Germline Mutations in *CDH23*, Encoding Cadherin-Related 23, Are Associated with Both Familial and Sporadic Pituitary Adenomas

Qilin Zhang,[1,2,15] Cheng Peng,[3,15] Jianping Song,[1,2,15] Yichao Zhang,[1,2,15] Jianhua Chen,[4,5,15] Zhijian Song,[4,15] Xuefei Shou,[1,2] Zengyi Ma,[1,2] Hong Peng,[3] Xuemin Jian,[4] Wenqiang He,[1,2] Zhao Ye,[1,2] Zhiqiang Li,[4] Yongfei Wang,[1,2] Hongying Ye,[2,6] Zhaoyun Zhang,[2,6] Ming Shen,[1,2] Feng Tang,[2,7] Hong Chen,[2,7] Zhifeng Shi,[1,2] Chunjui Chen,[1,2] Zhengyuan Chen,[1,2] Yue Shen,[1,2] Ye Wang,[1,2] Shaoyong Lu,[8] Jian Zhang,[8] Yiming Li,[2,6] Shiqi Li,[1,2] Ying Mao,[1,2,9,10] Liangfu Zhou,[1,2] Hai Yan,[11] Yongyong Shi,[4,12,13,14] Chuanxin Huang,[3,*] and Yao Zhao[1,2,9,10,*]

Pituitary adenoma (PA) is one of the most common intracranial neoplasms. Several genetic predisposing factors for PA have been identified, but they account for a small portion of cases. In this study, we sought to identify the PA genetic risk factors by focusing on causative mutations for PAs. Among the 4 affected and 17 asymptomatic members from one family with familial PA, whole-exome sequencing identified cosegregation of the PA phenotype with the heterozygous missense mutation c.4136G>T (p.Arg1379Leu) in cadherin-related 23 (*CDH23*). This mutation causes an amino acid substitution in the calcium-binding motif of the extracellular cadherin (EC) domains of CDH23 and is predicted to impair cell-cell adhesion. Genomic screening in a total of 12 families with familial PA (20 individuals), 125 individuals with sporadic PA, and 260 control individuals showed that 33% of the families with familial PA (4/12) and 12% of individuals with sporadic PA (15/125) harbored functional *CDH23* variants. In contrast, 0.8% of the healthy control individuals (2/260) carried functional *CDH23* variants. Gene-based analysis also revealed a significant association between *CDH23* genotype and PA ($p = 5.54 \times 10^{-7}$). Moreover, PA individuals who did not harbor functional *CDH23* variants displayed tumors that were larger in size ($p = 0.005$) and more invasive ($p < 0.001$). Therefore, mutations in *CDH23* are linked with familial and sporadic PA and could play important roles in the pathogenesis of PA.

With a prevalence of 14%–22%, pituitary adenoma (PA [MIM: 102200]) is one of the most common neuroendocrine tumors and varies in different populations. PAs manifest with a variety of clinical features, including local invasion of surrounding structures and excessive hormone secretion.[1] PAs are classified clinically as functional and non-functional (NF). According to the hormones produced by functional PAs, they are further classified into six subtypes: prolactin (PRL), growth hormone (GH), adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), gonadotropins hormone (GT), and multiple hormones (plurihormonal).[2]

Familial PAs are characterized by the development of PAs in multiple family members and are divided into (1) isolated groups, in which no other organs are involved in addition to the PA, and (2) syndromic groups. Each group can be caused by germline mutations in different genes

predisposing to PA. For example, mutations in arylhydrocarbon-receptor-interacting protein (*AIP* [MIM: 605555]) and G-protein-coupled receptor 101 (*GPR101* [MIM: 300393]) were discovered in familial isolated PAs, whereas mutations in multiple endocrine neoplastic type 1 (*MEN1* [MIM: 613733]) and cyclin-dependent kinase inhibitor 1B (*CDKN1B* [MIM: 600778]) were found in syndromic PAs.[3,4] However, individuals with these mutations account for ~20% of all cases of familial PA, and the genetic risk factors of most familial PAs remain unknown.[1,5,6] A recent genome-wide association study by our group revealed that common variants at three loci are significantly associated with the occurrence of sporadic PAs.[7] Genetic alterations of multiple tumor-suppressor genes, including *RB1* (MIM: 614041) and *MGMT* (MIM: 156569), have also been identified in sporadic PAs.[8] Moreover, some genetic risk factors for familial PA have been found in sporadic

[1]Department of Neurosurgery, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai 200040, China; [2]Shanghai Pituitary Tumor Center, Shanghai 200040, China; [3]Shanghai Institute of Immunology, Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China; [4]Bio-X Institutes, Ministry of Education Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders, Institute of Social Cognitive and Behavioral Sciences, Shanghai Jiao Tong University, Shanghai 200030, China; [5]Shanghai Key Laboratory of Psychotic Disorders, Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200030, China; [6]Department of Endocrinology, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai 200040, China; [7]Department of Pathology, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai 200040, China; [8]Department of Pathophysiology, Key Laboratory of Cell Differentiation and Apoptosis of Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China; [9]State Key Laboratory of Medical Neurobiology, Shanghai Medical College, Fudan University, Shanghai 200040, China; [10]Institute of Neurosurgery, Shanghai Medical College, Fudan University, Shanghai 200040, China; [11]Department of Pathology, Preston Robert Tisch Brain Tumor Center, Duke University Medical Center, Durham, NC 27710, USA; [12]Institute of Neuropsychiatric Science and Systems Biological Medicine, Shanghai Jiao Tong University, Shanghai 200030, China; [13]Department of Psychiatry, First Teaching Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054, China; [14]Biomedical Sciences Institute of Qingdao University, Qingdao Branch of SJTU Bio-X Institutes and the Affiliated Hospital of Qingdao University, Qingdao, Shandong 266003, China
[15]These authors contributed equally to this work
*Correspondence: huangcx@shsmu.edu.cn (C.H.), zhaoyaohs@vip.sina.com (Y.Z.)
http://dx.doi.org/10.1016/j.ajhg.2017.03.011.

cases, e.g., mutations in *AIP* were detected in approximately 5% of individuals with sporadic PA.[9] However, pathogenic variants in the majority of sporadic PAs have not yet been revealed.

