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GENETICS

Detection of candidate nectin gene mutations in infertile men with severe teratospermia

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Abstract
Purpose Approximately 40% of infertile men have an abnormal semen analysis, resulting from either abnormalities of sperm production (defective spermatogenesis) or sperm shape (defective spermiogenesis). This latter process is dependent upon the function of Sertoli cells, which maintain specialized junctional complexes with germ cells. Nectins, members of the immunoglobulin superfamily, participate in formation of these dynamic complexes. Male mice in which the nectin-2 or nectin-3 gene is knocked out are sterile. Their spermatozoa exhibit severe teratospermia, altered motility, and an impaired ability to fertilize eggs. We asked whether mutations in the protein coding regions of the nectin-2 (aka PVRL2) and nectin-3 (aka PVRL3) genes could be detected in men from infertile couples whose semen analysis revealed unimpaired sperm production, judged by normal sperm concentration, but severe abnormalities in sperm shape.
Methods Ejaculates were snap frozen in liquid nitrogen and later submitted for Sanger analysis of these two genes, to detect mutations in their protein coding regions.
Results Eighty-nine of 455 ejaculates (19.5%) met the inclusion criteria for study. Two of the 56 samples that were successfully analyzed for nectin-2 (3.6%) and one of 73 (1.3%) analyzed for nectin-3 possessed possibly damaging mutations.
Conclusions Despite the small-scale nature of the study, at least two low-frequency deleterious variants were identified. These results suggest the need for a larger-scale study of sequence variants in the nectins in severe teratospermia.

Keywords Spermatozoa · Teratospermia · Sertoli cells · Nectins · Genetics · Mutations

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Introduction

Approximately 15% of reproductive age couples experience a delay in conception. A male factor is found on semen analysis as the sole cause of infertility in approximately 20% of these couples and is contributory in an additional 30–40% [1]. These may consist of low sperm concentration resulting from abnormalities in the production of sperm (spermatogenesis), impaired sperm motility, or abnormalities of sperm shape (teratospermia) associated with defective spermiogenesis. This later process is dependent upon the function of Sertoli cells, which maintain specialized junctional complexes with the germ cells termed ectoplasmic specializations. These are responsible for linking the spermatid to the Sertoli cell, playing a role in promoting the normal shape changes that spermatozoa undergo during their maturation as well as their translocation through the seminiferous epithelium [2]. The etiology of teratospermia is varied and has been associated with the presence of varicoceles [3], prior febrile illness [4], and the smoking of tobacco [5]. There is clinical evidence that abnormally shaped spermatozoa exhibit a diminished ability to fertilize eggs, although sperm egg penetrating ability varies

markedly between individual men, perhaps reflective of the specific etiology leading to teratospermia [6, 7].

Recent studies in mice have revealed that the nectins, members of the immunoglobulin superfamily, participate in the formation of dynamic junctional complexes between sperm and Sertoli cells [8]. Male mice in which the nectin-2 gene is knocked out have been found to be sterile. Their spermatozoa exhibit severe abnormalities in shape, altered motility, and an impaired ability to fertilize eggs [9]. Sperm production in these mice, as judged by numbers of sperm recovered from the distal epididymis, is not impaired, when compared with wild-type mice. This phenomenon was traced to a defect in the association between spermatids and Sertoli cells within the testis, in the absence of nectin-2, which leads to abnormal spermiogenesis. Subsequent studies have demonstrated similar effects following knockout of the nectin-3 gene, which has been shown to be expressed on spermatozoa [10].

In this study, we asked whether mutations in the protein coding regions of the nectin-2 and nectin-3 genes could be detected in a select group of men from infertile couples who submitted their ejaculates for semen analysis. Given that male mice in which the nectin-2 gene is knocked out exhibit impaired spermiogenesis, but normal spermatogenesis, we asked whether men with similar phenotype (severe teratospermia in the face of normal sperm concentration) might be carriers of mutations in either the nectin-2 or nectin-3 genes.

