Truncating Mutations in the Adhesion G Protein-Coupled Receptor G2 Gene *ADGRG2* Cause an X-Linked Congenital Bilateral Absence of Vas Deferens

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In 80% of infertile men with obstructive azoospermia caused by a congenital bilateral absence of the vas deferens (CBAVD), mutations are identified in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*). For the remaining 20%, the origin of the CBAVD is unknown. A large cohort of azoospermic men with CBAVD was retrospectively reassessed with more stringent selection criteria based on consistent clinical data, complete description of semen and reproductive excurrent ducts, extensive *CFTR* testing, and kidney ultrasound examination. To maximize the phenotypic prioritization, men with CBAVD and with unilateral renal agenesis were considered ineligible for the present study. We performed whole-exome sequencing on 12 *CFTR*-negative men with CBAVD and targeted sequencing on 14 additional individuals. We identified three protein-truncating hemizygous mutations, c.1545dupT (p.Glu516Ter), c.2845delT (p.Cys949AlafsTer81), and c.2002_2006delinsAGA (p.Leu668ArgfsTer21), in *ADGRG2*, encoding the epididymal- and efferent-ducts-specific adhesion G protein-coupled receptor G2, in four subjects, including two related individuals with X-linked transmission of their infertility. Previous studies have demonstrated that *Adgrg2*-knockout male mice develop obstructive infertility. Our study confirms the crucial role of *ADGRG2* in human male fertility and brings new insight into congenital obstructive azoospermia pathogenesis. In men with CBAVD who are *CFTR*-negative, *ADGRG2* testing could allow for appropriate genetic counseling with regard to the X-linked transmission of the molecular defect.

The prevalence of couple infertility is roughly 15% worldwide.[1] The global rate of infertile men ranges from 2.5% to 12% depending on the geographic area,[1] and at least 15% of these infertile men have either a non-obstructive azoospermia caused by spermatogenic failure (SPGF1 [MIM: 258150]) or an obstructive azoospermia resulting from congenital or acquired reproductive tract abnormality.[2] Congenital bilateral absence of vas deferens (CBAVD [MIM: 277180]) is found in more than 25% of men with obstructive azoospermia.[2] CBAVD involves a complete or partial defect of the Wolffian duct derivatives, the pathogenesis of which remains elusive. In most cases of CBAVD, it is generally presumed that the genital tract abnormality is due to a progressive atrophy related to abnormal electrolyte balance and fluid transport in the male excurrent ducts rather than agenesis.[3] This model is supported by the well-established link between CBAVD and mutations of the gene encoding the cystic fibrosis transmembrane conductance regulator (*CFTR* [MIM: 602421]) chloride channel.[4] *CFTR* is expressed at the apical membrane of epithelial cells lining several organs, including the male excurrent ducts, where it plays a major role in the regulation of anion transport and fluid secretion.[5] Mutations in *CFTR* are mainly associated with cystic fibrosis (CF [MIM: 219700]), the most common life-limiting autosomal-recessive disease in populations of European descent. Nearly all men with CF are infertile due to CBAVD. Furthermore, at least one *CFTR* mutation is found in 80% of men with CBAVD but without other clinical manifestations of CF.[6,7] For the remaining 20%, the origin of the CBAVD is unknown. The co-occurrence of a solitary kidney in a subset of these CBAVD-affected subjects without *CFTR* mutations suggests different pathophysiological mechanisms and genetic heterogeneity.[8]

In contrast with the various genetic disorders identified in non-obstructive azoospermia, *CFTR* is the only identified gene whose mutations lead to obstructive azoospermia in humans.[2,9] Genetic studies on human infertility have severe limitations given that traditional family-based approaches are unworkable in practice and because of the psychological and cultural factors surrounding male infertility. To overcome these difficulties in the search for new genetic causes of congenital obstructive azoospermia, we opted for an alternative approach, using whole-exome

