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ORIGINAL ARTICLE

Deletion or underexpression of the Y-chromosome genes *CDY2* and *HSFY* is associated with maturation arrest in American men with nonobstructive azoospermia

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Maturation arrest (MA) refers to failure of germ cell development leading to clinical nonobstructive azoospermia. Although the azoospermic factor (AZF) region of the human Y chromosome is clearly implicated in some cases, thus far very little is known about which individual Y-chromosome genes are important for complete male germ cell development. We sought to identify single genes on the Y chromosome that may be implicated in the pathogenesis of nonobstructive azoospermia associated with MA in the American population. Genotype–phenotype analysis of 132 men with Y-chromosome microdeletions was performed. Protein-coding genes associated with MA were identified by visual analysis of a genotype–phenotype map. Genes associated with MA were selected as those genes within a segment of the Y chromosome that, when completely or partially deleted, were always associated with MA and absence of retrievable testicular sperm. Expression of each identified gene transcript was then measured with quantitative RT-PCR in testicular tissue from separate cohorts of patients with idiopathic MA and obstructive azoospermia. Ten candidate genes for association with MA were identified within an 8.4-Mb segment of the Y chromosome overlapping the AZFb region. *CDY2* and *HSFY* were the only identified genes for which differences in expression were observed between the MA and obstructive azoospermia cohorts. Men with obstructive azoospermia had 12-fold higher relative expression of *CDY2* transcript (1.33±0.40 *vs.* 0.11±0.04; *P*=0.0003) and 16-fold higher expression of *HSFY* transcript (0.78±0.32 *vs.* 0.05±0.02; *P*=0.0005) compared to men with MA. *CDY2* and *HSFY* were also underexpressed in patients with Sertoli cell only syndrome. These data indicate that *CDY2* and *HSFY* are located within a segment of the Y chromosome that is important for sperm maturation, and are underexpressed in testicular tissue derived from men with MA. These observations suggest that impairments in *CDY2* or *HSFY* expression could be implicated in the pathogenesis of MA.
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INTRODUCTION

Maturation arrest (MA) refers to the histological finding of germ cells throughout the seminiferous tubular epithelium that do not complete spermatogenesis. While it is believed that genetic abnormalities are more common in patients with MA than in men with other variants of nonobstructive azoospermia (NOA),[1] in the majority of patients, the cause of MA is not known. Many of these patients may have genetic anomalies that interfere with sperm production[2] which have not yet been discovered. Investigation of the genetic causes of MA is challenging due to several factors, including the absence of good animal models of MA, scarcity of human testicular tissue from men with MA for research and the likely polygenic nature of male infertility. Shotgun approaches to identification of genes important for completion of spermatogenesis with microarrays have been attempted using testicular tissue from men with NOA, but have been disappointingly unrevealing.[3]

The critical role of the Y chromosome in male fertility was first recognized by Tiepolo and Zuffardi[4] in 1976. One potential starting point in the search for genes associated with MA in humans, therefore, is the Y chromosome. Y-chromosome microdeletions involving complete or partial loss of the azoospermic factor (AZF) region are currently among the most common identifiable genetic causes of NOA, accounting for approximately 10% of cases.[5–7] Phenotypic characterization of infertile men with Y microdeletions provides insight into the spermatogenic function of genes within the deleted DNA segments, as men with Y microdeletions may be considered to be naturally occurring 'knockouts' for genes that have been deleted, most of which do not have functional autosomal homologues.

It is of particular interest that complete loss of the AZFb region causes MA and a poor prognosis for biological paternity.[5,8–10] This observation suggests that one or more genes within or surrounding AZFb may be critical for germ cell maturation, and that perturbation of expression of genes within AZFb or their function may underlie some cases of otherwise unexplained NOA associated with MA. However, despite our relatively advanced understanding of the genetic mechanisms and clinical sequelae of Y microdeletions, we still do not know which AZFb genes are essential for sperm production.