Materials and Methods

Human subject selection and semen analysis

The study was approved by the Stony Brook University Institutional Review Board [CORIHS No.177570-4], and informed consent was obtained from all individual participants included in the study. Ejaculates submitted for analysis to the Andrology Lab in the Department of Pathology at Stony Brook University Medical Center were obtained by masturbation into a sterile specimen container, following 2–6 days of sexual abstinence. A complete analysis, including sperm count, motility, viability, and morphology, was performed. The morphology of Spermac-stained (Cooper Scientific, Trumble, Conn) sperm was assessed using strict criteria established in the fourth edition of the WHO Laboratory Manual for the Examination of Semen and Semen-Cervical Mucus Interaction [11] prior to sperm freezing in liquid nitrogen. Men whose ejaculates exhibited unimpaired sperm production, as reflected in normal sperm concentration (at least 20 million spermatozoa/mL), but severe abnormalities in sperm shape (teratospermia) were asked to participate in the study. Individuals with a recent history of febrile illness during the prior 3 months, as well as men who were cigarette smokers, and men with known varicoceles were excluded. Nor were ejaculates obtained from men whose spermatozoa were morphologically normal, as controls in this pilot study, given the large sample size required to assess the significance of rare variants.

To validate our assessment of sperm morphology, freshly prepared smears from 21 consecutive ejaculates submitted to our laboratory for analysis were sent to the central reference lab for a multicenter study establishing semen parameters of fertile men in the USA [12]. These slides were analyzed independently and the results between laboratories then compared. In addition, the Andrology Laboratory undergoes periodic proficiency tests by the College of American Pathologists (CAP), during which semen samples are submitted for analysis as unknowns.

Two hundred and twenty-seven semen samples were analyzed during the collection period for the study of nectin-2 gene mutations, and analysis was performed on an additional 223 ejaculates during the subsequent collection period for nectin-3 analysis. Patient identity information was removed; samples were coded by a simple number, snap frozen by being plunged into liquid nitrogen, and stored at −278 °C until sperm DNA was extracted. Portions of ejaculates from 89 men were cryopreserved for future study. Fifty-nine samples possessed 6% or less normal spermatozoa and 30 possessed ≤9% normal spermatozoa. Sixty-one samples were first submitted for analysis of nectin-2 gene mutations and 89 subsequently for nectin-3 mutations, as an additional 28 samples that met the inclusion criteria had been obtained.

DNA isolation

Sperm DNA was isolated using Epicentre Quick Extract solution (Epicentre, Madison, WI). Briefly, 5 μl of sperm was combined with 100 μl of Epicentre Quick Extract solution and vortexed for 10 s. Then samples were heated at 65 °C for 6 min and vortexed for 10 s again. Next, incubation at 98 °C for 2 min was followed by another vortexing for 10 s. After that, samples were cooled to room temperature and stored at −20 °C. Next, exons of nectin-2 (gene name *PVRL2*) and nectin-3 (gene name *PVRL3*) were amplified by PCR and their sequences were analyzed using traditional Sanger sequencing in two directions—forward and reverse. Multiple sequence alignment was performed to compare patients' nectin-2 and nectin-3 amino acid sequences with reference nectin-2 and nectin-3 sequences from UCSC Genome Browser database (http://genome.ucsc.edu/, human genome assembly 2009).

Primer design, PCR amplification, quality assessment, and purification

Forward and reverse PCR primers were designed with Primer3 (v. 0.4.0) [13] at http://frodo.wi.mit.edu/primer3/.

Primers' annealing temperatures were recalculated with a Finnzymes/Thermo Scientific Tm calculator for use with Finnzymes' Phire Hot Start DNA polymerase (Table 1). For this study, Phire Hot Start DNA polymerase was chosen because it provides high fidelity of DNA amplification and minimizes amplification error. PCR was completed using Phire Hot Start DNA Polymerse kit (Thermo Fisher Scientific, Inc. , Waltham, MA) as outlined below. Typical PCR consisted of 40 cycles with 20 s at 98 °C, 20 s at 65 °C, and 20 s at 72 °C following initial 40 s enzyme heat activation at 98 °C. Reactions were assembled in a final volume of 30 μl as follows: 5× buffer—6 μl, 4 mM dNTP—1.5 μl, forward primer (100 μM)—1.5 μl, reverse primer (100 μlM)—1.5 μl, Phire Hot Start DNA polymerase—0.45 μl (~2 Units), and deionized water—17.05 μl. Amplification was carried out on an Eppendorf Mastercycler PCR device. PCR products were analyzed by agarose gel electrophoresis or more routinely on QIAxcel instrument (Qiagen, Germantown, MD). Prior to the sequencing reaction, PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) to remove unused primers and nucleotides. To cover all exons in nectin-2 and nectin-3 genes, a total of 14 PCR products were generated for every subject of the study. Every PCR product was sequenced in both directions (forward and reverse). Details of PCR primers are to be found in Table 1.