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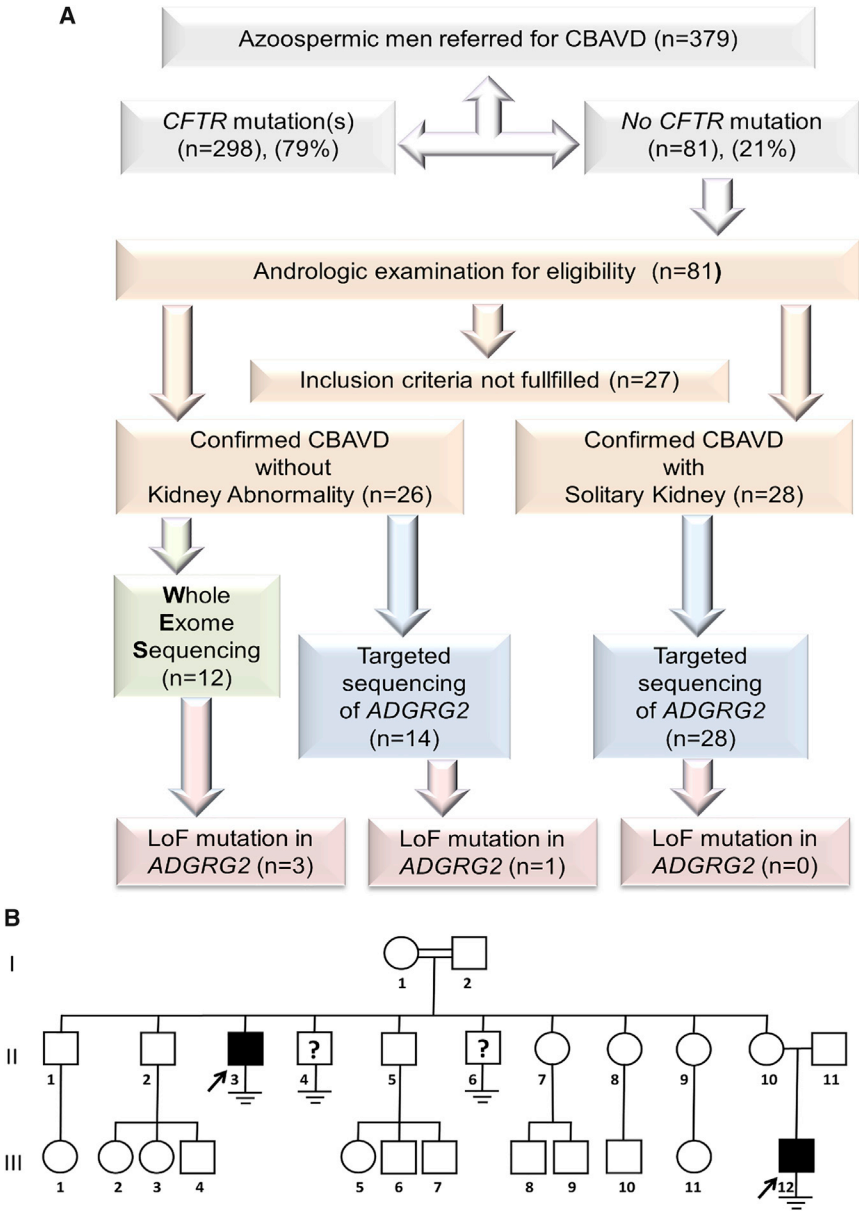

Figure 1. Study Flow Chart and Genealogic Tree of the Pedigree with Two Related Individuals with CBAVD

(A) Study flow chart showing the procedure for the identification of new male fertility genes from a cohort of azoospermic men with CBAVD. LoF, loss of function.

(B) Genealogic tree of the two related CBAVD-affected individuals included in the cohort (II-3 and III-12, arrow). ?, probable infertility.

sequencing (WES) data derived from massively parallel sequencing. The strategy, which consists of searching for causal private variants in a single gene by combining WES data from unrelated individuals sharing a similar phenotype, has demonstrated efficacy for several rare monogenic diseases.[10–12] In this study, we applied this so-called overlap strategy[10] to analyze exome data from a cohort of 12 well-phenotyped azoospermic men with CBAVD in whom extensive *CFTR* screening had failed to detect any mutations. Procedures for clinical screening and genetic investigation were in accordance with ethical standards and approved by the French Agency of Biomedicine (Agence de la Biomédecine).