In this study, we identified the germline genetic alterations in cadherin-related 23 (*CDH23* [MIM: 605516]) by performing whole-exome sequencing (WES) and follow-up analyses of 12 families and 125 individuals with familial and sporadic PAs, respectively. Individuals with PAs were recruited at the Department of Neurosurgery at Huashan Hospital, an affiliate of the Fudan University Shanghai Medical College, from 2008 to 2015. This study was approved by the ethics committee at Huashan Hospital, and informed consent was obtained from each individual involved in the study.

At first, we analyzed a PA family with four affected individuals (Figure 1A). Two of them (II-4 and III-8) were diagnosed with GH PAs, whereas the other two (II-7 and II-10) were diagnosed with NF PAs. An overview of the clinical findings is shown in Table S1 and Figure 1B. In brief, individual II-4 and III-8 suffered from high GH or IGF-1 levels and presented with acromegaly for at least 4 years. Neither of them received somatostatin analogs or any other medications before transsphenoidal surgery. Both clinical manifestations and hormone levels were significantly relieved 6 months after surgical resection. Individuals II-7 and II-10 were reported to experience occasional headaches for less than 1 year during the annual medical examinations and were further diagnosed with PAs according to the standard guidelines. No other symptoms, such as visual loss, acromegaly, amenorrhea, or menstrual abnormalities, were observed. Their hormone levels were within the normal ranges. Both individuals II-7 and II-10 declined to receive any surgical treatment, and no tumor enlargement was observed during the 9-month follow-up.

We performed WES in genomic DNAs from white blood cells of two affected (II-4 and III-8) and two asymptomatic (II-3 and III-12) family members to determine potential germline variants. In-solution exome capture was performed with the TruSeq Exome Enrichment Kit (Illumina) and the Agilent SureSelect Human All Exon V5+UTR Kit with 125-bp paired-end read sequences generated on a HiSeq 2500 (Illumina) and then analyzed by standard methods. UCSC Genome Browser build hg19 was used as the reference sequence. The mean sequencing depth was 84×, including >10× coverage for 88.2% of the target regions. Variants with allele frequencies greater than 0.1% in 1000 Genomes and the Exome Aggregation Consortium (ExAC) Browser were excluded from our analysis. A total of 383 nonsynonymous variants and 23 indels were shared by both PA individuals but not by the two unaffected family members. After filtration, we identified 25 variants and 5 heterozygous indels whose frequencies were less than 0.1% in 1000 Genomes and the ExAC Browser. Among them, three variants were predicted to affect the protein structure according to analyses by SIFT,[10] PolyPhen-2,[11]

CADD,[12] and MetaLR[13] (Figure 1C). The threshold was determined according to the criteria reported before.[14] Further Sanger sequencing of other family members ascertained that the heterozygous *CDH23* c.4136G>T (p.Arg1379Leu) (GenBank: NM_022124.5) mutation segregated with PA disease (Figure 1D). This variant site was not identified in either 1000 Genomes or the ExAC Browser. Two phenotypically unaffected relatives (IV-2 and III-22) were found to carry *CDH23* mutations. However, both of them are younger than 30 years old and could still develop PA in the future.

CDH23 is a member of the cadherin superfamily, which comprises calcium-dependent cell-cell adhesion glycoproteins.[15] Germline mutations in *CDH23* have been identified in people with Usher syndrome 1D (MIM: 601067) and nonsyndromic autosomal-recessive deafness (MIM: 601386), and *Cdh23* mutant mice develop a quite similar disease.[16,17] Deregulated expression and activity of some cadherin proteins are frequently observed in human PAs and are inferred to contribute to the pathogenesis of PAs.[18–20] In addition, CDH23 forms heterodimers and functions closely with protocadherin-related 15 (PCDH15), whose encoding gene is located near a genetic susceptibility locus for pituitary adenoma according to our previous work.[7] These findings further suggest mutations in *CDH23* as a potential genetic risk factor for this familial PA. The identified amino acid substitution is located in the second calcium-binding site of the extracellular cadherin 13 (EC13) domain, which is highly conserved among humans, mice, rats, and zebrafish (Figure 1E and Figure S1). Functions of cadherin members are typically mediated through their EC domains, especially through the highly conserved calcium-binding motifs.[21] Thus, this substitution could lead to loss of calcium-binding ability and the function of the EC13 domain.