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In the present study, our objective was to identify Y-chromosome genes that may be implicated in the pathogenesis of idiopathic NOA associated with MA. Two strategies were implemented. First, we used genotype–phenotype analysis of phenotypically well-characterized infertile men with precisely mapped Y-chromosome microdeletions to identify candidate Y-chromosome genes that may be associated with MA. Next, we investigated whether or not we could detect differences in RNA expression of the identified candidate genes in testicular tissue from patients with MA and negative Y microdeletion testing. Other groups have successfully used this approach for investigation of some Y-chromosome genes in patients with NOA,[11–14] though prior studies have not focused on MA.

MATERIALS AND METHODS

Patient selection for genotype–phenotype analysis

The Institutional Review Board of the Weill Cornell Medical College approved this study. The study population was derived from a cohort of 246 men with Y-chromosome microdeletions who were identified by consecutive screening of 1997 infertile men in our laboratory for Y-chromosome microdeletions. The ethnicity distribution in the screened population was representative of the population of the United States, including 69% Caucasians, 23% African American, 6% Asian, 1% native Hawaiian or Pacific Islander and 1% native American. One hundred and thirty-two patients with Y microdeletions met inclusion criteria for the genotype–phenotype analysis. All non-azoospermic patients for whom semen analysis results were available were included. For azoospermic patients, only patients who underwent simultaneous cytological evaluation by microdissection testicular sperm extraction (TESE) and histological evaluation by diagnostic testicular biopsy were included.

Microdissection TESE is a surgical technique for sperm retrieval in men with NOA whereby all seminiferous tubules can be visually examined with an operating microscope. Dozens of samples (biopsies) are taken during the procedure and cytologically examined for sperm as well as germ cells. A random biopsy is also taken for histology, allowing complete and detailed phenotypic classification of the typically heterogeneous testes. This approach minimizes the risk of phenotypic misclassification due to sampling error that is inherent to random testicular biopsy. Azoospermic patients who did not undergo microdissection TESE for therapeutic purposes were excluded.

Y microdeletion testing

Y microdeletion testing was performed by multiplex PCR of DNA extracted from peripheral blood leukocytes. For each patient, genomic DNA was extracted from peripheral blood using two commercially available DNA extraction kits. Thirty sequence-tagged sites (STSs) within the AZF region of Yq11 and the *SRY* gene (sY14) were targeted for PCR amplification using previously published primer sequences.[15–17] All patients were tested twice with multiplex PCR using DNA extracted with each method. DNA from a fertile male served as a positive control. Water and DNA from a female were used as negative controls. Single-primer PCR analyses were performed in duplicate for all deleted STSs and two flanking STSs to confirm multiplex PCR results that indicated a Y microdeletion. STS amplification patterns that reflect AZFa, AZFb, AZFb+c and AZFc microdeletions are indicated in **Figure 1**.

Microdissection TESE and testicular biopsy

Azoospermia was confirmed on the day of sperm retrieval by microscopic analysis of ejaculated semen after centrifugation. Microdissection TESE was performed utilizing the operating microscope and a transverse incision in the tunica albuginea until sperm were found or the entire volume of testicular tissue was dissected.[18] Extracted testicular tissue was cytologically examined for the presence of sperm by an experienced andrologist in the operating room and subsequently in the andrology laboratory. Microdissection TESE was considered successful if one or more sperm were found that were morphologically acceptable for intracytoplasmic sperm injection.

Tissue acquisition for histopathology and RT-PCR

Diagnostic testicular biopsies and seminiferous tubular tissue for research were taken during microdissection TESE after the tunica albuginea was widely opened. Randomly selected pieces of undisturbed seminiferous tubular tissue measuring 5–10 mm in greatest dimension were sharply excised. One piece of tissue was placed gently into Bouin's solution for pathological analysis. Tissue for research was placed without media into a cryovial, immediately snap frozen in liquid nitrogen and stored at −80 °C.