DNA sequencing

DNA sequencing was performed using an Applied Biosystems Model 3730 capillary DNA Analyzer with Big Dye Terminator v3.1 Sequencing Standard Kit (Life Technologies, Carlsbad, CA). DNA sequence analysis was performed using CodonCode Aligner of CodonCode Corporation (Centerville, MA). Potential SNP identities were checked using the UCSC Genome Browser with the latest SNP tracks. In silico translation was completed using CodonCode, the UCSC Genome Browser, or ExPASy Translate tool (http://web.expasy.org/translate/). PCR primers were designed on the basis of known nectin-2 exonic sequences, and PCR conditions were optimized by performing PCR on standard controls. In all, there were eight primer pairs used for sequencing analysis of nectin-2. One primer pair (exon 1) did not perform well, and a new pair was designed and synthesized. All available DNA sequences were analyzed using specialized software (CodonCode Aligner), which aligns sequences and detects variants. For all variants thus detected, analysis was performed using the software algorithm PolyPhen [14], available via a Web server (http://genetics.bwh.harvard.edu/pph2/), which helps to predict whether the given variant is likely to be deleterious or not. The prediction is based on a number of sequence, phylogenetic, and structural features characterizing the substitution. In addition, prediction of variant effects was obtained in ExAC and/or gnomAD.

Results

Nectin-2 sequencing

Out of 61 samples analyzed, five consistently failed for most primer pairs, suggesting an issue with the quality/yield of DNA obtained. For all other samples, the majority of exons could be amplified sufficiently well to yield usable DNA sequence data. Two examples of non-synonymous (amino acid changing) variants were found. One "possibly damaging" variant was found in sample 15, exon 6. One probably benign amino acid changing variant found in sample 78, exon 9. No other amino acid changing variants were found. All other variants found were synonymous (do not change an amino acid) and are likely benign.

Sample 15, exon 6

There was a T to C heterozygous change present. This changed an ATT codon to an ACT codon in one copy of the gene, which results in an isoleucine to threonine amino acid change (position 370 of the protein). This change is predicted to be "possibly damaging" on the basis of PolyPhen analysis [13], and deleterious on the basis of SIFT analysis. This variant has been reported before and is equivalent to rs374589262. The allele frequency in the ExAC browser in non-Finnish Europeans (NFE) is 0.0003028, which is equivalent to a heterozygote frequency of 1/1651. There were no homozygotes reported in the ExAC database. In the gnomAD browser, the allele frequency is 0.0001980 in NFE which is equivalent to a heterozygote frequency of 1/2526. There are no homozygotes reported in gnomAD (Fig. 1a).

>Ex6_15_Normal

CCACAGGCGGCATCATCGGGGGCATC
aTCGCCGCCATCATTGCTACTGCTGTGGCTGCC
ACGGGCATCCTTATCTGCCGGCAG

>Ex6_15_Mutated

CCACAGGCGGCATCATCGGGGGCATC
aTCGCCGCCATCACTGCTACTGCTGTGGCTGCC
ACGGGCATCCTTATCTGCCGGCAG

Sample 78, exon 9

There was a C to T heterozygous change present. This changes a CCG codon to a TCG codon in one copy of the gene, which results in a proline to serine amino acid change (position 479 of the protein). This variant has been reported before and is equivalent to rs145654351. This change is predicted to be "benign" on the basis of PolyPhen analysis and

Table 1 Primers for PCR amplification and sequencing of nectin-2 and nectin-3 genes