We performed further molecular modeling to evaluate the predicted functional effect of the discovered *CDH23* mutation (Figure 1F). In the homologous template of the CDH23 domain (pink model [PDB: 2WHV]), amino acid position 1,379 (corresponding to Arg73) forms hydrogen bonds with Asp102 and Glu74 and thus greatly contributes to the calcium-binding ability and conformational stability of the EC domain. In comparison, the blue and green models represent the protein structures encoded by wild-type and mutant *CDH23*, respectively. The amino acid alteration caused by c.4136G>T (p.Arg1379Leu) was predicted to prevent the formation of hydrogen bonds and thus impair the calcium-binding ability and stability of the EC domain, suggesting that it is an inactivating mutation. We next evaluated whether this *CDH23* mutation would affect expression and localization of CDH23. Immunohistochemistry staining showed that, regardless of the *CDH23* genotype, CDH23 was present on the membrane of normal pituitary glands, as well as PAs, with similar expression levels, suggesting that the *CDH23* mutation is not associated with reduced expression or mislocalization of the protein (Figure S2).
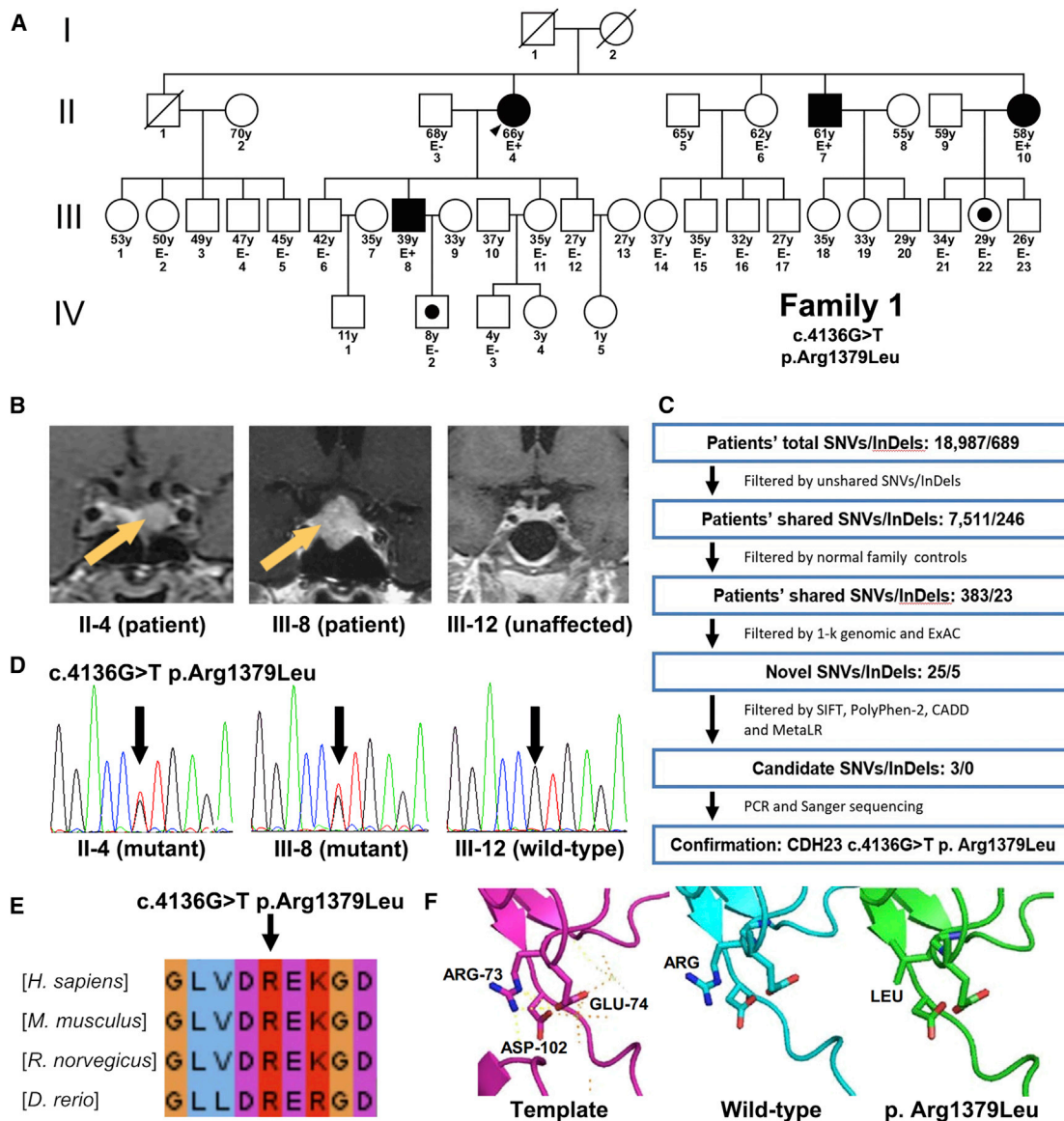
Figure 1. *CDH23* Mutations Identified in Familial PA

(A) Pedigree of family 1, who has familial PA. Two affected (II-4 and III-8) and two unaffected (II-3 and III-12) individuals were selected for exome sequencing. Black filled shapes represent affected siblings. White shapes represent asymptomatic subjects. White shapes with a black dot represent asymptomatic carriers of *CDH23* mutations. Symbols crossed with a diagonal line indicate deceased relatives. The age of each member is indicated under each symbol. Family members with available clinical data and DNA samples are indicated with E− or E+ under their symbols. E− indicates that the person had a negative evaluation for PA, and E+ indicates that the individual has PA.

(B) MRI data of two individuals with PA (II-4 and III-8) and one unaffected person (III-12) who were included for exome sequencing. Yellow arrows mark tumors.

(C) Filters applied to variants and indels detected by exome sequencing in this family.

(D) Sequencing chromatograms of *CDH23* in two individuals with PA (II-4 and III-8) and one unaffected person (III-12). All heterozygous variants were verified by Sanger sequencing (indicated by the black arrow). The mutation and amino acid substitution are shown on the top.

(E) Alignment of the CDH23 EC13 domain shows evolutionary conservation at the site of the identified nucleic acid changes (indicated by the black arrow).

(F) Protein pathogenicity prediction of wild-type and p.Arg1379Leu (c.4136G>T) CDH23 proteins. The pink model represents the homologous template for the EC domain (PDB: 2WHV). The blue model shows the structure of wild-type CDH23. The green model shows the structure of p.Arg1379Leu CDH23.

