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Expression profiles of NPHP1 in the germ cells in the semen of men with male factor infertility

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SUMMARY

NPHP1, the gene that encodes the protein nephrocystin-1 has been identified to be mutated in Juvenile Nephronophthisis, an autosomal recessive cystic kidney disorder which is the most frequent genetic cause of end-stage renal disease (ESRD) in children and young adults. *Nphp1*-targeted mutant mice studies have shown that it did not express renal manifestations of nephronophthisis; instead male mice were infertile with oligoteratozoospermia signifying the crucial role of *Nphp1* in relation to murine spermatogenesis. Whether an aberrant expression of NPHP1 in testis might lead to spermatogenic defects in human and ultimately male factor infertility is a possibility that have not been investigated so far. In this regard, characterization of NPHP1 in spermatozoa from fertile and infertile males was carried out by employing RT-PCR, western blotting, and immunofluorescence analysis. In all the 23 normozoospermic samples we screened, NPHP1 was significantly expressed at the target message and protein level and also prominent localization pattern of NPHP1 was observed at the head, midpiece, and tail segments of spermatozoa. Conversely, in majority of the 103 infertile samples we screened, aberrant pattern of NPHP1 expression was detected at the transcript and protein level and abnormal localization pattern of expression was observed in spermatozoa. Anomalies detected in infertile cases when compared with the normozoospermic controls points to the indispensable role of NPHP1 in relation to spermatogenesis. Thus, besides the decisive association with juvenile nephronophthisis, our study provides the first direct evidence that NPHP1 is associated with male factor infertility and also could be a possible biomarker for the assessment of male fertility status.

INTRODUCTION

Infertility is a major global health issue in which male factor infertility accounts for 30–55% of the infertile cases (Hamada *et al.*, 2013). Approximately, 30–40% of male infertile cases are diagnosed as idiopathic with abnormal seminal parameters but with normal physical and endocrine analysis results (Dohle *et al.*, 2012). Although the etiology behind idiopathic male infertility is clearly not defined till now, nevertheless, chromosomal abnormalities, gene mutations, genetic polymorphisms, and epigenetic errors in spermatogenesis are suggested to be the underlying causes of the idiopathic cases of male infertility (Ferlin *et al.*, 2007; O'Flynn O'Brien *et al.*, 2010; Boissonnas *et al.*, 2013). Furthermore, 6–27% of male factor infertility cases are classified as unexplained male infertility (UMI) when routine seminal analysis results are normal and physical, endocrine abnormalities and female factor infertility were ruled out (Hamada *et al.*, 2012; Esteves *et al.*, 2014). Low-level leukocytospermia, presence of antisperm antibodies, mitochondrial DNA polymerase gene polymorphism, sub-cellular sperm dysfunctions, and fertilization defects are recognized to be the etiologies in men diagnosed with UMI (Hamada *et al.*, 2012; Esteves, 2013).

The complete process of male germ cell development is synchronized by interactions between multitude of genes and signaling pathways which exemplifies the intricate nature of spermatogenesis and therefore alteration from the normal process at any stage could lead to aberrant spermatogenesis resulting in male infertility (Matzuk & Lamb, 2008). Aberrant expression of numerous genes including VASA (Guo *et al.*, 2007), Spermatogenesis Associated protein 16 (*SPATA 16*; Dam *et al.*, 2007), Ropporin (Chen *et al.*, 2011), brain-derived neurotrophic factor (Zheng *et al.*, 2011), methylenetetrahydrofolate reductase (Wu *et al.*, 2010), etc., have been implicated in human male infertility.

Juvenile nephronophthisis (nephronophthisis type 1, NPH) is a genetically heterogenous, autosomal recessive cystic kidney disorder and is the most frequent genetic cause of ESRD in children and young adults (Hildebrandt *et al.*, 1997; Jiang *et al.*, 2008). Mutations in 12 variants of NPHP (NPHP1-12) have been associated with nephronophthisis. NPHP1 mutations account for 20% of all cases of nephronophthisis (Hurd & Hildebrandt, 2011; Seeger-Nukpezah *et al.*, 2012). A prominent expression of nephrocystin-1 was observed in the meiotic and post-meiotic germ cells in mouse testis, implicating its role in male germ cell development (Otto *et al.*, 2000). *Nphp1*-targeted mutant mice did not express renal manifestations of nephronophthisis, instead male mice were infertile with oligoteratozoospermia suggesting the crucial role of *Nphp1* in relation to murine spermatogenesis (Jiang *et al.*, 2008). *Nphp4*-targeted mutant mice also did not express nephronophthisis, instead exhibited severe photo receptor degeneration and male infertility (Won *et al.*, 2011). Steroidogenic and spermatogenic impairments induced by ESRD raise the possibility of defective spermatogenesis in human nephronophthisis as a secondary defect (Palmer, 2003; Jiang *et al.*, 2008) besides the extra renal manifestations associated with nephronophthisis including cerebellar vermis hypoplasia, hepatic fibrosis, situs inversus, and skeletal defects (Simms *et al.*, 2011). Although not investigated, the possibility of direct disruption of NPHP1 which leads to impaired spermatogenesis in human have also been suggested (Jiang *et al.*, 2008). A recent study reported that human subjects with homozygous truncating mutation in NPHP4 had cerebello-oculo-renal syndrome and male infertility (Alazami *et al.*, 2014). Taking all this into account, whether an aberrant expression of NPHP1 might lead to spermatogenic defects in human and ultimately male infertility is thus a pre-requisite that needs to be investigated.