As a first step, the past ten years of medical records of azoospermic men with CBAVD referred to the Centers for Male Fertility at the University Hospitals of Toulouse and Lille were retrospectively reassessed. A standardized description form was designed by experts in clinical andrology to gather consistent phenotypic data and to provide a complete description of semen characteristics and excurrent ducts for each individual[13] (Tables S1 and S2). Selected subjects who fulfilled all CBAVD criteria were then divided into two groups on the basis of the presence or absence of associated kidney malformations (Figure 1A and Table S3). From the retrospective series of 379 infertile men referred for CBAVD, 81 (21%) were without *CFTR* mutations, despite a genetic testing whereby the 27 exons were scanned for point mutations and large rearrangements. Among these, 26 met all of the inclusion criteria for CBAVD and had no renal malformation. 12 of these individuals were selected for WES (Figure 1A and Table S1). With the exception of two (a nephew and his maternal uncle, II3 and III12, in Figure 1B) all subjects included in the study were unrelated. Genomic DNA was isolated from the blood after each subject provided proper informed consent for the exploration of the genetic cause of their infertility. Library preparation, sequencing, variant detection, and annotation were performed by IntegraGen. Exons were captured with Agilent's SureSelect All Exons V5 50 Mb, followed by sequencing on an Illumina HiSeq 2000 with paired-end 75-bp reads. Image analysis and base calling were performed with Illumina's RTA 2.1.3 and CASAVA 1.8.2 pipeline software. Sequence reads were aligned to the NCBI Genome browser (hg19) reference sequence with the ELANDv2 algorithm. The mean 10× coverage for the 12 exome-sequenced samples was more than 94%, with a mean depth of 75×. On average, WES identified 2,897 indels and 36240 SNVs per individual. The IntegraGen in-house ERIS pipeline (see Web Resources) was used for annotation of the variants (SNVs and indels) with reference to public databases (dbSNP, 1000 Genomes, NHLBI Exome Sequencing Project Exome Variant Server)

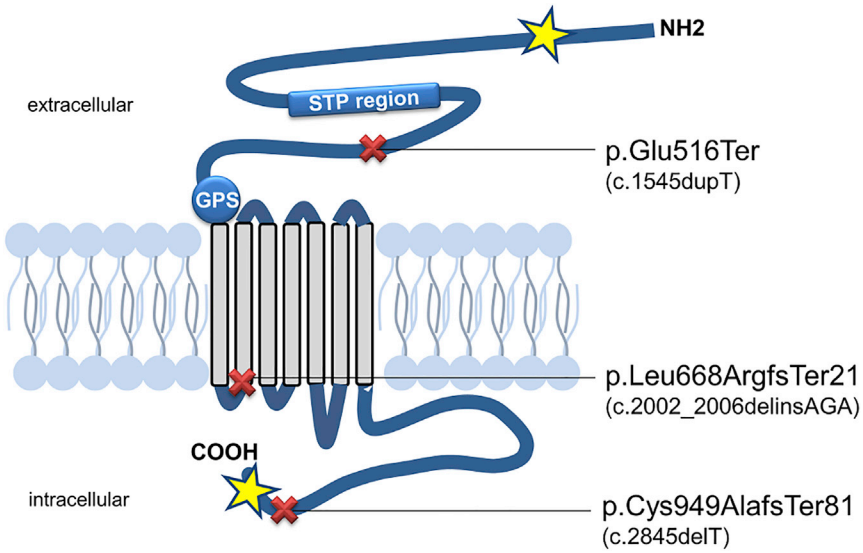

Figure 2. Schematic Representation of ADGRG2 Structure with the Position of the Amino Acid Changes Resulting from the Identified Truncating Mutations
Like the other members of the adhesion-GPCR family, ADGRG2 is composed of a seven-transmembrane domain and a highly conserved cystein-rich motif, named the GAIN domain, containing GPS, which is the site of autoproteolytic cleavage. The ADGRG2 extracellular domain contains a serine-threonine-proline-rich region (STP). Positions of C-terminal and N-terminal epitopes recognized by the populations of polyclonal antibodies used for immunohistochemistry are indicated by yellow stars.

