more cell(s) or animal(s) comprising a polynucleotide molecule comprising a mutant sequence of the protocadherin 19 (*PCDH19*) ORF nucleotide sequence shown as SEQ ID NO: 1.

In an eighth aspect, the present invention provides a kit for identifying an agent capable of treating a deficiency in functional protocadherin 19 (PCDH19) protein or altered PCDH19 protein function, wherein said kit comprises;

(i) a cell or animal comprising a polynucleotide molecule comprising a mutant sequence of the *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1; and optionally,

(ii) a control cell or animal comprising a polynucleotide molecule comprising a wild-type form of the complete *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1, said wild-type form encoding a functional PCDH19 protein or polypeptide, or a functional fragment or functional variant thereof.

The kit of the seventh or eighth aspect may further comprise means for comparing a response in said mutant cell or animal with a control response (eg as caused by a test agent), means for detecting a response (eg adhesiveness of PCDH19 or impaired calcium ion binding in the mutant cell or animal) and, for example, a test agent, and other components as are well known to persons skilled in the art including, for example, wash buffers, stabilisation buffers or other reagents.

In a ninth aspect, the present invention provides an isolated protein or polypeptide comprising an amino acid sequence encoded by a nucleotide sequence showing at least 70% sequence identity to a complete protocadherin 19 (*PCDH19*) ORF nucleotide sequence according to SEQ ID NO: 1, or a functional fragment or variant thereof.

As used herein, the term "isolated", when used in relation to a protein or polypeptide, or a functional fragment or variant thereof, or when used in relation to a polynucleotide molecule, is to be understood as referring to the protein, polypeptide, functional fragment, variant or polynucleotide molecule in a form that is essentially free of whole cells, components thereof and/or other exogenous cellular or biological materials such as exogenous proteins, polypeptides, peptides and nucleic acid molecules. As such, an isolated protein, polypeptide, functional fragment, variant or polynucleotide molecule, in accordance with the present invention, may be present in a preparation comprising no more than 10% (by weight) of

exogenous cellular or biological materials, and may be prepared by any of the methods well known to persons skilled in the art.

Preferably, the protein or polypeptide comprises an amino acid sequence showing at least 75% sequence identity to the complete PCDH19 amino acid sequence shown as SEQ ID NO: 2 and at least 65% sequence identity to the amino acid sequence corresponding to the extracellular cadherin (EC) domain.

More preferably, the protein or polypeptide, or functional fragment or variant thereof, comprises an amino acid sequence showing at least 85% sequence identity and, still more preferably, at least 95% sequence identity to the complete PCDH19 amino acid sequence shown as SEQ ID NO: 2. Most preferably, the protein or polypeptide comprises an amino acid sequence according to SEQ ID NO: 2.

In a preferred embodiment of the ninth aspect, the present invention provides a variant, preferably a non-functional variant, of the amino acid sequence shown as SEQ ID NO: 2 including one or more amino acid mutations. Particularly preferred mutations included within such a variant include one or more amino acid mutations selected from the following changes to the amino acid sequence shown as SEQ ID NO: 2; V441E, Q85X, S671X, L667fsX717, N557K, I119fsX122 and P364fsX375.

The isolated protein or polypeptide, functional fragment or variant thereof, may be isolated from tissues derived from whole organisms (eg biopsied tissues), from cultured tissues (eg cultured fibroblasts), or from other recombinant expression systems. This may involve any of the methods for isolating proteins or polypeptides well known to persons skilled in the art, including ion exchange, chromatography electrophoresis, isoelectric focusing, adsorption chromatography, paper chromatography, reverse-phase chromatography, hydrophobic interaction chromatography, dialysis, ultrafiltration, gel electrophoresis, gel filtration, and ultracentrifugation.

Suitable techniques for expressing a recombinant protein or polypeptide, functional fragment or variant thereof according to the present invention are well known to persons skilled in the art and include, for example, techniques for expressing recombinant His-tagged PCDH19 from a suitable expression vector or cassette using a suitable host cell (eg CHO cells and BL21 cells). Thereafter, the His-tagged expression products can be readily isolated using affinity chromatography (eg using a Ni-NTA column (Qiagen Inc, Valencia, CA, United States of America)). Where a functional fragment is to be provided, isolated recombinant protein or polypeptide may be cleaved using a proteolytic enzyme (eg trypsin).

Proteins, polypeptides, variants or, in particular, functional fragments according to the present invention may optionally be incorporated into synthetic proteins or polypeptides such as fusion proteins. Fusion proteins may include components that assist in the production or downstream processing (eg a protein,

polypeptide, functional fragment or variant thereof, may be linked to a secretory signal peptide, affinity purification tag or the like).

In a tenth aspect, the present invention provides an isolated polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete protocadherin 19 (*PCDH19*) ORF nucleotide sequence according to SEQ ID NO: 1 or a complementary sequence thereto.

Preferably, the polynucleotide molecule comprises a nucleotide sequence showing at least 85% sequence identity and, more preferably, at least 95% sequence identity to the complete *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1. Most preferably, the polynucleotide molecule comprises a nucleotide sequence as shown as SEQ ID NO: 1.

A polynucleotide molecule of the present invention may encode a protein or polypeptide comprising the amino acid sequence according to SEQ ID NO: 2, or a functional fragment or variant thereof. Alternatively, a polynucleotide molecule of the present invention may otherwise encode a non-functional *PCDH19* mRNA (eg an mRNA including a premature termination codon which is degraded by NMD processes) or altered *PCDH19* mRNA. Other examples of polynucleotide molecules according to the present invention are oligonucleotide probe/primer molecules which consist of 10 to 50 contiguous nucleotides and, more preferably, about 15 to 30 contiguous nucleotides of the complete *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1.

Where the polynucleotide molecule comprises a nucleotide sequence showing at least 70% sequence identity, preferably at least 85% sequence identity and, more preferably, at least 95% sequence identity to the nucleotide sequence shown as SEQ ID NO: 1, it will be appreciated that such a polynucleotide molecule may vary from that nucleotide sequence only in minor changes which, for example, do not result in a significant alteration in an encoded protein or polypeptide due to degeneracy in the DNA code or which may be required in order to enhance expression in a particular system (ie to comply with preferred codon usage). Further, it will be appreciated that such a polynucleotide molecule may otherwise encode a variant of a protein or polypeptide comprising the amino acid sequence shown as SEQ ID NO: 2 which shows enhanced or reduced biological activity (eg a variant including one or more amino acid mutations in the extracellular cadherin (EC) domain and showing reduced adhesiveness through impaired calcium ion binding). Indeed, in an embodiment of the tenth aspect, the present invention provides a polynucleotide molecule encoding a variant, preferably a non-functional variant, including one or more amino acid substitutions, additions or deletions in the extracellular cadherin (EC) domain and showing reduced adhesiveness through impaired calcium binding. Particularly preferred mutations encoded by such polynucleotide molecules include one or more of the following changes to the amino acid sequence shown as SEQ ID NO: 2; V441E, Q85X, S671X, L667fsX717, N557K, I119fsX122 and P364fsX375. With reference to the complete *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1, the

mutations responsible for such amino acid sequence changes may be, respectively; 1322 T>A, 253 C>T, 2012 C>G, 2030_2031insT, 1671 C>G, 357delC and 1091_1092insC.

The polynucleotide molecule of the present invention may be used to express an encoded protein or polypeptide, or functional fragment or variant thereof, by recombinant methodologies involving cloning of the polynucleotide molecule into a suitable expression cassette or vector and thereafter introducing the expression cassette or vector into a suitable host cell. Suitable expression vectors may include functional sequences such as a multiple cloning site, a detection tag (eg glutathione-S-transferase (GST) or green fluorescent protein (GFP)), a tag for downstream purification (eg a histidine tag (His)), linker and fusion sequences.

In an eleventh aspect, the present invention provides a cell transformed with the polynucleotide molecule of the tenth aspect.

The polynucleotide molecule may comprise a mutant sequence of the *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1, and thereby encode non-functional or altered *PCDH19* mRNA, non-functional PCDH19 protein or a PCDH19 protein with altered function, or which otherwise causes reduced expression of PCDH19 protein, or a complementary sequence thereto.

The polynucleotide molecule may also consist or encode an antisense RNA, ribozyme, DNAzyme or interfering RNA molecule (eg siRNA) targeted to *PCDH19*.

The transformed cell may be selected from bacterial cells, insect cells and mammalian cells. The cell may be transformed using any of the methods well known to persons skilled in the art including direct uptake, transduction, f-mating or electroporation. The transformed polynucleotide molecule may be maintained in a non-integrated form (eg in a non-integrated plasmid expression vector), or alternatively, may be integrated into the genome of the transformed cell.

The transformed cell can be employed in a variety of applications that will be readily apparent to persons skilled in the art, in particular, for the generation of an isolated recombinant protein or polypeptide, or functional fragment or variant thereof according to the present invention.

Where the transformed cell is intended for the production and harvest of a PCDH19 protein or polypeptide, functional fragment or variant thereof, the expression of the recombinant product can be determined by, for example, Western immunoblot assays for the direct detection of the protein or polypeptide, functional fragment or variant thereof, or for detection of an expression tag (eg a His tag).

In a twelfth aspect, the present invention provides a non-human animal comprising the polynucleotide molecule of the tenth aspect.

The polynucleotide molecule may comprise a mutant sequence of the *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1, and thereby encode non-functional or altered *PCDH19* mRNA, non-functional PCDH19 protein or a PCDH19 protein with altered function, or which otherwise causes reduced expression of PCDH19 protein, or a complementary sequence thereto.

The polynucleotide molecule may also consist or encode an antisense RNA, ribozyme, DNAzyme or interfering RNA molecule (eg siRNA) targeted to *PCDH19*.