In this study, we investigated whether aberrant expression of NPHP1 in spermatogenic cells is associated with male factor infertility by conducting expression profiling of NPHP1 in germ cells from the semen of fertile and infertile males. We report that aberrant expression of NPHP1 in ejaculated spermatozoa is associated with male factor infertility. We propose that NPHP1 could be a possible biomarker for the diagnosis with male infertility.

MATERIALS AND METHODS

Reagents and antibodies

Ready-To-Go T-Prime first strand cDNA synthesis kit, deoxy nucleotide triphosphates (dNTPs), PCR DNA and Gel Purification kit and Amersham Hybond-PVDF Membrane (GE Healthcare, Piscataway, NJ, USA); Taq DNA polymerase, 100 bp and 1 kb ladder (New England Biolabs, Ipswich, MA, USA), TRI reagent, propidium iodide, 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), ethylene glycol tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), phenyl methane sulfonyl fluoride (PMSF), Tris–HCl, Tween-20, Triton-X-100, sodium orthovanadate, glycerol, magnesium chloride ($MgCl_2$), agarose, ethidium bromide (EtBr), bovine serum albumin (BSA), acrylamide, bis acrylamide ammonium persulfate, ß-mercaptoethanol, and poly-L lysine (Sigma-Aldrich, St. Louis, MO, USA); Complete Mini EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA), Rabbit polyclonal anti-nephrocystin antibody (SC-33592), Goat polyclonal β-actin antibody, Goat anti-rabbit HRP antibody, and Donkey anti-goat HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Alexa Fluor 488 Rabbit IgG (H + L) antibody (Life Technologies, Carlsbad, CA, USA) and Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), Precision marker (Bio-Rad Laboratories, Hercules, CA, USA), and Nickel chloride (NiCl2) (SR Laboratories, Mumbai, India) were procured.

Subjects and methods

This study was approved by the human ethics committee of Rajiv Gandhi Centre for Biotechnology, Trivandrum. Subjects selected for the study program were recruited after obtaining their written informed and understood consent. Men who have fathered a child during the past 1 year and had normal seminogram (semen parameters falling between the 5th and 95th percentile values) as per WHO manual 2010 (Cooper *et al.*, 2010) were considered as fertile men. Men who could not help establish a pregnancy despite being sexually active during the past 1 year with a partner with no clinical indication of female factor infertility were recruited as infertile men. They were visiting either Samad IVF Hospital or KJK Infertility hospital, Thiruvananthapuram, India and were diagnosed with male factor infertility. Male partners of couples with either of them having urogenital tract infections or sexually transmitted diseases were excluded from this study. Infertile subjects were categorized according to the lower reference limits (5th centiles and 95% confidence intervals) as oligozoospermia (O), oligoasthenozoospermia (OA), oligoteratozoospermia (OT), oligoasthenoteratozoospermia (OAT), asthenozoospermia (A) asthenoteratozoospermia (AT), and teratozoospermia (T). The subjects were categorized as having UMI (U) when they were infertile although the routine semen analysis results were normal and physical and endocrine abnormalities were ruled out. In this study, 23 normal fertile males and 103 infertile patients were included. Mean values of semen parameters of the normal and infertile subjects (Table 1) and a detailed subject-wise listing of the semen analysis report (Table S1) are provided.

Semen collection and processing

Semen was collected by masturbation after 3 days of sexual abstinence. Semen was allowed to liquefy for 30–60 min at 37 °C and was analyzed on SQA-V Sperm Quality Analyzer using V-Sperm III Software (Media Electronic Systems, Perchtoldsdorf, Austria), following the WHO manual, 5th edition (Cooper *et al.*, 2010). The liquefied semen was diluted with an equal volume of sterile phosphate-buffered saline (PBS) and was layered over a 30–100% Percoll gradient. After centrifugation at 10 000 ***g*** for 30 min, immature spermatogenic cells were harvested from the 30–40% fractions. The fractions below 65% Percoll contained spermatozoa. These fractions were recovered and diluted with 10 mL PBS followed by centrifugation at 3000 ***g*** for 10 min at 37 °C. The pellet obtained was resuspended in 2 mL PBS and the sperm suspension was split apart for RNA isolation, protein extraction, and immunofluorescence analysis. The sperm suspension was then centrifuged at 800 ***g*** for 10 min at 37 °C. After centrifugation, the pellet obtained was stored at −80 °C for RNA and protein extraction and 100 μL of the resuspended sperm cells was used for immunofluorescence analysis.

