Genomics in Urologic Health and Disease

Whole-Exome Sequencing Identifies Novel Heterozygous Mutation in *RAF1* in Family With Neonatal Testicular Torsion

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OBJECTIVE	To investigate a genetic cause of neonatal testicular torsion in 2 siblings and paternal cryptorchidism in a Caucasian family without history of consanguinity, we performed whole exome sequencing.
PATIENTS AND METHODS	Targeted exon/whole-exome sequencing was performed in 2 siblings with testicular torsion. Potentially pathogenic variants passing filter criteria were validated with Sanger sequencing of parents to confirm familial segregation. Additionally, immunofluorescence staining for Raf-1, pERK (downstream from Raf-1) and c-Kit was performed on a testicular biopsy on the preserved testicle from the proband brother and compared with testicular biopsies from fertile men.
RESULTS	A potentially pathogenic variant was identified in the *RAF1* gene (serine/threonine-protein kinase) in exon 7 of chromosome 3: 12645786 G > C; both brothers and father were heterozygous for the variant, while the mother was negative for this mutation. This mutation in exon 7 (chr3:) of *RAF1* is predicted to be damaging as a highly conserved splicing site is disrupted. The mutation is not found in the single nucleotide polymorphism database, the 1000 Genomes Project, ExACT, or esp6500. Immunofluorescence of the testis biopsy from one of the brothers demonstrated markedly decreased expression of Raf-1 as well as pERK but similar expression of c-kit when compared with fertile controls.
CONCLUSION	We identified a novel nonsynonymous mutation in RAF1 in n Caucasian family with testicular torsion and cryptorchidism. We present the first human evidence that the *RAF/MEK/ERK* pathway is associated with testicular descent. UROLOGY 129: 60−67, 2019. © 2019 Elsevier Inc.

Testicular descent is a complex process unique to mammals. Cryptorchidism occurs in approximately 1%-9% of full-term boys, while 1% of boys have an undescended testicle at 3 months of age.[1] Initially, the testicle is attached intra-abdominally at the level of kidney via the cranial suspensory ligament.[2] By gestational week 35, the testicle reaches the scrotum and the gubernaculum becomes a fibrous remnant.[3] Abnormalities in gubernacular development can lead to both cryptorchidism[4] and

testicular torsion.[5] Several studies have suggested that cryptorchidism and testicular torsion are on a disease continuum, in which more severe gubernacular dysfunction results in cryptorchidism as the testicle never descends, while testicular torsion results from partial but incomplete descent.[6,7]

Testicular torsion is estimated to affect 1 in 4000 males, with 65% of cases occurring between the ages of 12 and 18.[8] Neonatal testicular torsion is a rare subtype of testicular torsion that is estimated to occur in less 1 in 16,000 males.[9] Neonatal testicular torsion is defined as torsion that occurs within 30 days of life, but often occurs in utero. Typically, neonatal testicular torsion is extravaginal[10] and several studies have suggested that testicular torsion in neonates maybe due to gubernacular dysfunction resulting in failed adhesion.[6,7]

Given that gubernacular dysfunction may be a common etiology of both cryptorchidism and testicular torsion, we hypothesized that a single genetic abnormality may be present in our family with both pathologies. As such, we performed whole-exome sequencing to investigate mutations,

in which 2 siblings had neonatal testicular torsion and the father had a history of unilateral cryptorchidism.

MATERIALS AND METHODS

Patients Ascertainment
Two male siblings with a history of unilateral and bilateral neonatal testicular torsion and whose father had a unilateral undescended testicle that was corrected at the age of 12 were identified at the University of Miami School of Medicine Urology Clinic. This study was approved by the Institutional Review Board (research project number—20150740). Informed consent was obtained from all subjects (including fertile men who underwent biopsies for fertility preservation) prior to enrollment in the project, and the study was performed in accordance to the World Medical Association's Declaration of Helsinki.[11]

Whole Exome Sequencing
DNA was extracted from peripheral venous blood samples from the 2 brothers, their father, and their mother using the QIAamp DNA blood maxi kit (Qiagen, Germantown, MD). Whole exome sequenced was performed at the John P. Hussman Institute for Human Genomics at the University of Miami for both the siblings. There, 3 μg of DNA was prepared using the SureSelect Human All Exon V6 (Agilent, Santa Clara, CA). Resulting libraries were sequenced on an Illumina HiSeq 2500 (Illumina, San Diego, CA) for 50 million paired end 100−bp reads according to published protocols.[12] Methods for data analysis can be found in Appendix I.