The polynucleotide molecule is preferably uniformly integrated throughout the animal's tissues. Where a chimeric animal is provided, the polynucleotide molecule is preferably present in cells of the animal's nervous tissues.

The polynucleotide molecule may be introduced into the animal by any of the methods of transformation or transgenesis well known to persons skilled in the art. Such transformation methods include DNA transfection (via electroporation, liposome or protoplast fusion, or microinjection), infection with viral delivery vectors (ie vectors that facilitate genomic integration such as adenoviral and retroviral vectors), or via random mutagenesis followed by the selection of desired mutations by screening. However, the animal of the present invention will generally be preferably generated by micro-injection methodologies. To ensure the genetic uniformity of resulting transgenic animals, micro-injection is preferably performed at the one-cell embryo stage by any of the methods well known to persons skilled in the art. Preferred transgenic animals include rodents, in particular mice and rats.

The animals of the eleventh aspect can be employed in a variety of applications that will be readily apparent to persons skilled in the art, in particular, for use as *in vitro* or *in vivo* disease models for use in methods or kits for screening potential agents to compensate for PCDH19 protein deficiency or altered PCDH19 protein function.

In a further aspect, the present invention provides an antibody or fragment thereof which specifically binds to the protein or polypeptide, functional fragment or variant thereof, of the ninth aspect.

Suitable antibodies include monoclonal and polyclonal antibodies. Suitable antibody fragments include fragments produced by enzymatic cleavage of antibodies such as Fab and $F(ab')_2$ fragments, and recombinant antibody fragments such as single chain Fv (scFv) fragments.

The antibody or fragment thereof may be capable of distinguishing between, for example, a wild-type PCDH19 protein (ie comprising the complete PCDH19 amino acid sequence shown as SEQ ID NO: 2) and a variant thereof, particularly, a non-functional variant thereof. Thus, the present invention extends to an antibody or fragment thereof that specifically binds to a variant of the complete PCDH19 amino acid sequence shown as SEQ ID NO: 2.

The present invention is hereinafter further described by way of the following, non-limiting example and accompanying figures.

EXAMPLE

Materials and Methods

Patient and family details

Individuals from seven families with family members suffering from epilepsy and mental retardation limited to females (EFMR) were admitted. The clinical details of Families 1-4 are described in Scheffer IE *et al.* (2007). Family 5 was screened on the basis of one sister having infantile seizures and Asperger syndrome and her sister having epilepsy and mild intellectual disability. The clinical details of members of Family 6 and Family 7 are described elsewhere (Juberg RC and Hellman CD, 1971; Fabisiak K and Erickson RP, 1990; and Ryan SG *et al.*, 1997).

Northern Blotting

Human brain (MTN) blot II and V (acquired from Clontech Laboratories) were hybridised according to the manufacturer's instructions. Detection assays utilised a probe containing nucleotides 2884-3257 of human *PCDH19* ORF (NCBI accession number 921478). The primers used to generate the probe were:

forward primer - 5'CCGGATTCTTGGCCACTCTGAC3' (SEQ ID NO: 3); and
reverse primer - 5'CAATGGTGTAAGACACGGAAG3' (SEQ ID NO: 4).

The 374 bp probe was labelled with radioactive α32P-dCTP (Perkin Elmer, Waltham, MA, United States of America) using the Mega prime DNA labelling system (GE Healthcare, Piscataway, NJ, United States of America).

RT-PCR Analyses

Total RNA was extracted from fibroblast cells using the RNeasy mini kit (Qiagen, Doncaster, VIC, Australia), and treated with DNase I (Qiagen). 2 μg of RNA was primed with 1μg of random hexanucleotides and subjected to reverse-transcription for 90 minutes at 42°C using Superscript II according to the maufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, United States of America). The efficiency of the reaction was tested by PCR using primers specific to the ubiquitously

expressed *ESD* gene (Saviozzi *et. al.*, 2006). cDNAs were amplified with Taq DNA polymerase (Roche, Basel, Switzerland) and specific single-stranded DNA primers for 35 cycles (denaturation, 94°C for 30 seconds; annealing, at specific Tm for each pair of primers for 30 seconds; extension, 72°C for 30 seconds). PCR products were separated by agarose gel electrophoresis and stained with 1% ethidium bromide for visualisation under UV.

Tissue culture – primary skin fibroblast lines

A 3 mm skin biopsy excised from the upper arm of each subject was cut finely and transferred to a tissue culture flask. The biopsy was cultured in RPMI medium with 20% foetal calf serum (FCS) (further supplemented with 4 mM L-Glutamine, 0.017 mg/ml benzylpenicillin) and grown at 37 ^{0}C with 5% CO_2. Once established, fibroblasts where cultured in RPMI with 10% FCS (also including the supplements described above).

Cycloheximide treatment of skin fibroblast cell lines

Primary fibroblast cells were seeded 1x 10 ^{4}/cm^2 in RPMI with 10% FCS and incubated with 50 µg/ml cycloheximide (Sigma-Aldrich Co, St Louis, MO, United States of America), or media alone, for 6 hrs. Fibroblasts were harvested using a sterile cell scraper (Techno Plastic Products AG, Trasadingen, Switzerland), then washed once in phosphate buffered saline (PBS) prior to total RNA extraction and reverse transcription to generate cDNA as described above.

Mouse *in situ* hybridisation analysis

15.5 days postcoital (dpc) embryonic heads and dissected postnatal day 2 (P2) brains from c57xCBAF1 mice were fixed in 4% paraformaldehyde at 4°C, cryoprotected in 30% sucrose and frozen in optimal cutting temperature (OCT). *In situ* hybridisation of 16µM sections was performed as described previously (Wilson LD *et al.*, 2005) using a digoxygenin-labelled *PCDH19* antisense RNA probe, prepared as previously described (Gaitan Y and Bouchard M, 2006). A total of three neonates and two embryos were analysed and representative sections were documented. No signal was detected in negative controls, which utilised a sense control probe. Images were taken on a Zeiss Axiophot microscope, compiled and minimally processed (adjusted for colour and light/dark) using Adobe Photoshop CS®.

Semi-Quantitative RT-PCR

Gene expression profiles were generated using Rapid-Scan Gene Expression Human Brain cDNA panels (Origene Technologies, Inc, Rockville, MD, United States of America) containing first strand cDNA prepared from polyA+ RNA. The cDNA panels allow semi-quantitative analysis due to the cDNAs being serially diluted over a 4-log range. The profiles were obtained from panels containing approximately 1 ng of first strand cDNA. The PCR primer pair;

X-RT4F2 - 5' GTA ACA AGT GTA CCT GGT ATG GAC T 3' (SEQ ID NO: 5) and

X-RT5R2 - 5' TCA ACC TTT ACT TTC ATC ACG 3' (SEQ ID NO: 6), were used to amplify the *PCDH11X* sequence to yield a 683 bp product, and the primer pair;
Y-RT4F - 5' TAC AAC AAA CTG TCA CAA GTG TTT 3' (SEQ ID NO: 7), and
Y-RT5R2 - 5' TCA ACC TTT ACT TTC ATC ACA 3' (SEQ ID NO: 8), were used to amplify *PCDH11Y* to yield a 681 bp product. The primers;
WLF - 5' AAC CAG AAT ACC CGC AAC AC 3' (SEQ ID NO: 9) and
WLR - 5' CTG CAG ATG GTC ACA TCG AC 3' (SEQ ID NO: 10), were used to amplify *PCDH19* to yield a 626 bp product.

The PCR conditions used to amplify the *PCDH19* product comprised an initial step at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 60° for 30 sec and 72° C for 2 mins. The *PCDH11X* and *PCDH11Y* products were amplified using Hotstar Taq (Qiagen) according to the Hotstar recommended cycling conditions (94°C for 15 minutes followed by 10 cycles at [94° for 30 sec, 60° for 30 sec, 72° for 1 min] and then 30 cycles at [94° for 30 sec, 55° for 30 sec, 72° for 1 min] followed by 72°C for 10 min).

GenBank accession numbers

Where available, partial nucleotide and amino acid sequences were accessed from the GenBank library. The accession numbers corresponding to these sequences are:

incomplete human *PCDH19* mRNA accession number NM_020766.1;
incomplete human PCDH19 protein accession number NP_065817.1; and
complete human *PCDH19* mRNA and protein accession number GenBank EF676096.

The GenBank library can be accessed at the following URLs;
NCBI: http://www.ncbi.nlm.nih.gov/, or Ensembl: http://www.ensembl.org/.

Nucleotide and amino acid sequences were determined by standard di-deoxy chain termination sequencing methods as described in Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY, Vol 1, 2, 3 (1989).

Results and Discussion

Genetic linkage analysis and identification of EFMR gene

As a follow up to previous studies in which a single large American family with EFMR was described (Juberg RC and Hellman CD, 1971; Fabisiak K and Erickson RP, 1990; and Ryan SG *et al.*, 1997), four new EFMR families were identified based on the inheritance pattern of EFMR, the electroclinical features of family members and the localisation of the gene responsible for EFMR (Scheffer IE *et al.*, 2004; Scheffer IE *et al.*, 2007).

Pedigrees of the seven EFMR families in total were generated, which showed the characteristic inheritance pattern of affected females and transmitting males (Figure 1). Further linkage analysis within families showed that the disease condition in each family consistently localised to Xq22.

The EFMR gene was identified from re-sequenced 737 VEGA annotated X-chromosome genes in probands from three families. In all three families, each of the X chromosomes encoded protocadherin 19 (PCDH19) mutations (X^m) which were found to co-segregate with the EFMR clinical phenotype. The *PCDH19* gene was known to be located at Xq22 (Ensembl) within the original linkage region (Ryan SG *et al.*, 1997). An example of a sequence chromatogram of a *PCDH19* mutation as detected in an affected female from each family is shown alongside the pedigrees in Figure 1.