Table 1 Mean values of the seminal parameters of the samples included in the study

Samples	Count ($\times 10^6$ cells/mL)	Motility (%)	Normal forms (%)	Head defect (%)	Neck defect (%)	Tail defect (%)
N	55.52 ± 10.67	54.91 ± 5.86	17 ± 4.95	39.08 ± 5.68	9.39 ± 2.79	34.56 ± 6.08
O	4.2 ± 4.11	50.33 ± 8.52	8 ± 2	49.33 ± 2.31	14 ± 3.46	28.67 ± 4.16
OA	5.93 ± 4.62	22.84 ± 9.45	17.46 ± 8.89	37.68 ± 11.17	21.62 ± 12.89	23.93 ± 8.09
OT	5.10 ± 2.68	51 ± 12.72	2.50 ± 0.70	48 ± 0	9 ± 1.41	39 ± 1.41
OAT	4.65 ± 0.91	27.5 ± 3.53	3 ± 1.41	40 ± 2.82	12 ± 5.65	44 ± 8.48
A	44.55 ± 22.24	30.66 ± 10.77	15.88 ± 11.75	38.27 ± 14.96	18.33 ± 9.92	26.22 ± 8.88
AT	43.14 ± 15.72	19.85 ± 13.18	2.8 ± 0.37	53.14 ± 3.23	14.28 ± 4.19	29.71 ± 4.02
T	16.50 ± 0.70	60.50 ± 6.36	3 ± 0	50 ± 2.82	10 ± 2.84	36 ± 5.65
U	75.30 ± 40.87	52.43 ± 8.04	15.82 ± 9.48	39.04 ± 12.22	15.15 ± 6.64	28.74 ± 6.30

Data shown are the mean ± SD. N, normozoospermia, O, oligozoospermia; OA, oligoasthenozoospermia; OT, oligoteratozoospermia; OAT, oligoasthenoteratozoospermia; A, asthenozoospermia; AT, asthenoteratozoospermia; T, teratozoospermia; U, unexplained male infertility.

RT-PCR analysis

Total RNA from immature germ cells collected from the semen was extracted using TRIzol reagent following the protocol recommended by the manufacturer (Sigma Chemical Company, Milwaukee, WI, USA). Five micrograms of total RNA was reverse transcribed using Ready-To-Go T-Prime first strand synthesis kit (Amersham Biosciences, Piscataway, NJ, USA) and the cDNA was further amplified by PCR with the primers designed using Primer3 Software. PCR primers for *NPHP1* corresponding to the partial coding sequence of *NPHP1* NM_000272.3 (565 bp), *ACTB* (419 bp), and *GAPDH* (79 bp) were designed using Primer3 Software (http://bioinfo.ut.ee/primer3-0.4.0/) and were synthesized (Sigma-Aldrich Corporation, Bangalore, India). The sequences of primers used in this study are listed (Table S2). *ACTB* and *GAPDH* served as internal controls. PCR reaction was performed in 22 μL reaction volume containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 units Taq polymerase, 1 μg cDNA and 1 μM each forward and reverse primers. Thermal cycling conditions were as follows: initial denaturation step of 2 min at 94 °C followed by 35 cycles of 94 °C at 30 s, 58 °C for 45 s, 72 °C for 1.30 min, and at 72 °C for 10 min in an ABI Gene-AMP PCR system 9700 (Applied Bio Systems, Carlsbad, CA, USA). The PCR products were analyzed by electrophoresis at 100 V constant voltage on 2.0% agarose gels containing 0.5 μg/mL EtBr. The gels were imaged on a Versa Doc MP-3000 system with Quantity One Software (Bio-Rad). The band intensities of proteins were quantified using the Phoretix Gel Analysis Software Version (Phoretix International, Newcastle upon Tyne, UK). The band intensities of *NPHP1* were normalized to the *ACTB* or *GAPDH* levels. Automated sequencing based on Dye termination reaction was performed in an ABI Gene-AMP PCR system 9700 (Applied Biosystems) in which an initial denaturation at 94 °C for 2 min was followed by 25 cycles of 94 °C for 15 s, 60 °C for 15 s and a final extension at 60 °C for 4 min. The dye terminated products were precipitated and sequenced on Applied Biosystem 3730 48-capillary automated DNA sequencer (Applied Biosystems). The sequences were analyzed using NCBI nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) followed by ClustalW alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Western blot analysis

The spermatozoa were resuspended in lysis buffer (100 mM Tris–HCl, 2% SDS, 0.05% CHAPS, 10% glycerol, 1 mm PMSF, 1 mM EGTA, protease inhibitor cocktail (1 : 100), and 1 mM sodium orthovanadate), homogenized at 5000 ***g*** using a Polytron homogenizer, (Kinematica GMBH, Munstertaler, Germany) followed by centrifugation at 9000 ***g*** for 10 min at 4 °C. The supernatant fraction containing the protein extract was collected, denatured at 95 °C for 5 min and then separated at a constant voltage (100 V) on 12% SDS-PAGE gels. Following electrophoresis, the separated proteins were electroblotted onto PVDF membranes. The membranes were blocked with 3% gelatine in PBS containing 0.1% (v/v) Tween-20 (PBST), washed with PBST and were subsequently incubated in PBST containing anti-nephrocystin primary antibody (1 : 1000 dilution) for 2 h at room temperature. The membranes were washed with PBST followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody in PBST (1 : 2000) for 1 h at room temperature. Beta-actin was used as the internal control for which the membranes were probed with goat polyclonal anti-actin antibody (1 : 1000) and then by incubation with HRP-conjugated rabbit anti-goat secondary antibody (1 : 2000), both diluted in PBST. A negative control was run probing the membrane with secondary antibody alone. The membranes were developed using 0.05% diaminobenzidine (DAB), 0.1% hydrogen peroxide, and 0.1% nickel chloride in 50 mM Tris–HCl until the desired contrast was obtained. The membranes were imaged on a Versa Doc MP-5000 imager with the Quantity One Software (Bio-Rad). The band intensities of proteins were quantified using the Phoretix Gel Analysis Software Version (Phoretix International, Newcastle upon Tyne, UK). The levels of NPHP1 were normalized corresponding to the ACTIN levels.