Pathological analysis of testicular biopsies

Histopathological analysis was performed as previously described.[19] Sections were stained with hematoxylin and eosin and examined with a light microscope under ×100 to ×400 magnification. Biopsies were classified according to the most advanced pattern of spermatogenesis observed anywhere within the tissue biopsied. We classified biopsies as Sertoli cell only (SCO) when germ cells were completely absent ('pure SCO'), and as MA when germ cells were identified anywhere in the biopsy specimen but oval sperm heads were completely absent (**Figure 2**). For example, a biopsy that was comprised of 95% SCO pattern and rare tubules containing spermatocytes was classified as MA, not SCO.

Phenotypic characterization

Combined results of semen analyses, diagnostic testicular biopsies and microdissection TESE were used to classify patients with Y microdeletions by testicular histopathological phenotype. Patients were classified as either capable or incapable of mature sperm production. The 'capable of mature sperm production' group included oligozoospermic patients and those for whom spermatozoa were identified on testicular biopsy or in tissue extracted during microdissection TESE. Therefore, a man with sperm production so poor that sperm were not present in the ejaculated semen sample but could only be found in focal areas of the testes would be classified as 'capable of mature sperm production' despite his quantitatively very impaired production. This group included patients with histological hypospermatogenesis, as well as patients with diagnostic biopsies that showed SCO or MA but in whom sperm were successfully retrieved. Azoospermic patients in whom microdissection TESE failed were considered incapable of mature sperm production. This cohort was subclassified based on the most advanced spermatogenic pattern evident on testicular biopsy as either SCO or MA.

Genotype–phenotype analysis

A genotype–phenotype map was constructed to enable visual analysis of genotype–phenotype correlations (**Figure 1**). Patients were placed into three histopathological phenotypic categories: (i) incapable of mature sperm production/SCO; (ii) incapable of mature sperm production/MA; and (iii) capable of mature sperm production. The Y microdeletion of each patient was mapped according to its STS amplification pattern and the published sequence of the Y chromosome