Immunohistochemistry and Fluorescence Staining
For immunohistochemical staining, tissue sections of the testis biopsy (fertile controls and proband brother, brother A) were stained with hematoxylin and eosin. A genitourinary pathologist that was blinded to the samples independently analyzed the sections. For immunostaining, Bouin-fixed specimens from the testis of brother A was processed and embedded into a paraffin block. Control tissue from 2 fertile men (who underwent testicular sperm extraction for fertility preservation during vasectomy reversal) was also processed for fixed specimens. The slides prepared from these samples and blocks were rehydrated and heated in a boiling water bath for antigen retrieval (10 mmol/L citrate buffer, pH 6.0, 20 minutes). The sections were allowed to cool in citrate buffer for 20 minutes, washed twice in PBS for 5 minutes. After washing again with PBS (3 × 5 minutes), the slides were incubated for 1 hour in blocking solution (serum from the animal that the secondary antibody was made in). The sections were then incubated in either primary rabbit polyclonal anti-RAF1 or anti-pERK antibody (Abcam, ab137435 and ab65142, respectively) and anti c-Kit (Cell Signaling, ab 81), in blocking solution overnight at 4°C. Next, the slides were washed with PBS (2 × 5 minutes) and incubated in secondary antibody (Alexa Fluor 568 dye) for 2 hours at room temperature. After being washed with PBS (3 × 5 minutes) slides were mounted using 4',6-diamidino-2-phenylindole (DAPI) containing mounting media and stored at −20° overnight. All samples were assessed under a fluorescence microscope (Leica Microsystem, Wetzlar, Germany)—60×. Images were acquired using MetaMorph version 4.6 (Molecular Devices, Sunnyvale, CA).

RESULTS

Case Report
The proband in this study, brother A, was a 25-year-old male referred to our Andrology Department for evaluation of male infertility. The patient had a history of unilateral neonatal extravaginal testicular torsion and underwent left testicle orchiopexy to preserve his remaining testicle. His 27-year brother, brother B, had a history of bilateral neonatal extravaginal testicular torsion and remains anorchid. A further family history revealed that the father of both brothers, aged 62 years, had been diagnosed at the age of 10 with an undescended testicle (groin) and underwent orchiopexy (Fig. 1A).

Brother A was evaluated by an andrology-trained clinician for male infertility and was found to have 25 cc left testis with indurated epididymis on physical exam. Luteinizing hormone (LH) levels and follicle-stimulating hormone (FSH) levels were 2.5 and 2.8 mIU/mL, respectively. Serum total testosterone was 680 ng/dL. Semen analysis revealed azoospermia. In an effort to retrieve sperm, an epididymal exploration and testicular sperm extraction was performed. Motile sperm was retrieved from the epididymis and was cryopreserved for future use for wife with assisted reproduction.

Whole Exome Sequencing Results
Whole exome sequencing identified a total of 135,042 single nucleotide variants and small insertion-deletions (indels) with variation in at least 1 of the 2 brothers or father. Of the shared identical genotypes in 20,260 positions, filtering for putatively functional coding variants revealed 11,013 exonic variants. These exonic variants comprised 5837 synonymous or nonframeshift alterations, 4826 nonsynonymous single nucleotide variants, and 129 likely gene-disrupting events (frameshift indels, stop gains, stop losses or splice site alterations). We expected that the underlying causative variant would be (1) shared by the brothers, (2) missense or gene disrupting, and (3) rare in the overall population. Thus, we further applied filtering criteria that required both brothers and father to be sharing a variant while the mother lacks the variant and with less than 1% frequency in the EVS, 1000 Genomes, and ExAC database. Only 2 variants met these criteria and contained the genes KIAA2026 and RAF1.

Among the 2 candidate variants, we identified a nonsynonymous missense G > C variant in RAF1 at chr3: 12645786 (Fig. 1B). This induces a c.228 S > C change in Raf-1, which modifies a highly conserved splicing site and therefore likely destabilizes the protein structure impairing function. This heterozygous mutation was confirmed in both brothers and the father while this mutation was absent in the mother by Sanger sequencing at GENEWIZ (http://www.genewiz.com; Fig. 2). This RAF1 mutation is predicted as "Damaging" in SIFT, "Probably Damaging" by Polyphen2, and "Disease Causing" in Mutation Taster (each of these are the most severe category for the respective prediction program).