Immunocytochemistry

Spermatozoa smeared on the surface of poly-L-lysine coated cover slips were fixed with 4% paraformaldehyde for 15 min at room temperature. The sperm cells were then permeabilized with 0.1% Triton-X-100 for 10 min and blocked for 30 min in 1% BSA. Following PBS wash, the cells were incubated with rabbit polyclonal anti-nephrocystin primary antibody (1 : 200) overnight at 4 °C. The cells were then washed with PBS and probed with Alexa Fluor 488 Rabbit IgG (H + L) secondary antibody (1 : 100) for 1 h. Nuclei were subsequently counter-stained with propidium iodide at a final concentration of 0.50 μg/mL for 7 min. The cover slips were washed in PBS, dried and mounted on a glass slide using 60% glycerol in PBS and the cells were imaged on Nikon A1R Confocal Microscope using NIS Elements AR 4.00.04 Software (Nikon Instruments, Shizuoka-Ken, Japan).

SMART analysis

The full-length sequence of NPHP1 (accession No. NM_000272.3) was submitted to SMART server (http://

smart.embl-heidelberg.de/) and a domain structure analysis was performed choosing the options outlier homologues, PFAM domains, signal peptides, and internal repeats.

Statistical analysis

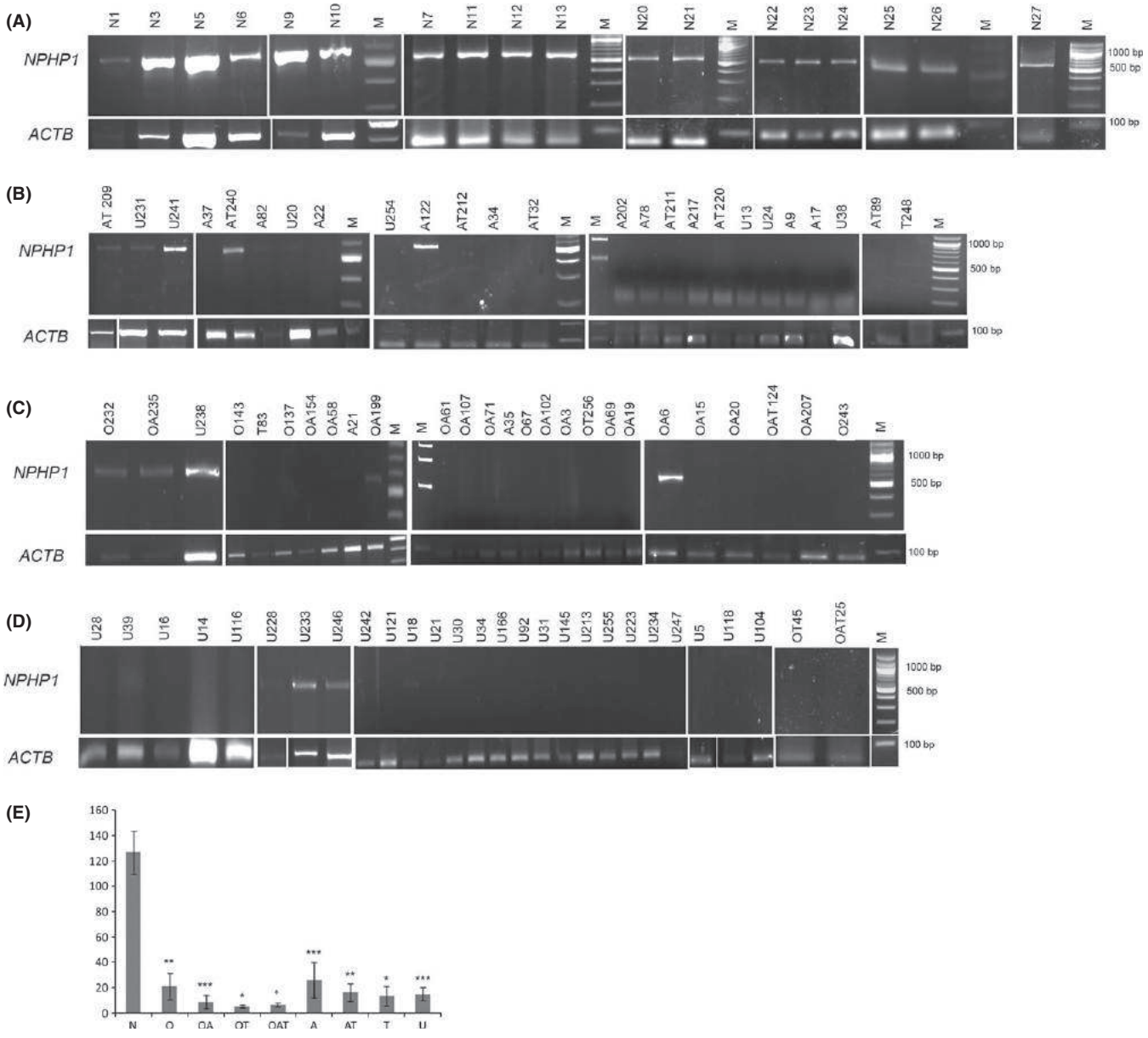
Reverse-transcriptase PCR and western blot data are expressed as mean ± SD of the normalized band intensities from subjects of various categories as mentioned earlier. The differences in NPHP1 expression between normozoospermic and infertile cases included in the study were examined using two-tailed Student's *t*-test and $p < 0.05$ was considered statistically significant.

