HHS Public Access

Author manuscript

Kidney Int. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:

Kidney Int. 2019 June ; 95(6): 1494–1504. doi:10.1016/j.kint.2019.01.025.

SON haploinsufficiency causes impaired pre-mRNA splicing of CAKUT genes and heterogeneous renal phenotypes

Jung-Hyun Kim, Ph.D.[1], **Eun Young Park, Ph.D.**[1], **David Chitayat, M.D.**[2,3,4], **David L. Stachura, Ph.D.**[5], **Jörg Schaper, M.D.**[6], **Kristin Lindstrom, M.D.**[7], **Tamison Jewett, M.D.**[8], **Dagmar Wieczorek, M.D.**[9,10], **Jos M. Draaisma, M.D.**[11], **Margje Sinnema, M.D.**[12], **Christianne Hoeberigs, M.D.**[13], **Maja Hempel, M.D.**[14], **Kristine K. Bachman, M.S.**[15], **Andrea H. Seeley, M.D.**[15], **Joshua K. Stone, Ph.D.**[1], **Hyun Kyung Kong, Ph.D.**[1], **Lana Vukadin, B.S.**[1], **Alexander Richard, B.S.**[1], **Deepali N. Shinde, Ph.D.**[16], **Kirsty McWalter, M.S.**[17], **Yue Cindy Si, M.D., Ph.D.**[17], **Ganka Douglas, Ph.D.**[17], **Ssang-Taek Steve Lim, Ph.D.**[18], **Lisenka E.L.M. Vissers, Ph.D.**[19], **Mathieu Lemaire, M.D.C.M., Ph.D.**[20,21,*], and **Eun-Young Erin Ahn, Ph.D.**[1,18,*]

[1]Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36604, USA [2]Division of Clinical and Metabolic Genetics, Department of Pediatrics, The Hospital for Sick Children, University of Toronto, Toronto, ON, Canada [3]The Prenatal Diagnosis and Medical Genetics Program, Department. of Obstetrics and Gynecology, Mount Sinai Hospital, University of Toronto, Toronto, ON, Canada [4]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada. [5]Department of Biological Sciences, California State University Chico, Chico, CA 95929, USA [6]Institute of Diagnostic and Interventional Radiology, University of Düsseldorf, Düsseldorf, Germany [7]Division of Genetics and Metabolism, Phoenix Children's Hospital, Phoenix, AZ 85016, USA [8]Department of Pediatrics, Section on Medical Genetics, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA [9]Institute of Human Genetics, University Clinic Düsseldorf, Heinrich-Heine-University, Düsseldorf, Germany [10]Institute of Human Genetics, University Clinic Essen, University Duisburg-Essen, Essen, Germany [11]Department of Pediatrics, Radboudumc Amalia Children's Hospital, 6500 HB, Nijmegen, The Netherlands [12]Department of Clinical Genetics and School for Oncology & Developmental Biology (GROW), Maastricht University Medical Center, 6202 AZ, Maastricht, The Netherlands [13]Department of Radiology and Nuclear Medicine, Maastricht University Medical Center, 6202 AZ, Maastricht, The Netherlands [14]Institute of Human Genetics, University Medical Center Hamburg-Eppendorf, Hamburg, Germany [15]Geisinger Medical Center, Danville, PA 17822, USA [16]Ambry Genetics, Aliso Viejo, CA 92656, USA [17]GeneDx, Inc., Gaithersburg, MD 20877, USA [18]Department of Biochemistry and Molecular Biology, College of Medicine, University of South

[*]Corresponding authors: Eun-Young Erin Ahn, Ph.D., Mitchell Cancer Institute, University of South Alabama, 1660 Springhill Ave, Mobile, AL 36604, USA, Phone: (251) 445-9805, Fax: (251) 460-6994, eahn@health.southalabama.edu, Mathieu Lemaire, M.D.C.M., Ph.D., FAAP, FASN, Department of Pediatrics, Division of Nephrology, The Hospital for Sick Children, 686 Bay Street, Toronto, M5G 4A0, Ontario, Canada, Phone: (416) 813-7654, Fax: (416) 813-6271, Mathieu.lemaire@sickkids.ca.

DISCLOSURE

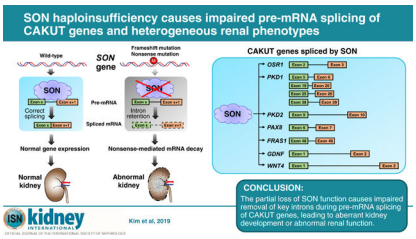
D.N.S. is an employee of Ambry Genetics, Inc. K.M., Y.C.S. and G.D. are employees of GeneDx., Inc., a wholly-owned subsidiary of OPKO Health, Inc. All other authors declared no competing interests.

Alabama, Mobile, AL 36688, USA [19]Department of Human Genetics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, 6500 HB, Nijmegen, The Netherlands [20]Division of Nephrology, Department of Pediatrics, The Hospital for Sick Children, Toronto, ON, Canada. [21]Cell Biology Program, SickKids Research Institute, University of Toronto, Toronto, ON, Canada

Abstract

Although genetic testing is increasingly used in clinical nephrology, a large number of patients with congenital abnormalities of the kidney and urinary tract (CAKUT) remain undiagnosed with current gene panels. Therefore, careful curation of novel genetic findings is key to improving diagnostic yields. We recently described a novel intellectual disability syndrome caused by *de novo* heterozygous loss-of-function mutations in the gene encoding the splicing factor SON. Here, we show that many of these patients, including two previously unreported, exhibit a wide array of kidney abnormalities. Detailed phenotyping of 14 patients with SON haploinsufficiency identified kidney anomalies in 8 patients, including horseshoe kidney, unilateral renal hypoplasia, and renal cysts. Recurrent urinary tract infections, electrolyte disturbances, and hypertension were also observed in some patients. SON knockdown in kidney cell lines lead to abnormal pre-mRNA splicing, resulting in decreased expression of several established CAKUT genes. Furthermore, these molecular events were observed in patient-derived cells with SON haploinsufficiency. Taken together, our data suggest that the wide spectrum of phenotypes in patients with a pathogenic SON mutation is a consequence of impaired pre-mRNA splicing of several CAKUT genes. We propose that genetic testing panels designed to diagnose children with a kidney phenotype should include the *SON* gene.

Graphical Abstract



Keywords

Pediatric nephrology; gene expression; genetic mutations; *SON* haploinsufficiency; pre-mRNA splicing

INTRODUCTION

Genetic mutations are the primary causes that directly account for early onset of kidney diseases.[1,2] Recent progress in next-generation sequencing (*e.g.* whole-exome and whole-genome sequencing) and genome-wide copy number variant (CNV) analysis has dramatically increased our knowledge on the genetic causes of kidney abnormalities.[1,3,4] In

particular, this led to the identification of mutations in candidate genes that are important for kidney development in patients with congenital anomalies of the kidney and urinary tract (CAKUT).[1,5] Nevertheless, the mechanisms by which many of these mutations conspire to cause specific renal phenotypes are often unclear. A better understanding of disease pathophysiology is crucial to clarify prognosis, devise new therapies, and improve patient outcomes.