Primer's target and orientation	Primer name	Primer sequence, 5′ to 3′ orientation
Nectin3/PVRL3 exon1, left primer	Nec3ex1L	GGCGCTAGAGCTGGGAGCTG
Nectin3/PVRL3 exon1, right primer	Nec3ex1R	AAAAGTTTCCAGCCAAAGTCCTC
Nectin3/PVRL3 exon2, left primer	Nec3ex2L	GGGTCAGGAAGGGAGGAGAGT
Nectin3/PVRL3 exon2, right primer	Nec3ex2R	AGACAAGATTTTATTTACAGGGAACCT
Nectin3/PVRL3 exon3, left primer	Nec3ex3L	TGCAGTTGTCCTTAAGCTTGTGA
Nectin3/PVRL3 exon3, right primer	Nec3ex3R	TGAAATTTCCATGAAAACAAACAAA
Nectin3/PVRL3 exon4, left primer	Nec3ex4L	TTTCAGTCAGCCAATACTTGTGGA
Nectin3/PVRL3 exon4, right primer	Nec3ex4R	GGTGGGGAAGGATAAGACAGAAA
Nectin3/PVRL3 exon5, left primer	Nec3ex5L	TGCTATTTTGGAAGAATGGGTGTT
Nectin3/PVRL3 exon5, right primer	Nec3ex5R	GACATTTCAATAAAGGATGCCACA
Nectin3/PVRL3 exon6, left primer	Nec3ex6L	CAGCCTGCATTGACATTCTTTG
Nectin3/PVRL3 exon6, right primer	Nec3ex6R	AGCTTGAAAATCAACTTGAAAAAGC
Nectin2/PVRL2 exon1, left primer	PVRL2e1L	GGGGCGTGGCTAGACCTTAG
Nectin2/PVRL2 exon1, right primer	PVRL2e1R	CCAGCTCGGAAGGTAAGGTTG
Nectin2/PVRL2 exon2, left primer	PVRL2e2L	CGAAGCTGCCTACGTTGCAT
Nectin2/PVRL2 exon2, right primer	PVRL2e2R	GAGAGAAACTGACCCGCAAGG
Nectin2/PVRL2 exon3, left primer	PVRL2e3L	TGTTGAATGACTGCCGGTGAG
Nectin2/PVRL2 exon3, right primer	PVRL2e3R	TCAGGGAACCCACGTAGACAA
Nectin2/PVRL2 exons4&5, left primer	PVRL2e45L	GCTAACTTGTCCACCCGCTCT
Nectin2/PVRL2 exons4&5, right primer	PVRL2e45R	CCAAGTCCTGAAGGGCAGAAC
Nectin2/PVRL2 exon6alternative, left primer	PVRL2e6aL	GCGATCCTCGTGATCTTGTGT
Nectin2/PVRL2 exon6alternative, right primer	PVRL2e6aR	CACCCTCTCTTTCCCCAACAT
Nectin2/PVRL2 exon6, left primer	PVRL2e6L	TAGACAGGCAGGCGATGATGT
Nectin2/PVRL2 exon6, right primer	PVRL2e6R	TCAGTTCTTCGACCCCTCTCC
Nectin2/PVRL2 exons7&8, left primer	PVRL2e78L	CAGAGGGATGCCTGGGTCTTA
Nectin2/PVRL2 exons7&8, right primer	PVRL2e78R	GGACACACCCTTGACCTTGAG
Nectin2/PVRL2 exon9, left primer	PVRL2e9L	GGATTTTGGGGTCAAGAGCAG
Nectin2/PVRL2 exon9, right primer	PVRL2e9R	GCCCACTCCAGCAGACCTATT

"tolerated_low_confidence" on the basis of SIFT. ExAC allele frequency is reported as 0.00015, equivalent to a heterozygote frequency of 1/3333. No homozygotes reported in ExAC. Data from gnomAD is similar, with an allele frequency of 0.0001604, equivalent to a heterozygote frequency of 1/3118 (Fig. 1b).