and the IntegraGen in-house WES database. After variant filtration by exclusion of those reported as common (i.e, minor allele frequency > 0.01) or synonymous, we combined the data from all exomes of the cohort to retain candidate genes with variants in at least two unrelated subjects, in accordance to the so-called overlap strategy.[10] We then focused on nonsense, frameshift, or canonic splice-site variants, hereafter referred to as presumed loss-of-function (LoF) mutations, and on genes highly or specifically expressed in the male reproductive organs according to the Human Protein Atlas.[14] The most promising variants were then prioritized based on functional data concerning the candidate gene from the literature review and without excluding any mode of Mendelian inheritance. With this restrictive approach, the overlap for a number of three subjects or more pointed to only five candidate genes for the autosomal-recessive model, two for the X-linked model, and six for the autosomal-dominant model (Tables S4–S8). Among all these 13 candidate genes, *ADGRG2* (MIM: 300572 [GenBank: NM_001079858.2]) was the only one whose expression was restricted to the excurrent ducts of the male reproductive tract.[15,16] Two different *ADGRG2* hemizygous frameshift mutations were identified: c.2845delT (p.Cys949AlafsTer81) in one subject (L5 in Table S3) and c.2002_2006delinsAGA (p.Leu668ArgfsTer21) in two maternally-related individuals (II-3 and III-12 in Figure 1B, T3 and T4 in Table S3), demonstrating an X-linked transmission (Figures 1B and 2). One of these two related men (II-3 in Figure 1B, T3 in Table S3) had had a right-sided surgical epididymal sperm extraction, including a head-epididymal biopsy, for assisted reproductive technologies; therefore histological analysis was available for this CBAVD-affected subject. Immunohistochemical analysis was performed on sections of epididymal and efferent ducts obtained from paraffin-embedded biopsies of the proband carrying the *ADGRG2* truncating mutation and of an azoospermic control individual who had undergone a scrotal exploration with testicular biopsy demonstrating spermatid maturation arrest. Slides were deparaffinized, rehydrated, and pretreated for 20 min at 94°C in Target-Retrieval Solution, High pH (Dako). Primary rabbit polyclonal antibodies (Abcam) directed against the C-terminal (ab188908, dilution 1/100) and N-terminal (ab198032, dilution 1/50) parts of human ADGRG2 were incubated for 30 min at room temperature. Staining was visualized with the Envision FLEX/HRP system and diaminobenzidine (Dako), followed by hematoxylin counterstain. Non-specific staining was systematically evaluated by the use of a negative reagent control without primary antibodies. Histological examination of the biopsy from the proband revealed enlarged sections of the epididymal head containing spermatic material, lined by a cuboidal epithelium as a result of sperm stasis (Figure 3B). Some sections of the efferent ducts also appeared dilated with spermatozoa in the lumen (data not shown), whereas other sections appeared normal. With the anti-N-terminal antibody, we observed apical staining both in the control subject and the CBAVD-affected subject, whose mutation predicted a C-terminal truncated ADGRG2. We hypothesize that this expression was the result of escape from nonsense-mediated decay of the mutated mRNA. By contrast, with the anti-C-terminal antibody, no ADGRG2 staining was detected in any tissue sections from the CBAVD-affected subject, whereas staining was present in the control subject (Figure 3). Interestingly, in the efferent ducts sections of the control subject, we observed more staining with the N-terminal antibody, particularly outside of the crypt-like grooves, than with the C-terminal antibody, raising the question of whether this finding reflects different maturation processes of the N-terminal and the C-terminal fragments occurring in efferent ducts or whether its origin is artifactual. Further studies are required to assess this question properly.

These findings prompted us to analyze *ADGRG2* by targeted sequencing in the remaining subjects of the cohort not selected for WES. For this purpose, we