To further investigate whether *CDH23* variants exist in other familial PAs, we recruited 11 additional families with familial PAs. Each family had two individuals with PA, except families 3 and 4 each had three affected individuals (Figures 2A–2C and Figure S3). WES was per-formed in one person per family. We found that three individuals with familial PA harbored *CDH23* variants (c.6344G>A [p.Arg2115His], c.9412C>T [p.Arg3138Trp], and c.9886G>A [p.Asp3296Asn] in families 2, 3, and 4, respectively; GenBank: NM_022124.5; Figure 2D). All of
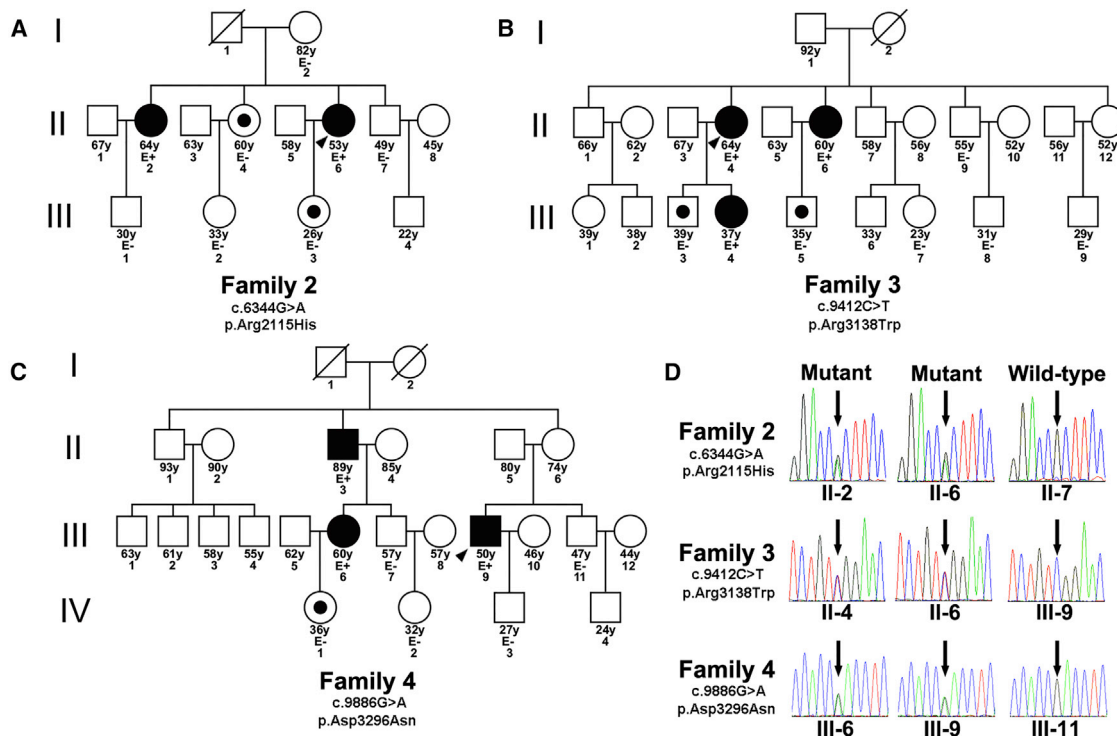
Figure 2. Three Additional Families Affected by *CDH23* Mutations

(A–C) Pedigrees of three families carrying *CDH23* mutations. Black filled shapes represent affected siblings. White shapes represent asymptomatic subjects. White shapes with a black dot represent asymptomatic carriers of *CDH23* mutations. Symbols crossed with a diagonal line indicate deceased relatives. The age of each member is indicated under each symbol. Family members with available clinical data and DNA samples are indicated with E− or E+ under their symbols. E− indicates that the person had a negative evaluation for PA, and E+ indicates that the individual has PA.

(D) Sanger sequencing showing *CDH23* mutations in three affected individuals from different families. The black arrows indicate the mutated nucleotides. The mutations and amino acid substitutions are shown on the left.

these mutations were heterozygous, and the allele frequency of each variant in the ExAC Browser was less than 0.05% (Table S1). PolyPhen-2 scores of all three variants were greater than 0.99, indicating potential pathogenicity. Sanger sequencing of 8 affected and 17 unaffected relatives with available blood DNA samples verified the presence of the variants and showed that the *CDH23* mutations co-segregated with clinical phenotypes (Figures 2A–2D and Figure S4). Notably, all three discovered alternative residues in CDH23 are highly conserved among humans, mice, rats, and zebrafish (Figure S5).

To identify whether *CDH23* mutations are associated with sporadic PAs, we used WES to analyze genomic DNAs of white blood cells from 125 individuals with sporadic PAs. Subjects with any potential previous malignancies or family history of PAs were excluded. These PAs included 20 NF PAs, 20 PRL PAs, 20 GH PAs, 20 ACTH PAs, 20 GT PAs, 10 TSH PAs, and 15 plurihormonal PAs. The plurihormonal PAs in this study secreted GH and PRL (n = 11), GH and ACTH (n = 2), or GH and TSH (n = 2). We found that 15 (12.0%) individuals harbored *CDH23* mutations, including one NF PA, three PRL PAs, two GH PAs, four ACTH PAs, one GT PA, three TSH PAs, and one plurihormonal (GH and TSH) PA (Figure 3A and Table S1). Most of these variants were heterozygous, but

two were homozygous. Allele frequencies of all variants were less than 0.1% in 1000 Genomes and the ExAC Browser (Table S1). All 15 variants were predicted to be pathogenic by at least one in silico method (SIFT, PolyPhen-2, CADD, or MetaLR). Although the spectrum was diverse, all amino acid substitutions were located in the EC domains of CDH23 and were close to the conserved site of each domain (Figure 3B),[21,22] which could affect protein folding or calcium-binding affinity.

In contrast, among the 260 local healthy control individuals, two (0.8%) carried *CDH23* mutations, one of which was predicted to be pathogenic (Table S2). The frequencies of *CDH23* mutations were significantly different between people with sporadic PA and normal control individuals (p = 2 × 10^{-6}, Pearson's χ^2 test). Sequence kernel association testing identified significant gene-based associations between PAs and *CDH23* variants (p = 5.54 × 10^{-7}), suggesting a significantly increased risk of PAs in subjects with *CDH23* mutations.[23] Very interestingly, none of the sporadic PAs harbored somatic *CDH23* mutations on the basis of previous genomic studies.[4] Similar to *AIP* variants, germline *CDH23* variants are genetic risk factors for both familial and sporadic Pas.[6,24]

