Lack of association of *KATNAL1* gene sequence variants and azoospermia in humans

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Abstract

Purpose A recent experiment indicated that a loss of function mutation in the murine *Katnal1* gene resulted in male factor infertility due to premature exfoliation of spermatids. This study investigated the relevance of this gene to infertility in humans.

Methods Multiple methods of genetic analysis were employed to investigate whether mutations in human *KATNAL1* have a causative role in male infertility. This was a genetic association study, which included DNA samples from 105 men with non-obstructive azoospermia (NOA) and 242 anonymous sperm donor controls. 28 commercially available TaqMan SNP assays were used to haplotype samples from both groups and genetically tag regions of interest across the entire gene. AmpliSeq primers were then designed for identified regions so that targeted next-generation sequencing (NGS) could be used to identify causative variants.

Results Four SNPs in the 3'UTR demonstrated a putative association with NOA. The AmpliSeq primers designed for the 3'UTR provided 83 % coverage of the 7,202 basepairs within the regions of interest. Variant sites were analyzed

against genetic models to identify sequence polymorphisms which associated with NOA. No variants met standard criteria for significance when tested between the groups.

Conclusions This study suggests a lack of association of *KATNAL1* gene sequence variants and azoospermia in humans.

Keywords *KATNAL1* · Azoospermia · Genetic association · Next generation sequencing · Tagging SNP

Introduction

Male factor infertility can stem from numerous pre-, post-, and testicular etiological factors, among which are genetic mutations [1, 2]. Chromosomal abnormalities such as Y-chromosome microdeletions and aneuploidy are the most common genetic abnormalities associated with male infertility, responsible for around 5 % of cases in males and 15 % in azoospermic males [3]. Single gene and polygenic mutations can be causative of various infertility phenotypes as well, and genetic association studies using knockout models and ENU mutagenesis screens, as well as microarrays and hybridization arrays for whole genome analyses, have been conducted in attempts to identify responsible genes [4–6]. The advent of targeted next generation sequencing has furthered these attempts by allowing for large scale studies to be conducted at base pair resolution for both mutations and their surrounding sequences.

The relevance of associated SNPs and genes involved in male specific infertility can be difficult to discern since more than 2,300 genes have been predicted to be involved in spermatogenesis alone [7]. Investigating genes based on specific phenotypes, however, can help narrow the number of candidate genes. For example, the human katanin p60 subunit A-like 1 (*KATNAL1*) and katanin p80 subunit B-like 1 (*KATNB1*) genes have been identified in murine models as

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Capsule Investigation of the *KATNAL1* gene for an association with the human phenotype non-obstructive azoospermia using genetic association and next generation sequencing yielded no significant findings.

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causative of male-specific infertility due to two different loss-of-function point mutations. Katanin is a member of the AAA ATPase super family that uses energy from nucleotide hydrolysis to sever and disassemble microtubules through the catalytic p60 subunit and centrosome-targeting regulatory p80 subunit [8–11]. In *Katnbl1* mutant mice, a guanine to thymine mutation in exon 9 caused oligoasthenoteratozoospermia due to decreased sperm production, motility, and abnormal sperm morphology [12], while in *Katnal1* mutant mice, Sertoli cell microtubules were disrupted causing the premature exfoliation of spermatids due to a thymine to guanine mutation in exon 7 resulting in the absence of mature sperm [13].

Spermatogenesis maturation arrest in humans is most typically seen during meiosis, indicating that mutations in genes essential for meiosis are potentially causative of infertility [14]. Katanin has a conserved functional role in meiosis, with mutations in functional homologs (MEI-1 and MEI-2) revealing roles in meiotic spindle function and regulation of microtubules in *C. elegans* [15–17], and flagellar central apparatus assembly and microtubule severing in *Chlamydomonas* [18]. Based on the murine mutation and the conserved functional role of Katanin, the *KATNAL1* gene was investigated for further associations with infertility by using a combination of genetic association and next generation sequencing. Specifically, this study aimed to identify tagging SNPs associated with azoospermia across *KATNAL1* through a genetic association study and then used a targeted next generation sequencing approach to further investigate regions of interest for novel or functional polymorphisms associated with this phenotype.

Materials and methods

Ethics

This study was done under IRB approval. The samples were selected from an existing repository consisting of individuals seeking fertility treatment.

Population

Samples were identified from a cohort of patients undergoing IVF treatment in Northern New Jersey. Cases consisted of men with non-obstructive azoospermia (NOA) and severe oligospermia, while controls consisted of men with normal sperm concentration. Cases ($n=105$) had at least one semen specimen submitted for analysis that revealed azoospermia without a known cause. Samples were excluded from this cohort if they had: undescended testicles, vasectomy/vasovasostomy, cancer treatments (chemotherapy, radiation or radical orchiectomy), Y-chromosome microdeletions, aneuploidy (including the sex chromosomes), retrograde

ejaculation, congenital bilateral absence of the vas deferens, bilateral vas deferens occlusion as a result of inguinal hernia repair, etc. The control group ($n=242$) consisted of anonymous sperm donor samples.