In exploring the KIAA2026 mutations, while expressed in the testis—the literature does not report any variant-causing disease despite several known sequence variations and has never been associated with testicular torsion. Therefore, according to the American College of Medical Genetics guidelines, we conclude that this is a pathogenic variant and the cause of neonatal testicular torsion and cryptorchidism in this family.[13]

Immunofluorescence Staining Results
To evaluate Raf-1 expression in the human testis, immunostaining signals in both the proband and the fertile male controls (who underwent testis biopsies for fertility preservation during vasectomy reversal) were assessed using immunofluorescence. Expression of Raf-1 in brother A (Fig. 3A-E), with the history of unilateral right testicular torsion and left unilateral orchiopexy,
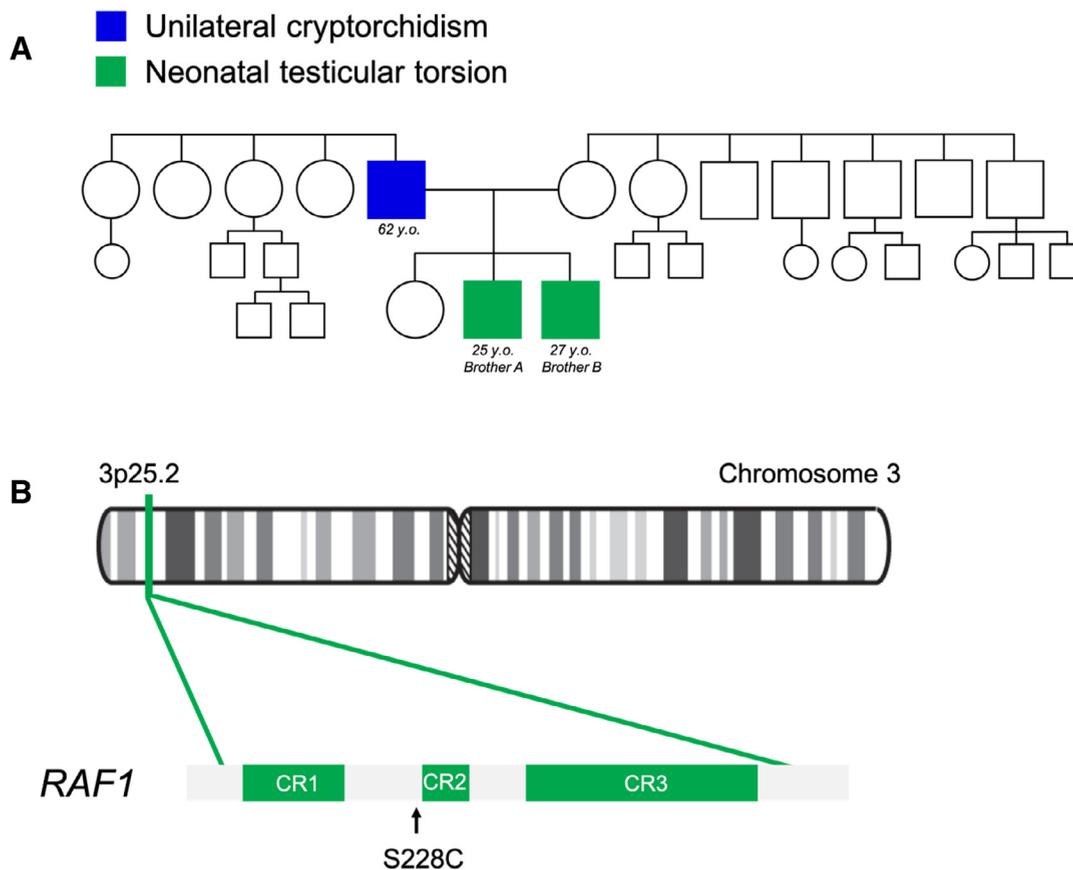
Figure 1. Family pedigree.
(A) Familial pedigree demonstrating cryptorchidism in the 62-year-old father and neonatal testicular torsion in the now 25-year-old and 27-year-old sons. (B) Chromosome 3 demonstrating the locus of *RAF1* and the three conserved regions (CR) as well as the mutation found in this family. (Color version available online).

was markedly decreased compared with Raf-1 expression in the fertile controls (Fig. 3G-L). c-Kit expression was found to be equivalent in the proband and control tissue demonstrating that the decreased expression of Raf-1 in the proband was likely pathologic (Supplemental Fig. 1). Together, these demonstrate that our patient with history of extravaginal neonatal torsion of the testis had markedly decreased expression of Raf-1 despite the presence of normal spermatogenesis (Fig. 3F,L).

To determine if decreased Raf-1 expression affects *MEK/ERK* pathway signaling, immunostaining for pERK was performed on the testis samples from the proband and fertile male. The pERK was decreased in the testis section from the proband (Fig. 4A-D) compared with the fertile control (Fig. 4E-H), suggesting that this mutation of Raf-1 likely affects the signaling of the MEK/ERK pathway in the testis.