Sequence analysis of family members showed a single nonsense nucleotide change 2012C>G (residue S671X) in the *PCDH19* gene in Family 3, while Families 1 and 2 initially did not show any changes (Figure 1). There were no other deleterious nucleotide changes identified in the three families for which the other 736 genes were screened. Subsequent comparative sequence analysis of the annotated *PCDH19* open reading frame (ORF) (accession number NM_020766.1) revealed that it was incomplete. This prompted the sequencing of the additional N-terminal 1.5 kb of *PCDH19* ORF.

N-terminal sequencing of family members identified a missense change, 1322T>A (residue V441E) in Family 1, a nonsense change, 253C>T (residue Q85X) in Family 2 and a putative frameshift change, 2030_2031 insT (residues L667fsX717) in Family 4. The N-terminal *PCDH19* region in affected females from an unreported Irish EFMR family (Family 5) was also sequenced resulting in the identification of a frameshift change, 375delC (residues I119fsX122). The *PCDH19* N-terminal region from the original, large American EFMR family (Family 6) was re-sequenced resulting in the identification of a further frameshift mutation 1091_1092insC (residue P364fsX375) (Juberg RC and Hellman CD, 1971; Fabisiak K and Erickson RP, 1990; Ryan SG *et al.*, 1997) (Figure 1). Finally, a nucleotide change 1671 C>G was identified in Family 7, coding for an amino acid substitution of N557K.

Once aligned, silent nucleotide changes were further identified between family members and localised to positions 6 (G>A), 348 (G>A), 402 (C>A), 1137 (C>T), 1627 (C>T) and 1683 (G>A) (frequencies shown in Table 2).

Table 2 **Summary of the nucleotide changes found in *PCDH19* (GenBank accession number EF676096) with allele frequencies are indicated in parentheses.**

exon	base change	amino acid position
1	c.6G(99.8%)>A(0.2%)	E2E
1	c.348G(99.8%)>A(0.2%)	K116K
1	c.402C(97.1%)>A(2.9%)	I134I
1	c.1137C(99.0%)>T(1.0%)	G379G
1	c.1627C(68%)>T(32%)	L543L
1	c.1683G(99.8%)>A(0.2%)	P561P

Partial alignment of the human PCDH19 amino acid sequence with other human PCDH sequences and orthologues of PCDH19 from other species demonstrates high conservation of residues affected by the two missense mutations, V441E and N557K, across other species and across other functionally similar proteins (Figure 3). Mutation V441E was observed in Family 1 and N577K was observed in Family 7.

Accordingly, *PCDH19* ORF nucleotide changes were identified in all seven of the assessed EFMR families. All seven nucleotide changes segregated with the clinical phenotype in each EFMR family and were not identified in 250 male probands from families with putative X-linked mental retardation (XLMR) or in 750 control X chromosomes. The positions of the *PCDH19* mutations in conjunction with alignments showing the location and conservation of the two missense mutations further demonstrate that *PCDH19* mutation is causative of EFMR.

Relationship between PCDH19 mutation and Rett Syndrome or Autism

To determine whether *PCDH19* mutations also contribute to the presentation of disease conditions with similar phenotypes to EFMR, subjects presenting with Rett syndrome or autism, where no known cause had been determined, were tested for mutations at *PCDH19*.

Rett syndrome is a female specific disease known to present with a similar phenotype to EFMR. 46 females with apparent Rett syndrome, who were negative for mutations in the two Rett associated genes, *MECP2* and *CDKL5*, were investigated. No nucleotide changes were found in the Rett syndrome cohort, suggesting that *PCDH19* mutations are unlikely to commonly contribute to Rett syndrome.

Since autistic features were commonly seen in affected EFMR females, a cohort of 55 females with autism and seizures were screened for changes in *PCDH19*. The absence of mutations in this cohort suggests that *PCDH19* mutations also do not commonly contribute to autism.

Characterisation of the *PCDH19* gene

The complete 3.447 kb ORF of the *PCDH19* gene which consists of 6 exons was annotated (GenBank accession number EF676096). The full-length processed *PCDH19* mRNA is 9.765 kb long, this was confirmed by Northern blot analysis which showed a transcript size of approximately 9.8 kb using a *PCDH19* specific probe on combined male and female mRNA from various areas of the adult human brain (Figure 4, *PCDH19* mRNA is indicated by an asterisk). *PCDH19* exon 2 was found to be alternatively spliced (results not shown).

PCDH19 encodes an 1148 amino acid protein belonging to the protocadherin (PCDH) δ2 subclass within the cadherin superfamily. The PCDH19 protein contains a signal peptide, six extracellular cadherin (EC) repeats, a transmembrane (TM) domain and a cytoplasmic region with conserved CM1 and CM2 domains. At Figure 2, a schematic diagram is shown, illustrating the locations of the PCDH19 amino acid sequence changes of each family with respect to the signal peptide, the extracellular cadherin domain (comprising EC1, EC2, EC3, EC4, EC5 and EC6), the transmembrane domain (TM) and the cytoplasmic (CM1 and CM2) domains of the *PCDH19* protein. All seven EFMR mutations identified are located in the large extracellular domain.

The biological role of PCDH19 is not known; however, members of the PCDH family are predominantly expressed in the nervous system and are postulated to be involved in the establishment of neuronal connections and in signal transduction at the synaptic membrane (Wu Q and Maniatis T, 1999; Yagi T and Takeichi M, 2000).

The partial alignments of human and orthologues of PCDH19 from other species, shown at Figure 3, shows high conservation of residues affected by the two missense mutations, V441E (top panel) and N557K (bottom panel). The N557K mutation affects an invariant asparagine (N) residue within the EC5 domain (Figure 2). The equivalent asparagine residue of EC1 of classical cadherins (eg N100 of N-cadherin; Patel SD *et al.*, 2006) and protocadherins (eg N101 of CNR/Pcdhα; Morishita H *et al.*, 2006) is essential for calcium ion binding and for adhesive function of the EC1 domain, thus it is expected that tissue mosaicism of PCDH19 negative and PCDH19 wild-type cells scrambles cell-cell communication manifesting clinically as EFMR. The valine residue at position 441 (EC4 in Figure 2, or the equivalent of V96 of EC1 of N-cadherin; Patel SD *et al.*, 2006; or V97 of EC1 of CNR/Pcdhα; Morishita H *et al.*, 2006) is also highly conserved (Figure 3) and in close proximity to the calcium binding site (indicated by a bracket against both alignments). Thus, the two missense mutations, V441E and N557K are predicted to lead to loss of *PCDH19* function.

Thus, it is predicted that both *PCDH19* missense variants adversely affect PCDH19 adhesive function through impaired calcium binding. Given the similarity of the clinical phenotype associated with all seven *PCDH19* mutations, it is reasonable to suggest that they all represent loss of function mutations.

Stability of mutant *PCDH19* mRNA transcripts

The *PCDH19* mutations 253C>T, Q85X (Family 2) and 2012C>G, S671X (Family 3) introduce a premature termination codon (PTC) into the *PCDH19* mRNA. Such PTC-containing mRNAs are usually recognised by the NMD surveillance complexes and efficiently degraded (Maquat LE, 2004). The consequences of the *PCDH19* mutations 253C>T, Q85X (Family 2) and 2012C>G, S671X (Family 3) were examined on the stability of their respective mRNAs by detecting *PCDH19* mRNA in primary skin fibroblasts collected from biopsied patients by RT-PCR.

Figure 5 shows a sequence chromatogram from an EFMR affected female from Family 2 showing the detection of the mutation 253C>T, Q85X, in the genomic DNA (gDNA) (top panel), the absence of the mutant sequence in fibroblast cDNA (middle panel) and the presence of both mutant and wild-type cDNA after the treatment of fibroblasts with cyclohexamide (bottom panel), which inhibits the pioneer round of translation and leading to NMD. Similar results were found in tissues collected from EFMR affected members of Family 3 (2012C>G mutation, S671X) (data not shown). The inhibition of NMD by cycloheximide treatment of skin fibroblast cells was found to preserve PTC mutation-containing mRNA.

To confirm that the absence of mutant *PCDH19* mRNAs was not a consequence of skewing of X-inactivation, random X-inactivation in DNA isolated from blood and skin fibroblast cultures of each affected female available were assessed (data not shown). The absence of X inactivation skewing is in agreement with the published data (Ryan SG *et al.*, 1997; Scheffer IE *et al.*, 2007).

The results demonstrate that the PTC mutations in Families 2 and 3 lead to mRNA removal by NMD. It is anticipated that the mutations at 2030_2031 insT (residues L667fsX717) found in Family 4, 375delC (residues I119fsX122) found in Family 5, and 1091_1092insC (residue P364fsX375) found in Family 6 will also lead to a complete loss of functional mRNA as a consequence of NMD degradation of their respective PTC-containing mRNA.