De novo heterozygous loss-of-function mutations in the gene encoding for the splicing factor *SON* were recently identified by our group[6] and others[7–9] as pathogenic in patients with intellectual disability and developmental delay. We showed that *SON* haploinsufficiency causes aberrant pre-mRNA splicing in many genes critical for brain development and metabolism,[6] resulting in "SON deficiency syndrome", a unique syndrome characterized by intellectual disability, developmental delay, brain malformation, seizure, and hypotonia (also referred to as ZTTK syndrome; Zhu-Tokita-Takenouchi-Kim syndrome; OMIM: 617140). While the original reports provided detailed descriptions of the patients' neurological phenotypes, many other extra-neurological abnormalities were also noted. For example, a subset of patients had various facial dysmorphisms, musculoskeletal abnormalities and/or CAKUT-like findings.[6–9] It is important to delineate more systematically the complete phenotype associated with SON deficiency syndrome to better define this disease condition and improve diagnosis.

In this report, we define the array of possible renal phenotypes exhibited by patients with SON deficiency syndrome, which includes a broad spectrum of structural and functional kidney defects. We also identify the likely molecular mechanisms that explain these abnormalities by demonstrating for the first time that *SON* haploinsufficiency leads to aberrant pre-mRNA splicing and subsequent downregulation of genes that are crucial for kidney development or function. These genes include *OSR1, PKD1, PKD2, PAX8, FRAS1, GDNF*, and *WNT4*. Our study strongly indicates that it is important to start testing for the presence of a *SON* mutation in all patients presenting with neurological and any renal abnormalities.

RESULTS

Renal phenotypes in patients with *SON* haploinsufficiency

Previously, our group and others reported SON deficiency syndrome (ZTTK syndrome), a novel type of intellectual disability syndrome caused by heterozygous loss-of-function mutations in *SON*.[6–9] *De novo* SON mutations were identified from diagnostic whole-exome sequencing. all patients with *SON* haploinsufficiency have mild-to-severe intellectual disabilities. Around 90% display various brain malformations and many of these patients have other congenital abnormalities, which include defects in the heart, intestines, and kidneys. From our current cohort of SON deficiency syndrome patients, which include two new patients in addition to twenty patients previously identified by our group,[6] we identified that 14 out of 22 patients have had renal examination (*e.g.* ultrasound or MRI) and 8 patients have not been examined for their kidneys. Among 14 patients who have had renal exams, 8 patients (8/14; 57%) have kidney abnormalities (Table 1 and Figure 1). None of these patients has a family history of renal disease or anatomic abnormalities of the genitourinary

system. The renal phenotypes exhibited by these patients are heterogeneous. Anatomic anomalies include the horseshoe kidney (patient-1 and patient-2, see Figures 2a and 2b, respectively), hydronephrosis (patient-1, Figure 2a) and bilateral malrotated kidneys (patient-3, Figure 2c). Unilateral renal hypoplasia was described in 2 patients (patient-4 and patient-7; Table 1). Patient-5 was found to have a simple cyst (which can be a normal variant) whereas patient-1 and patient-6 had multiple cysts (Figures 2d and 2e for patient-6). Compensatory hypertrophy of the contralateral kidney was observed in patient-6 (Table 1). Patient-8 is unusual because she exhibits persistent, unexplained hyperkalemia associated with inappropriately low kaliuresis (Table 1). While she always had very small kidneys (3rd percentile), they tracked with her small body size (Figure 2f). Patient-1 and patient-8 were also found to have recurrent urinary tract infections and hypertension. It is important to acknowledge that two patients reported by Tokita *et al.* also showed kidney agenesis (single kidney) and dysplastic kidney.[7] Collectively, these findings suggest that congenital kidney anomalies are frequently associated with SON deficiency syndrome.

Our previous study showed that reduced son expression triggered by a transcript-specific morpholino (MO) injected into the yolk of one-cell stage zebrafish embryos accurately recapitulated the phenotypes of human patients with a heterozygous SON loss-of-function mutation.[6] We used the same morpholino-based approach to determine if reduced son expression affects the development of the zebrafish pronephric kidney which is a commonly used experimental model of nephron development.[10] We found that *son* morpholinos caused a reduction in the staining of the pronephric tubule marker cadherin-17 (cdh17) at 24 hours post-fertilization; reduced cdh17 expression at this stage has been associated with abnormal pronephros development[11] (Supplementary Figure S1). These data suggest that *SON* knockdown indeed leads to disruption of the kidney development and morphogenesis *in vivo*.

SON knockdown causes downregulation of a group of genes required for kidney morphogenesis

SON is a splicing co-factor facilitating intron removal and exon inclusion at weak splice sites.[12–14] Our previous study demonstrated that heterozygous loss-of-function mutations in *SON* leads to downregulation of multiple genes required for brain cortex development and metabolism.[6] However, whether *SON* loss-of-function mutation is a causative factor of kidney abnormalities in these patients remains unknown.

We therefore set out to investigate if *SON* loss-of-function mutations affect the expression of genes required for kidney development. To do so, we examined the impact of son knockdown on the expression pattern of a set of genes required for kidney development. We used two human kidney cell lines because primary kidney cells from patients with SON deficiency syndrome are unavailable (and there are no clinical indications for kidney biopsy). We have previously shown that brain genes identified in cell lines as SON-mediated splicing targets also had reduced expression when tested in blood samples of patients.[6] *In vitro* cell line models of SON deficiency thus provides valuable insights regarding the identity of SON target genes.

We selected 14 candidates CAKUT-associated genes based on their functional importance in kidney morphogenesis: *OSR1*, *PKD1, PKD2, SALL1, PAX8, SIX1, SIX2, FRAS1, GRIP1, HNF1B, GDNF, BMP4, WNT4* and *NOTCH2*.[1,5,15–19] Importantly, mutations in many of these genes have been reported from the analyses of the patients with congenital kidney diseases (*PKD1, PKD2, SALL1, SIX2, FRAS1, HNF1B, WNT4* and *GDNF*).[20–24] In order to accurately detect the expression change of these genes, quantitative real-time PCR was performed after *SON* knockdown in HK-2 cells (a human kidney proximal tubule epithelial cell line). As shown in Figures 3a and b, the expression level of 11 out of 14 genes was significantly downregulated upon *SON* knockdown (*OSR1, PKD1, PKD2, PAX8, SIX1, SIX2, FRAS1, GDNF, BMP4*, *WNT4* and *NOTCH2*). Downregulation of most of these genes was also confirmed at the mRNA and protein levels in HEK 293T cells (a human embryonic kidney cell line) transfected with *SON* siRNA (Figures 3c–e). We note that *SIX1*, *BMP4* and *NOTCH2* were downregulated in HK-2 cells, but not in HEK 293T, suggesting that splicing factors other than SON may act on these genes in HEK 293T cells.

To address whether downregulation of these CAKUT genes also occurs in patients with a heterozygous SON loss-of-function mutation, we collected peripheral blood mononuclear cells (PBMCs) from patient-8 and two unrelated healthy donors. Our quantitative RT-PCR data showed that SON expression levels in the patient-derived cells were about 50% lower than those of healthy controls (Figure 3f). Because of sample quantity limitations, we could only assess gene expression of 3 out of the 8 SON-targeted CAKUT-associated genes identified in the kidney cell lines. We decided to focus on *PKD1, PKD2* and *PAX8* based on the evidence of their expression in PBMCs documented in the R2 database (R2: Genomics Analysis and Visualization Platform; http://r2.amc.nl; Supplementary Table S1). Our data demonstrate that the expression levels of *PKD1, PKD2* and *PAX8* mRNA are significantly decreased in the patient's PBMCs (Figure 3g). These results indicate that SON haploinsufficiency causes downregulation of SON-targeted, CAKUT-associated genes in patients with a heterozygous SON loss-of-function mutation.