DISCUSSION

We identified a nonconsanguineous family with 2 siblings who experienced neonatal extravaginal testicular torsion and a father with a history of unilateral cryptorchidism. Both the siblings and the father each have a heterozygous nonsynonymous mutation in *RAF1* (c.C683G; p.S228C, RefSeq ID: NM_002880; chr3: 12645786 G > C). This variant is not reported in the 3 genomic databases studied, but other variants in *RAF1* are associated with Noonan

Syndrome—a genetic condition in which 77% of boys present with cryptorchidism.[14] This is the first human study to identify a genetic variant in a family with testicular torsion and cryptorchidism.

Our findings suggest that *RAF1* and the *MEK/ERK* pathway are integral to the descent of the testicle and this variability in phenotype may be a result of gubernacular dysfunction, as failure of attachment and descent could result cryptorchidism while a free-floating cord without secure attachment may also result in *in utero* neonatal testicular torsion. This variability in phenotype has been demonstrated in mouse models with the gene *INSL3*, wherein heterozygous mice presented with both cryptorchidism and testicular torsion. Sozubir et al found in knockout $Insl3^{-/-}$ mice that 94% had intra-abdominal testes, while in heterozygous $Insl3^{+/-}$ mice 24% had intraabdominal testes and 27% experienced spermatic cord twisting. Overall, 42% of the testicles of $Insl3^{+/-}$ mice were found to hypermobile and not fixed. Testes were normally descended without spermatic twisting in the wild-type mice $Insl3^{+/+}$.[15] Zimmermann et al similarly reported intra-abdominal testes in double knockout $Insl3^{-/-}$ mice and a failure of the gubernaculum to develop during embryogenesis.[7] Insl3 expression will likely be similar between proband and fertile controls since it is upstream of Raf-1 (Fig. 5).
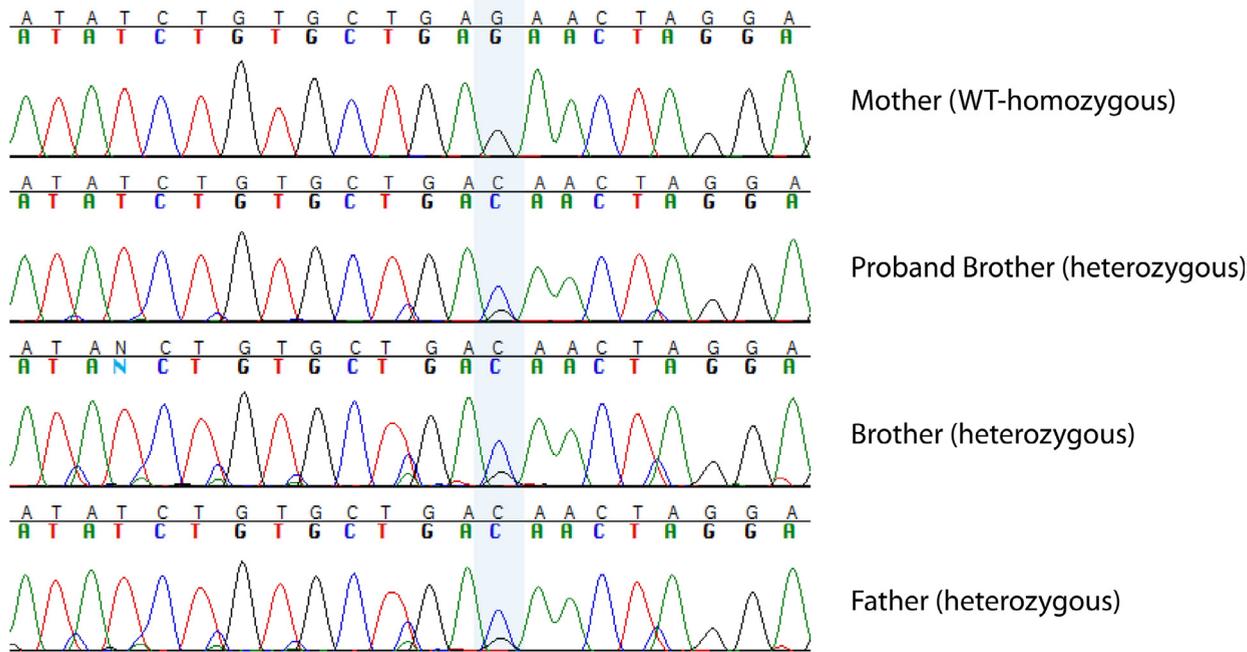
Figure 2. Sanger sequencing.
Sanger chromatographs of the entire family for segregation analyses. Both sons and father were found to have heterozygous mutations while the mother was found to be wild type homozygous. The highlighted nucleotide sequence demonstrates only 1 signal for the mother—indicating a homozygous gene without the mutation of interest, while brother A, brother B, and the father demonstrate 2 signals—indicating heterozygous mutation. (Color version available online).