PCDH19 expression in the developing brain

To investigate the expression of *PCDH19* in the developing murine CNS, *in situ* hybridisation analysis *PCDH19* mRNA in embryonic (15.5 days post coitum (dpc)) and postnatal day 2 tissue was undertaken. Figure 6 shows the expression of *PCDH19* in the developing murine CNS at 15.5 dpc (Figures 6a to 6f) and P2 (Figures 6g to 6l). Figures 6a and 6b show adjacent sections stained with Haemotoxylin and Eosin and processed for *PCDH19* mRNA *in situ*, respectively. *PCDH19* mRNA was expressed in a widespread,

symmetrical pattern in the embryonic forebrain and frequently localised to discrete cell clusters within the cortex (CxP, cortical plate), thalamus (Th) and hypothalamus (Hy). The lateral ventricle (lv) and hippocampal neuroepithelium (Hn) are also indicated. Figures 6c, 6d and 6e show higher magnification images of the boxed regions in Figure 6b. The arrowheads in Figure 6c indicate PCDH19-expressing cells within the cortex. In the cortex, expression was restricted to the cortical plate and extended medially into the intercerebral fissure (icf) (Figure 6d). The asterisk in Figure 6e highlights the dorsolateral wall of the lateral ventricle, robust expression was also detected in the ganglionic eminence that abuts the dorsolateral wall of the lateral ventricles.

At this stage, hippocampal expression was not observed on the medial edge of the lateral ventricle in the presumptive hippocampus (Figures 6b and 6e). However, subsequent analysis of anterior forebrain sections revealed PCDH19 expression in the epithelial lining of the nasal cavity (consistent with a previous report (Gaitan Y and Bouchard M, 2006)) and in the olfactory bulbs (see Figure 6f, olfactory bulbs indicated at Ob and nasal epithelium at Ne).

Figures 6g and 6h show adjacent sections stained with Haemotoxylin and Eosin and processed for *PCDH19* mRNA by *in situ* hybridisation, respectively. Figure 6i shows a brain section posterior with respect to the brain section shown at Figure 6h, each highlighting *PCDH19* mRNA expression. Figures 6j, 6k and 6l show higher magnification images of the boxed regions in Figures 6g and 6h, respectively.

At postnatal day 2, PCDH19 expression was maintained in discrete regions of the cortex and the thalamus however, unlike the embryonic brain, expression was also observed in the hippocampus (Figures 6g, 6h, and 6i). In the cortex, expression was restricted to a band of cells that spanned layers II-IV (indicated by arrows in Figures 6j and 6k) whilst the most prominent *PCDH19* signal was observed in the CA1 and CA3 regions of the hippocampus (Figures 6h and 6l).

Consistent with previous Northern blot analyses (Figure 2), *PCDH19* transcripts were not detected in white matter tracts including the corpus callosum (Figure 6h). Together these data indicate that *PCDH19* has widespread expression in both the embryonic and adult brain including the developing cortex and hippocampus and are consistent with the finding that mutation of this gene in humans is associated with cognitive impairment.

Mechanism for the observed disease inheritance patterns

Analysis of the EFMR family pedigrees showed that within the seven EFMR families, there are 2 obligate carrier females (Family 6, individual III.2 and Family 7, individual IV.15) who have not been diagnosed with EFMR, indicating the incomplete penetrance of the disorder. Having identified *PCDH19* mutations, a mechanistic explanation was sought for the remarkable inheritance pattern observed with EFMR.

One of the hypotheses, considered by Ryan SG *et al.*, 1997, suggests that a dominant negative effect of mutant protein on wild-type protein in females may be responsible for expression of the phenotype being limited to females. In this example, it has been demonstrated that mutant *PCDH19* mRNA is removed by NMD in affected females and a carrier male (Figure 2 and data not shown), which is inconsistent with a dominant negative hypothesis. However, in consideration of an alternative hypothesis (Ryan SG *et al.*, 1997) involving a Y chromosome compensatory gene rescuing transmitting males from the EFMR phenotype, it was noted that while there is no *PCDH19* paralogue on the human Y chromosome, there is the related protocadherin gene *PCDH11Y* within a block of X-Y homology at Xq22. The *PCDH11Y* gene is thought to have arisen by transposition from Xq after the divergence of chimpanzees and humans (Lambson B *et al.*, 1992; Page DC *et al.*, 1984) and therefore, the *PCDH11Y* gene is only found in humans and in males.

A Northern blot analysis of *PCDH19* and *PCDH11X/Y* was conducted in human cerebellum, cerebral cortex, medulla, spinal cord, occipital pole, frontal lobe, temporal lobe, putamen, amygdale, caudate nucleus, corpus callosum, hippocampus, thalamus and whole brain tissues. Figure 6c shows the presence of *PCDH19* transcripts (indicated by an asterisk, approximately 9.8kb band), and the presence of *PCDH11X/Y* mRNAs (indicated by an arrowhead, approximately 9.5kb band). Figure 8 also shows expression profiles of *PCDH19, PCDH11X* and *PCDH11Y* in the adult human Frontal lobe, temporal lobe, cerebellum, hippocampus, substantial nigra, caudate nucleus, amygdale, thalamus, hypothalamus, pons, medulla, and spinal cord. Like many other members of the protocadherin family (Kim SY *et al.*, 2007) *PCDH19*, *PCDH11Y* and *PCDH11X* are expressed in human brain. *PCDH11X* and *PCDH11Y* show high sequence similarity, being 98.1% identical at the nucleotide level and 98.3% identical at the amino acid level (Blanco P *et al.*, 2000) and show similar expression profiles in brain regions (Blanco P *et al.*, 2000 and Figure 8). However, *PCDH11X* and *PCDH11Y* have undergone sequence divergence at the 5' and 3' ends of their ORF sequences, are regulated differently and show slight differences in their regions of expression in the brain (Blanco P *et al.*, 2000). It is therefore possible that *PCDH11X* and *PCDH11Y* have evolved different functions.

Sequence comparisons show that the extracellular cadherin (EC) domains of both PCDH11X and PCDH11Y have some similarity to the EC domains of PCDH19; a higher level of similarity than that seen between the EC domains of PCDH19 and fellow PCDH d2 subclass members (PCDH-8, 10, 17 and 18).

The high sequence identity and overlap in expression patterns between PCDH11X and PCDH11Y provides support for the hypothesis that PCDH11X compensates for *PCDH19* loss of function mutations in females and that both PCDH11X and PCDH11Y compensate for *PCDH19* mutations in males.

A uniquely evolved function of PCDH11Y may enable the protein to provide greater rescue of *PCDH19* mutations than PCDH11X, which provides rationale for the greater frequency of spared male carriers than females presenting with EFMR.

A diagrammatic representation of the proposed mechanism underlying the inheritance of EFMR is illustrated in Figure 7. The *PCDH19* gene is located at Xq22.1 and is now known to harbour EFMR mutations. Within a homology region between the X and Y chromosomes there are two very similar PCDH genes, *PCDH11X* on the X chromosome and *PCDH11Y* on the Y chromosome. The results provided herein indicate that PCDH11Y may functionally rescue *PCDH19* mutations in transmitting males, while in females PCDH11X is unable to carry out rescue, explaining the EFMR phenotype being limited to females.

The loss of function of all seven of the *PCDH19* changes characterised herein, their absence from control chromosomes, the absence of evidence for potential disease-causing variants elsewhere on the X chromosome and the mRNA studies conclusively show that the identified *PCDH19* mutations are causative of EFMR. The identification of nucleotide and amino acid sequences corresponding to a complete *PCDH19* ORF provide for the development of diagnostic and therapeutic agents for EFMR and similar disorders associated with deficiencies in functional *PCDH19* protein. Further, the elucidation of the suspected mechanism of *PCDH19* rescue by PCDH11Y provides for the possibility of identifying and developing alternative therapeutic agents for the treatment of illnesses associated with PCDH19 protein-deficiency.

All seven of the characterised EFMR mutations are located in the large extracellular domain and five of these are predicted to be complete loss of function mutations as a consequence of NMD degradation of their respective PTC containing mRNA. The remaining two missense mutations, V441E and N557K are predicted to lead to a loss of PCDH19 function. Loss of function may be the result of impaired calcium ion binding through a lack of PCDH19 adhesiveness. Thus, genetic and functional targets are provided for use in methods for diagnosis of illnesses associated with PCDH19 protein deficiency, methods for the identification of a predisposition to such illnesses and methods of screening to identify carriers of such illnesses, and methods and kits for screening candidate agents for potential therapeutic use in the treatment of illnesses associated with PCDH19 protein deficiency.

Although a preferred embodiment of the method of the present invention has been described in the foregoing detailed description, it will be understood that the invention is not limited to the embodiment disclosed, but is capable of numerous rearrangements, modifications and substitutions without departing from the scope of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

REFERENCES

Blanco P, Sargent CA, Boucher CA, Mitchell M, Affara NA. Conservation of PCDHX in mammals; expression of human X/Y genes predominantly in brain. *Mamm Genome* 11, 906-14 (2000).

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Del Rev* 46, 3-26 (2001).

Fabisiak K, Erickson RP. A familial form of convulsive disorder with or without mental retardation limited to females: extension of a pedigree limits possible genetic mechanisms. *Cli. Genet* 38, 353-358 (1990).

Gaitan Y, Bouchard M. Expression of the δ-protocadherin gene Pcdh19 in the developing mouse embryo. *Gene Express Patterns* 6, 893-899 (2006).

Juberg RC, Hellman CD. A new familial form of convulsive disorder and mental retardation limited to females. *J Pediatr* 79, 726-732 (1971).

Kim SY *et al.* Spatiotemporal expression pattern of non-clustered protocadherin family members in the developing rat brain. *Neuroscience* 147, 996-1021 (2007).

Lambson B, Affara NA, Mitchell M, Ferguson-Smith MA. Evolution of DNA sequence homologies between the sex chromosomes in primate species. *Genomics* 14, 1032-40 (1992).

Maquat LE. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nature Rev Mol. Cell Biol* 5, 89-99 (2004).

McKee S. X-linked epilepsy affecting females and sparing males: report of a second family. *J Med Genet* 43, Suppl 1, S48 (2006).

Morishita H. *et al.* Structure of the cadherin-related neuronal receptor/protocadherin-alpha first extracellular cadherin domain reveals diversity across cadherin families. *J Biol Chem* 281, 33650-33663 (2006).