On the basis of the fact that tumor growth and invasion are crucial for the treatment of PAs and that the cadherin
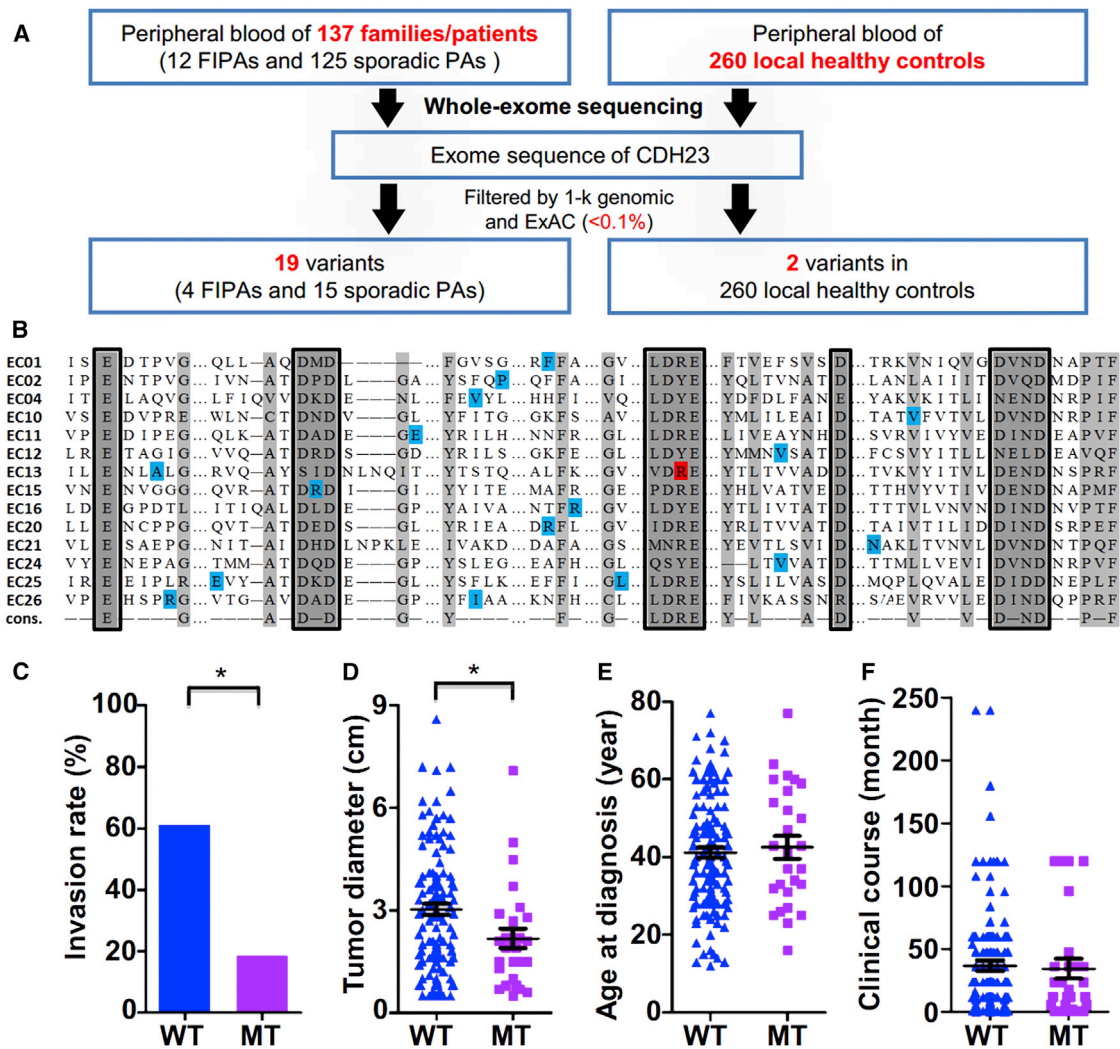
Figure 3. *CDH23* Mutations in a Large PA Cohort
(A) The frequency of *CDH23* mutations in familial PAs, sporadic PAs, and local healthy control individuals.
(B) Mutant *CDH23* variant sites in familial and sporadic PAs. All 14 EC domains with detected amino acid substitutions are shown. The ordinal of each EC domain is shown on the left. Corresponding EC domains are indicated to the left of the lineup. Light gray indicates conserved residues. Highly conserved calcium-binding elements are in gray and boxed. The residue in red indicates the mutant site discovered in family 1, and other variants are marked in blue.
(C) Percentages of individuals with invasions in both wild-type (WT) and mutant (MT) groups. The p values were calculated by Pearson's χ^2 test.
(D–F) Maximal tumor diameters of the WT and MT groups (D), ages at diagnosis of the WT and MT groups (E), and the clinical course of each person in the WT and MT groups (F). Each symbol represents an individual case. Error bars show the mean and standard errors of the data. The p values were calculated by the Mann-Whitney U test (*p < 0.05 considered significant).

superfamily is involved in cell-cell adhesion, we focused on the correlation between *CDH23* genotypes and clinical characteristics, especially tumor diameter and invasiveness.[25] Among the 145 cases considered (20 familial and 125 sporadic PAs), *CDH23* mutations were identified in all subtypes. On the basis of the contrast-enhanced MRI and intra-operative findings, 72 of 118 (61.02%) *CDH23* wild-type PAs extended into the parasellar spaces, including the cavernous sinus and the sphenoidal sinus. In contrast, the vast majority of *CDH23* mutant PAs diffusely distributed within the sella: 5 of 27 cases (18.52%) displayed parasellar invasion (p < 0.001, Pearson's χ^2 test; Figure 3C and Table S3). This morphological

characteristic is further supported by the fact that *CDH23* mutant PAs are generally smaller than *CDH23* wild-type PAs (1.90 versus 2.90 cm, p = 0.005, Mann-Whitney U test; Figure 3D and Table S3). To exclude the possibility that this finding was due to closer surveillance MRI scans detecting tumors in familial cases at earlier stages of their natural history, we analyzed only sporadic cases. As shown in Table S4, *CDH23* mutations were significantly negatively related to both the invasiveness and tumor diameter in sporadic PAs (p = 0.003 and 0.046, respectively). CDH23 has been reported to play a role in tumor progression through regulating cell-cell adhesion. For example, CDH23 is related to the early stages of tumor metastasis

and is upregulated in breast cancers (MIM: 114480).[26] All of the identified *CDH23* mutations seem to be inactivating mutations and could impair cell-cell adhesion because they are located close to or within the regions encoding the conserved calcium-binding motifs of CDH23, partially explaining why *CDH23* mutations are related to the invasiveness of PA. Other clinical features, including age at diagnosis, clinical course, and radical resection rate, showed no significant differences between wild-type individuals and those with *CDH23* mutations (Figures 3E and 3F and Tables S3 and S4).