Impaired RNA splicing is induced as a direct consequence of partial reduction of SON and results in intron retention within the target pre-mRNA

We previously reported that *SON* knockdown results in downregulation of a group of genes associated with cell cycle, microtubule organization, brain development and cellular metabolism due to impaired RNA splicing.[6,12] Many SON-dependent introns contain weak splice sites with non-canonical splice site sequences or dual specificity sites (that can be recognized as both 5' and 3' splice site sequences).[12] Therefore, we next set out to identify direct targets of SON-mediated RNA splicing among the 11 kidney development-controlling genes (*OSR1*, *PKD1, PKD2, PAX8, SIX1, SIX2, FRAS1, GDNF, BMP4*, *WNT4*, and *NOTCH2*) that showed reduced expression after *SON* knockdown in HK-2 cell lines. We analyzed the 378 donor or acceptor splice sites present in these 11 genes using ESEfinder 3.0 to identify splice sites with a high probability of being targets for SON-mediated splicing (splice site score < 6.6 and/or dual-specificity splice sites; Supplementary Table S2).[25] Overall, our *in silico* analysis showed that 9/11 genes (*OSR1, PKD1, PKD2, PAX8, FRAS1, GDNF, BMP4, WNT4* and *NOTCH2)* have at least one splice site that could be acted upon by SON at the pre-mRNA stage. We could not predict potential target sites for SON-

mediated splicing in the *SIX1* and *SIX2* genes (each with a single intron) based on the splice site analysis results.

Among these weak splice sites, we first selected short introns (<500 bp) because previous observations revealed that the average length of SON-targeted intron is shorter than that of non-targets.[13] To detect retention or removal of these short introns, we designed a series of primers that target flanking exons to amplify the retained intron in the pre-mRNAs by RT-PCR. The results demonstrate that short introns with weak splice site(s) were retained in the *PKD1, PAX8*, and *FRAS1* pre-mRNAs when SON expression is reduced (Figure 4a). For example, we found that *PKD1*, the gene mutated in autosomal dominant polycystic kidney disease (ADPKD),[17,18] possesses 20 short introns with weak splice sites (2 weak 5' splice sites, 5 weak3' splice sites, 13 dual specificity sites). We provide experimental evidence that confirming intron retention at splice sites between exons 5–6, 19–20, 25–26 and 38–39. Intron retention was also clearly observed in intron 6 of *PAX8* and intron 48 of *FRAS1* (Figure 4a) in both HK-2 and HEK 293T cells. Retention of intron 27 in *NOTCH2* was only observed in HK-2 cells after *SON* knockdown, which is consistent with the quantitative real time-PCR data showing that *NOTCH2* expression is affected only in HK-2 cells (Figures 3b and 3d). As a negative control, we show that a short intron with strong splice sites in the gene encoding for tubulin-α 1A (TUBA1A) is not retained.

In *OSR1, PKD2, GDNF, BMP4* and *WNT4* genes, the predicted weak splice sites are in longer introns, with more than 500 bases.[6,12,13] To test for intron retention in these segments, we designed primer sets to amplify the unspliced 5' splice sites (exon-intron junction; primers indicated as *gene name-1*) and unspliced 3' splice sites (intron-exon junctions; primers indicated as *gene name-2*), flanking both ends of the target introns (Figure 4b). Our quantitative RT-PCR data show that unspliced junctions were indeed significantly increased after *SON* knockdown in HK-2 cells (Figure 4c). These results were confirmed in HEK 293T cells (Figure 4d) for the same splice sites except for *BMP-4*. Increased amounts of unspliced junctions is clearly shown by the density differences between the PCR-amplified bands (Supplementary Figure S2).

Most importantly, we confirm the presence of 4 retained introns in 3 of these genes in PBMCs obtained from patient-8 using the same primer sets. Indeed, we detected impaired splicing of short introns in the transcripts of *PKD1* (introns 19 and 25) and *PAX8* (intron 6), but not in the negative control *TUBA1A* (Figure 4e). We also observed retention of the long intron between exons 9 and 10 of *PKD2* (Figure 4f).

Taken together, these data reveal that partial loss of SON function directly causes impaired removal of many introns during RNA splicing of *OSR1, PKD1, PKD2, PAX8, FRAS1, GDNF, BMP4, WNT4 and NOTCH2*. It also suggests that this process is more dependent on predicted splice site strength than intron length. These results unveil molecular mechanisms that provide a link between *SON* haploinsufficiency and the renal phenotypes observed.

DISCUSSION

In this study, we provide clinical and functional evidence supporting the notion that finding a *de novo* heterozygous loss-of-function mutation in the *SON* gene in a patient should trigger investigations aimed at documenting the extent of renal involvement. Indeed, we demonstrate that this scenario is frequently associated with various structural and functional abnormalities of the kidneys such as horseshoe kidney, incomplete rotation of the kidney, kidney cysts, recurrent urinary tract infections, or hypertension. It is important to acknowledge that our report may be underestimating the overall burden of renal disease experienced by these patients because many are still very young. If a patient is diagnosed by a non-renal team (most likely scenario), a referral to a kidney specialist for initial renal evaluation and long-term follow-up is recommended since the phenotype may be heterogeneous and subtle, or may gradually emerge over time. The heterogeneity of the renal phenotypes uncovered in our cohort of SON deficiency syndrome patients is likely due to the presence of multiple downstream targets of SON-mediated splicing, stochastic functional compensation by other splicing factors and/or the varying levels of functional efficiency of the nonsense-mediated RNA decay machinery in each patient, which can result in varying degrees of mRNA reductions when intron retention occurs in pre-mRNA. A similar phenomenon has been described in patients with ADPKD caused by the same PKD1 or PKD2 pathogenic genotype.[17]

Since SON regulates both RNA splicing and transcription,[12,26] heterozygous *SON* loss-of-function mutations could alter the expression of a myriad of genes, thus potentially impairing normal development and/or functions of multiple organs. As such, it is important to identify direct SON targets in cells relevant to the clinical phenotypes observed. We reveal for the first time a novel connection between *SON* haploinsufficiency and dysregulation of RNA splicing of several genes that are important for kidney development and function. This finding highlights the potential link between aberrant RNA splicing and renal diseases.

PKD1 and *PKD2* are the most notable SON target genes identified in our study. The products of both genes are expressed in epithelial cells of the developing and mature renal tubules.[27,28] We show that SON is a critical RNA splicing regulator of *PKD1* and *PKD2*, and downregulation of SON severely impairs the intron removal process at multiple splice sites during *PKD1* and *PKD2* pre-mRNA splicing, thereby reducing gene expression. Patients with a pathogenic mutation in either gene develop ADPKD, the most common genetic disease implicating the kidney.[17,18] *PKD1* gene expression in renal tubular cells appears to be tightly regulated since both PKD1 deficiency (in both human and mice[29,30]) and overexpression (in mice) lead to cyst formation.[31] PKD2-deficient mice exhibit a similar phenotype.[32] Formation of aberrantly spliced PKD1 and PKD2 mRNA is a common pathway to disease since 10–20% of disease-causing mutations affect acceptor or donor splice sites (ADPKD Mutation Database [http://pkdb.mayo.edu], accessed June 2018). Recent data suggest that the importance of this process is likely underappreciated since several exonic synonymous and non-synonymous variants were found to results in abnormal *PKD1* splicing.[33] This finding reveals another layer of the regulatory mechanism of *PKD1* and *PKD2* gene expression. This is the first time that mutations in a gene encoding for a member of the spliceosome are implicated in a form of cystic kidney diseases.