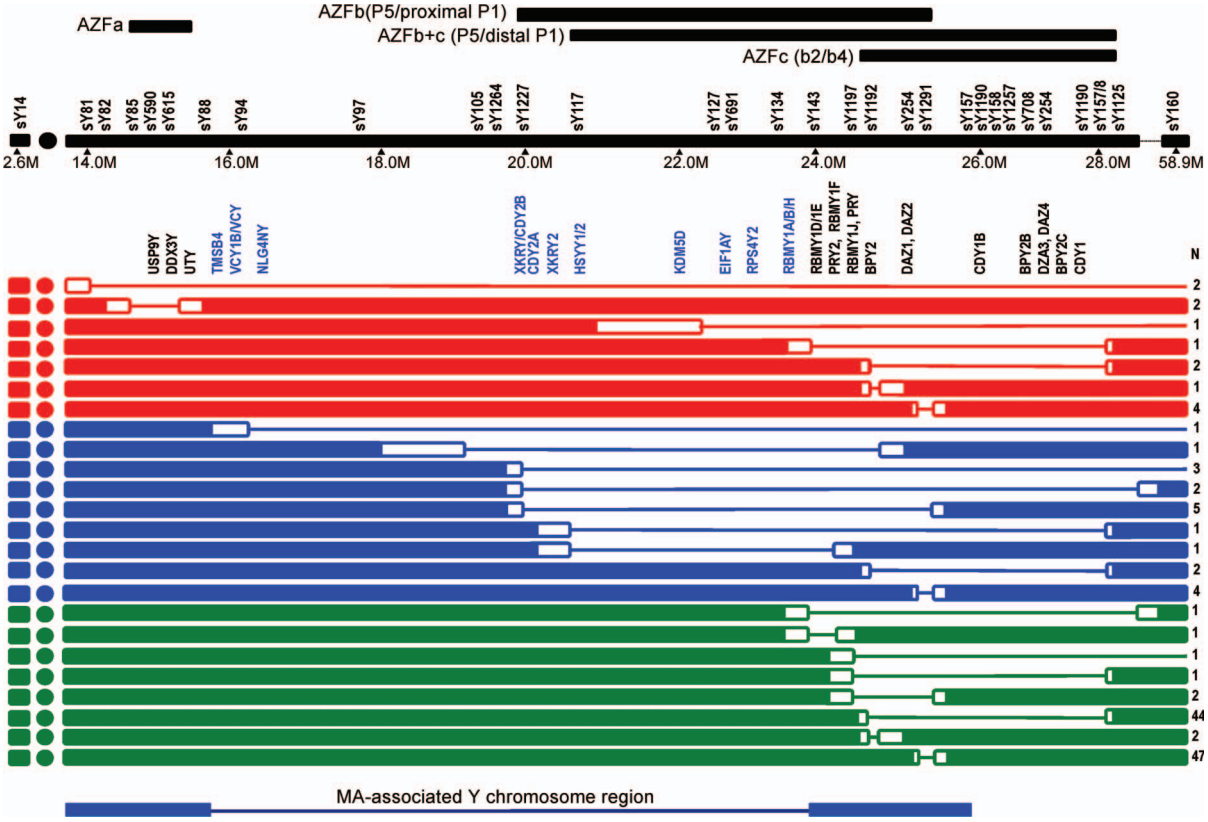

Figure 1 Genotype–phenotype map constructed to enable visual analysis of genotype–phenotype correlations. STSs used for Y microdeletion screening in our laboratory and the protein-coding genes within the AZF region are indicated in their respective positions on Yq11. Black bars indicate the STS amplification patterns used in our laboratory to diagnose the AZFa, AZFb (P5/proximal P1), AZFb+c (P5/distal P1) and AZFc (b2/b4) Y microdeletions. Y microdeletion breakpoints based upon STS amplification patterns observed during Y microdeletion testing are indicated for the 132 patients with Y microdeletions analyzed. Patients were placed into one of three phenotypic categories for mapping: (i) incapable of mature sperm production/SCO histology (red color), (ii) incapable of mature sperm production/MA histology (blue color), and (iii) capable of mature sperm production (green color). The number of patients with each Y microdeletion pattern depicted is indicated on the far right. Solid bar, STS presence; thin line, STS absence; hollow bar, DNA segment located between STSs for which there is insufficient information to determine DNA segment presence or absence. The AZF segment that we identified as associated with MA is indicated at the bottom of the map. AZF, azoospermic factor; MA, maturation arrest; SCO, Sertoli cell only; STS, sequence-tagged site.

(http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606).

The region of the Y chromosome associated with MA was identified by visual analysis of the genotype–phenotype map by the following criteria: (i) immature germ cells were histologically identified in the majority of patients with complete or partial deletions of the region; and (ii) all patients with complete or partial deletions of the region were incapable of sperm production (i.e., ejaculated or testicular sperm were never found in any patient in whom the region was completely or partially deleted). The borders of the MA-associated region were defined by the nearest flanking nondeleted STSs. The protein-coding genes within the MA-associated region of the Y chromosome were determined by online query of the NCBI HuRef-primary assembly database (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606).

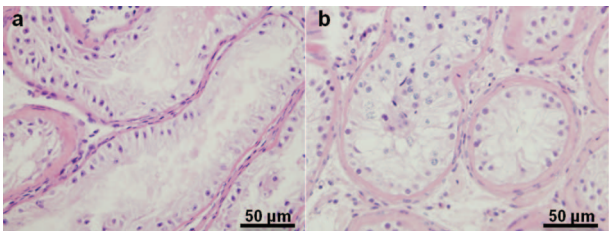

Figure 2 Representative testicular biopsies from patients with idiopathic NOA and failed microdissection TESE. Hematoxylin and eosin staining. (**a**) SCO pattern. (**b**) MA pattern at the level of the pachytene spermatocyte. Rare cells with condensed nuclei are present and may represent apoptotic germ cells or early spermatids. MA, maturation arrest; NOA, nonobstructive azoospermia; SCO, Sertoli cell only; TESE, testicular sperm extraction.