RESULTS

RT-PCR analysis

Reverse-transcriptase PCR with the primers designed to amplify the partial CDS of *NPHP1* detected a band at the expected 565 bp position indicating the presence of *NPHP1* transcripts in the spermatogenic cells from all the 18 normozoospermic men screened (Fig. 1A). The authenticity of *NPHP1* was confirmed by automated dideoxy sequencing followed by BLAST analysis and ClustalW alignment (Figure S2). Of the infertile cases screened, one oligozoospermic (O232), three oligoasthenozoospermic (OA6, OA199, and OA235), one asthenozoospermic (A122), two asthenoteratozoospermic (AT209, AT240), and nine

Figure 1 Reverse-transcriptase PCR (RT-PCR) analysis of *NPHP1* expression in spermatozoa of fertile and infertile males. Reverse-transcriptase PCR analysis with 331 Forward and 896 Reverse primers specific to 565 bp fragment of *NPHP1* in spermatogenic cells from normozoospermic (A) and infertile (B–D) men. *ACTB* or *GAPDH* served as internal controls. Bar diagram (E) represents the average band intensities of *NPHP1* in ejaculated spermatozoa from normal ($n = 18$), oligozoospermic ($n = 5$), oligoasthenozoospermic ($n = 13$), oligoteratozoospermic ($n = 2$), oligoasthenoteratozoospermic ($n = 2$), asthenozoospermic ($n = 9$), asthenoteratozoospermic ($n = 5$),teratozoospermic ($n = 2$), and unexplained male infertility ($n = 31$). Values presented are mean ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.



UMI (U18, U39, U228, U231, U233, U241, U238, U246, and U255) cases were positive for *NPHP1* expression. The remaining cases were negative for *NPHP1*, as interpreted from RT-PCR results (Fig. 1B–D). *GAPDH* or *ACTB* were used as the internal control. The averaged relative band intensities showing the expression levels of NPHP1 in infertile men are significantly lower when compared with that of fertile men. The results are represented as a bar diagram (Fig. 1E).

Western blot analysis

NPHP1 was detected below 75 kDa position in all the 12 normozoospermic samples screened (Fig. 2A). ACTIN served as the internal control. On the other hand, barring OA6, U241, U231, U233, T248, T83, AT209, and AT240, all the infertile subjects showed very faint or undetectable level of NPHP1 expression in spermatozoa when compared with the normozoospermic controls (Fig. 2B–D) Secondary antibody alone (GAR-HRP) served as the negative control (Figure S3). The band intensities of proteins were quantified using Phoretix Software and the relative protein levels of NPHP1 were normalized corresponding to the ACTIN levels which are represented as bar diagrams. The averaged relative band intensities which shows the expression levels of NPHP1 in infertile men are significantly lower when compared with the fertile men. The results are represented as a bar diagram (Fig. 2E).

Immunofluorescence analysis

The representative images of the localization of NPHP1 on spermatozoa are shown in Fig. 3. In the spermatozoa from normozoospermic men (N7), a prominent localization of NPHP1 was observed at the head, middle piece segments and at the principal, end piece tail segments of spermatozoa (N7). The spermatozoa from infertile men screened for immunofluorescence analysis showed weak localization of NPHP1 toward the midpiece or tail segments (OA61, OT256, OAT124, A34, AT211, O28, T248) or at the post-acrosomal region and at the neck region (U11).

SMART analysis

SMART (simple modular architecture research tool) analysis was carried out to identify the domain architecture of *NPHP1* which revealed the presence of coiled-coil region (amino acids 3–67), Src homology 3 (SH3) domain (amino acids 155–211), and the presence of 14-3-3 domain (amino acids 433–533) (Figure S1).

DISCUSSION

A well-orchestrated process of spermatogenesis leads to the production of healthy spermatozoa which represent an important parameter in determining the male fertility status. Therefore, understanding the molecular mechanisms regulating gene expression during male germ cell development could unravel the subtle nature of male factor infertility.

NPHP1, the gene identified to be mutated in Juvenile nephronophthisis, comprises of 20 exons and a 4.5 kb transcript which is located on chromosome 2q-13 (Hildebrandt *et al.*, 1997). Nephrocystin-1 with a predicted molecular weight of 83 kDa is embedded with three putative distinct domains which includes N-terminal coiled-coil domain, SH3 domain flanked by two E-rich domains and a C-terminus nephrocystin homology domain (NHD; Donaldson *et al.*, 2002; Jiang *et al.*, 2008). The N-terminal 105 amino acid residues form coiled-coil architecture which possesses a characteristic amphipathic α-helical heptad repeat pattern known to mediate highly stable dimerization or oligomerization interactions. SH3 domain mediates interactions with proline-rich motifs of $p130^{Cas}$ and Pyk2 which