Page DC, Harper ME, Love J, Botstein D. Occurrence of a transposition from the X-chromosome long arm to the Y-chromosome short arm during human evolution. *Nature* 311, 119-123 (1984).

Patel SD *et al.* Type II cadherin ectodomain structures: implications for classical cadherin specificity. *Cell* 124, 1255-1268 (2006).

Redies C, Vanhalst K, van Roy F. Protocadherins: unique structures and functions. *Cell Mol Life Sci* 62, 2840-2852 (2005).

Ryan SG, Chance PF, Zou CH, Spinner NB, Golden JA, Smietana S. Epilepsy and mental retardation limited to females: an X-linked dominant disorder with male sparing. *Nature Genet* 17, 92-95 (1997).

Sambrook, J., Fritsch, E.F., and Maniatis, T., in *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, NY, Vol 1, 2, 3 (1989).

Saviozzi S, Cordero F, Lo Iacono M, Novello S, Scagliotti GV, Calogero RA. Selection of suitable reference genes for accurate normalization of gene expression profile studies in non-small cell lung cancer. *BMC Cancer* 6, 200 (2006).

Scheffer IE, Harkin LA, Grinton BE, Dibbens LM, Turner SJ, Zielinski MA, Xu R, Jackson G, Adams J, Connellan M, Petrou S, Wellard RM, Briellmann RS, Wallace RH, Mulley JC, Berkovic SF. Temporal lobe epilepsy and GEFS+ phenotypes associated with SCN1B mutations. *Brain* 130, 100-109 (2007).

Scheffer IE, Turner SJ, Turelli L, Haan E, Mazarib A, Neufeld MY, Korczyn AD, Mulley JC, Berkovic SF. Epilepsy and mental retardation limited to females (EFMR): Underrecognition of a remarkable phenotype and confirmation of linkage. *EPILEPSIA* 45 (Suppl 7), 226 (2004).

Vanhalst K, Kools P, Staes K, van Roy F, Redies C. Proto-cadherins: a gene family expressed differentially in the mouse brain. *Cell Mol Life Sci* 62, 1247-1259 (2005).

Wilson LD, Ross SA, Lepore DA, Wada T, Penninger JM, Thomas PQ. Developmentally regulated expression of the regulator of G-protein signaling gene 2 (*Rgs2*) in the embryonic mouse pituitary. *Gene Expression Patterns* 5, 305-311 (2005).

Wu Q, Maniatis T. A striking organization of a large family of human neural cadherin-like cell adhesion genes. *Cell* 97, 779-790 (1999).

Yagi T, Takeichi M. Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes Dev* 14, 1169-1180 (2000).

CLAIMS

1. A method of diagnosing an illness related to functional protocadherin 19 (PCDH19) protein deficiency or altered PCDH19 protein function, or assessing a predisposition to an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or screening to identify carriers of illnesses related to functional PCDH19 protein deficiency or altered PCDH19 protein function, wherein said method comprises the step of:

(i) detecting in a suitable biological sample from a subject, a loss of PCDH19 protein function or altered PCDH19 protein function.

2. The method of claim 1, wherein said PCDH19 protein is encoded by a nucleotide sequence showing at least 70% sequence identity to a complete *PCDH19* open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1.

3. The method of claim 1 or 2, wherein said illness is characterised by epilepsy and/or mental retardation.

4. The method of claim 3, wherein said illness is EFMR (Epilepsy and Mental Retardation limited to Females).

5. The method of any one of claims 1 to 4, wherein step (i) comprises detecting a mutant sequence in a region of *PCDH19* encoding an extracellular (EC) domain of PCDH19 protein.

6. The method of claim 5, wherein said mutant sequence comprises one or more of the following nucleotide sequence changes: 1322 T>A, 253 C>T, 2012 C>G, 2030_2031insT, 1671 C>G, 357delC and 1091_1092insC.

7. A kit for diagnosing an illness related to functional protocadherin 19 (PCDH19) protein deficiency or altered PCDH19 protein function, or assessing a predisposition to an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or screening to identify carriers of illnesses related to functional PCDH19 protein deficiency or altered PCDH19 protein function, wherein said kit comprises one or more of the following: an antibody or fragment thereof which specifically binds to PCDH19 protein or polypeptide, or a fragment or variant thereof; and an oligonucleotide probe/primer molecule which specifically hybridises to a polynucleotide molecule encoding PCDH19 protein or polypeptide, or a fragment or variant thereof under high stringency conditions.

8. The kit of claim 4, further comprising instructions for the operation of the method and, optionally, for thereafter diagnosing an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or assessing a predisposition to an illness related to functional PCDH19 protein deficiency or altered PCDH19 protein function, or identifying carriers of illnesses related to functional PCDH19 protein deficiency or altered PCDH19 protein function, together with one or more containers or vessels containing said antibody or fragment thereof and/or said oligonucleotide probe/primer molecule.

9. A use of: a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1, wherein said nucleotide sequence encodes a functional PCDH19 protein or polypeptide, or functional fragment or functional variant thereof; or a functional PCDH19 protein or polypeptide, or functional fragment or functional variant thereof encoded by a polynucleotide molecule comprisng a nucleotide sequence showing at least 70% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1; in the treatment of PCDH19 protein deficiency or altered PCDH19 protein function in a subject.

10. The use of claim 9, wherein the functional PCDH19 protein or polypeptide comprises an amino acid sequence according to SEQ ID NO: 2.

11. A method for the therapeutic or prophylactic treatment of protocadherin 19 (PCDH19) protein deficiency or altered PCDH19 protein function in a subject, wherein said method comprises the step of:

(i) administering to the subject: a polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to the complete protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1, wherein said nucleotide sequence encodes a functional PCDH19 protein or polypeptide, or functional fragment or functional variant thereof; or a functional PCDH19 protein or polypeptide, or functional fragment or functional variant thereof encoded by a polynucleotide molecule comprisng a nucleotide sequence showing at least 70% sequence identity to the complete *PCDH19* ORF nucleotide sequence according to SEQ ID NO: 1; and/or an agent that compensates for PCDH19 function, optionally in combination with a pharmaceutically-acceptable carrier.

12. The method of claim 11, wherein step (i) comprises administering a functional PCDH19 protein or polypeptide comprising an amino acid sequence according to SEQ ID NO: 2.

13. An agent capable of treating a deficiency in functional protocadherin 19 (PCDH19) protein or altered PCDH19 protein function in a subject.

14. A method for identifying an agent capable of treating a deficiency in functional protocadherin 19 (PCDH19) protein or altered PCDH19 protein function, wherein said method comprises the steps of;

(i) providing a cell or animal comprising a polynucleotide molecule comprising a mutant sequence of the *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1;

(ii) contacting a test agent with said cell or administering a test agent to said animal; and

(iii) comparing a response in said cell or animal with a control response.

15. A kit for use in the method of claim 14, wherein said kit comprises instructions for the operation of the method together with one or more containers and/or vessels containing one or more cell(s) or animal(s) comprising a polynucleotide molecule comprising a mutant sequence of the protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence shown as SEQ ID NO: 1.

16. A kit for identifying an agent capable of treating a deficiency in functional protocadherin 19 (PCDH19) protein or altered PCDH19 protein function, wherein said kit comprises;

(i) a cell or animal comprising a polynucleotide molecule comprising a mutant sequence of the *PCDH19* open reading frame (ORF) nucleotide sequence shown as SEQ ID NO: 1; and optionally,

(ii) a control cell or animal comprising a polynucleotide molecule comprising a wild-type form of the complete *PCDH19* ORF nucleotide sequence shown as SEQ ID NO: 1, said wild-type form encoding a functional PCDH19 protein or polypeptide, or a functional fragment or functional variant thereof.

17. An isolated protein or polypeptide comprising an amino acid sequence encoded by a nucleotide sequence showing at least 70% sequence identity to a complete protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1, or a functional fragment or variant thereof.

18. The isolated protein or polypeptide of claim 12 comprising an amino acid sequence showing at least 95% sequence identity to the complete PCDH19 amino acid sequence shown as SEQ ID NO: 2, or a functional fragment or variant thereof.

19. A variant according to claim 17 or 18, wherein the amino acid sequence of said variant includes an amino acid substitution within a calcium binding site or within 20 amino acids of a calcium binding site.

20. The variant of claim 17 or 18, wherein the amino acid substitution is selected from V441E, Q85X, S671X, L667fsX717, N557K, I119fsX122 and P364fsX375.

21. An isolated polynucleotide molecule comprising a nucleotide sequence showing at least 70% sequence identity to a complete protocadherin 19 (*PCDH19*) open reading frame (ORF) nucleotide sequence according to SEQ ID NO: 1 or a complementary sequence thereto.

22. The molecule of claim 21, wherein the nucleotide sequence comprises one or more mutations in a region encoding an extracellular (EC) domain of PCDH19 protein.

23. The molecule of claim 22, wherein the nucleotide sequence comprises one or more of the following mutations: 1322 T>A, 253 C>T, 2012 C>G, 2030_2031insT, 1671 C>G, 357delC and 1091_1092insC.

24. A cell transformed with the polynucleotide molecule of any one of claims 21 to 23.

25. A non-human animal comprising the polynucleotide molecule of any one of claims 21 to 23.

26. An antibody or fragment thereof which specifically binds to the protein or polypeptide, functional fragment or variant thereof, of any one of claims 17 to 20.