Several mutations in *CDH23* are associated with inherited hearing loss and blindness.[16] However, none of the variants found in this study were linked to any symptoms of deafness or blindness. This could be explained by the observation that no shared variants were identified in either hearing loss or PA. Another possible explanation is that deafness-related *CDH23* mutations are mostly homozygous or compound heterozygous,[16,27,28] whereas most *CDH23* mutations in this study were heterozygous. It is conceivable that carriers of *CDH23* mutations are at a high risk of developing hearing loss. Because most individuals included in this study are younger than 65 years old, age-related hearing loss (MIM: 612448) cannot be excluded.[29] Extensive clinical investigation should be undertaken to determine whether pituitary MRI scans should be adopted in the screening of *CDH23*-related diseases, including Usher syndrome 1D and age-related hearing loss.

It is common that familial and sporadic human diseases, including PA, share the same susceptibility genes. For example, *APC* (MIM: 611731) mutations are found in both familial and sporadic human colorectal cancers (MIM: 114500).[30] *CDH23* is another case, further supporting the notion that sporadic and familial PAs can be attributed to similar genetic predisposing factors.

In summary, we have identified *CDH23* mutations as a genetic risk factor for both familial and sporadic PAs. Further functional studies of this gene are necessary for gaining insights into the pathogenic mechanisms involved in PAs.

Supplemental Data

Supplemental Data include five figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2017.03.011.

Acknowledgments

Web Resources

1000 Genomes, http://www.1000genomes.org/
CADD, http://cadd.gs.washington.edu/download
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
ExAC Browser, http://exac.broadinstitute.org/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
OMIM, http://www.omim.org
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
Protein Data Bank, http://www.rcsb.org/pdb/home/home.do
UCSC Genome Browser, https://genome.ucsc.edu/

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Supplemental Data

Germline Mutations in *CDH23*, Encoding

Cadherin-Related 23, Are Associated with Both

Familial and Sporadic Pituitary Adenomas

Qilin Zhang, Cheng Peng, Jianping Song, Yichao Zhang, Jianhua Chen, Zhijian Song, Xuefei Shou, Zengyi Ma, Hong Peng, Xuemin Jian, Wenqiang He, Zhao Ye, Zhiqiang Li, Yongfei Wang, Hongying Ye, Zhaoyun Zhang, Ming Shen, Feng Tang, Hong Chen, Zhifeng Shi, Chunjui Chen, Zhengyuan Chen, Yue Shen, Ye Wang, Shaoyong Lu, Jian Zhang, Yiming Li, Shiqi Li, Ying Mao, Liangfu Zhou, Hai Yan, Yongyong Shi, Chuanxin Huang, and Yao Zhao

Figure S1.The location of *CDH23* c.4136G>T (p.Arg1379Leu) mutation.
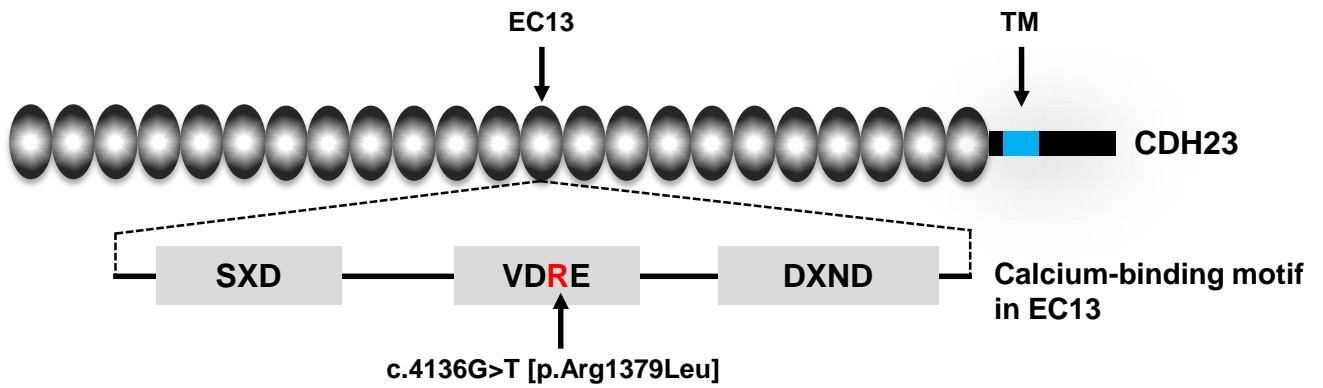

The identified c.4136G>T (p.Arg1379Leu) amino acid substitution located in the second calcium-binding site of extracellular cadherin (EC) 13 domain.

Figure S2. Immunochemistry staining of CDH23 protein.
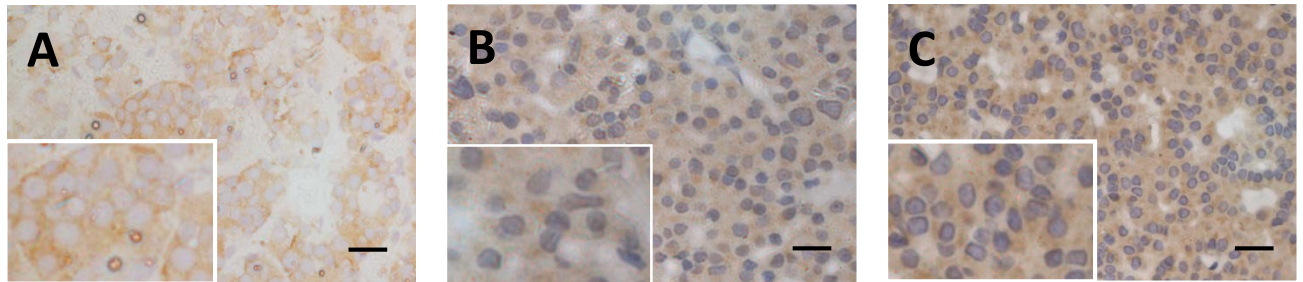
Immunochemistry staining using anti-CDH23 (1:100, Abcam, ab131135) showed that CDH23 protein presented in normal pituitary gland (A), pituitary adenomas with wild-type *CDH23* gene (B) and pituitary adenomas with mutant *CDH23* gene (C). Scale bar, 20 μm.

Figure S3. Family pedigrees of eight *CDH23* wild-type families with familial PA.