While strong evidence supports the role of *Insl3* and *RXFP2* in testicular descent, the downstream pathway remained unknown. A recent study assessing *Insl3* signaling in human osteoblasts identified that this downstream pathway mainly signals via the *MEK/ERK* pathway.[16] *RAF1* is an important contributor to the *MEK/ERK* pathway, thus it is highly likely that decreased expression of *RAF1* decreases production of *MEK/ERK* and downstream transcription factors.[17] Our familial model provides additional support for the theory that reduced signaling via *Insl3* and the subsequent *MEK/ERK* pathway results in varying degrees of impaired testicular descent—with
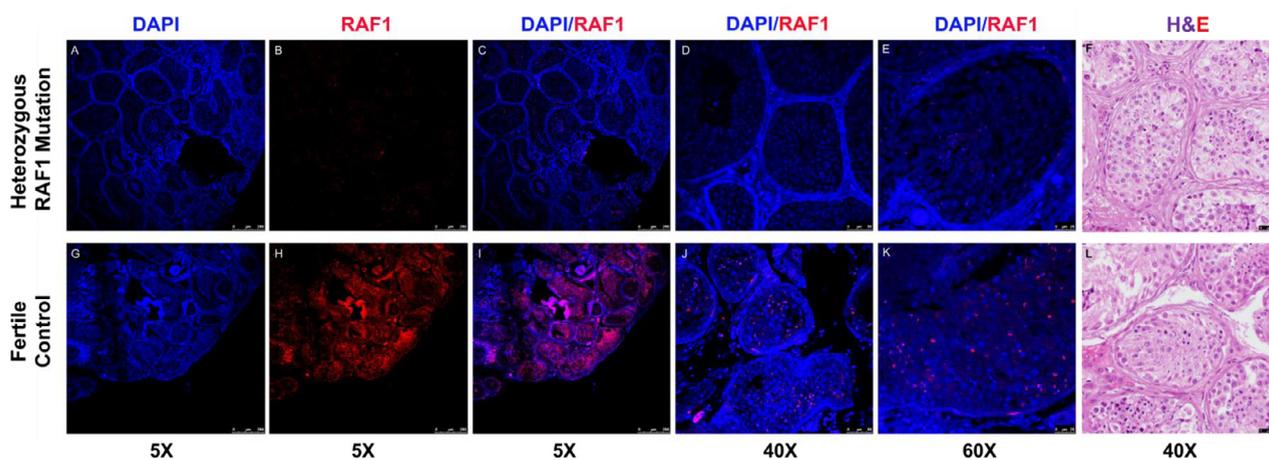
Figure 3. RAF1 immunohistochemistry staining expression pattern of Raf1 in the testis. **(A-E)** Expression of Raf1 in testis biopsy from son with unilateral neonatal testis torsion and unilateral orchiopexy. **(G-K)** Expression of Raf1 in a patient with normal spermatogenesis who underwent testis biopsy during vasectomy reversal—positive control. **(F,L)** H&E sections of testis biopsy demonstrating normal spermatogenesis in both control as well as patient. Scale bar in bottom right. Markedly decreased expression of Raf1 is evident in brother A with history of unilateral testicular torsion despite the presence of normal spermatogenesis. (Color version available online).
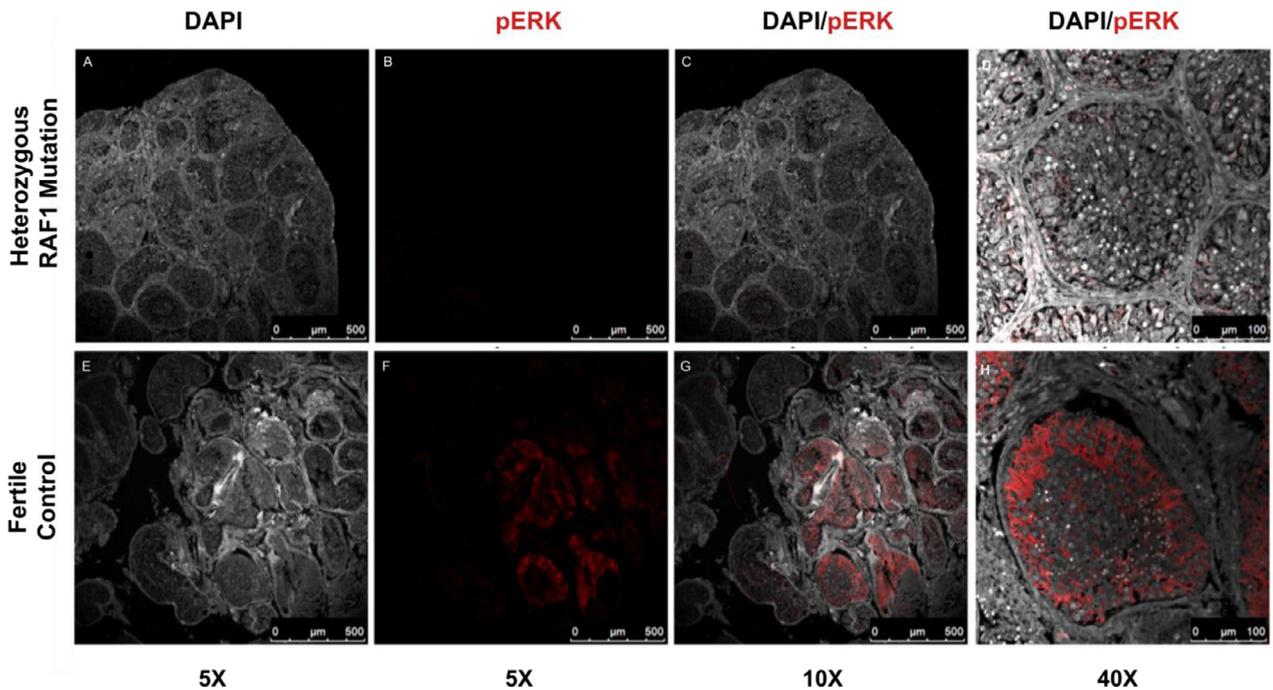
Figure 4. pERK immunohistochemistry staining expression pattern of pERK in the testis. **(A-D)** Expression of pERK in testis biopsy from brother A with unilateral neonatal testis torsion and unilateral orchiopexy. **(E-H)** Expression of pERK in a patient with normal spermatogenesis who underwent testis biopsy during vasectomy reversal—positive control. Scale bar in bottom right. Markedly decreased expression of pERK is evident in brother A with history of unilateral testis torsion. (Color version available online).