Besides *PKD1* and *PKD2* gene, we also found that SON impacted on the splicing of many other genes crucial for kidney development, such as *OSR1, PAX8, FRAS1, GDNF, BMP4* and *WNT4*. It is interesting to note that a single splicing factor can modulate the expression of so many genes at once. Of these genes, *PAX8* and *FRAS1* have been directly linked to a human condition affecting the kidneys; other genes (*OSR1, GDNF, BMP4* and *WNT4*) are crucial for murine kidney development (Supplementary Table S4).

It may unfortunately be very challenging to provide formal evidence supporting a causal link between SON deficiency and a specific phenotype exhibited by a particular patient. The problem lies in the inherent complexity of SON biology: SON can affect the expression of many genes and other splicing factors may partially compensate for SON deficiency. In addition, the effects of both SON itself and the compensating splice factors may vary depending on the cell type and/or organ, timing of development, or even perhaps in different environmental contexts. In turn, the functions of SON target genes are exquisitely linked to them being expressed at precise developmental stages and/or in specific cell types. The compounded effects of these complex interactions probably explains why “meta-phenotypes” exhibited by patients are so broad, and why individual “sub-phenotypes” could, in principle, change over time. On that basis, we suggest that showing the negative impact of SON deficiency on the expression of a collection of genes related to the phenotype under scrutiny in distinct model systems (including patient-derived samples) provides sufficient evidence of biological plausibility. It also motivates us to emphasize the potential importance of continuous phenotype monitoring for all patients with SON deficiency syndrome during their lifetime (with a particular focus on organ systems already known to be affected in many patients, such as the brain and kidneys).

We further analyzed previously published microarray and RNA-sequencing data from HeLa cells and human embryonic stem cells transfected with SON siRNA or shRNA[12–14] to identify additional SON-targeted CAKUT genes. However, many of the CAKUT-associated genes were poorly expressed in the cell types used previously and we could not validate additional SON targeted CAKUT-associated genes besides *PKD1, PAX8, FRAS1* and *SIX1*. To identify the complete set of SON-targeted CAKUT genes, further transcriptome analyses with kidney cells will be required.

In conclusion, our study strongly suggests that sequencing for a *SON* gene mutation should be prioritized for children with any renal phenotype accompanied by intellectual disability, developmental delay, mild-to-moderate facial dysmorphisms, and/or muscular-skeletal phenotypes. It will be important to also add the *SON* gene to all genetic test panels focused on any kidney disease because it is unlikely that nephrologists will be well aware of the literature on SON deficiency syndrome. Prompt and accurate molecular confirmation is beneficial for patients and their family since they often experience delays in diagnosis[34] that cause anxiety, frustration, stress, delays in starting treatment, or even inappropriate therapies. We have no doubt that the protocols elaborated in prior studies and refined in the current report will facilitate future investigations aimed at defining the role of SON deficiency in triggering phenotypes in other organ systems.

METHODS

Study approval for using genetic and clinical data of human patients with SON deficiency syndrome

Whole exome sequencing of patients was done as described previously.[6] Before molecular diagnosis and using clinical information, written informed consent for participation/publication was received from all patients or parents. The study was approved by the Institutional Review Board Committees of the participating institutions under the realm of diagnostic whole exome sequencing.

Splice site motif analysis

Splice site motif scores of SON target genes were calculated using the matrices for splice sites available in ESEfinder version 3.038 (http://rulai.cshl.edu/tools/ESE/).[25] The exon and intron sequences that possess low splice site scores (threshold score: 6.6) or dual-specificity splice sites are shown in Supplementary Table S2.

Cell culture and siRNA transfection

Human embryonic kidney (HEK) 293 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and penicillin/streptomycin. HK-2 (an immortalized proximal tubule epithelial cell line from normal adult human kidney) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA; CRL-2190) and were cultured in keratinocyte-serum free medium (K-SFM; Thermofisher, Waltham, MA) containing 5% FCS, 5 ng/ml recombinant epidermal growth factor (rEGF), and 0.05 mg/ml bovine pituitary extract (BPE). We used the Silencer Select siRNA (Life Technologies, Carlsbad, CA) directed against human *SON* (GCAUUUGGCCCAUCUGAGAtt) and a negative control siRNA (UAACGACGCGACGACGUAAtt) as described previously.[26] Cells were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) with 100–200 pmol of siRNA according to the manufacturer's instructions.

RNA extraction, cDNA synthesis, PCR and Real-Time qPCR

Total RNA was extracted from cell lines using the RNeasy Mini Kits (Qiagen, Valencia, CA) and contaminating genomic DNA was removed with RNase-free DNase (Qiagen). The patient's blood was collected in a PAXgene Blood RNA Tube (PreAnalytiX, Hilden, GE). Total RNA from PBMCs was extracted using PAXgene Blood RNA kit (PreAnalytiX, Hilden, GE), following the supplier's instructions. Total RNA was reverse-transcribed using SuperScript III RT (Life Technologies). PCR reactions were performed in the following cycle conditions: 95°C for10 min; followed by 35 cycles of 95°C for 30 sec, 5 7°C for 30 sec, and 72°C for 1 min; and final extension of 10 min at 72°C. To detect splicing efficiency, the final PCR products were electrophoresed on 2% agarose gels. All quantitative real-time PCR reactions were performed using the CFX96 real-time PCR system, and amplifications were done using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Real-time qPCR was carried out under the following conditions: one cycle of denaturing at 95°C for 10 min followed by 40 cycles of 95°Cfor 15 sec and 60°C for 1 min. *GAPDH* was used as the internal control for normalization for qPCR data analysis. Relative quantification of

gene expression was calculated using according to the 2-$\Delta\Delta$Ct method. Splicing of *TUBA1A* intron 2 (between exons 2 and 3) was examined as a negative control that is not affected by SON knockdown and to verify an equal amount of cDNA in each PCR reaction. All primer sequences are listed in the Supplementary Tables S3 and S4.

Western Blot Analysis

Cell lysates were prepared using lysis buffer (50 mM Tris-HCl, pH 8.0/150 mM NaCl/0.5% NP-40/10% glycerol) supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, St Louis, MO). Samples were subjected to a SDS/PAGE and transferred to an PVDF. Blots were incubated overnight at 4 °C with S ON antibody (ab121759; Abcam, Cambridge, UK), PAX8 antibody (sc-81648; Santa Cruz,Dallas, TX) and PKD2 antibody (A302–470A; Bethyl, Montgomery, TX). Actin antibody (A5228; Sigma-Aldrich, St. Louis, MO) was used as a loading control. The blots were washed, incubated with HRP-conjugated secondary antibody, and detected using Bio-Rad Laboratories' Clarity Western ECL Substrate (Hercules, CA).

Statistics

For Real-Time qPCR, all assays were performed in triplicates and independently repeated three times. Experimental data were plotted as mean values with standard deviation (SD) using the Student's t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We are deeply grateful to the patients and their families for participating in this study. We thank Margot R.F. Reinders (Radboud University Medical Center, The Netherlands) for her help in summarizing clinical features of a patient and Megan T. Cho for her help in connecting collaborators. This work was supported by the US National Institutes of Health Grants (CA190688 to E.-Y.E.A) and Institutional fund from the University of South Alabama Mitchell Cancer Institute (to E.-Y.E.A.). M.L. is supported by New Investigators Awards from the Kidney Research Scientist Core Education and National Training Program (KRESCENT) and the Canadian Child Health Clinician Scientist Program (CCHCSP).