Family 5 Family 6 Family 7 Family 8

Family 9 Family 10 Family 11 Family 12
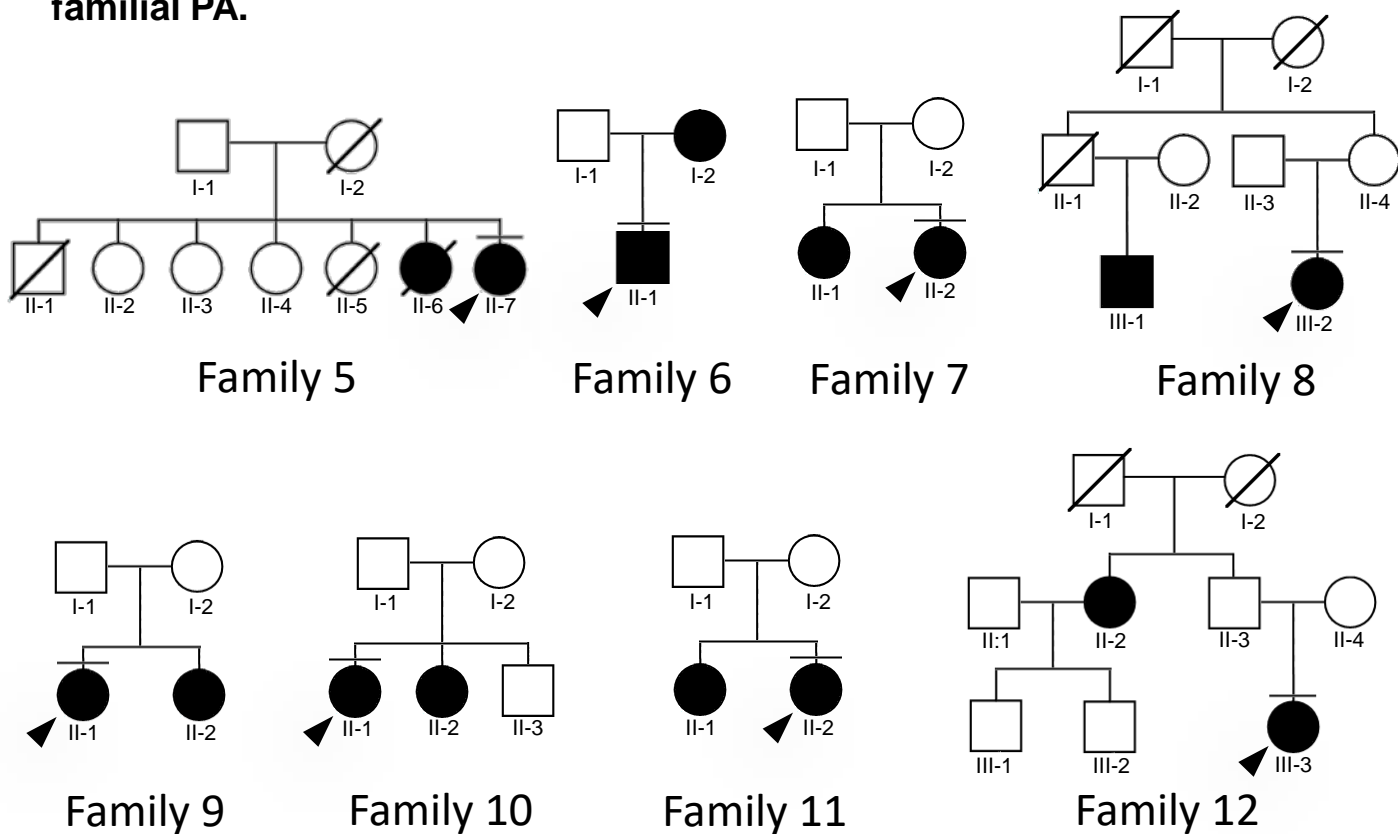
Another eight families with familial PA were included for further exome sequencing. None of these families carried mutant *CDH23*. Black filled shapes represented affected siblings. White shapes were asymptomatic subjects. The family members selected for exome sequencing were noted with a horizontal bar above their respective symbols.

Figure S4. MRI scan of patients and asymptomatic relevants in three families.
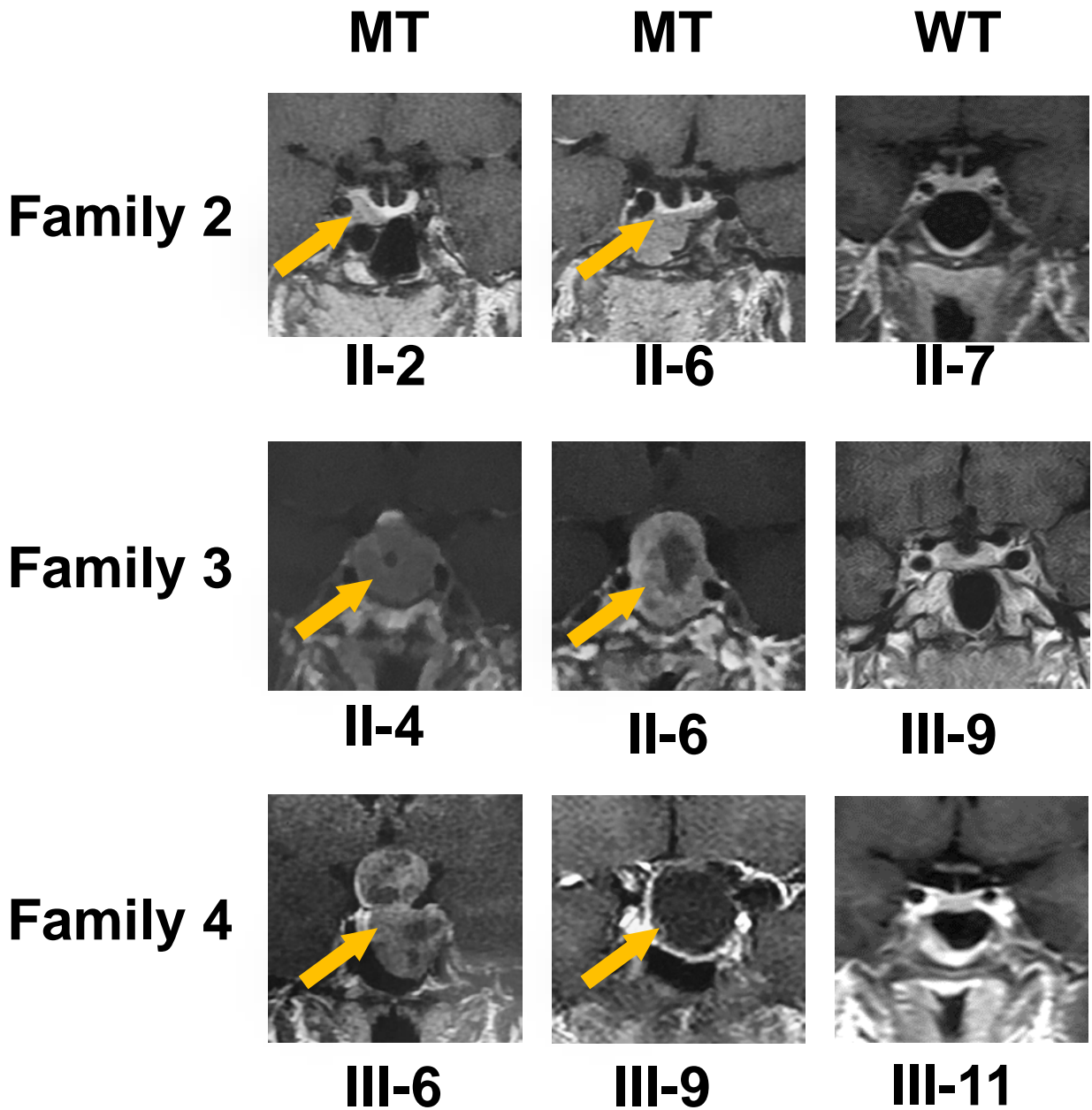
The MRI data of individuals with *CDH23* mutations (MT) were shown in the first two columns, while that of asymptomatic *CDH23* wild-type (WT) individuals were shown in the last column. Yellow arrows marked tumors.