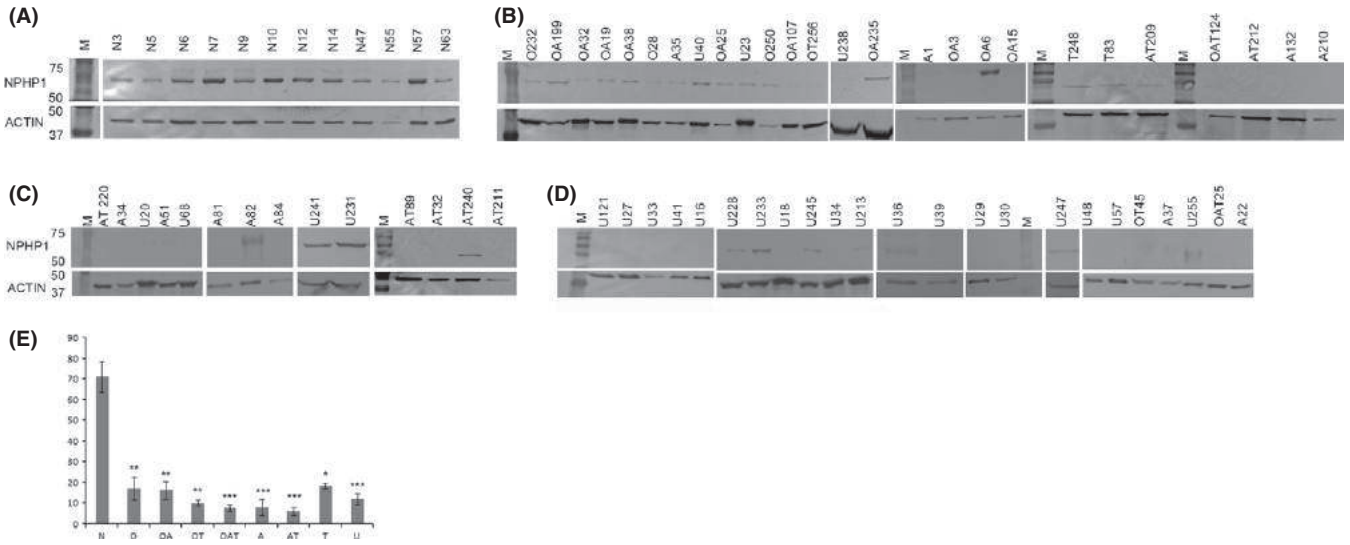
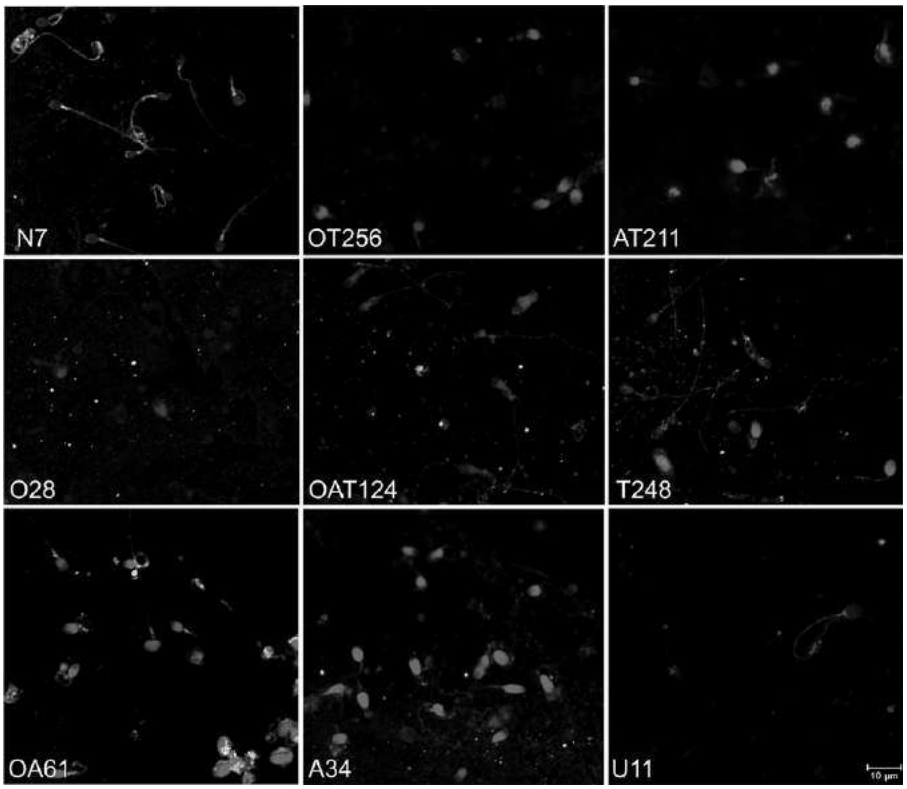