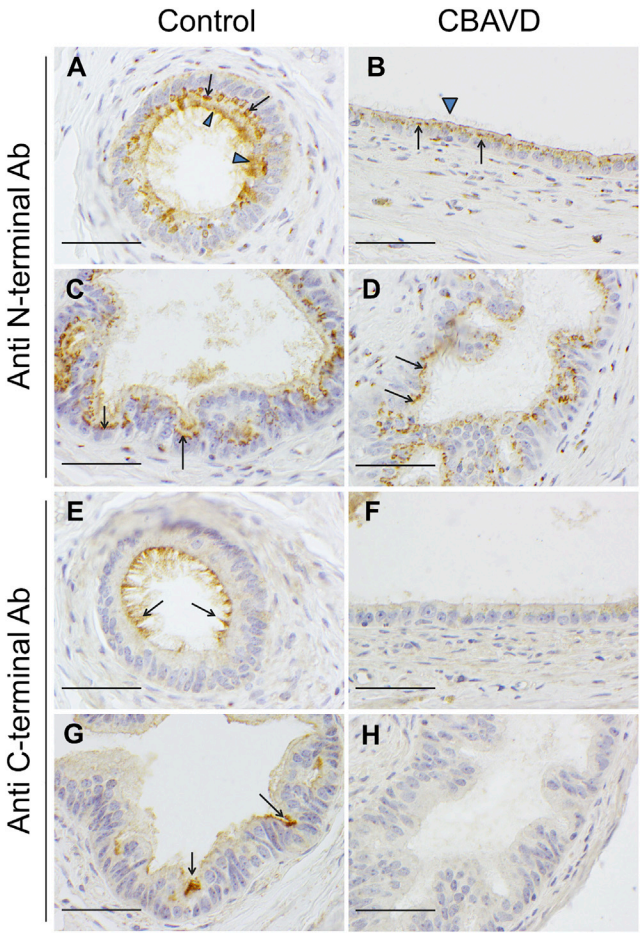

Figure 3. Immunohistochemical Staining of *ADGRG2* in Sections of Epididymal and Efferent Ducts Obtained from a Control Individual with Non-obstructive Azoospermia and from the CBAVD-Affected Proband Carrying the p.Leu668Argfs*21 *ADGRG2* Truncating Variant

With the antibody directed against the N-terminal portion of *ADGRG2*, subapical staining (arrows) was observed on the control epididymal (A) and efferent (C) ducts. The same staining was observed on sections from the proband, respectively (B and D). In the control subject, staining of stereocilia and apical membrane is observed on the epididymis section (A) (arrowhead), whereas stereocilia are not stained in the proband (B) (arrowhead). With the antibody directed against the C-terminal portion of *ADGRG2*, positive staining was observed on the control subject at the apical level and stereocilia of cells from the epididymis (E) (arrows) or at the apical level of efferent ducts, especially in crypt-like grooves (G) (arrows). In contrast, in the CBAVD-affected proband whose mutation is predicted to delete the *ADGRG2* C-terminal region, such staining was not observed on the epididymal (F) or efferent (H) ducts. Scale bars indicate 50 μm.

performed in-house PCRs for each coding exon, followed by sequencing of the barcode-tagged amplicons on a MiSeq sequencer (Illumina) with a Nano flow cell and 2 × 250 v.2 chemistry. Results were analyzed with the bioinformatic pipelines MiSeq Reporter v.2.3.32 and SeqNext v.4.1.2. An additional *ADGRG2* truncating mutation, c.1545dupT (p.Glu516Ter), was identified in one of the 14 subjects with CBAVD without renal involvement. In contrast, no mutations were found in the *ADGRG2* sequence of 28 CBAVD-affected men with associated solitary kidney, supporting the assumption that the association of CBAVD and unilateral renal agenesis results from a different pathophysiological mechanism.[8] Identification of the three *ADGRG2* truncating mutations (Figure 2) was confirmed by Sanger sequencing. These mutations were absent from any public databases, including the Exome Aggregation Consortium (ExAC), in which only 7 out of 40,000 individuals were found with a LoF variant. Based on the difference between the number of expected and observed LoF variants, *ADGRG2* is predicted in ExAC as extremely intolerant toward LoF variants,[17] which is consistent with X-linked male infertility (see Web Resources).

ADGRG2 (previously named *GPR64, or HE6*)[15] encodes for the adhesion G protein-coupled receptor G2, one of the 33 members of the adhesion-class G protein-coupled receptors (adhesion-GPCRs), which is the second largest GPCR subfamily in humans.[18] Adhesion-GPCRs are believed to play essential physiological roles in a variety of organ systems, but their cellular functions and signaling mechanisms are poorly understood. These highly conserved receptors are tightly regulated and most of them have a cell-type- or tissue-restricted pattern of expression, making them promising targets for novel therapies.[19] ADGRG2 is specifically expressed within the efferent ducts,[15] in which most of the testicular fluid carrying immature spermatozoa is reabsorbed.[20] The involvement of ADGRG2 in the regulation of this fluid reabsorption process was strongly suggested in mice by the pathophysiological effect of the targeted *Adgrg2* deletion. Indeed, the mutant mice develop fluid accumulation in the testis with stasis of spermatozoa within the efferent ducts, leading to an obstructive infertility phenotype.[21] In view of this, ADGRG2 was identified as a potential post-testicular target for future male contraceptives.[22,23] Similarly, the targeted disruption of *Adgrf5*, another adhesion-GPCR expressed in alveolar type II pneumocytes, results in a marked dysregulation of fluid homeostasis with accumulation of abnormal surfactant rich in lipids and proteins, reminiscent of alveolar proteinosis.[24]