Sources of Support: The US National Institutes of Health Grants (NIH CA190688 to E.-Y.E.A), Institutional fund from the University of South Alabama Mitchell Cancer Institute (to E.-Y.E.A.), New Investigators Awards from the Kidney Research Scientist Core Education and National Training Program (KRESCENT to M.L.) and the Canadian Child Health Clinician Scientist Program (CCHCSP to M.L.).

REFERENCES

1. Nicolaou N, Renkema KY, Bongers EM, Giles RH, Knoers NV. Genetic, environmental, and epigenetic factors involved in CAKUT. Nat Rev Nephrol. 2015;11(12):720–731. [PubMed: 26281895]
2. Hildebrandt F. Decade in review--genetics of kidney diseases: Genetic dissection of kidney disorders. Nat Rev Nephrol. 2015;11(11):635–636. [PubMed: 26324198]
3. Capone VP, Morello W, Taroni F, Montini G. Genetics of Congenital Anomalies of the Kidney and Urinary Tract: The Current State of Play. Int J Mol Sci. 2017;18(4).
4. Hildebrandt F Genetic kidney diseases. Lancet. 2010;375(9722):1287–1295. [PubMed: 20382325]

5. Short KM, Smyth IM. The contribution of branching morphogenesis to kidney development and disease. Nat Rev Nephrol. 2016;12(12):754–767. [PubMed: 27818506]
6. Kim JH, Shinde DN, Reijnders MRF, et al. De Novo Mutations in SON Disrupt RNA Splicing of Genes Essential for Brain Development and Metabolism, Causing an Intellectual-Disability Syndrome. Am J Hum Genet. 2016;99(3):711–719. [PubMed: 27545680]
7. Tokita MJ, Braxton AA, Shao Y, et al. De Novo Truncating Variants in SON Cause Intellectual Disability, Congenital Malformations, and Failure to Thrive. Am J Hum Genet. 2016;99(3):720–727. [PubMed: 27545676]
8. Takenouchi T, Miura K, Uehara T, Mizuno S, Kosaki K. Establishing SON in 21q22.11 as a cause a new syndromic form of intellectual disability: Possible contribution to Braddock-Carey syndrome phenotype. Am J Med Genet A. 2016;170(10):2587–2590. [PubMed: 27256762]
9. Zhu X, Petrovski S, Xie P, et al. Whole-exome sequencing in undiagnosed genetic diseases: interpreting 119 trios. Genet Med. 2015;17(10):774–781. [PubMed: 25590979]
10. Drummond IA. The zebrafish pronephros: a genetic system for studies of kidney development. Pediatr Nephrol. 2000;14(5):428–435. [PubMed: 10805474]
11. Horsfield J, Ramachandran A, Reuter K, et al. Cadherin-17 is required to maintain pronephric duct integrity during zebrafish development. Mech Dev. 2002;115(1–2):15–26. [PubMed: 12049763]
12. Ahn EY, DeKelver RC, Lo MC, et al. SON controls cell-cycle progression by coordinated regulation of RNA splicing. Mol Cell. 2011;42(2):185–198. [PubMed: 21504830]
13. Lu X, Goke J, Sachs F, et al. SON connects the splicing-regulatory network with pluripotency in human embryonic stem cells. Nat Cell Biol. 2013;15(10):1141–1152. [PubMed: 24013217]
14. Sharma A, Markey M, Torres-Munoz K, et al. Son maintains accurate splicing for a subset of human pre-mRNAs. Journal of cell science. 2011;124(Pt 24):4286–4298. [PubMed: 22193954]
15. Blake J, Rosenblum ND. Renal branching morphogenesis: morphogenetic and signaling mechanisms. Semin Cell Dev Biol. 2014;36:2–12. [PubMed: 25080023]
16. Dressler GR. The cellular basis of kidney development. Annu Rev Cell Dev Biol. 2006;22:509–529. [PubMed: 16822174]
17. Harris PC, Rossetti S. Molecular diagnostics for autosomal dominant polycystic kidney disease. Nat Rev Nephrol. 2010;6(4):197–206. [PubMed: 20177400]
18. Harris PC, Torres VE. Polycystic kidney disease. Annu Rev Med. 2009;60:321–337. [PubMed: 18947299]
19. Chapin HC, Caplan MJ. The cell biology of polycystic kidney disease. J Cell Biol. 2010;191(4):701–710. [PubMed: 21079243]
20. Weber S, Moriniere V, Knuppel T, et al. Prevalence of mutations in renal developmental genes in children with renal hypodysplasia: results of the ESCAPE study. J Am Soc Nephrol. 2006;17(10):2864–2870. [PubMed: 16971658]
21. Hwang DY, Dworschak GC, Kohl S, et al. Mutations in 12 known dominant disease-causing genes clarify many congenital anomalies of the kidney and urinary tract. Kidney Int. 2014;85(6):1429–1433. [PubMed: 24429398]
22. Saisawat P, Tasic V, Vega-Warner V, et al. Identification of two novel CAKUT-causing genes by massively parallel exon resequencing of candidate genes in patients with unilateral renal agenesis. Kidney Int. 2012;81(2):196–200. [PubMed: 21900877]
23. Chatterjee R, Ramos E, Hoffman M, et al. Traditional and targeted exome sequencing reveals common, rare and novel functional deleterious variants in RET-signaling complex in a cohort of living US patients with urinary tract malformations. Hum Genet. 2012;131(11):1725–1738. [PubMed: 22729463]
24. Wu H, Xu Q, Xie J, et al. Identification of 8 Novel Mutations in Nephrogenesis-Related Genes in Chinese Han Patients with Unilateral Renal Agenesis. Am J Nephrol. 2017;46(1):55–63. [PubMed: 28618409]
25. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: A web resource to identify exonic splicing enhancers. Nucleic Acids Res. 2003;31(13):3568–3571. [PubMed: 12824367]
26. Kim JH, Baddoo MC, Park EY, et al. SON and Its Alternatively Spliced Isoforms Control MLL Complex-Mediated H3K4me3 and Transcription of Leukemia-Associated Genes. Mol Cell. 2016;61(6):859–873. [PubMed: 26990989]

27. Ward CJ, Turley H, Ong AC, et al. Polycystin, the polycystic kidney disease 1 protein, is expressed by epithelial cells in fetal, adult, and polycystic kidney. Proc Natl Acad Sci U S A. 1996;93(4): 1524–1528. [PubMed: 8643665]

28. Markowitz GS, Cai Y, Li L, et al. Polycystin-2 expression is developmentally regulated. Am J Physiol. 1999;277(1 Pt 2):F17–25. [PubMed: 10409293]

29. Wu G, Somlo S. Molecular genetics and mechanism of autosomal dominant polycystic kidney disease. Mol Genet Metab. 2000;69(1):1–15. [PubMed: 10655152]

30. Boucher C, Sandford R. Autosomal dominant polycystic kidney disease (ADPKD, MIM 173900, PKD1 and PKD2 genes, protein products known as polycystin-1 and polycystin-2). Eur J Hum Genet. 2004;12(5):347–354. [PubMed: 14872199]

31. Thivierge C, Kurbegovic A, Couillard M, Guillaume R, Cote O, Trudel M. Overexpression of PKD1 causes polycystic kidney disease. Mol Cell Biol. 2006;26(4):1538–1548. [PubMed: 16449663]

32. Wu G, D'Agati V, Cai Y, et al. Somatic inactivation of Pkd2 results in polycystic kidney disease. Cell. 1998;93(2):177–188. [PubMed: 9568711]