Figure 2 Western blot analysis of NPHP1 expression in spermatozoa of fertile and infertile cases. NPHP1 is detected below 75 kDa in all the normozoospermia cases (A). The samples from infertile men showed very faint or undetectable level of NPHP1 expression when compared with that of normozoospermic controls, with the exception of eight men OA6, U241, U231, U233, T248, T83, AT209, and AT240 showing normal levels of NPHP1 expression (B–D). Bar diagram (E) represents the average band intensities of NPHP1 expression in spermatozoa from normal (n = 12), oligozoospermic (n = 3), oligoasthenozoospermic (n = 10),oligoteratozoospermic (n = 2), oligoasthenoteratozoospermic (n = 2), asthenozoospermic(n = 10), asthenoteratozoospermic (n = 7),teratozoospermic (n = 2), and unexplained male infertility (n = 26). The band intensities of NPHP1 were normalized to the corresponding ACTIN levels. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Figure 3 Confocal images showing the localization pattern of NPHP1 in spermatozoa of fertile and infertile men as detected by anti-nephrocystin and Alexa Flour 488 Rabbit IgG (H + L) antibody. Propidium iodide is the nuclear stain used. The panel shows the representative images of NPHP1 expression in spermatozoa of normozoospermic (N7), oligozoospermic (O28), oligoasthenozoospermic (OA61), oligoteratozoospermic (OT256), oligoasthenoteratozoospermic (OAT124), asthenozoospermic (A34), asthenoteratozoospermic (AT211), teratozoospermic (T248), and unexplained male infertility (U11) conditions. ×63.



are essential proteins involved in cell–cell and cell-matrix signaling cascades indicating that nephrocystin is a key regulator of focal adhesion signaling complex (Benzing *et al.*, 2001). NHD mediates interactions with filamins that organize actin cytoskeleton at sites of epithelial cell–cell adhesion (Benzing *et al.*, 2001; Donaldson *et al.*, 2002). Although the role of *NPHP1* in kidney development and function in human has been well explored, a critical role in spermatogenesis has also been reported (Otto *et al.*, 2000; Jiang *et al.*, 2008). Although *Nphp1* knock-out studies have provided valuable information regarding its decisive role in relation to murine spermatogenesis, whether an aberrant expression of NPHP1 might leads to impaired spermatogenesis in human and ultimately male factor infertility had not been investigated so far. Our study has provided significant results from the expression profiling of NPHP1 in spermatozoa from fertile and infertile males and is the first direct evidence regarding the role of NPHP1 in relation to human male factor infertility.

SMART analysis carried out to identify the domain architecture of NPHP1 revealed the presence of coiled-coil region (amino acids 3–67), SH3 domain (amino acids 155–211), and the presence of 14-3-3 domain (amino acids 433–533). The 14-3-3 proteins are essential regulators of various intracellular processes including cell growth control, cell cycle control, control of gene transcription and apoptosis (Neukamm *et al.*, 2012). During the complete process of male germ cell development, germ cells interact with Sertoli cells which provide necessary nourishment, support, and guidance for the developing germ cells. The site of interactions between Sertoli cells and germ cells are termed as the apical ectoplasmic specialization (ES), a cell–cell actin-based adherens junction regulated by an array of adhesion proteins which includes junctional adhesion molecule-C, cadherins, nectins, and $\alpha 6\beta 1$-integrin-laminin complex that are anchored onto the F-actin scaffold through corresponding adaptors (Yan *et al.*, 2007). It has been reported that disruption to the adhesion proteins before spermiation would cause the premature germ cell release from the seminiferous epithelium culminating in male infertility (Gliki *et al.*, 2004; Yan & Cheng, 2006; Jiang *et al.*, 2008). Jiang *et al.* have reported that in *Nphp1*-knock-out mice, maturation defect was observed at the spermatid stage of spermatogenesis and also an abnormal F-actin distribution was detected at the apical ES signifying a possible role of *NPHP1* in apical ES organization events (Jiang *et al.*, 2008). Donaldson *et al.* (2002) have shown that nephrocystin colocalize with e-cadherin at adherens junctions of cell–cell contacts in Madin–Darby canine kidney (MDCK) cells. Moreover, *NPHP1* is regarded as a docking protein in regulating actin cytoskeleton at cell–cell adhesion sites (Donaldson *et al.*, 2002; Jiang *et al.*, 2008). As the SH3 domain of NPHP1 mediates protein–protein interaction at sites of cell–cell and cell-matrix signaling, absence of NPHP1 expression in majority of the infertile cases we screened might be because of the disruption of NPHP1 at the apical ectoplasmic specialization (EPS) dynamics which in turn affects Sertoli–Spermatid adhesion events resulting in spermatogenic failure. Indeed, the discrepancies observed at the protein level and the anomalous localization pattern of NPHP1 in

spermatozoa from infertile males when compared with the normozoospermic controls also points to the indispensable role of NPHP1 in relation to spermatogenic differentiation. In addition, in normozoospermic conditions we screened, NPHP1 was seen significantly expressed at the target message and protein level and the prominent localization pattern in spermatozoa deserves special mention. However, the possibility of multitude of factors which leads to aberrant expression of NPHP1 in the infertile cases we screened cannot be ruled out but is worth to be validated.

Nephrocystin-1 has been reported to be localized to the transition zone of primary cilia (Fliegauf *et al.*, 2006; Jiang *et al.*, 2008) which are microtubule based sensory organelles that orchestrates various signaling pathways including Notch, Sonic Hedgehog, canonical and non-canonical Wnt signaling mechanisms (May-Simera & Kelley, 2012). Recent reports have shown that JADE-1, a candidate renal tumor suppressor colocalizes with NPHP1 at the transition zone of primary cilia and mediates interactions with NPHP4 and also possess E3 ubiquitin ligase activity to negatively regulate β-catenin signaling for proteasomal degradation (Borgal *et al.*, 2012). It has been reported that spermatid-specific deletion of β-catenin in EPS in testis resulted in acrosomal defects and loss of Sertoli–germ cell adhesion resulting in infertility (Chang *et al.*, 2011; Lombardi *et al.*, 2013). Because impaired spermatogenesis is because of the alteration of many genes involved in Sertoli cell–germ cell adhesion events and germ cell differentiation events (Jeays-Ward *et al.*, 2004; Lombardi *et al.*, 2013), it is speculated that NPHP1 might be an essential component of Wnt/β-catenin signaling events during male germ cell development.