FIGURE 1

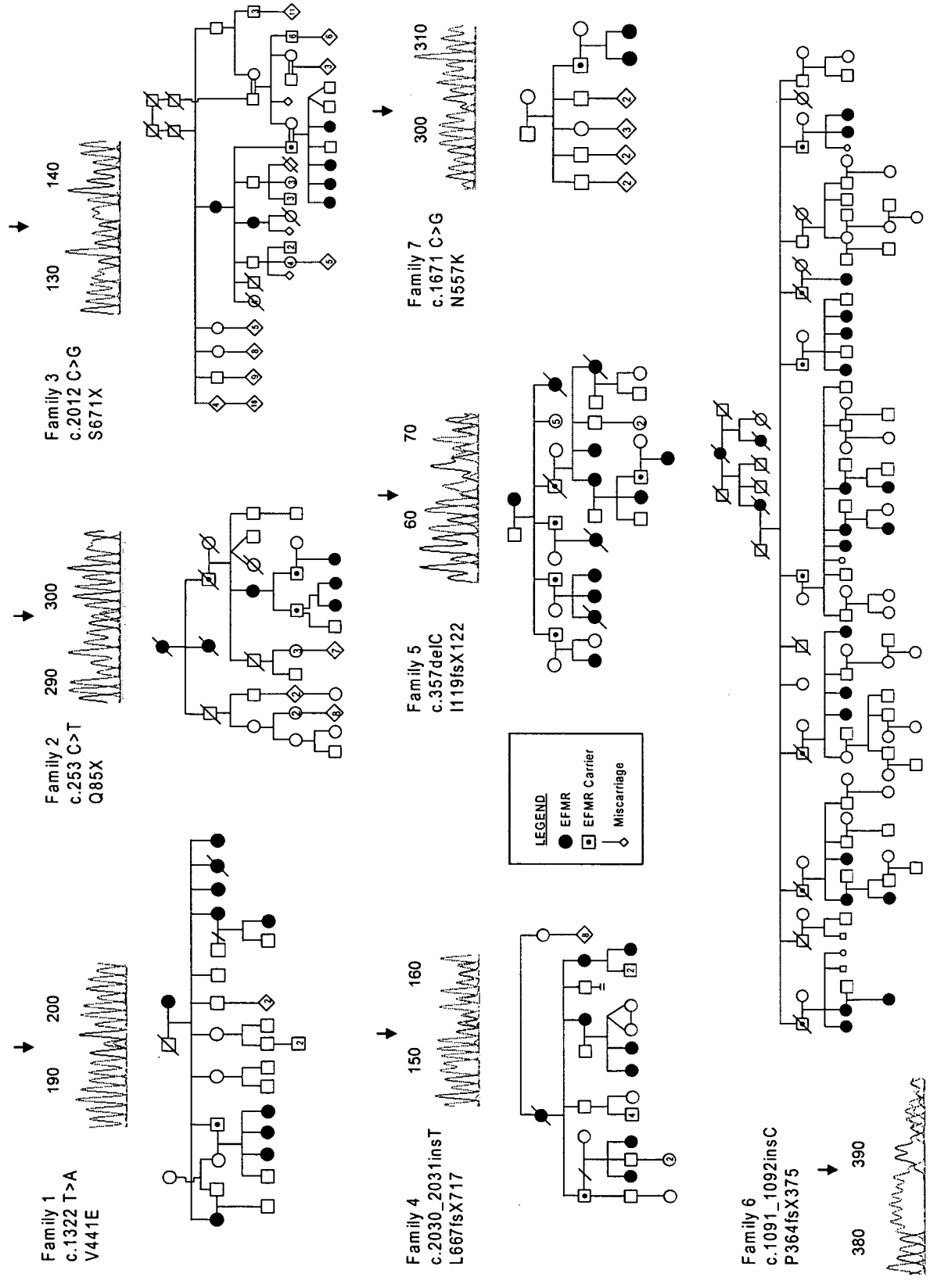

Family 1
c.1322 T>A
V441E
190
200
Family 2
c.253 C>T
Q85X
290
300
Family 3
c.2012 C>G
S671X
130
140
Family 4
c.2030_2031insT
L667fsX717
150
160
Family 5
c.357delC
I119fsX122
60
70
Family 7
c.1671 C>G
N557K
300
310
Family 6
c.1091_1092insC
P364fsX375
380
390
LEGEND
EFMR
EFMR Carrier
Miscarriage