Interestingly, the three private *ADGRG2* truncating mutations reported here were found in azoospermic men phenotypically indistinguishable from those with CBAVD caused by *CFTR* mutations, suggesting that mutations of these two genes might result in obstructive azoospermia through a similar pathophysiological mechanism. Like ADGRG2, the CFTR chloride channel is expressed in efferent ducts at the apical edge of the principal non-ciliated cells, responsible for major fluid reabsorption in this organ.[20,25] Moreover, CFTR is recognized as the hub of a complex protein-protein network implicated in transepithelial electrolyte transport, fluid secretion, and pH regulation.[25] The cAMP-dependent protein kinases A and C regulate the activation of CFTR, which interacts via its PDZ domain with several intracellular proteins, including, notably, the apically co-localized Na(+)/H(+) exchanger

regulating factor 1 (NHERF1 [MIM: 604990]).[26] This prominent partner contributes, along with ezrin activation, to maintaining CFTR at the cell membrane by tethering it to the actin cytoskeleton.[27] NHERF1 is also involved in cAMP regulation of other ion transporters, and it interacts with aquaporin 9, believed to actively participate in water and solute transport in the male excurrent ducts.[28]

ADGRG2, like the majority of adhesion-GPCRs, is still an orphan receptor regarding its endogenous agonist and signal transduction. This seven-transmembrane domain receptor has a long extracellular N-terminal structure that contains multiple glycosylated mucin-like domains and a GPCR proteolysis site (GPS) within the highly conserved GPCR autoproteolysis inducing (GAIN) domain (Figure 2). It is assumed that the cleavage of the N-terminal fragment modulates the activity of adhesion-GPCRs. Recently published experimental evidence suggests that ADGRG2 is able to activate G-protein-mediated signal transduction through a tethered agonist sequence located immediately downstream of the GPS domain.[23] On the other hand, findings from studies on some other adhesion-GPCRs, such as GPR126 (MIM: 612243), indicate that N-terminal and C-terminal fragments resulting from autoproteolysis might have separate functions.[29] Thus, with regard to the residual expression of the N-terminal fragment observed in the subject with a truncating mutation in the first transmembrane domain (Figure 3), we cannot rule out the hypothesis of a gain of function consisting in a more active or constitutively activated N-terminal fragment of the protein. The c.1545dupT (p.Glu516Ter) mutation, which is thought to leave only two-thirds of the N-terminal fragment of ADGRG2, could also fit this model of pathogenicity. However, the three reported mutations that spread all over the gene, predicting premature truncations in both the N-terminal and the C-terminal fragments of ADGRG2, have an identical pathophysiological impact. Moreover, similarities between the excurrent ducts' obstruction phenotype of men bearing these mutations and that of *Adgrg2*-knockout mice strongly support the hypothesis that the identified truncating mutations lead to ADGRG2 activity disruption, and therefore to a loss of function rather than a gain of function.

Studies in the last decade indicate that the various physiological functions of adhesion-GPCR result from distinct modes of signaling pathway activation and from multiple extra- and intracellular interaction partners.[18,30] Thus, inactivation of *ADGRG2* might indirectly affect CFTR activity and thereby affect luminal fluid homeostasis in the excurrent ducts through some of the already reported adhesion-GPCR cellular functions such as cytoskeletal organization, cAMP level regulation,[31] or recruitment of PDZ-domain-containing proteins such as NHERF1.[19,32] Functional studies are needed to further explore the potential links between CFTR and ADGRG2.

Lastly, as in all eutherian species, human *ADGRG2* is located on the X chromosome (non-pseudoautosomal region), and therefore mutations in this gene that cause male infertility are maternally transmitted as we have shown in the reported pedigree. Thus, *ADGRG2* testing in *CFTR*-negative CBAVD-affected individuals could allow for appropriate genetic counseling for infertile men with this type of obstructive azoospermia. In summary, our findings confirm the pivotal role of *ADGRG2* in male fertility and provide new insight into CBAVD pathogenesis.

Accession Numbers

The accession numbers for the DNA variant data reported in this paper are ClinVar: SCV000268120, SCV000268121, and SCV000268122

Supplemental Data

Supplemental Data include eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.06.012.

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Web Resources

1000 Genomes, http://www.1000genomes.org
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
ERIS, http://eris.integragen.com/
ExAC Browser, http://exac.broadinstitute.org/
NCBI Genome, http://www.ncbi.nlm.nih.gov/genome/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
The Human Protein Atlas, http://www.proteinatlas.org/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq

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