33. Gonzalez-Paredes FJ, Ramos-Trujillo E, Claverie-Martin F. Defective pre-mRNA splicing in PKD1 due to presumed missense and synonymous mutations causing autosomal dominant polycystic disease. Gene. 2014;546(2):243–249. [PubMed: 24907393]

34. Zurynski Y, Deverell M, Dalkeith T, et al. Australian children living with rare diseases: experiences of diagnosis and perceived consequences of diagnostic delays. Orphanet J Rare Dis. 2017;12(1): 68. [PubMed: 28399928]

35. Rosenbaum DM, Korngold E, Teele RL. Sonographic assessment of renal length in normal children. AJR Am J Roentgenol. 1984;142(3):467–469. [PubMed: 6607625]

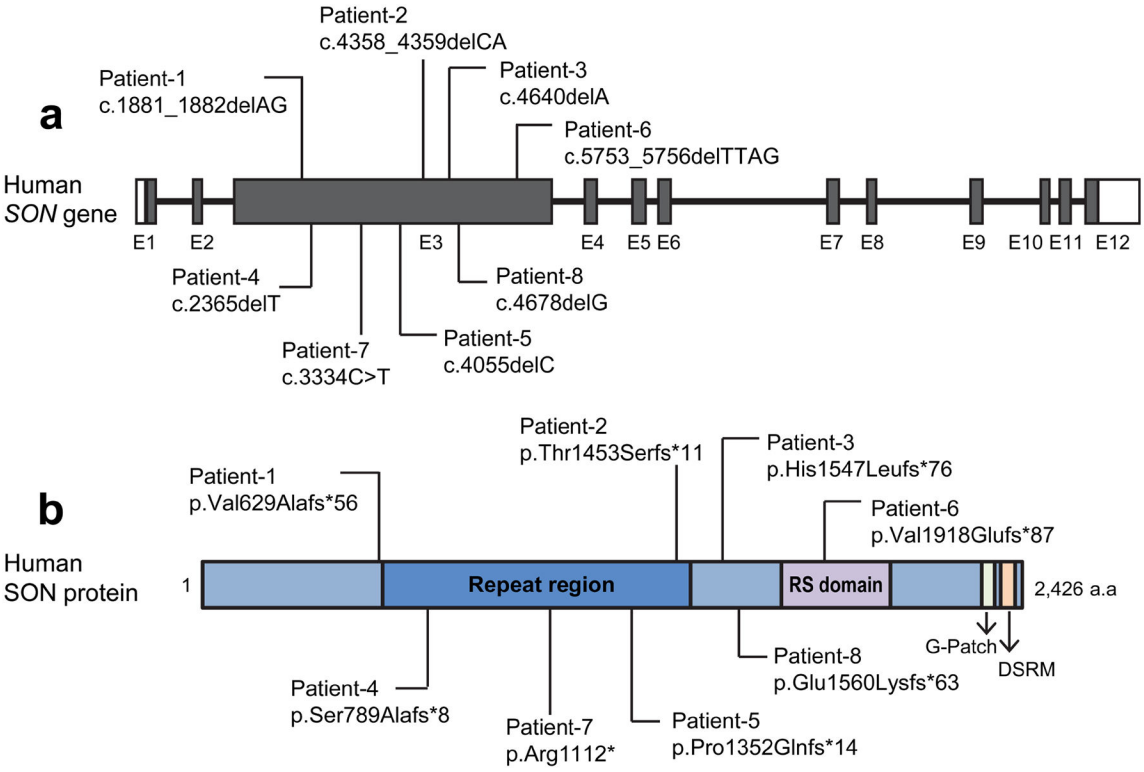


Figure 1. Locations of the *SON* mutations found in eight SON deficiency syndrome patients with kidney phenotype.

The figure represents the relative location of *SON* mutations in the mRNA (GenBank: NM_138927) including non-coding (white) and coding (gray) exons **(a)** and encoded protein (GenBank: NP_620305) domains **(b)**. Two variants newly identified in this study (patient-7 and patient-8) and six published variants cluster within exon 3 region. The mutations include seven heterozygous insertion mutations which lead to frameshift and a premature termination codon and one non-sense mutation. RS domain, Ser/Arg-rich domain; G-patch, glycine-rich motif; DSRM, double-stranded RNA-binding motif.

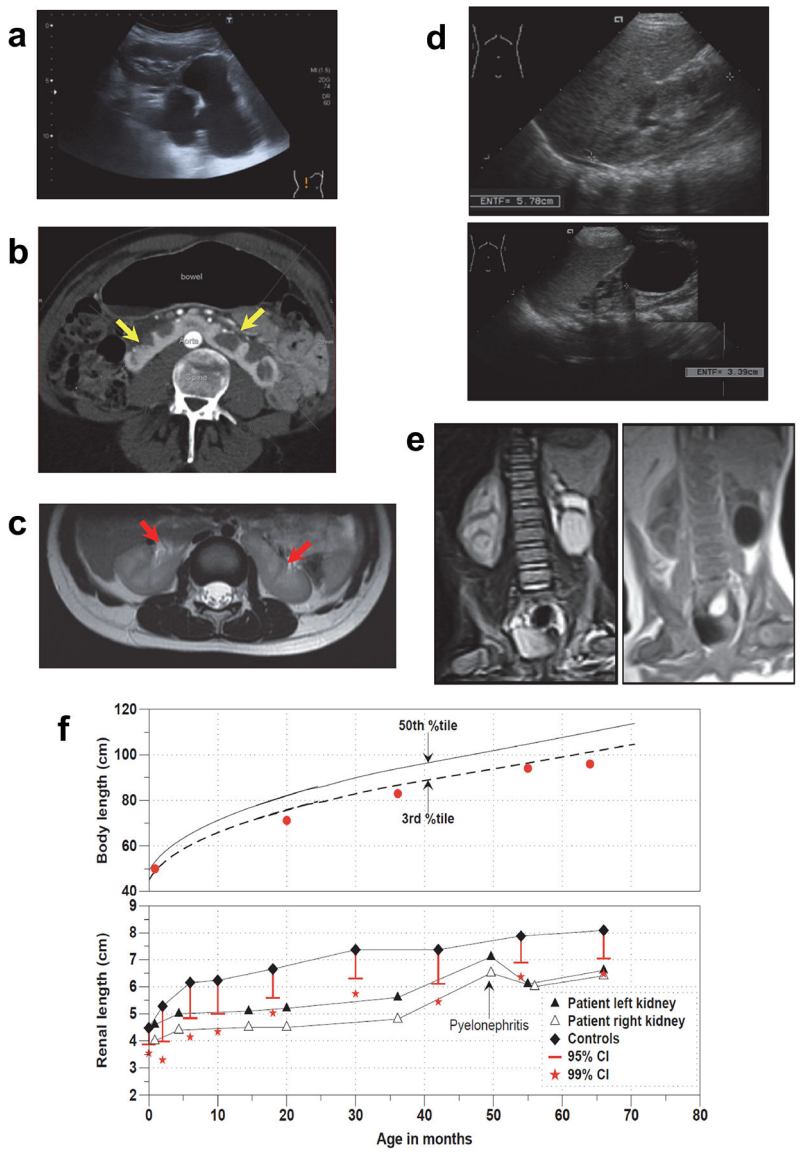


Figure 2. Renal phenotypes of the patients with SON deficiency syndrome.