Dysfunction of primary cilia often results in pleiotropic and genetically heterogenous ciliopathies including nephronophthisis (Ware *et al.*, 2011). Sha *et al.* (2014) have reported that because sperm flagellum is a ciliary type, abnormal cilia leads to the loss of flagellar ability to swing resulting in male infertility. Furthermore, primary cilia dyskinensia, a ciliopathy often associated with male infertility occurs because of dysfunction in the sperm flagellar dynamics (McIntyre *et al.*, 2013). As both primary cilia and sperm flagella extend from the basal body and also the components of the intraflagellar transport machinery and ciliary cargo assemble near the transition zone, nephrocystyin-1 is suggested to be a component of the protein complex at the transition zone in the organization events of primary cilia and sperm flagella (Jiang *et al.*, 2008).

Mergen *et al.* (2013) have shown that NPHP1 interacts with Aurora A kinase, one of the canonical regulator of mitosis and controls ciliary disassembly and cell cycle progression in MDCK cells. Nephrocystin-1 has also been shown to be colocalized with polo-like kinase (PLK1), a regulator of centrosome maturation and spindle assembly, to the transition zone of primary cilia where PLK1 mediates phosphorylation of the nephrocystin N-terminus and a crucial role of PLK1 in the pathogenesis of nephronophthisis has been suggested (Seeger-Nukpezah *et al.*, 2012). Aberrant expression of Aurokinase A and PLK have been reported in prostate cancer cells which causes mitotic check point defects and deregulation of anaphase-promoting complex leading to chromosomal instability and aneuploidy (Weichert *et al.*, 2004; Buschhorn *et al.*, 2005; Wang & Kung, 2012). Moreover, PLK has been demonstrated to be an important component of the synaptonemal complex disassembly during the prophase to metaphase-1 transition in mouse spermatocytes (Jordan *et al.*, 2012). Thus, interactions of nephrocystin-1 with the components of cell cycle regulation, Aurora A kinase and PLK indicate that NPHP1 might be a component of cell cycle progression in male germ cell development which needs to be explored.

In our study, NPHP1 was seen expressed in human spermatozoa from all the normozoospermic individuals, whereas uncharacteristic expression pattern was detected in most of the infertile cases we screened indicating the possibility that aberrant expression pattern of NPHP1 is associated with impaired spermatogenesis in infertile conditions. The presence of coiled-coil region known to mediate highly stable oligomerization interactions, SH3 domain which regulates focal adhesion signaling complex and 14-3-3 domain which are key regulators of cell cycle control and apoptosis as revealed by SMART analysis points to the essential role of NPHP1 in various intracellular processes. Furthermore, this signifies that NPHP1 might interacts with distinct protein complexes and orchestrates cell–cell adhesion, cell cycle regulation, sperm flagellar formation, and signaling cascades during various stages of male germ cell development and is thus a critical regulator of spermatogenic events which necessitates extensive research. Indeed, the presence of NPHP1 transcripts in human spermatozoa raises the possibility of NPHP1 in fertilization events as the potential role of spermatozoal RNAs in genome recognition and embryonic development has been reported (Jodar *et al.*, 2013; Sendler *et al.*, 2013). In addition, the use of paternal transcriptome as biomarker be worthy of special mention in view of the reports regarding the variable transcript profile between fertile and infertile males (Jodar *et al.*, 2013). In conclusion, besides the imperative association with juvenile nephronophthisis, our study suggests that NPHP1 is associated with male factor infertility and thus could be a possible biomarker for the assessment of male fecundity status.

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DISCLOSURES

None of the authors have declared conflict of interest.

AUTHOR CONTRIBUTIONS

PGK conceived the idea, designed the experiments and edited the manuscript. DAN and ATR executed the experiment and wrote the manuscript. SMP and KJ performed semen analysis and diagnosed the subjects. All the authors have approved the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. SMART analysis of NPHP1 which shows the presence of coiled-coil region (amino acids 3–67), SH3 domain (amino acids 155–211), and the presence of 14-3-3 domain (amino acids 433–533).

Figure S2. (A) A portion of chromatogram confirming the expression of NPHP1 transcripts in spermatozoa from normozoospermic individual (N5) deduced using four color dye termination reaction and automated sequencing with specific forward primer (331F). (B) ClustalW alignment of NPHP1 sequence obtained by di-deoxy sequencing with NPHP1 [NM_000272.3, Homosapiens nephronophthisis 1(juvenile) (NPHP1), transcript variant 1] mRNA sequence in the data base which authenticates NPHP1 expression in spermatozoa.

Figure S3. Western Blot analysis results of NPHP1 expression in spermatozoa from normal fertile males (N9, N6). Secondary antibody alone (GAR-HRP) which served as the negative control for the same (N9, N6) is also shown.

Table S1. Category-based listing of the semen samples included in the study.

Table S2. List of primers included in the study.