Selection of patients with idiopathic NOA for RNA expression analysis

We queried our prospectively maintained database of NOA patients who underwent microdissection TESE and simultaneous diagnostic testicular biopsy. We identified patients with idiopathic NOA associated with MA or SCO histology who had normal 46XY karyotypes, negative Y microdeletion testing and absence of any identifiable clinical factors associated with infertility such as prior orchitis, cryptorchidism, hypogonadotropic hypogonadism and chemotherapy or radiation exposure. Testicular sperm were not retrieved by microdissection TESE in any of the included patients. Tissue for research was available for 19 patients with NOA associated with MA, 13 patients with NOA associated with 'pure' SCO and eight patients with OA (who have normal sperm production and served as controls). The

MA group included 11 patients with early MA, in whom germ cell development was arrested at the primary spermatocyte or spermatogonial stages, and eight patients with late MA, in whom germ cell development was arrested at the stage of the round spermatid. Additionally, RNA expression of all candidate genes studied was analyzed in testicular tissue from one patient with an AZFb deletion.

RNA extraction and RT-PCR

Snap-frozen seminiferous tubular tissue for RNA extraction was thawed, weighed and homogenized. RNA was extracted with a commercially available monophasic solution of phenol and guanidine isothiocyanate (Life Technologies, Carlsbad, CA, USA). To remove contamination with genomic DNA, extracted RNA was incubated with RNase-free DNase (Qiagen, Hilden, Germany) for 30 min purified with a commercially available RNA-binding spin column (Qiagen). RNA concentration was measured spectrophotometrically at 260 nm and the purity was confirmed by measurement of the A260/A280 ratio. cDNA was synthesized from 1 μg of purified total RNA with random hexamer primers and stored at −20 °C until use.

Candidate gene mRNA level was measured using dual-color, multiplex qRT-PCR with the Universal Probe Library (UPL) hydrolysis probe set on a LightCycler 480 instrument (Roche Diagnostics Corp., Basel, Switzerland). *PBGD*, a gene encoding porphobilinogen deaminase was used as the housekeeping gene for relative quantification based upon observations in our laboratory of consistent *PBGD* RNA expression in human testis irrespective of histology (data not shown). The multiplex assays were designed using the UPL Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). Primer sets and detection probes for each candidate gene are indicated in **Table 1**. *PBGD* mRNA was detected with a proprietary Human *PBGD* Gene Assay (Roche Diagnostics Corp.).

All qRT-PCR reactions were run in duplicate on 96-well plates. The 20-μl reaction mixture contained 5 μl of 1 : 5 diluted cDNA and 200 nmol l^{-1} UPL probe, 200 nmol l^{-1} *PBGD* probe, 200 nmol l^{-1} forward and reverse primers for *HSFY*, 500 nmol l^{-1} forward and reverse primers for *PBGD* and ×1 LightCycler 480 Probes Master mix. The cycle protocol used was: denaturation at 95 °C for 10 min, 45 cycles of 95 °C for 10 s and 60 °C for 30 s, and a cooling cycle to 55 °C with single fluorescence acquisitions at the end of each cycle. Candidate gene/*PBGD* expression ratios were determined with LightCycler 480 Relative Quantification software (Roche Diagnostics Corp.) using crossing points that were determined by the second derivate maximum method and standard curves that were generated during each PCR run for both the candidate gene and *PBGD*. Standard curves were generated by running the multiplex reactions in triplicate with serially diluted cDNA from a patient with OA. PCR-efficiency corrections and color compensation were applied by the software based on the standard curves and the calculated efficiencies for each candidate gene and *PBGD*.

Statistical analysis

Statistical differences in candidate gene expression between patients with MA and OA were assessed by the Mann–Whitney test. Two-tailed *P* values less than 0.05 were considered statistically significant. For the candidate genes with observed differences in expression between the MA and OA cohorts, one-way analysis of variance with Bonferroni post-test analysis was performed to investigate differences in expression between patients with SCO, early MA, late MA and OA.