(a) Ultrasound of patient-1 at age 5, showing the horseshoe kidney. Hydronephrosis in the left side of the horseshoe kidney is shown. The right side of the horseshoe kidney shows a normal pelvicalyceal system (upper left side of the image). **(b)** A computing tomography (CT) of patient-2 demonstrating the horseshoe kidney (yellow arrows). **(c)** Magnetic resonance imaging (MRI) of patient-3, showing bilateral malrotation of the kidneys (red arrows). **(d)** Ultrasound of patient-6 at age 4 months, showing large and normal right kidney (top) and dysplastic left kidney with a large cyst at lower pole and small cysts in a small dysplastic upper pole (bottom). **(e)** Magnetic resonance imaging (MRI) of patient-6. Magnetom open, STIR coronal image (left) and T1-weighted coronal image (right), showing large normal right kidney and smaller dysplastic left kidney with cysts. **(f)** The body size and the kidney size of patient-8 over time. Note that 0 on the graph is a corrected age. Normative renal length values were derived from data presented by Rosenbaum and colleagues.[35]

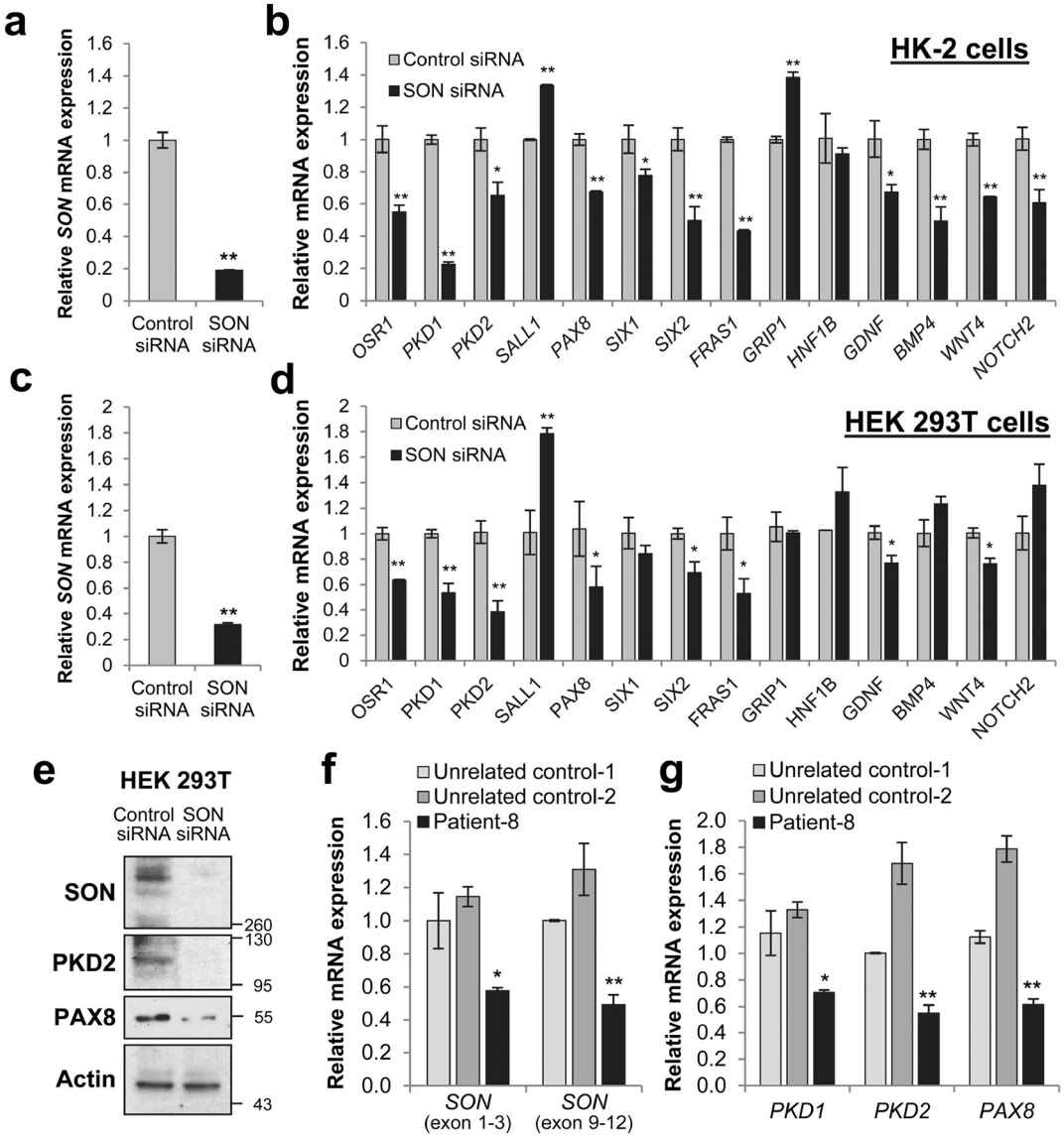


Figure 3. Several kidney development-related genes are downregulated in kidney cell lines upon siRNA-mediated SON knockdown and in PBMCs from a patient with SON haploinsufficiency.

(a-e) HK-2 **(a, b)** and HEK 293T cells **(c-e)** were transfected with control or *SON*-specific siRNA and harvested after 48hr for RNA purification and cDNA preparation. Quantitative real-time PCR was carried out with gene-specific primers listed in Supplementary Table S3 (exon 1–3 primers for *SON*) and each gene expression was normalized to endogenous *GAPDH*. Western blots demonstrated reduced expression of SON, PKD2 and PAX8 in HEK 293T cells **(e)**. **(f and g)** Analyses of patient-derived PBMCs for SON and target CAKUT gene expression. PBMCs from patient-8 as well as from two unrelated, healthy donors were analyzed by real-time qPCR with two sets of SON primers (targeting the exon 1–3 region and the exon 9–12 region **(f)** and target gene-specific primers for *PKD1, PKD2* and *PAX8* **(g)**. Data from three independent experiments, each of which was done in triplicate, are presented as the mean ± SE. *$p < 0.05$, **$p < 0.01$ (Student-t test).