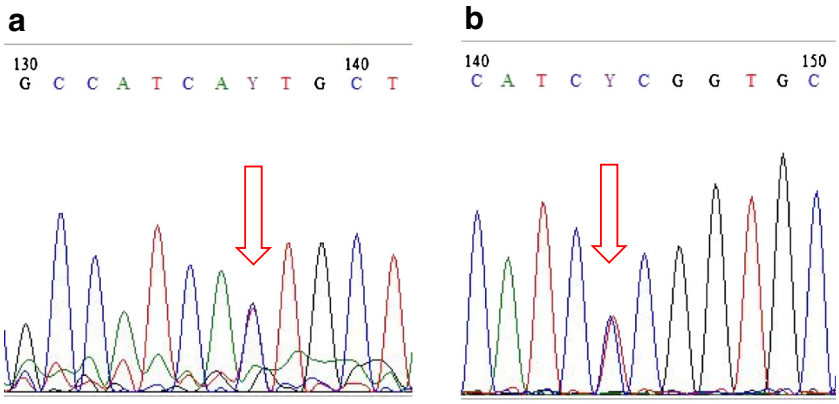

Fig. 1 Sequence alterations identified in patients with severe teratospermia. **a** Sample 15, exon 6 sequence shows a heterozygous base (Y, i.e., C or T) at the polymorphism site (indicated by *red arrow*). **b** Sample 78, exon 9 also shows a heterozygous base (Y, i.e., C or T) (indicated by *red arrow*)

>Ex9_78_Normal

GGACCCTTGCACCCTGGAGCCACAAGCCTGGG GTCCCCCATCCCGGTGCCTCCAGGGCCACCTGCTGT GGAAGACGTTTCCCTGGATCTAGA

>Ex9_78_Mutated

GGACCCTTGCACCCTGGAGCCACAAGCCTGGG GTCCCCCATCTCGGTGCCTCCAGGGCCACCTGCTGT GGAAGACGTTTCCCTGGATCTAGA

Nectin-3 sequencing

In all, 89 samples were analyzed. Exon 1 proved extremely difficult (probably due to its high GC content), despite repeated attempts and a variety of different primer sequences and PCR conditions and was thus abandoned. Exon 3 was also problematic, but multiple primer sequences and PCR conditions were tried, which eventually resulted in partial success (approximately 50% success compared to the other exons). Out of 89 samples analyzed, 16 failed to yield a PCR product for all primer pairs and a small number of other samples only yielded products for a small subset of the primer pairs, suggesting an issue with the quality/yield of DNA obtained. For all other samples, the majority of exons were amplified sufficiently well to yield usable DNA sequence data. One example of a "probably damaging" non-synonymous (amino acid changing) variant was found. All other variants found were predicted to be benign.

Sample 46, exon 4

There was an A to C heterozygous change present. This changes an AAT codon to a CAT codon in one copy of the gene, which results in an asparagine to histidine amino acid change (position 296 of the protein—Asn296His). This variant has been reported before and is equivalent to rs79006549. This change is predicted to be "probably damaging" on the basis of PolyPhen analysis and "deleterious" on the basis of SIFT. ExAC allele frequency is reported as 0.002766, equivalent to a heterozygote frequency of 1/181. No homozygotes reported in ExAC. Data from gnomAD is similar, with an allele frequency of 0.002891, equivalent to a heterozygote frequency of 1/173. One homozygote is reported in gnomAD (Fig. 2).

>Exon4_Normal

GAAAAGGTGTTAATCTCAAATGTAATGCTGAT GCAAATCCACCACCCTTCAAATCTGTGTGG AGCAGGTAATGTTATA

>Exon4_Mutated

GAAAAGGTGTTAATCTCAAATGTAATGCTGAT GCACATCCACCACCCTTCAAATCTGTGTGG AGCAGGTAATGTTATA

This change is predicted to be "probably damaging" with a score of 1 (which is high) on the basis of PolyPhen-2 analysis.

In summary, 89 of 455 ejaculates (19.5%) met the inclusion criteria for study. Two of the 56 samples that were successfully analyzed for nectin-2 (3.6%) and one of 73 (1.3%) analyzed for nectin-3 possessed possibly damaging mutations.

Discussion

Approximately 15% of reproductive age couples experience a delay in conception. It has been estimated that in approximately 30–40% of these couples, semen quality is abnormal. Testicular production of spermatozoa can be divided into two separate processes: (1) the generation of large numbers of spermatocytes through meiosis and mitosis, termed spermatogenesis, and (2) the morphologic changes that spermatozoa undergo during their ascent toward the lumen of the seminiferous tubules, during which they change from round immotile cells to tadpole-like motile cells. This process, termed spermiogenesis, is dependent upon the function of Sertoli cells. These cells maintain two types of specialized cell-cell junctional complexes termed ectoplasmic specializations. The basal junctional complexes between Sertoli cells create a "blood-testis barrier" sequestering within the adluminal compartment of the seminiferous tubule the developing germ cells, which display new developmental antigens to which the immune system is not tolerant [15]. Those junctional complexes near the heads of spermatids, the apical ectoplasmic specializations, are characterized by hexagonal arrays of actin filaments intimately linked to both the cell membrane and endoplasmic reticulum cisternae of the Sertoli cell. They are responsible for adhering the spermatid to the Sertoli cell, playing a role in promoting the normal shape changes that spermatozoa undergo during their maturation, for their translocation though the seminiferous epithelium and their release into the tubular lumen on completion of spermiogenesis [2].