RESULTS

Results of the genotype–phenotype analysis are depicted in **Figure 1**. We identified an 8.4-Mb DNA segment as a Y-chromosome region containing candidate genes for association with MA. This region spans the 4.4 Mb centromeric to the AZFb region and the centromeric 4.0 Mb of the AZFb region itself (from the centromeric border of AZFb to sY143). This region was completely or partially deleted in 15 patients, all of whom were classified as incapable of mature sperm production (azoospermic with absence of sperm upon bilateral microdissection TESE). Fourteen of the patients with deletions involving this region had MA histology and one had SCO. In contrast, we found complete sperm production in six patients with deletions involving the telomeric 1.6 Mb of the AZFb region (from sY143 to the telomeric border of AZFb).

This region of the Y chromosome that was identified as associated with MA contains 10 protein-coding genes (*TMSB4*, *VCY*, *NLGN4Y*, *XKRY*, *CDY2*, *HSFY*, *KDM5D*, *EIF1AY*, *RPS4Y2* and *RBMY*) that were designated as candidate genes for association with MA. Of these 10 genes, only *RBMY* exists in additional copies outside of this AZF segment (multiple *RBMY* copies are present within the AZFc region). We analyzed testicular RNA levels for eight of the 10 identified candidate genes. We did not study *XKRY* because we could not design a valid qRT-PCR assay, and we elected not to study *RBMY*, because it exists in multiple copies outside of the Y-chromosome region of interest.

The clinical characteristics of the patients with idiopathic MA and OA included in the transcript expression analysis are presented in **Table 2**. The relative transcript expression of each candidate gene was evaluated in testicular tissue derived from one patient with an AZFb deletion. The transcript ratios were negligible for each of the genes located within the known deleted interval in this patient

Table 1 Primers and UPL probes used in dual-color, multiplex real-time PCR assays for detection of candidate gene RNA transcript expression

Gene	*Forward primer*	*Reverse primer*	*UPL number*
TMSB4	5′-tgctccctacggcttcct-3′	5′-cttgcctctcctgttcgatag-3′	46
VCY[a]	5′-ggccaaggagacaggaaag-3′	5′-cggccaccttggtagtctt-3′	45
CDY2[b]	5′-ggcgaaagctgacagcac-3′	5′-gggtgaaagttccagtcaaaa-3′	54
NLG4NY	5′-aagaacgacgtcatgctcagt-3′	5′-tggttggtttggatcacca-3′	9
EIF1AY	5′-catgctaaaatcaatgaaacagaca-3′	5′-tgtcatgtaaaaacacttggttca-3′	80
HSFY[b,c]	5′-gtcaatgaggctccatatcgt-3′	5′-gatcgtaggcatttgcaacc-3′	40
KDM5D	5′-tctggagccaaccatgtg-3′	5′-gaaggctgcacagactgtctaa-3′	89
RPS4Y2	5′-tgggaagataaccagctttatca-3′	5′-accaggatgtctttccctgtt-3′	67

Abbreviation: UPL, Universal Probe Library.
[a] *VCY* primers targeted both transcripts.
[b] Nonintron spanning assays.
[c] *HSFY* primers targeted transcript variant 1.

Table 2 Clinical characteristics of patients included in the testicular RNA transcript expression analyses[a]

	MA (n=*19*)	*OA* (n=*8*)	P-*value* (*MA* vs. *OA*)
Age (year)	33.7±6.3	37.2±7.7	NS
FSH (IU/l)	12.8±11.6	3.7±2.2	0.04
Average testicular volume (ml)	12.8±4.4	13.9±3.7	NS

Abbreviations: FSH, follicle-stimulating hormone; MA, maturation arrest; NS, not statistically significant; OA, obstructive azoospermia.
[a] Data given as mean±s.d.

(**Figure 3**). We observed differences in expression between the MA and OA cohorts for *CDY2* and *HSFY*. Men with OA had 12-fold higher relative expression of *CDY2* transcript (1.33±0.40 *vs.* 0.11±0.04; P=0.0003) and 16-fold higher expression of *HSFY* transcript (0.77±0.32 *vs.* 0.05±0.02; P=0.0005) compared to men with MA. We did not observe significant differences in testicular transcript expression ratios for any of the other candidate genes (**Table 3** and **Figure 4**).