FIGURE 2

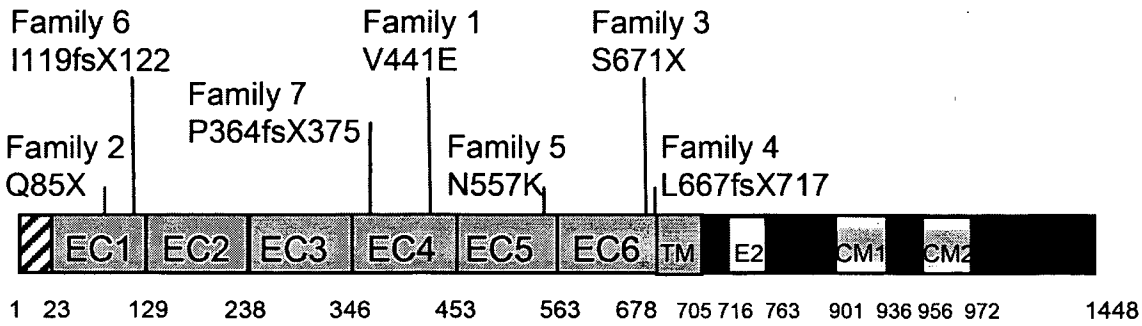


FIGURE 3

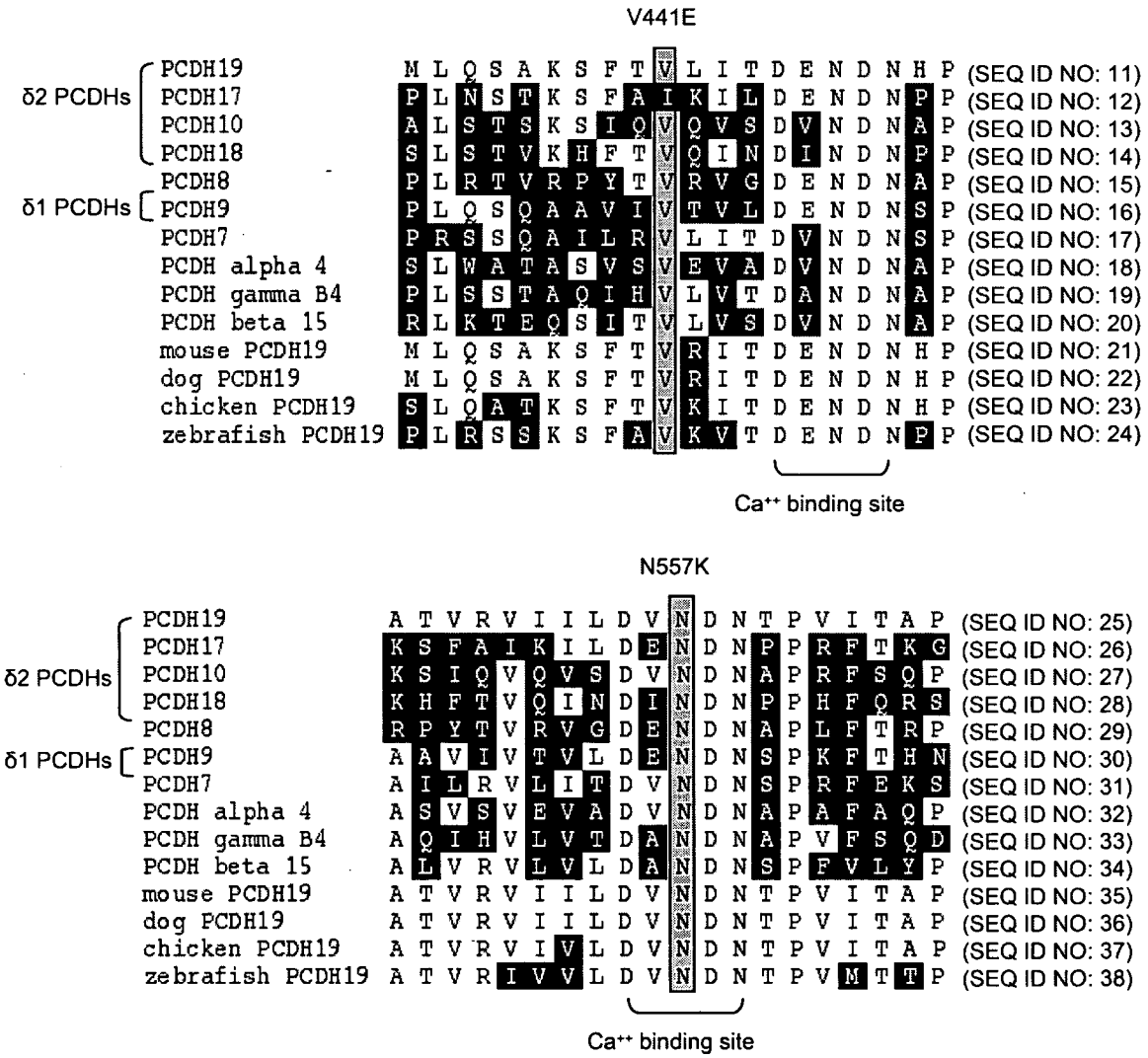


FIGURE 4

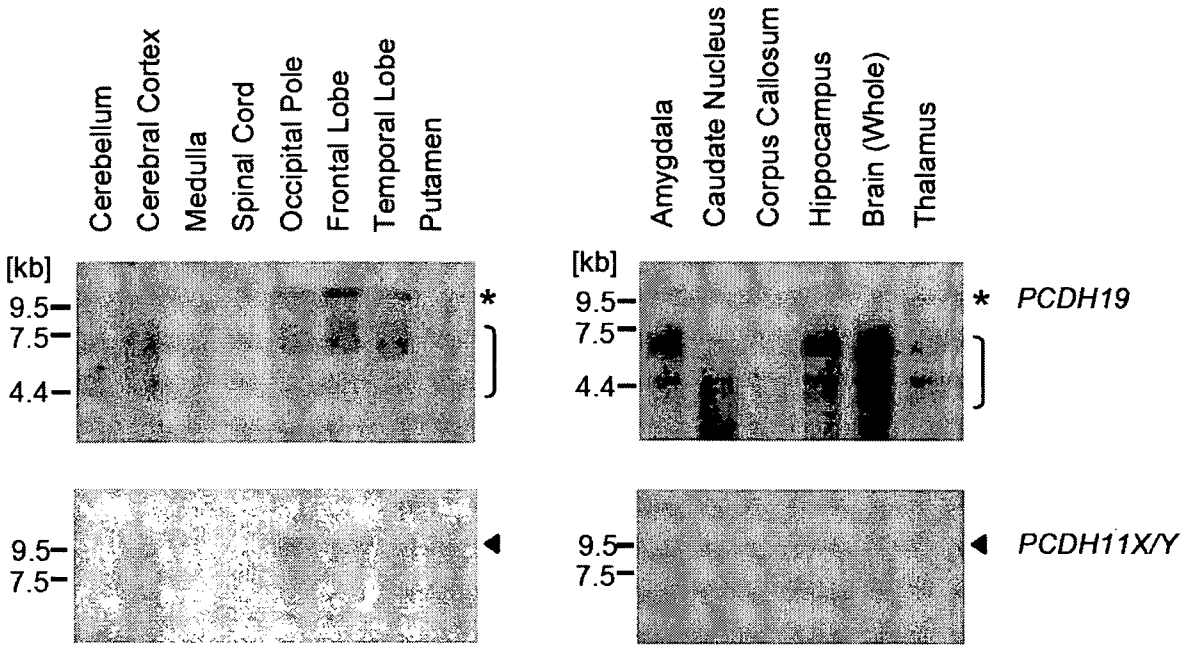


FIGURE 5

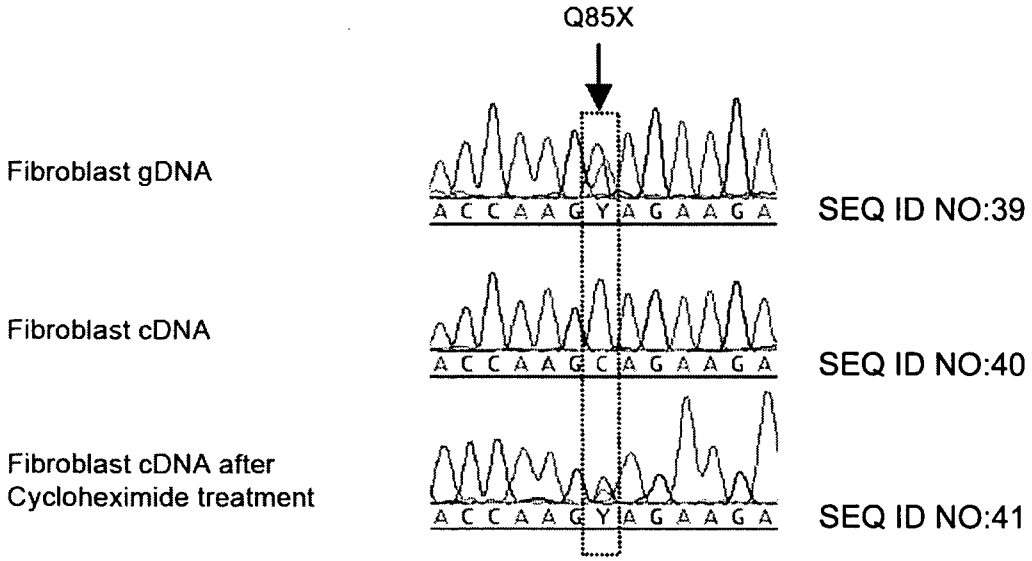


FIGURE 6

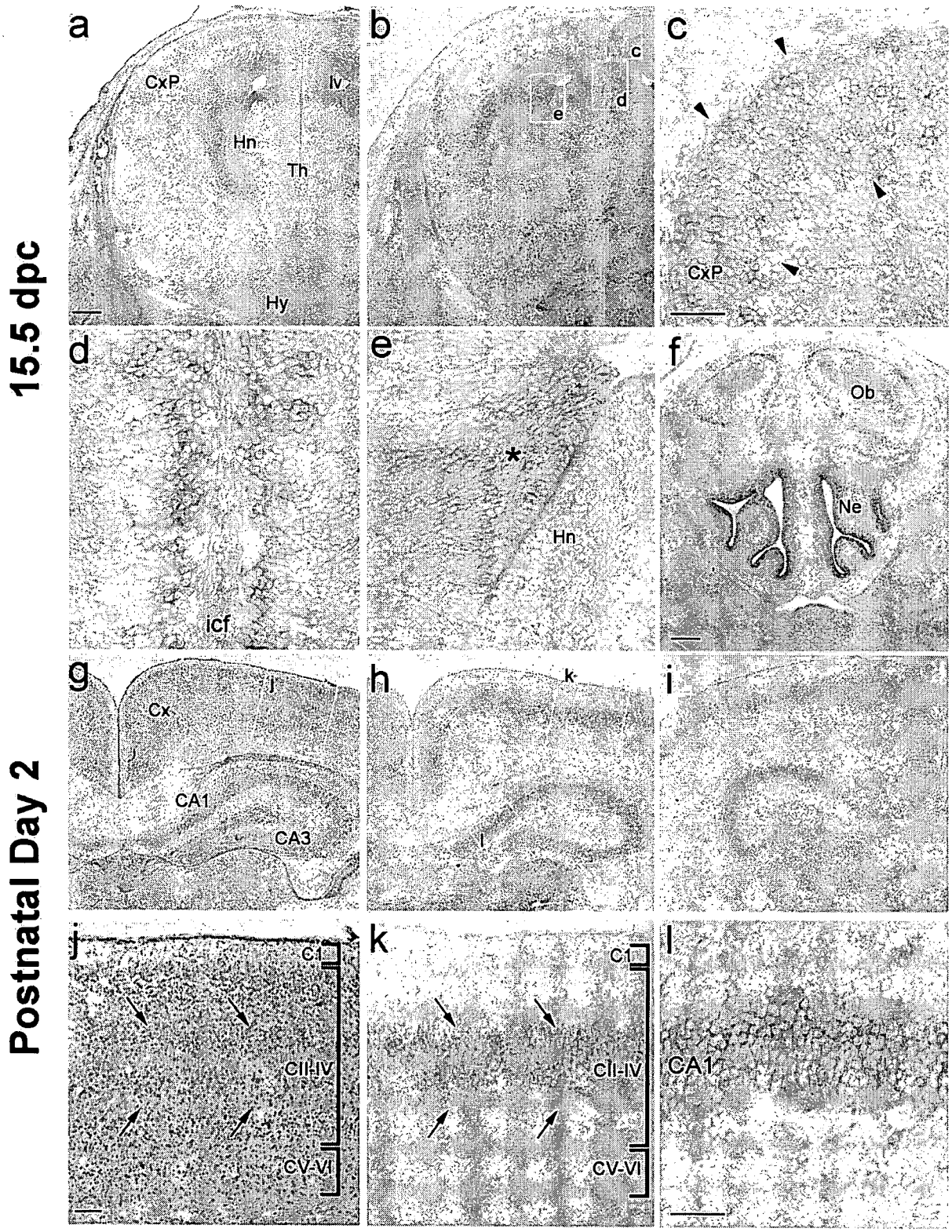

15.5 dpc
Postnatal Day 2
a
CxP
Iv
Hn
Th
Hy
b
c
e
d
c
CxP
d
icf
e
*
Hn
f
Ob
Ne
g
Cx
j
CA1
CA3
h
k
l
i
j
CI
CII-IV
CV-VI
k
CI
CII-IV
CV-VI
l
CA1