A number of prior studies have addressed the genetic basis of abnormal spermatogenesis [16]. Mutations occurring within the long arm of the Y chromosome, comprising the azoospermia factor (AZF) are associated with severe spermatogenic failure [17]. AZF mutations are caused by recombination between retroviral homologous sequences. AZFa deletions are associated with complete absence of germ cells within the testes leading to Sertoli cell only syndrome. AZFb and AZFc lesions result from recombination between palindromic sequences. Complete AZFb deletions lead to meiotic arrest of germ cells. The AZFc region contains two genes, including four copies of the deleted in azoospermia gene (DAZ) leading to a more heterogeneous phenotype ranging from azoospermia to severe oligospermia (<5 million sperm/mL). Bashamboo et al. sequenced the nuclear receptor, NR5A1, a transcription regulator of genes involved in the hypothalamic-pituitary-steroidogenesis axis, in 315 men with idiopathic spermatogenic failure [18]. Seven men (4%) with otherwise

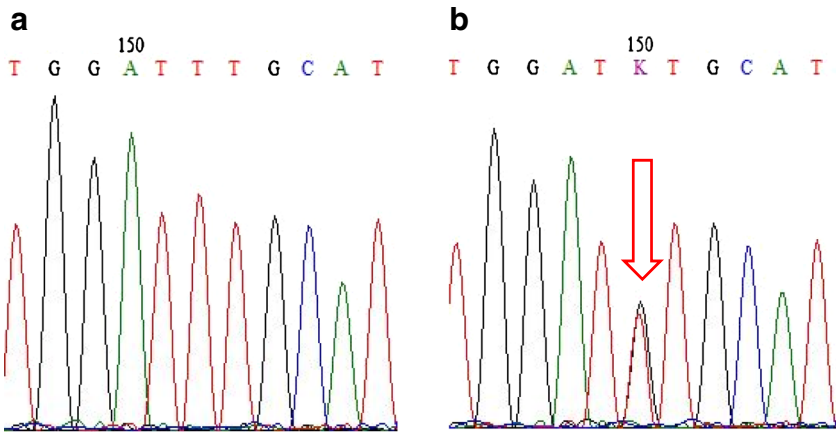

Fig. 2 The heterozygous change is clearly seen in sample 46 (**b**) (sample 47 (**a**) is included for comparison). Double peaks of the same height at the sixth position from the left indicate a heterozygous mutation, or the presence of both alleles containing T and G bases in this position in 50/50% ratio (indicated by *red arrow*). *Red color* corresponds to T, whereas the *black color* corresponds to G. Note that the traces shown are for the reverse sequencing reaction, as these were cleaner (hence, a T/G heterozygous base is seen, instead of A/C) but the change was also visible on the forward strand

unexplained severe spermatogenic failure were identified who carried missense mutations. These were not observed in more than 4000 control alleles, including the entire coding sequence of 389 normospermic men and 370 fertile men. Imken et al. [19] examined the DNA variants present in four protamine coding genes within the protamine locus and transition nuclear protein gene TNP2 of men who had an intact Y chromosome. A total of 160 control and 125 infertile men with idiopathic azoospermia or oligospermia were sequenced. Twenty-eight variants were identified, of which 21 had been previously described. Novel variants were observed in the infertile group, including the SNP c65G>A mutation, which resulted in an amino acid change in codon 22 in the PM1 gene, a mutation in the promoter region of PRM2, and a non-sense mutation in the PM3 gene. Montjean et al. [20] performed global sperm DNA methylation studies on 62 normospermic and 30 oligoasthenospermic men presenting for an infertility evaluation. A significant positive association was found between sperm global DNA methylation and sperm concentration and motility, but not morphology. The methylenetetrahydrofolate reductase (MTHFR) enzyme is one of the main regulatory enzymes in folate metabolism and in methylation reactions utilized in the epigenetic regulation of genes. Eloualid et al. [21] analyzed the distribution of MTHFR C677T and A1298C gene mutation frequencies using PCR fragment length polymorphism analysis in 344 men with unexplained reduced sperm counts. The A1298C variant was present in a statistically significant level when compared with controls (OR = 3.32).