CDY2 and *HSFY* expressions were further analyzed with respect to testicular histopathological phenotype by comparison of gene/*PBGD* transcript ratios in testicular tissue derived from patients with NOA associated with SCO histology, NOA associated with early MA, NOA associated with late MA, and OA. Both *HSFY* (P<0.0001) and *CDY2* (P<0.0001) were significantly underexpressed in tissue derived from SCO patients (P<0.0001 and P<0.001, respectively) when compared to tissue derived from patients with OA. We did not observe significant differences in expression between the SCO, early MA and late MA cohorts (**Table 4** and **Figure 5**).

DISCUSSION

Better understanding of the molecular mechanisms that regulate spermatogenesis and the genetic disturbances that cause MA will allow us to develop novel diagnostic tests and may ultimately lead to more forms of therapy in the future. We elected to focus our search for candidate MA genes on the Y chromosome, which has a well-established but incompletely understood role in germ cell maturation. Thus far, no single Y-chromosome gene has been definitively linked with MA.

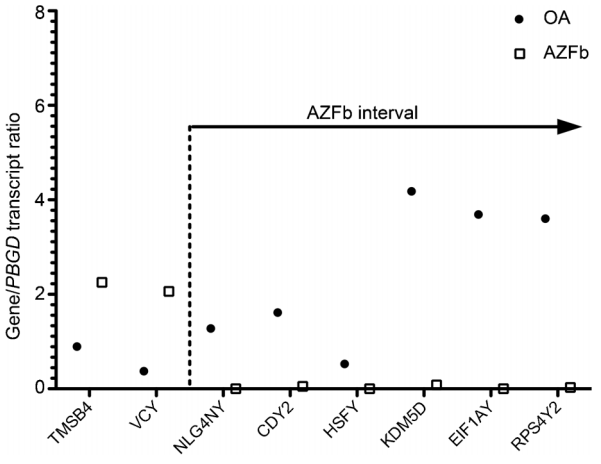

Figure 3 Relative testicular RNA transcript expression of each candidate gene for MA in one patient with an AZFb deletion, and in a representative patient with OA. The genes within the known deleted AZFb interval are indicated. AZF, azoospermic factor; MA, maturation arrest; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase.

Table 3 Expression of each candidate gene RNA transcript relative to expression of the housekeeping gene *PBGD* in patients with MA and OA[a]

Gene/PBGD transcript expression ratio	*MA*	*OA*	P-*value*
TMSB4	0.99±0.16	0.84±0.11	0.62
VCY	0.70±0.12	0.35±0.05	0.17
NLG4NY	0.96±0.28	0.87±0.29	1.00
CDY2	0.11±0.04	1.33±0.40	0.0003
HSFY	0.05±0.02	0.77±0.32	0.0005
KDM5D	6.50±0.60	5.06±1.69	0.09
EIF1AY	2.26±0.59	3.19±049	0.44
RPS4Y2	2.84±0.39	2.38±0.43	0.62

Abbreviations: MA, maturation arrest; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase.
[a] Data given as mean±s.e.

Deletions of the AZF region genes, such as occurs in Y microdeletions, cause multiple downstream effects on the testicular transcriptome involving altered expression of hundreds of genes.[20] This observation suggests a central, regulatory role of the AZF gene products in spermatogenesis. Mutations of genes in the AZF regions themselves or in genes that regulate their expression would be expected to have significant downstream consequences. Such genetic alterations would not be detectable by Y microdeletion testing and might underlie same cases of MA that are presently considered to be idiopathic. We identified an 8.4-Mb region including the 4.4-Mb DNA segment centromeric to the AZFb region and the centromeric 4.0 Mb of AZFb as the Y-chromosome region containing genes that may be associated with MA. Our genotype–phenotype analysis confirms the well-established importance of the AZFb region for germ cell maturation, and adds to the present understanding of AZFb by suggesting that the centromeric 4.0-Mb section of this region (from the centromeric border of AZFb to sY143) is the critical AZFb subregion.