SNP selection

HapMap was used to identify 59 SNPs present in *KATNAL1*. 28 of these SNPs were selected based on haplo-block correlation and distribution across *KATNAL1*. Only tagging SNPs that had primers commercially available through Life Technologies (Life Technologies [LTI], Carlsbad, CA, USA) were considered for inclusion. The selected SNPs (see Fig. 1) captured 86 % of the targeted alleles, with a mean r -squared value of 0.999. The 28 SNPs also did not have high marker-to-marker linkage disequilibrium with their neighboring selected SNPs, with r -squared values ranging from 0–0.477 [19]. After the initial results were viewed, four SNPs were excluded from further analysis; SNPs rs12866391 and rs7327725 were excluded due to complete homozygosity, and rs7328946 and rs9550539 were excluded for violating Hardy-Weinberg equilibrium across both the case and control sample groups. Samples were then filtered to include only those with ≥ 90 % call rates before statistical analysis was performed.

Genotyping through allelic discrimination

Genotyping was done on the QuantStudio™12 K Flex Real-Time PCR System (LTI). 347 samples were normalized to 50 ng/uL. Three samples did not have enough starting gDNA, so whole genome amplification with the REPLI-g Mini Kit (QIAGEN Inc, Germantown, MD, USA) was performed prior to use. 2.5 uL of TaqMan® OpenArray® Genotyping Master Mix (LTI) and 2.5 uL of each gDNA sample were premixed in a 384 well plate and loaded onto the genotyping plates using the QuantStudio™ 12 K Flex OpenArray® AccuFill™ System. The samples were plated in duplicate, and results were analyzed in TaqMan Genotyperv1.2 (LTI). Samples that were called unamplified or undetermined were rerun using duplex real time PCR reactions followed by allelic discrimination on the ABI PRISM® 7900 HT Sequence Detection System using SDS 2.3 software (LTI). A no template control (NTC) consisting of water was included in each run.

SNP statistical analysis

Association analysis was performed in Golden Helix SNP & Variation Suite (SVS) v7.x software (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com). Additive, Dominant, Recessive, and Basic Allele associations were performed, as well as Principal component analysis.

Fig. 1 The locations of the 28 tagging SNPs used in the genetic association study are depicted spanning the *KATNAL1* gene. The magnification of the 3'UTR shows the regions that were covered by the AmpliSeq design (green) for next generation sequencing

Next generation sequencing data acquisition

Sample library preparation was done according to the procedure specified in the Ion AmpliSeq Library Preparation User Guide (LTI). Custom AmpliSeq primers were designed using Ion AmpliSeq Designer to cover the region stretching from the 3'UTR to the last exon in *KATNAL1*

(Fig. 1). Forty-nine amplicons 200 bp in length were designed to cover the 7.2 kilobase region (chromosome 13: 30,776,500–30,783,702) (hg19). The primer design gave 83.48 % coverage, excluding 1,190 base pairs due to repetitive sequences and low GC content (between 0–19.685). Input DNA consisted of 2 different DNA pools each at 20 ng, with sequence targets amplified for 21 cy-

Table 1 Genetic Association Values

Marker	Corr/Trend P	BonferroniCorrected P	Corr/Trend Full Scan Permutation* P	Odds Ratio ((Dd) vs. (dd))**
rs17074416	0.025	0.589	0.351	1.392
rs17632858	0.068	1	0.666	1.445
rs17074420	0.004	0.098	0.070	2.546
rs9506275	0.060	1	0.611	0.686
rs2031998	0.884	1	1	0.914
rs169332	0.386	1	1	1.319
rs9551875	0.764	1	1	1.004
rs2277450	0.368	1	1	1.301
rs17724817	0.585	1	1	0.999
rs648538	0.050	1	0.571	0.797
rs202093	0.310	1	0.997	1.380
rs10492463	0.749	1	1	0.835
rs17633078	0.109	1	0.821	1.228
rs846491	0.196	1	0.959	0.705
rs9579551	0.286	1	0.993	1.426
rs7317962	0.634	1	1	0.563
rs17074507	0.947	1	1	0.773
rs7358807	0.312	1	0.998	1.398
rs202091	0.397	1	1	0.734
rs2077011	0.600	1	1	1.301
rs9508700	0.093	1	0.765	0.671
rs185694	0.564	1	1	0.791
rs9508701	0.468	1	1	0.806
rs1952607	0.646	1	1	1.038

* Permutation analysis done at 10,000 permutations

** dd=Major Allele

cles using the 2X primer pool protocol, followed by adapter and barcode ligation. Molar concentrations for each amplicon were obtained using the Bioanalyzer and Agilent High-Sensitivity DNA microfluidic chips (Agilent Technologies), and the samples were then normalized to 22 pmol/L. Template preparation was done according to the Ion One Touch protocol (LTI), which used the Ion PGM^{TM} Template OT2 200 Kit for template preparation and the Ion PGM^{TM} Sequencing 200 Kit v2.0 for sequencing. 318 Chips were used, with 12–24 samples barcoded per chip (with each sample having 2 barcodes for each primer pool).