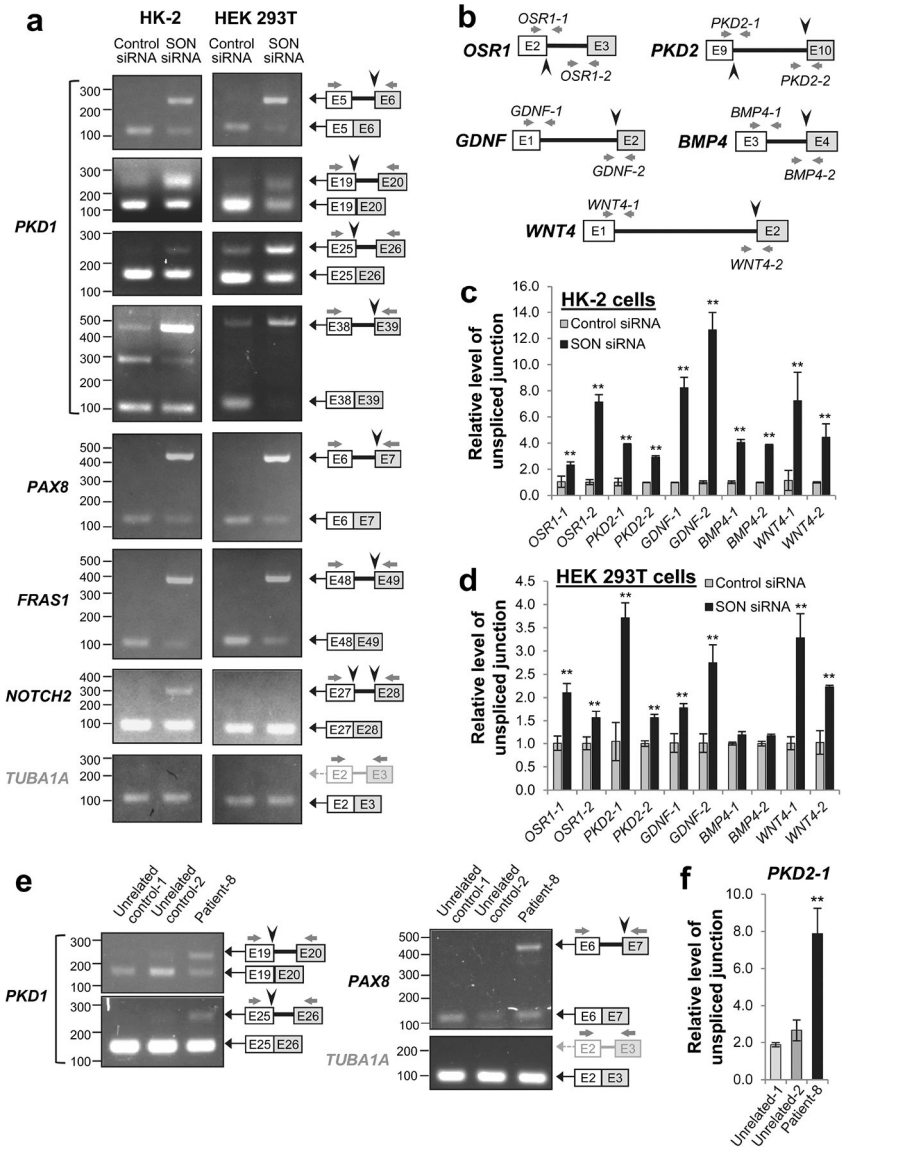


Figure 4. SON depletion impairs the removal of the weak splice site from the several target transcripts.

Removal and retention of weak splice site-bearing short introns **(a and e)** or long introns **(b, c, d and f)** were analyzed by PCR amplifying the retained introns. **(a)** HK-2 and HEK 293T cells were transfected with control or *SON*-specific siRNA and harvested after 48hr for RNA purification and cDNA preparation. Spliced and unspliced status of indicated areas within the *PKD1*, *PAX8*, *FRAS1*, and *NOTCH2* transcripts were examined by RT-PCR, using the indicated gene specific primers targeting two adjacent exons flanking a short intron. *TUBA1A* exon 2–3 region was analyzed as a negative control since it is not regulated by SON; it also acts cDNA loading control for each reaction. Schematic diagrams on the right side of the gel image illustrate the spliced and unspliced forms of each transcript and the gray arrows indicate the primers used in quantitative RT-PCR. Black arrow heads indicate the locations of weak splice sites or dual specificity sites predicted by ESEFinder. **(b)** Schematic diagrams showing the exon and intron regions and the primers (gray arrows)

designed for measuring exon-intron junctions (*gene name*-1) or intron-exon junctions (*gene name-2*) within in the *OSR1, PKD2, GDNF, BMP4 and WNT4* transcripts. **(c, d)** HK-2 **(c)** and HEK 293T cells **(d)** were transfected with control or *SON*-specific siRNA and harvested after 48hr for RNA purification and cDNA preparation. Quantitative RT-PCR was done with the primer sets indicated in **(b)** for each gene to determine the level of unspliced junction present in the cells. **(e and f)** PBMCs were collected from patient-8 as well as two unrelated, healthy donors, and used for the analyses of retentions of SON-regulated short introns present in the *PKD1* and *PAX8* transcripts **(e)** and a long intron present in the *PKD2* transcript **(f)**. Primer sequences are listed in Supplementary Tables S3 and S4. Data from three independent experiments, which had been done in triplicate, are presented as the mean ± SE. $^{**}p < 0.01$ (Student-t test).

Table 1.

Clinical features of the patients with SON deficiency syndrome.

Characteristics	Patient IDs [patient IDs in Kim et al. 2016]							
	Patient-1 [2]	**Patient-2 [8]**	**Patient-3 [9]**	**Patient-4 [13]**[a]	**Patient-5 [16]**[b]	**Patient-6 [18]**	**Patient-7 [new]**	**Patient-8 [new]**
Gender	**F**	**F**	**F**	**M**	**M**	**F**	**M**	**F**
Age (y)	12	34	9	18	6.8	14	2.5	5.7
SON mutation (cDNA; protein)	c.1881_1882delAG p.Val629Alafs*56	c.4358_4359delCA p.Thr1453Serfs*11	c.4640delA p.His1547Leufs*76	c.2365delT p.Ser789Alafs*8	c.4055delC p.Pro1352Glnfs*14	c.5753_5756delTTAG p.Val1918Glufs*87	c.3334C>T p.Arg1112*	c.4678delG p.Glu1560Lysfs*63
Anatomic abnormality	Horseshoe kidney Kidney cysts Hydronephrosis in the left side of the horseshoe kidney	Horseshoe kidney	Bilateral renal malrotation	At 7 y, both R and L kidneys were 50th. At 11 y, R kidney 20th & L kidney no data.	Single cyst on R kidney	Dysplastic and polycystic L kidney[c] Parenchymal volume <3rd%tile with atrophy in the course of time Contralateral R kidney hypertrophy[d]	Small R kidney (<3rd%tile)	Bilateral small kidneys but proportional to body size (3rd %tile)
Creatinine (μmol/L) Creatinine (mg/dL)	46 (30–60) 0.52 (0.34–0.69)	82 (55–95) 0.93 (0.62–1.10)	-	35 (18–57) 0.4 (0.2–0.65)	36 (18–57) 0.41 (0.20–0.65)	26 (9–27) 0.3 (0.10–0.30)	29 (9–62) 0.33 (0.10–0.70)	27 (29–52) 0.31 (0.33–0.59)
Na (mmol/L)	142 (135–145)	139 (135–145)	-	140 (135–145)	140 (135–145)	-	146 (134–143)	146 (135–143)
K (mmol/L)	4.2 (3.5–4.7)	3.5 (3.2–4.5)	-	4.2 (3.5–4.5)	3.2 (3.5–4.5)	-	3.8 (3.6–5.2)	5.9 (3.5–5.0)[e]
Proteinuria (mg protein/gcreat)	No	Albumin/creat ratio: 10.7 mg/mmol (normal < 2.5 mg/mmol)	-	-	-	-	250 (15–170)	No
Hematuria	No	-	-	-	-	-	No	No
Hypertension	Yes[f]	No	No	No	No	No	No	Yes[g]
Urinary tract infections	Yes, recurrent	No	No	No	No	No	No	Yes, two episodes of urosepsis
Anthropometry (percentile)	<1st	7th	<1st	3rd	2nd	1st	N/A	3rd

L kidney, left kidney; R kidney, right kidney.

None of these patients has family history of kidney abnormalities.

[a] A heterozygous mutation in the TERT gene (c.544A>T, p.T182S) has been identified.

[b] A deletion of 258 kb fragment in 7q31.33 was identified. This deletion does not affect any RefSeq genes.

[c] One cyst at lower part and at least 3 small cysts in the dysplastic upper part.

[d] At the age of 8 years and a body weight of 19 kg and the renal volume was 90 ml.

[e] Patient oral formula is pre-treated with 40g of sodium polystyrene (potassium binder)

[f] The patient was treated with Enalapril and has a normal blood pressure with this treatment.

[g] The patient is treated with 2.5 mg amlodipine PO qD.