Figure S5. Conservation of the alternative residues in CDH23 protein in three families.
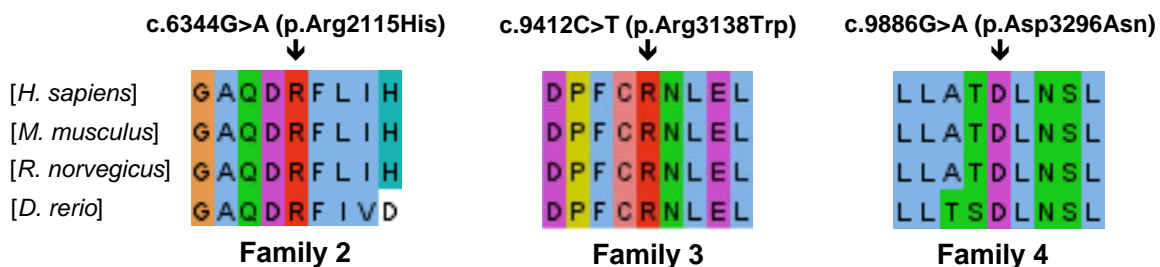
Three partial protein alignments of CDH23 showed evolutionary conservation at the site of the identified nucleic acid changes (noted by the black arrow).

Alignments of the CDH23 protein sequences were created using Clustal X, version 2, and checked visually using Jalview, version 2.7.

Table S2: The number of individuals carrying *CDH23* mutations in PA patients(cases) and normal controls

CDH23 Genotype	Pituitary adenoma		Normal controls
	Familial	Sporadic	
Mutant	4	15	2
Wild-type	8	110	258
Total	12	125	260

It should be notated that some of the sporadic cases might be masked familial cases because clinical phenotypes of their family members had not been manifested by the time of the diagnosis of these affected persons

Table S3: Comparison of clinical characteristics and *CDH23* genotypes of 145 PA cases

Charateristic		WT (n = 118)	Mutant (n = 27)	*P* value*
Gender				
	Male	68	11	0.113†
	Female	50	16	
Age at diagnosis (Year)				
	Median	40.00	41.00	0.759‡
	Interquartile range	29.75-52.25	31.00-57.00	
Clinical course (Month)				
	Median	24.00	18.00	0.772‡
	Interquartile range	6.00-60.00	6.00-36.00	
Diameter (cm)				
	Median	2.90	1.90	0.005‡
	Interquartile range	1.60-4.00	1.00-2.70	
Invasiveness#				
	Yes	72	5	<0.001†
	No	46	22	
Radical resection				
	Yes	85	18	0.509†
	No	28	4	

* *P*<0.05 was considered to be statistically significant.
† Values were compared by Pearson's χ2 test.
‡ Values were compared by Mann-Whitney U-test.
# Invasive adenomas were defined by fulfilling at least one of the following conditions:
    (1) Hardy's modified classification grade III or IV and/or stage C, D, and E;
  or (2) Knosp classification grade III and IV.
  Intra-operative findings and pathological examinations were also considered as evidence for the identification of invasive PAs

Table S4: Comparison of clinical characteristics and *CDH23* genotypes of 125 sporadic PA cases

Charateristic		WT (n = 110)	Mutant (n = 15)	*P* value*
Gender				
	Male	67	7	0.296†
	Female	43	8	
Age at diagnosis (Year)				
	Median	33.00	40.50	0.187‡
	Interquartile range	26.00-47.00	28.75-53.00	
Clinical course (Month)				
	Median	24.00	24.00	0.625‡
	Interquartile range	12.00-36.00	6.00-60.00	
Diameter (cm)				
	Median	2.90	1.90	0.046‡
	Interquartile range	1.68-4.10	1.00-2.20	
Invasiveness#				
	Yes	67	3	0.003†
	No	43	12	
Radical resection				
	Yes	82	12	0.649†
	No	28	3	

* *P*<0.05 was considered to be statistically significant.

† Values were compared by Pearson's χ2 test.

‡ Values were compared by Mann-Whitney U-test.

Invasive adenomas were defined by fulfilling at least one of the following conditions:

    (1) Hardy's modified classification grade III or IV and/or stage C, D, and E;

  or (2) Knosp classification grade III and IV.

  Intra-operative findings and pathological examinations were also considered as evidence for the identification of invasive PAs