NGS data analysis

Fastq files [20] for all of the barcoded samples were obtained from the Ion Torrent Server. Ion Reporter Software 1.4 was used to analyze the variants. The reference file was generated from the .BED file that the Ion AmpliSeq primers were designed from, and the variant caller parameters were the same as for the built-in CHPv2 workflow. The output vcf files contained the SNP and INDELs annotated for each sample.

Results

Association analysis using the additive model with the NOA samples ($n=90$) compared against the control

samples ($n=226$) was performed for twenty-four SNPs to explore possible associations with NOA (overall call rate 97.06 %). The statistical results are listed in Table 1. As indicated, SNP rs17074420 had a significant uncorrected p -value, $p=0.004$, while rs17074416, rs17632858, and rs9506275, showed putative associations at $p=0.025$, $p=0.068$, and $p=0.060$, respectively. While none of the p -values survived Bonferroni correction, the corrected p -value for rs17074420 showed a putative association at $p=0.098$. Dominant and basic allele models yielded similar results, while the recessive model did not have any significant associations (results not shown).

In order to ensure that results were not affected by population stratification, a PCA was performed for the twenty-four SNPs across all samples. While there was no difference observed in the distribution of cases and controls in the PCA space ($p>0.05$), there was not enough power to definitively rule out PCA without examining additional SNPs. However the observed results suggest that population stratification was not a strong confounding factor.

Since rs17074420 was located in the 3'UTR of *KATNAL1*, the entire 3'UTR (7.2 kilobases, chromosome 13: 30,776,500–30,783,702) was selected for further investigation via next generation sequencing. Samples carrying rs17074420 were roughly matched at a 2:1 ratio with randomly selected samples that did not carry the SNP, yielding an overall sample size of 194 samples (105 cases and 89 controls). Ion Reporter Software 1.4 was used to identify variant sites.

From the entire sequenced 3'UTR region, four SNP sites had both genotyping data from the original

Table 2 Novel variants detected in the 3'UTR region

Chr 13 Position	No. Cases (Het)	No. Controls (Het)	Ref Allele	Alt Allele
30,776,977	1	2	A	G
30,777,467	0	1	C	A
30,777,656–30,777,666	0	1	AAGAGGGAAAC	-
30,778,287	1	0	A	C
30,778,292	0	1	C	T
30,779,639	0	1	T	C
30,779,734	0	1	C	A
30,779,755	2	0	G	C
30,779,960	0	1	A	ATCGTGGTTGCC
30,780,749	0	1	A	C
30,780,773	1	0	T	C
30,781,443	1	0	T	C
30,781,636–30,781-642	1	0	AATGTAA	-
30,781,682	0	1	A	C
30,781,712	1	0	T	A
30,782,331	1	0	A	G
30,783,284	1	0	A	G
30,783,564	1	0	C	T

Table 3 Known Variants Detected in the 3'UTR Region

rs#	Cases		Controls		Fisher <i>P</i> -Values	Bonferroni Corrected <i>P</i>
	Het Carrier	Homo Affected	Het Carrier	Homo Affected		
rs182105027	0	0	1	0	0.918	1
rs11839654	0	0	1	0	0.918	1
rs147850458	0	0	1	0	0.918	1
rs186665940	0	0	1	0	0.918	1
rs76781079	0	0	1	0	0.918	1
rs145250193	0	0	1	0	0.918	1
rs148949899	0	0	1	0	0.918	1
rs76940165	0	0	1	0	0.918	1
rs113998014	0	0	1	0	0.918	1
rs188065499	0	0	1	0	0.918	1
rs55836900	0	0	1	0	0.918	1
rs9508679	0	0	1	0	0.918	1
rs140253752	0	0	1	0	0.918	1
rs61946916	0	7	1	3	0.337	1
rs182453378	1	0	0	0	>0.999	1
rs114243171	1	0	1	0	>0.999	1
rs61435763	1	0	2	0	0.876	1
rs17689385	1	0	3	0	0.504	1
rs117247354	1	0	3	0	0.504	1
rs78699770	2	0	0	0	0.585	1
rs114829192	2	0	0	0	0.585	1
rs77772670	2	0	0	0	0.585	1
rs9579542	2	0	1	0	>0.999	1
rs144510457	2	0	2	0	>0.999	1
rs117991635	2	0	2	0	>0.999	1
rs75680479	5	0	9	0	0.257	1
rs45507700	6	0	7	0	0.758	1
rs9508678	9	1	7	0	0.719	1
rs11620056	16	4	19	0	0.944	1
rs1023104	17	1	22	0	0.372	1
rs12428633	17	9	28	10	0.019	1
rs9508676	21	0	16	0	0.873	1
rs12430537	21	1	15	1	0.779	1
rs17074420	21	1	20	1	0.783	1
rs45484093	23	1	14	1	0.445	1
rs9551868	25	7	33	2	0.885	1
rs2031998	27	7	34	2	0.749	1
rs3825533	28	5	34	3	0.345	1
rs7328946	28	9	37	5	0.360	1
rs112146305	33	0	24	0	0.637	1
rs17632858	44	7	37	2	0.360	1
rs9506275	52	18	45	15	>0.999	1