phenotypes ranging from bilateral cryptorchidism to complete testicular torsion. Importantly, our study is unique in that all previous studies have been performed in mouse models without knowledge of whether this effect occurs in humans.

Our findings also unify two known genetic phenomena associated with cryptorchidism. First, mutations in *RAF1* are estimated to cause 3%-17% of Noonan syndrome—a disorder that typically involves unusual facial features, hypertrophic cardiomyopathy, developmental delays, short stature, and cryptorchidism.[18] Cryptorchidism can be unilateral or bilateral but occurs in 77% of boys with Noonan syndrome who have mutations at various loci in *RAF1*.[14] Second, one study examined the relative expression of 3 genes in undescended vs normally descended testes. Assessing testicular biopsies of 16 cryptorchid and 6 descended testicles, higher levels of *RAF1*, *FGFR1*, and *SOS1* were identified in descended testicles.[19] Given that individuals with known *RAF1* mutations frequently have cryptorchidism and that *RAF1* has been shown to be underexpressed in the cryptorchid testis, our study provides the first familial evidence that an inherited *RAF1* variant is associated with both cryptorchidism and testicular torsion, reinforcing that *RAF1* is a key mediator in the pathway for testicular descent.

Additionally, our study is the first to identify any gene associated with familial testicular torsion. Several studies have been published detailing familial cases of testicular torsion[20,21]; these studies, including a meta-analysis, suggest the presence of a genetically-determined component in familial testicular torsion, but none of these studies has performed genetic analysis to identify a candidate gene. While we were unable to assess the gubernaculum of our patient, the occurrence of siblings with neonatal testicular torsion is consistent with the dysfunction of the gubernaculum identified in the *Insl3* mouse models. Thus, given the shared MEK/ERK downstream pathway between *Insl3* and *RAF1*, we assume a similar gubernacular dysfunction is present in our familial cases of bilateral intravaginal testicular torsion with an identified *RAF1* variant.