Although a number of genes have been discovered in mice through mutagenesis experiments, which play a role in abnormalities of the shape of the spermatozoon head [22], few orthologues have been identified in humans. Globospermia, a rare form of severe teratospermia, was discovered in infertile men characterized by round-headed spermatozoa. This has been found to be caused by homologous deletions in the DPY19L gene [23, 24]. Pierre et al. have shown, in mice, that this gene encodes a protein expressed on the inner nuclear membrane facing the acrosomal vesicle that is necessary for sperm head elongation and acrosome formation [25]. Evidence has recently been presented that the syndromes of macrospermia and of multiple morphologic abnormalities of the flagellum (MMAF) also have a genetic basis [26]. We believe our study is the first to ask whether altered spermiogenesis in humans could be due to mutations in the nectin-2 and nectin-3 genes, as suggested by studies carried out in mice. Nectin-2 and nectin-3, members of the immunoglobulin superfamily, play a role in calcium-independent cadherin-based adhesions junctions [8]. They have been detected within the apical ectoplasmic specialized junction complexes between Sertoli cells and germ cells, creating a heterotypic adhesion system. The elimination of nectin-2, expressed on Sertoli cells in mice, leads to improperly formed Sertoli-spermatid junctional complexes exhibiting disturbed localization of nectin-3 on spermatozoa and loss of co-localization with actin and abnormal spermiogenesis [9]. Similarly, the knockout of the nectin-3 gene results in a disturbance of spermiogenesis leading to abnormal sperm morphology [10].

The ejaculates of 89 men with normal sperm concentrations whose spermatozoa exhibited severe teratospermia were available for analysis. Fifty-six out of 61 initial specimens were successfully analyzed for nectin-2 mutations. An additional 25 samples were then obtained so that 86 were submitted for analysis of nectin-3 mutations. Of these samples, 73 where successfully analyzed. Sample failures were due to insufficient product generated in PCR. This was due either to insufficient material obtained from DNA extraction or else the presence of PCR inhibitors.

Through classical Sanger analysis sequencing the nectin-2 gene in 56 sperm samples, we found two examples of non-synonymous (amino acid changing) variants in the nectin-2 gene. All other variants detected were found to be benign, that is did not change an amino acid. Sequencing of the nectin-3 gene was carried out in 73 samples, and a single probably damaging non-synonymous variant was found. All other variants were predicted to be benign. Analysis of publicly available data (ExAC (http://exac.broadinstitute.org) and gnomAD (http://gnomad.broadinstitute.org)) reveals that there are individuals reported with variants at this site. While this may cast doubt on the likely pathogenicity of this variant, it should be noted that only ~1/180 "normal" individuals have this variant present (heterozygously). It should also be appreciated that individuals classified as "normal"' in public databases will not have been screened for many conditions, including sperm abnormalities.

Our study was limited to analyzing the protein coding regions of the nectin genes. Although coding variants in the nectin-2 and nectin-3 genes were found in only a few of the samples analyzed, the possibility of alterations in the regulatory regions of these nectin genes has not been explored. Further, we analyzed only variations in DNA sequence that may affect function of nectin-2 and nectin-3 genes, but there are other mechanisms that potentially may affect expression levels of these genes and protein function. Thus, methylation of genomic DNA in promoter regions may lead to significant decrease of messenger RNA (mRNA) expression of these genes, or microRNA regulation may significantly reduce the number of functional mRNA templates for nectin-2 and nectin-3 translation. Future work, using a variety of platforms, is likely to yield further insights into the potential role of nectin-2 and nectin-3 in the etiology of severe teratospermia.

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Compliance with ethical standards All procedures performed in studies involving participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Stony Brook University Institutional Review Board [CORIHS No.177570-4], and informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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