association study done by qPCR and allelic discrimination, and from the second phase by next-generation sequencing, allowing for comparisons to be made between the genotype calls assigned to each sample. The

similarity between the two methods ranged from 90.2 % to 94.8 %, which is within the expected range identified for cross-platform replication of next-generation sequencing data [21–23].

A total of 60 SNPs were identified in the 3'UTR region (42 variants with dbSNP identification numbers [24] and 18 variants that were novel). Most variants were covered at an average depth of ~500 reads. All of the novel variants were observed only in the heterozygous state, with seventeen of these variants appearing only once. Because of this, Fisher exact tests (2-tailed, 95 % CI) indicated that these results were insignificant (Fisher p-value = >0.9 for variants present in only one sample, and 0.585 or 0.876 when present in more than one sample). A Fisher exact test was also done comparing the total number of minor alleles observed between the cases and controls for these novel variants, but the result was also insignificant (Fisher p-value=1.00). Table 2 shows a summary of these novel SNPs and their locations in *KATNAL1*. None of the regions where the SNPs were located were known to have roles in regulation.

The 42 variants that did have dbSNP identification numbers had been previously reported as untranslated or intron variants, and had minor allele frequencies available from the 1000 genome project. Fisher p-values were calculated for these variants as well, however none of the variants were found to be significant after Bonferroni correction, with all values driven to p=1.00 (Table 3). Therefore no variants met the standard criteria for significance when tested between the cases and controls.

Discussion

The lack of associated infertility phenotypes found in *KATNAL1* suggests that the genotype-phenotype correlation observed for this gene in murine models may not apply to humans. While many spermatogenesis genes and processes are conserved between mice and humans [25–27], studies have shown that functional orthologs are not always representative of their corresponding human genes [28, 29], and even if they are, the expected phenotype severity can vary between species [14]. It is also possible however that there could have been a novel variant in this region that was not picked up by the association study that could have been causative of the NOA phenotype. Since sequencing was not done across the entire *KATNAL1* gene, it is possible that such a variant was missed. The leucine to valine [UUA to GUA] change reported in exon 7 of murine *Katnal1* [13] corresponds to position 30805474 on human chromosome 13. In terms of reported variants, the USC Genome Browser reports no SNPs at this position, and no significant association was observed when utilizing the TaqMan SNPs in the association study.

With the exception of mutations that cause very specific phenotypes, such as globozoospermia [30, 31], very few genes have been definitively linked to infertility. The numerous candidate genes identified as causative of these specific phenotypes suggest genetic heterogeneity, indicating that

male factor infertility could be caused by patient-specific mutations in different genes or can result from various genetic interactions. Genomic instability may also be causative of infertility [4], with studies showing that men with good semen quality (normal and motile sperm) and concentration had dose-response decreased mortality [32] and comorbidity rates [33] than infertile men. The lack of association of *KATNAL1* with the NOA phenotype may also reflect the relatively small sample size (n=105 NOA patients) utilized in this study.

In summary, 28 tagging SNPs that spanned *KATNAL1* were originally analyzed for their potential association with the NOA phenotype. Only 4 of those SNPs demonstrated a putative association with the NOA phenotype and all of them were located in the 3'UTR. Since the 3'UTR is important for various regulatory processes, including transcript cleavage, stability, and polyadenylation, as well as translational activation, mRNA localization, and binding sites for microRNAs and regulatory proteins [34, 35], the entire region was sequenced in attempts to identify SNPs that could affect functional variants located in *KATNAL1* or in neighboring genes. The lack of any significant variants identified in the NOA patients in both the 3'UTR and across *KATNAL1* indicates that this gene is not likely to be responsible for the NOA phenotype in male infertility patients.

Ethical Standards

The manuscript does not contain clinical studies or patient data.

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