Our study has significant limitations. All familial studies are limited by participation of extended family members. In our case, we were unable to test any additional male relatives on the father's side of the family to establish a negative control because there were none. While we have demonstrated that this mutation of *RAF1* is likely disease-causing via (1) modeling software, (2) immunofluorescent staining that demonstrated decreased expression of *RAF1* and downstream factors, and (3) additional studies cited above implicating the role of *RAF1* in cryptorchidism, we can only demonstrate correlation between our whole exome sequencing data and the phenotype present in this family. Other potential causes of poor testicular descent, such as low androgen levels during development, cannot be definitively ruled out.[22] While there is no immediate clinical application for these findings, as the purpose of our study was to provide further evidence in the search for the genetic pathway for testicular descent, understanding this pathway may be useful in the
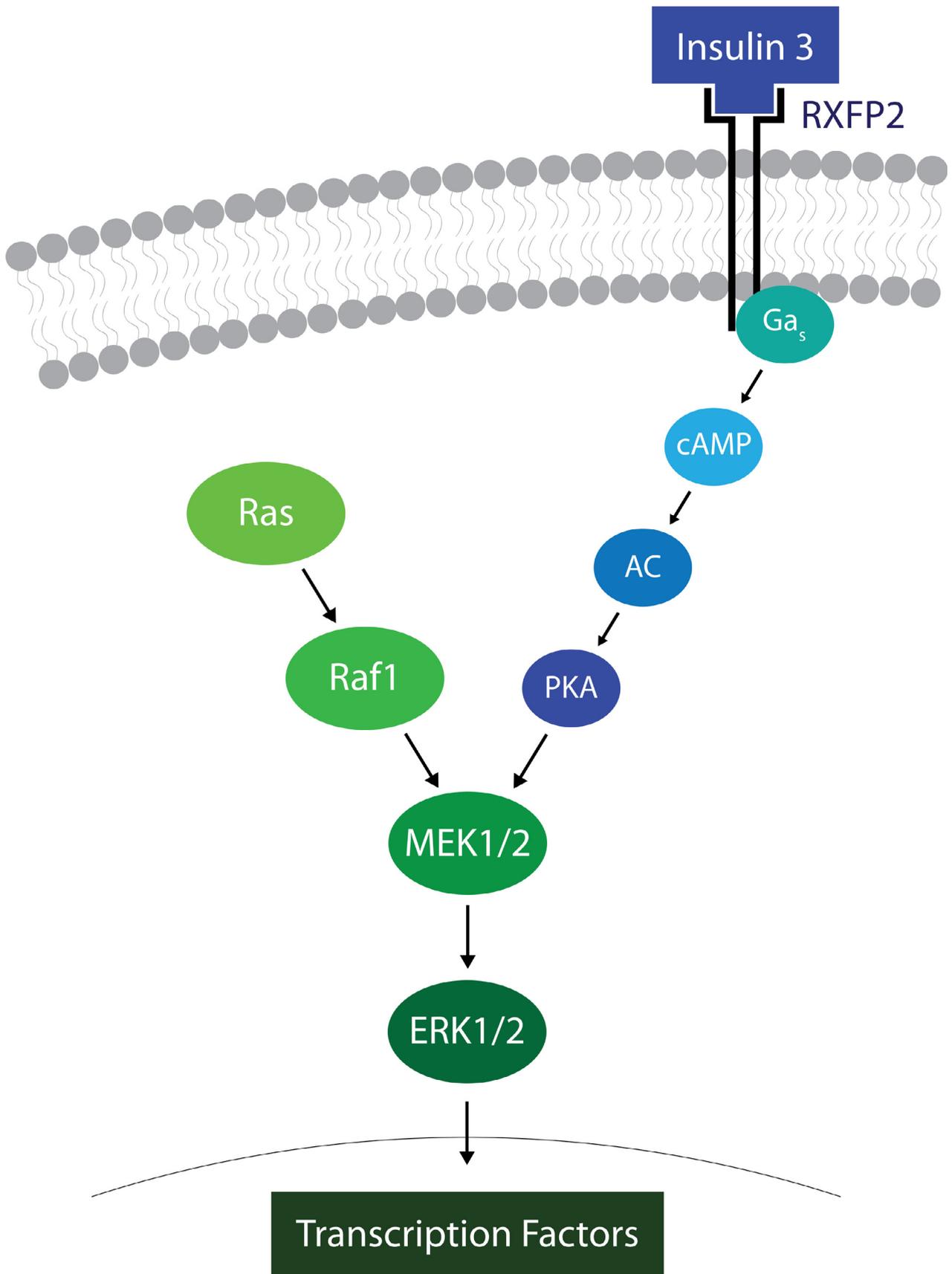
Figure 5. RAF1 pathway.
    Role of Raf1 in connection with MEK/ERK pathway as well as insulin 3 and RXFP2 receptor. (Color version available online).

future for determining when to intervene in cases of cryptorchidism if genetic causes can be identified. Further work is still required to elucidate the pathway for testicular descent and future studies are planned to investigate the role of *RAF1* and the *MEK/ERK* pathway in a mouse knockout model.