FIGURE 7

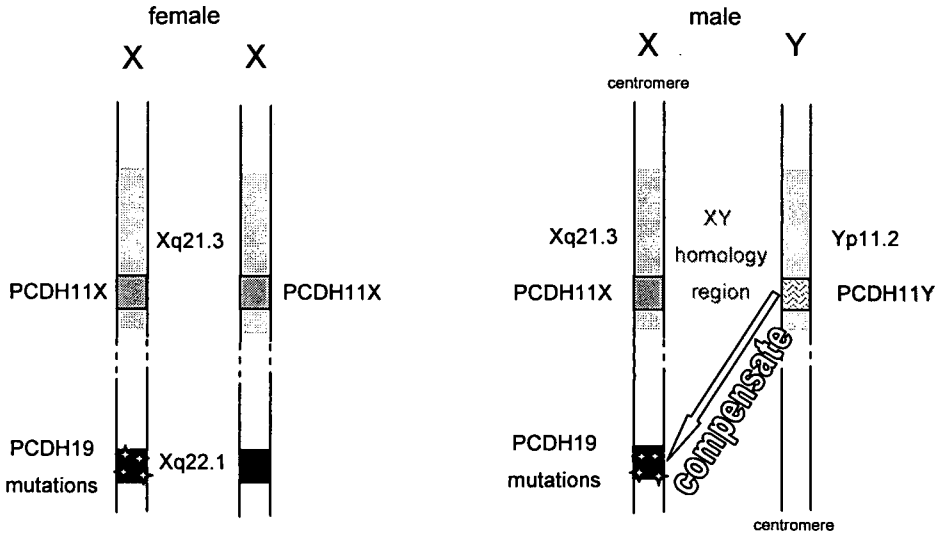
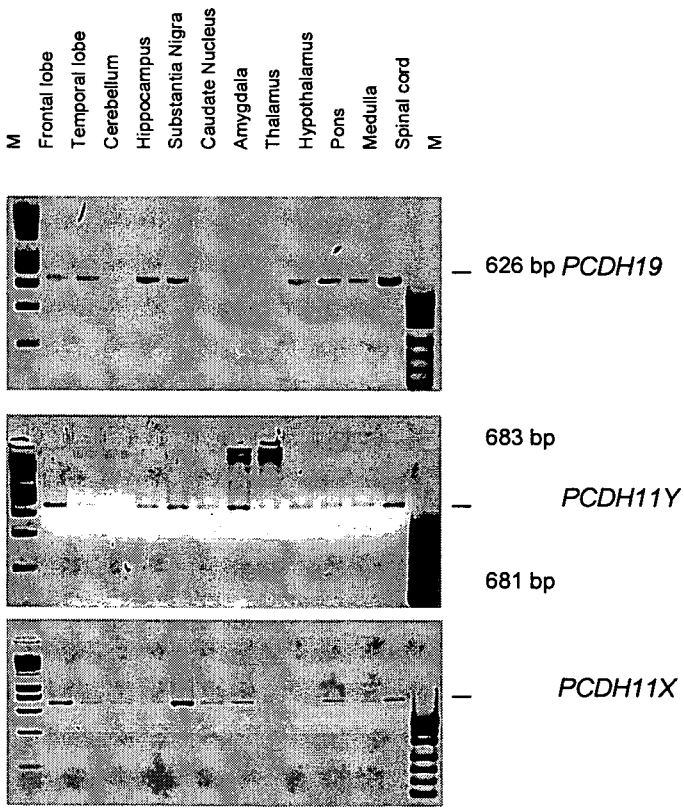


FIGURE 8



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2009/000008

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

G01N 33/68 (2006.01) *A61P 25/00* (2006.01) *C12N 15/12* (2006.01)
A01K 67/00 (2006.01) *C07K 14/435* (2006.01) *G01N 33/53* (2006.01)
A61K 38/17 (2006.01) *C07K 16/18* (2006.01)
A61K 48/00 (2006.01) *C12N 15/01* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GENOMEQUEST sequence search of SEQ ID NO:1 and SEQ ID NO:2. CA, BIOSIS, MEDLINE keyword search based on: female, mental, cognitive, impairment, retardation, restricted, limited, EFMR, PCDH19, protocadherin 19, KIAA1313 and DKFZP686P1843.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2002/088322 A2 (INCYTE GENOMICS, INC.) 7 November 2002 See SEQ ID NO:5; SEQ ID NO:16; page 1, lines 4-9; page 13, line 33-page 14, line 2; page 17, lines 12-18; page 49, lines 14-21 and page 49, line 32-page 50, line 1; page 50, lines 19 and 28; page 53, line 2-page 54, line 7; page 69, lines 5-18 and claims 48-55	1-3, 7-9, 11, 13, 17, 18, 21, 22, 24-26
X	WO 2007/047796 A2 (INSTITUTE FOR SYSTEMS BIOLOGY) 26 April 2007 See SEQ ID NO:5795; page 4, lines 8-18; page 7, lines 22-page 8, line 8; page 78, line 25-page 79, line 6	1, 2, 7, 8, 17, 18, 21, 26
X	DATABASE SwissProt Acc. No. Q8TAB3, Pub.Date: 2000 See sequence	17, 18, 21

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 March 2009	24 MAR 2009
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **KATE HOLDEN** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6225 6129

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2009/000008

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE NCBI GenPept Acc. No. NP_001098715, Pub. Date: 2003 & DATABASE NCBI GenBank Acc. No. NM_001105245, Pub. Date: 2003 See sequences	14-17, 18, 19, 21, 22
X	DATABASE NCBI GenPept Acc. No. XP_228429, last update: 22 June 2006 & DATABASE NCBI GenBank Acc. No. XM_228429, last update: 22 June 2006 See sequences	14-17, 18, 19, 21, 22
X	DATABASE SwissProt Acc. No. Q80TF3, Pub. Date: 2003 See sequence	17-19, 21, 22
X	DATABASE NCBI GenPept Acc. No. BAC65774; Pub. Date: 2003 & DATABASE NCBI GenBank Acc. No. AK122492, Pub. Date: 2003 See sequences	14-17, 19, 21, 22
A	Ryan, S. G. et al. Epilepsy and mental retardation limited to females: an X-linked dominant disorder with male sparing. Nature Genetics (1997) Vol. 17, No. 1, page 92-95 Cited in the application See whole document	1-26
P,A	Scheffer, I. E. et al. Epilepsy and mental retardation limited to females: an under-recognized disorder. Brain (29 January 2008) Vol. 131, pages 918-927 See whole document	1-26
P,X	Dibbens, L. M. et al. X-linked protocadherin 19 mutations cause female-limited epilepsy and cognitive impairment. Nature Genetics (online: 11 May 2008) Vol. 40, No. 6, pages 776-781 See whole document	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2009/000008

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
WO 02088322	AU	2002248723	AU	2002255733	AU	2002257088
	AU	2002258520	AU	2002258657	AU	2002303843
	AU	2002320026	AU	2002324915	AU	2002326910
	AU	2002327419	AU	2002327533	AU	2002330157
	AU	2002336529	AU	2002342010	AU	2002343515
	AU	2002343516	AU	2002347778	AU	2002352912
	AU	2002354803	AU	2002357850	AU	2002357860
	AU	2002358273	AU	2002359242	AU	2002359567
	AU	2002360513	AU	2002362586	AU	2002364699
	AU	2002364890	AU	2002366606	AU	2003205353
	AU	2003211112	AU	2003212475	AU	2003215369
	AU	2003218238	AU	2003218321	AU	2003220607
	AU	2003222189	AU	2003222225	AU	2003224811
	AU	2003225282	AU	2003228869	AU	2003230760
	AU	2003231245	AU	2003231531	AU	2003238930
	AU	2003239871	CA	2434944	CA	2437398
	CA	2437571	CA	2438235	CA	2439941
	CA	2440272	CA	2440618	CA	2442062
	CA	2443334	CA	2443713	CA	2443897
	CA	2444675	CA	2445338	CA	2445366
	CA	2446023	CA	2447338	CA	2447340
	CA	2447647	CA	2448116	CA	2448146
	CA	2452082	CA	2452501	CA	2453985
	CA	2456369	CA	2458445	CA	2458625
	CA	2458645	CA	2458648	CA	2459022
	CA	2459137	CA	2459140	CA	2459323
	CA	2459442	CA	2460476	CA	2460480
	CA	2460625	CA	2460808	CA	2460812
	CA	2460953	CA	2460959	CA	2462795
	EP	1355935	EP	1368472	EP	1368473
	EP	1383789	EP	1383798	EP	1385977
	EP	1390396	EP	1390410	EP	1409550

EP	1409655	EP	1417224	EP	1421111
EP	1423415	EP	1423697	EP	1429806
EP	1432801	EP	1434785	EP	1434788
EP	1434796	EP	1434860	EP	1436383
EP	1444254	EP	1451212	EP	1456365
EP	1456655	EP	1461446	EP	1478745
EP	1480994	EP	1481081	EP	1485474
EP	1487989	EP	1497319	EP	1506303
EP	1506304	EP	1519741	EP	1521822
EP	1521824	EP	1537138	EP	1572875
EP	1578899	EP	1578902	US	7348139
US	7378492	US	2003198975	US	2004063924
US	2004087773	US	2004097711	US	2004101882
US	2004101884	US	2004106125	US	2004115687
US	2004132043	US	2004152157	US	2004152877
US	2004158039	US	2004166501	US	2004171009
US	2004198651	US	2004203014	US	2004203097
US	2004248249	US	2004248251	US	2004249128
US	2004254350	US	2005019763	US	2005033018
US	2005048490	US	2005064543	US	2005069876
US	2005107293	US	2005107588	US	2005118594
US	2005130145	US	2005176944	US	2005191627
US	2006051836	US	2006121459	US	2006127894
US	2006194275	US	2007225218	US	2008076117
US	2008213782	WO	02062841	WO	02063006
WO	02066646	WO	02070669	WO	02070709
WO	02072794	WO	02074913	WO	02078420
WO	02079441	WO	02081636	WO	02083705
WO	02083712	WO	02086069	WO	02088316
WO	02092759	WO	02094780	WO	02094988
WO	02094990	WO	02096951	WO	02097032
WO	02097035	WO	03002610	WO	03004615
WO	03012065	WO	03014322	WO	03016493
WO	03016497	WO	03016506	WO	03018612
WO	03023003	WO	03023009	WO	03025129
WO	03025130	WO	03025131	WO	03025150
WO	03025542	WO	03027228	WO	03027263

		WO	03029437	WO	03031568	WO	03031595
		WO	03031939	WO	03031940	WO	03042357
		WO	03046152	WO	03046196	WO	03048305
		WO	03050084	WO	03050253	WO	03051902
		WO	03052049	WO	03052075	WO	03054219
		WO	03062391	WO	03063688	WO	03068943
		WO	03070902	WO	03072723	WO	03074726
		WO	03077875	WO	03080805	WO	03083081
		WO	03083082	WO	03083085	WO	03087300
		WO	03091419	WO	03093427	WO	03093439
		WO	03093444	WO	03100016	WO	03104410
WO	2007047796	AU	2006304605	CA	2625403	EP	1938104
		US	2007099251				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX