



The potential impact of tumor suppressor genes on human gametogenesis: a case-control study

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Abstract

Purpose To study the incidence of tumor suppressor gene (TSG) mutations in men and women with impaired gametogenesis. **Methods** Gene association analyses were performed on blood samples in two distinct patient populations: males with idiopathic male infertility and females with unexplained diminished ovarian reserve (DOR). The male study group consisted of men with idiopathic azoospermia, oligozoospermia, asthenozoospermia, or teratozoospermia. Age-matched controls were men with normal semen analyses. The female study group consisted of women with unexplained DOR with anti-Müllerian hormone levels ≤ 1.1 ng/mL. Controls were age-matched women with normal ovarian reserve (> 1.1 ng/mL). **Results** Fifty-seven male cases (mean age = 38.4; mean sperm count = 15.7 ± 12.1 ; mean motility = 38.2 ± 24.7) and 37 age-matched controls (mean age = 38.0; mean sperm count = 89.6 ± 37.5 ; mean motility = 56.2 ± 14.3) were compared. Variants observed in CHD5 were found to be enriched in the study group ($p = 0.000107$). The incidence of CHD5 mutation c.*3198_*3199insT in the 3'UTR (rs538186680) was significantly higher in cases compared to controls ($p = 0.0255$). 72 DOR cases (mean age = 38.7; mean AMH = 0.5 ± 0.3 ; mean FSH = 11.7 ± 12.5) and 48 age-matched controls (mean age = 37.6; mean AMH = 4.1 ± 3.0 ; mean FSH = 7.1 ± 2.2) were compared. Mutations in CHD5 (c.-140A>C), RB1 (c.1422-18delT, rs70651121), and TP53 (c.376-161A>G, rs75821853) were found at significantly higher frequencies in DOR cases compared to controls ($p \leq 0.05$). In addition, 363 variants detected in the DOR patients were not present in the control group. **Conclusion** Unexplained impaired gametogenesis in both males and females may be associated with genetic variation in TSGs. TSGs, which play cardinal roles in cell-cycle control, might also be critical for normal spermatogenesis and oogenesis. If validated in larger prospective studies, it is possible that TSGs provide an etiological basis for some patients with impaired gametogenesis.

Keywords Tumor suppressor genes · Gametogenesis · Male factor infertility · Diminished ovarian reserve · Idiopathic infertility

Introduction

Gametogenesis is the biological process in which cells undergo meiosis and differentiation to form functional gametes that are haploid and can go on to join with gametes of the opposite sex to form an offspring. In humans, gametogenesis begins early in fetal development. In males, germ cell populations called spermatogonia undergo mitosis resulting in two cell populations: one that maintains stem cell characteristics and another that initiates meiosis [1]. After completing both meiosis I and meiosis II, the resulting cells are referred to as spermatids that differentiate into mature spermatozoa. Spermatogenesis occurs continuously throughout the male's adult lifetime, and men are thus able to father children until death. In females, primordial germ cells migrate to the

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embryonic gonad to become oogonia, which go on to proliferate via mitosis [2]. These cells differentiate into primary oocytes with a single layer of granulosa cells surrounding the oocyte. While females are still in utero, their primary oocytes duplicate their DNA and arrest in prophase I. Only those oocytes that are recruited to the follicular pool and ultimately selected as the dominant follicle resume meiosis I, continue to metaphase II, and will complete meiosis II once fertilized by a spermatozoa. In contrast to spermatogenesis in males, oocyte production is finite in females; thus, women are fertile from puberty through menopause.

Gametogenesis in both males and females is a highly coordinated process requiring a number of internal and external signaling pathways to ensure the production of mature and functional gametes. Disruption of sperm or oocyte production can result in infertility. Endocrine and autocrine signaling provides cues for timing of entering the various phases of differentiation and meiosis [2, 3]. The influence of altered hormone signaling on infertility has been well studied [4–6]. Throughout the process of mitosis and meiosis, cell-cycle checkpoints are necessary to guarantee the duplication and segregation of DNA with high fidelity [7–11]. The hypothesized sources of DNA damage in sperm and oocytes differ. It is thought that reactive oxygen species (ROS), sperm chromatin packing, and apoptosis are at the root of DNA damage during spermatogenesis [12–18]. In females, it is postulated that oxidative stressors and replication errors are the main causes of DNA damage requiring DNA repair [19]. Depending on the type of DNA damage, several different repair pathways, including nucleotide excision repair, base excision repair, mismatch repair, double-strand break repair, and post-replication repair, can be activated in order to maintain DNA integrity [1, 19]. In cases where repair is not appropriate, cells can initiate apoptosis.

DNA repair and apoptotic pathways require internal signaling pathways that result in either the restoration of the correct DNA sequence or in programmed cell death. Many of the genes involved in both of these processes have been identified as tumor suppressor genes (TSGs). TSGs are genes that aid in controlling cell proliferation and survival; mutations altering their function can result in development of tumors. As these genes are known to be involved in processes related to gametogenesis, we hypothesized that mutations in TSGs affect fertility potential in addition to increasing the risk for cancer. It has been shown that the tumor suppressor P53 controls DNA repair during spermatogenesis; however, its involvement in female oogenesis is sparse [33]. In fact, a number of studies in mice have demonstrated infertility phenotypes (reviewed in [4]) However, association of mutations in TSGs with infertility in humans has not been extensively studied. In some instances, case-control studies have set out to identify TSG mutations associated with specific infertility diagnoses, including poor ovarian reserve and idiopathic male infertility.

Researchers have found that women carrying BRCA1 pathogenic mutations demonstrated poorer ovarian responses to controlled ovarian stimulation protocols and lower anti-Müllerian hormone (AMH) levels compared to healthy controls [20–22]. Other studies focusing on TP53 (P53) have failed to show an association with infertility in both females and males [23–27]. Thus, while there seems to be a clear connection between TSGs and infertility in model systems, it has yet to be rigorously addressed in humans.

Here, we investigate associations between a panel of nine TSG genes and infertility through a retrospective case-control study in two populations of infertility patients—females with diminished ovarian reserve and males with idiopathic male infertility. Cases were compared to age-matched controls. Targeted exome sequencing identified a number of single nucleotide polymorphisms (SNPs) that were overrepresented in each case group. These findings suggest that TSGs may contribute to the etiology of infertility in humans.

Materials and methods

Study population

Patients who presented to a single, academic, large-volume fertility center (Northwell Health Fertility, Manhasset, NY) between August 2015 and February 2016 were considered for inclusion in the study. All study procedures were reviewed and approved by the Northwell Health Institutional Review Board.

Males were included in the case group for idiopathic male factor infertility based on a semen analysis, which was assessed for a male factor diagnosis using the World Health Organization (WHO) criteria, including azoospermia, oligozoospermia (< 15 million/mL), asthenozoospermia (< 40% motility), and teratozoospermia (< 4% normal oval) [28]. Exclusion criteria included males with more than one type of male factor diagnosis (e.g., oligozoospermia and asthenospermia) and males with a known etiology of the diagnosis (e.g., anatomic, hormonal, genetic). Standard of care evaluative tests in addition to semen analyses were used to identify known etiologies, including hormone levels, genetic testing, and scrotal ultrasound. Using these criteria, 57 cases were identified and age-matched to 37 controls with normal semen analysis parameters. For both cases and controls, age and semen analysis parameters (total volume, sperm count, motility) were recorded.

Women included in the DOR study group presented with no clear etiology (i.e., genetic, environmental). With no established standard definition of DOR [29], we define it as women with AMH levels ≤ 1.1 ng/mL, which is one of the inclusion criteria for poor ovarian response listed in the Bologna criteria [30]. Using these criteria, 72 DOR cases were

selected and age-matched to 48 controls with normal AMH levels (> 1.1 ng/mL). Additional information including significant past medical history and a full hormone panel (follicle stimulating hormone (FSH), estradiol, thyroid-stimulating hormone (TSH), and prolactin) was recorded for each eligible participant. Women with a current diagnosis or history of polycystic ovary syndrome (PCOS) were excluded from the study.

Genetic testing

For each study subject, a blood sample previously provided for genetic screening was used to sequence for 9 TSGs: *CDKN2A*, *TP53*, *TP53BP1*, *TP63*, *TP73*, *RBI*, *BRCA1*, *BRCA2*, and *CHD5*. Sequencing of these genes were performed using a targeted approach, in which all exons (including 100 base pairs into the surrounding introns) as well as the regions 1 kb upstream and downstream of each gene were sequenced. In addition to these regions, for all included genes, putative CpG islands were sequenced for *CHD5*.

Probes to enrich for sequencing of the nine genes were designed using NimbleGen SeqCap EZ System (Roche NimbleGen, Switzerland). Sequencing was performed using the NextSeq500 platform (Illumina, USA). Reads were processed and aligned to the GRCh37/hg19 reference genome assembly. Genetic variants were detected using DRAGEN (Edico Genome, United States) algorithms.

Statistical analysis

Differences in semen analysis parameters and hormone levels between cases and controls were calculated using Welch's *t* test. R software was utilized to calculate these statistical differences (v3.3.3) [31]. *p* Values ≤ 0.05 were considered significant.

Case and control groups were compared by analyzing differences in enrichment of identified SNPs in each of the TSGs included in the study. SNPs were analyzed individually or grouped by gene. For each comparison, a chi-square test was performed to assess statistical enrichment. Multiple hypothesis testing was corrected using the FDR Benjamini-Hochberg method [32]. Statistical significance was defined at $p \leq 0.05$ after multiple hypothesis corrections.

Results

Study population demographic and clinical characteristics

Chart review of 494 male patients was completed to identify cases and controls. Of these, 163 did not have semen analysis reports available. Of these, 98 were diagnosed with infertility.

Excluding those subjects with a known etiology resulted in 57 cases included in the study. Of the 162 fertile males, 37 subjects were selected as controls for comparison. The case and control groups differed in several measured semen analysis parameters, including mean sperm concentration, motility, and forward progression (Table 1).

A total of 868 female patients' charts were reviewed in order to select cases and controls. Of these, 115 women had AMH levels < 1.1 ng/mL and 72 were selected for inclusion in the study. Of the 302 women with normal hormone evaluations and no history of PCOS, 48 subjects were selected to serve as controls for comparison. The case and control groups differed in several measured hormone levels, including AMH and FSH (Table 2).

Genetic associations

Enrichment of TSG variants were assessed using two different approaches. First, each individual SNP identified was compared between cases and controls. Second, for each gene included in the study, all variants were grouped together in both the study group and the control group and enrichment of SNPs were measured. These comparisons were conducted in the males, the females and in the entire population (combining both males and females).

In males, comparison of individual variants identified one significant difference between cases and controls; the incidence of *CHD5* mutation c.*3198_*3199insT was found to be significantly higher in cases compared to controls ($p = 0.0255$) (Table 3). Comparing cases and controls at the gene level revealed that *CHD5* had an enrichment of genetic variants in the case group ($p = 0.000107$). In addition to these comparisons, there were 422 variants that appeared at a low frequency in cases only, prohibiting statistical analysis.

In females, the frequency of three individual genetic variants was identified as significantly different when comparing cases and controls: *CHD5* SNP c.-140A>C ($p = 0.0273$), *RBI* SNP c.1422-18delT ($p = 0.0273$), and *TP53* SNP c.376-161A>6 ($p = 0.0273$) (Table 3). No statistical differences were observed when assessing enrichment of variants at the gene level in DOR cases compared with age-matched controls. An additional 363 SNPs were observed only in the female cases.

Table 1 Comparison of male cases and controls

Parameter	Case group	Control group	<i>p</i> value
Age	38.4	38	0.76
Volume (mL)	2.55	2.59	0.93
Count (mil/mL)	15.7	89.7	2.7×10^{-14}
Motility (%)	38.2	56.2	3.6×10^{-5}
pH	8.36	8.39	0.70
Forward progression	1.81	2.44	1.3×10^{-6}

Table 2 Comparison of female cases and controls

Parameter	Case group	Control group	<i>p</i> value
Age	38.8	37.7	0.14
AMH	0.57	4.05	1.4×10^{-10}
FSH	10.3	7.1	0.00042
Estradiol	46.4	45.4	0.88
TSH	2.00	1.94	0.76
Prolactin	14.0	15.4	0.34

When all cases and controls (males and females) were combined together and compared, analysis of individual genetic variants resulted in one SNP with statistically different frequencies in the case and control group: *RBI* c.1422-18delT ($p=0.016$) (Table 3). Two genes contained variants that were overrepresented in the cases compared to controls: *TP53* ($p=0.00566$) and *CHD5* ($p=0.00566$) when the analysis was completed at the gene level.

Discussion

To our knowledge, this pilot study represents the first time targeting next generation sequencing (NGS) has been utilized to investigate the association between TSG variants and infertility in humans. This approach, compared to a candidate SNP approach, allows for discovery of novel SNPs that may not have been included in previous studies. In fact, we identify novel variants that may influence male factor infertility or poor ovarian reserve (Table 3). The four unique SNPs identified in this study have yet to be characterized elsewhere; however, variants in TSGs known to influence infertility—*RBI* and *TP53*—were found to be significantly enriched in our study populations. [33, 34]

We identified genetic variants in a third gene that has yet to characterize any variants associated with impaired gametogenesis or infertility in humans—*CHD5*. Recent gene targeted disruption of *CHD5* (the mouse equivalent of human *CHD5*) in mice demonstrate not only an increased risk for cancer, but also abnormal sperm morphology and infertility in males [35, 36, 37]. Li et al. defined a functional role for *CHD5* in the histone-to-protamine transition and in DNA repair during

sperm maturation [35]. This study used a gene expression dataset comparing testes biopsies of men with testicular pathology to males with normal spermatogenesis, revealing that *CHD5* expression was decreased by 75–95% in males with varying levels of testicular pathology compared to the normal males, suggesting that *CHD5* plays a role in human spermatogenesis. Here, we find that SNPs within *CHD5* are enriched in our male cases compared to controls. In addition, we find that the frequency of an individual *CHD5* genetic variant is statistically different between the case and control populations. This SNP (c.*3918_3199insT; rs538186680) can be found in the 3'UTR (untranslated region); while this variant does not affect a predicted microRNA binding site [38], it is possible that it impacts translation of *CHD5* transcripts by perturbing 3'UTR function [39]. Additional functional studies are required to determine the extent that this variant affects spermatogenesis in males diagnosed with idiopathic male factor infertility.

No studies outside the present investigation have explored the role of *CHD5* in females. Here, we find a genetic variant within *CHD5* to be associated with females in the poor ovarian reserve case group compared to the female controls (c.-140A>C). This SNP has not been characterized elsewhere. However, this variant is conserved in the mouse genome. It is possible that the nucleotide change at this position alters expression of *CHD5* as it may impact transcription factor binding or histone placement. Given that mouse *CHD5* has been shown to play a role in DNA repair during spermiogenesis (the sperm differentiation process), it is possible that it also plays a role in oogenesis. There is evidence that DNA double-strand breaks, if not repaired in a timely manner, may induce chromatin remodeling [40, 41], processes known to be regulated by *CHD5*. Further functional studies both in model organisms and in humans should be pursued to determine the significance of *CHD5* perturbations in oogenesis.

CHD5 is a member of the chromodomain helicase DNA-binding (*CHD*) protein family, which belong to the SNF2 superfamily of ATP-dependent chromatin remodelers (reviewed in [41, 42]). In addition to the chromatin binding domain, *CHD5* also has additional domains including paired PHD zinc finger like domains and DNA binding domains. Bagchi et al. identified *CHD5* as a TSG in 2007 by engineering mouse models of human 1p36 copy number variations

Table 3 Genetic variants significantly different between cases and controls

Case/control comparison	Gene	cDNA	rsID	Population frequency	<i>p</i> value
Males	<i>CHD5</i>	c.*3198_3199insT	rs538186680	0.02133	0.0255
Females	<i>CHD5</i>	c.-140A>C	N/A	0.15	0.0273
	<i>RBI</i>	c.1422-18delT	rs70651131	0.05–0.17	0.0273
	<i>TP53</i>	c.376-161A>G	rs75821853	No data	0.0273
Male + female	<i>RBI</i>	c.1422-18delT	rs70651131	0.05–0.17	0.016

prevalent in human cancers, and by assessing its deletion in human gliomas [43]. In male mice, *CHD5* is essential for removing canonical histones and repackaging the genome during sperm maturation [44], coordinating both the histone-to-protamine transition and resolution of the double-stranded DNA breaks that are generated during this transition. It is possible that *CHD5* is also a regulator of human gametogenesis, as our current findings suggest.

In addition to studying the male and female populations separately, we also examined the association of TSGs with gametogenesis, combining the two case-control groups. As tumor suppressors are involved in both spermatogenesis and oogenesis, our findings suggest that these proteins influence gametogenesis overall. While the processes differ in terms of timing and differentiation steps, meiosis and subsequent differentiation must be completed with fidelity in both males and females in order to have normal sperm and oocytes that can go on to produce a normal haploid gamete. While the results from such analyses may be due to a strong correlation in one subpopulation, it may also provide insights to shared processes critical for both genders. Analysis of each group should be conducted separately as well to help determine the contribution of each population to any overall findings.

Limitations of this study include population size and the nature of retrospective study designs. The small sample size for each subpopulation might have affected the findings of the study. A larger sample size would increase the statistical power of the analysis and may even result in additional variants that are significantly associated with idiopathic male factor infertility or poor ovarian reserve. Additionally, this was designed as a retrospective study and we encountered missing data for some individuals that we were unable to ascertain or follow up to have additional testing done. As a result, this may have led us to exclude truly eligible study subjects or have a mischaracterization of the overall study population. However, the cases in which data points used for inclusion in the study were rarely missing and clear documentation of exclusion criteria were also sought in order to ensure that the study populations met the strictest criteria possible. Future studies with larger sample sizes that are designed prospectively should overcome the limitations encountered in this study.

Conclusions

The findings from this pilot study demonstrate an association between TSGs and abnormal sperm production and DOR. It is the first study to utilize NGS to identify genetic variants in these patient populations and resulted in establishing a relationship between four variants not yet associated with infertility. Such results warrant larger prospective studies that investigate the association of TSGs with both idiopathic male factor infertility and poor ovarian reserve in women. Additional

applications to other types of infertility that are known to disrupt gametogenesis may shed further light on the influence of TSGs on infertility in humans.

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