CONCLUSION

This is the first familial series to identify a specific gene associated with impaired testicular decent and testicular torsion. This heterozygous *RAF1* variant in a nonconsanguineous family presented with neonatal testicular torsion in two sons and cryptorchidism in their father. Impaired *RAF1* may hinder expression of the shared *MEK/ERK* pathway and may result in dysfunction of the gubernaculum potentially explaining both cryptorchidism and neonatal testis torsion associated with this mutation.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urology.2019.01.052.

APPENDIX I

Data Analysis

DNA reads were processed according to the Genome Analysis Toolkit (GATK) best practices guidelines.[23-25] Reads were aligned to the human GRCh37 genome with Burrows Wheeler Aligner.[26] Duplicates were marked with Picard (http://broadinstitute.github.io/picard) and base-quality recalibration was performed by GATK. DNA variants were called with the GATK HaplotypeCaller for each sample. Variants were filtered for sites at a minimum depth of 8×, alternate allele fraction >0.3, and alternate genotype likelihood (PL score) >100. The Feb2017 version of ANNOVAR[27] was used for annotation against the RefGene transcript model. Information regarding population frequency was added from the Exome Variant Server,[28] 1000 Genomes project,[29] and the Exome Aggregation Consortium.[30]

Variants were interpreted as pathogenic or likely pathogenic according to the American College of Medical Genetics and the Association for Molecular Pathology guidelines,[13] prioritized by being shared in both brothers and the father but absent in the mother, present in protein coding regions or within 20 bp of introns, and introducing nonsense, frameshift, splicing, or nonsynonymous amino acid changes. These variants were compared with population frequencies in the public databases and filtered for those present at less than 1% in the overall population or not reported the aforementioned databases (Exome Variant Server, 1000 Genomes Project and ExAC). Validation of identified likely-pathogenic variants was performed by targeted Sanger sequencing (GENEWIZ, http://www.genewiz.com) of the surrounding 310 base pairs in the variant of interest. Sequencing was performed with the primers 3′ CCCAAGGTGCCCTAT-TACCT 5′ and 3′ AAAGAATCAAACATTTTGTCAAAGG 5′. We analyzed the possible pathogenic functional effects of the variants using three types of prediction programs (Mutation Taster: http://www.mutationtaster.org, SIFT: http://sift.jcvi.org, and PolyPhen2[15]). These tools predict the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.

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EDITORIAL COMMENT

The basic science of this article is likely beyond the scope of the average practicing urologist, but nevertheless raises interesting genetic and clinical issues.

It is not entirely clear that the disordered genes of RAF-1 and MEK/ERK are the cause of the torsions and cryptorchidism in this family or merely coincidental. And even if this genetic testing was unfailingly specific and sensitive, it is unlikely to be available for use in the acute setting of scrotal pain. It seems to me the main utility of this genetic testing would be to get this test in infancy or even in utero and educate males who test positive of the risk of torsion in the future. And if the tests were found to be exceedingly predictive of testis torsion, would there be a role for prophylactic orchidopexy to prevent later torsion, or (and this suggestion would surely be controversial) earlier delivery to prevent neonatal torsion?

The authors would hopefully continue this line of investigation by testing for abnormalities of RAF-1 and MEK/ERK in the larger populations of controls versus patients with cryptorchidism and testis torsion.

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AUTHOR REPLY

We identified a novel nonsynonymous mutation in RAF1 in a Caucasian family whose father experienced cryptorchidism while both sons had bilateral testicular torsion shortly after birth. Testing our hypothesis that *RAF1* is part of the *MEK/ERK* pathway, we demonstrated in testicular tissue sample that decreased expression of RAF/MEK/ERK proteins. A limitation to all familial genetic studies is that causal relationships cannot be established rather only associations can be reported; however, the screening of nonaffected individuals and tissue analysis further strengthens these associations.

Although our study may not have immediate clinical application at this time, we expect our findings can add to the growing body of literature describing the molecular pathway for testicular descent. Several studies have suggested that cryptorchidism and testicular torsion are on a continuum of impaired testicular descent.[1,2] Understanding the pathway involved in testicular descent may aid in identifying those males who have a dysfunctional gene required for normal descent. While genetic screening during a testicular torsion presentation would be impractical, genetic screening may be useful in cases of cryptorchidism to help decide whether natural descent of the testis would occur with age. Taken together, this familial genetic study provides further evidence that impaired testicular descent may result in testicular torsion or cryptorchidism phenotypes.

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