Interestingly, we found complete sperm production in six patients with deletions involving the telomeric 1.6 Mb of the AZFb region (from sY143 to the telomeric border of AZFb), suggesting that this segment of AZFb is nonessential for sperm production. This nonessential segment contains both copies of the *PRY* gene and four of the

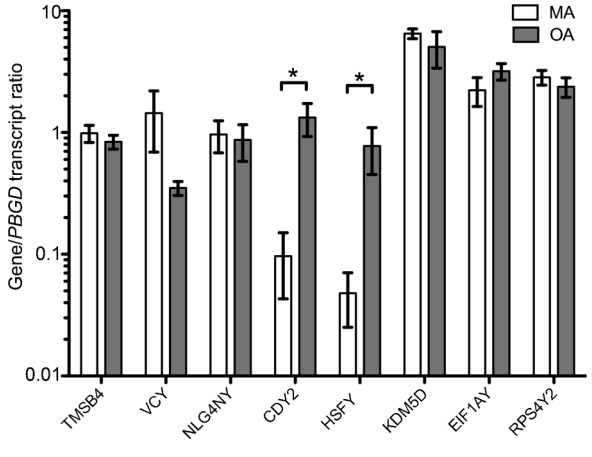

Figure 4 Relative testicular RNA transcript expression of each candidate gene for MA in patients with idiopathic NOA due to MA, and in patients with OA. Gene expression is normalized to expression of the housekeeping gene *PBGD*. *P<0.0001 on one-way ANOVA with Bonferroni *post hoc* analysis. MA, maturation arrest; NOA, nonobstructive azoospermia; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase.

Table 4 Expression of each candidate gene RNA transcript relative to expression of the housekeeping gene *PBGD* in patients with SCO, early MA, late MA and OA[a]

Gene/PBGD transcript expression ratio	*SCO*	*Early MA*	*Late MA*	*OA*
CDY2	0.02±0.003	0.05±0.02	0.21±0.08	1.33±0.40*
HSFY	0.002±0.0004	0.06±0.04	0.03±0.02	0.77±0.32^#

Abbreviations: MA, maturation arrest; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase; SCO, Sertoli cell only.
*$P<0.0001$ *vs.* SCO, early MA and late MA.
^$P<0.0001$ *vs.* SCO.
# $P<0.001$ *vs.* early MA and late MA.
[a] Data given as mean±s.e.

six copies of *RBMY*, demonstrating that PRY and four of the six copies of *RBMY* are not necessary for complete sperm production in all men. These findings are supported by a recent case report of a microdeletion involving this region that was naturally transmitted from father to son.[21]

The Y-chromosome region that we identified as associated with MA includes both copies of *CDY2* (*CDY2*A and *CDY2*B), which was one of two Y-chromosome genes found to be underexpressed in testicular tissue derived from men with NOA and MA. *CDY2* belongs to the family of human chromo domain proteins that includes two copies of *CDY1* within the more telomeric section of the AZFc region of the Y chromosome, as well as the autosomal genes *CDYL* and *CDYL2*. In contrast to the autosomal chromo domain proteins that are ubiquitously expressed in humans, *CDY2* and *CDY1* are exclusively expressed in testis.[22]

The function of the *CDY* genes in spermatogenesis remains incompletely understood. They encode proteins that contain both a chromatin-binding domain and a catalytic domain that is often found in acylation enzymes, suggesting that CDY proteins may interact with histones during chromatin remodeling.[22,23] Indeed, CDY proteins have been shown *in vitro* to exhibit histone acetyltransferase activity, and CDY protein expression has been localized in human testis to the nuclei of maturing spermatids.[12,24] However, the recently elucidated crystalline structure of CDY2 is surprisingly dissimilar from the structure of other known histone acetyltransferases.[25]

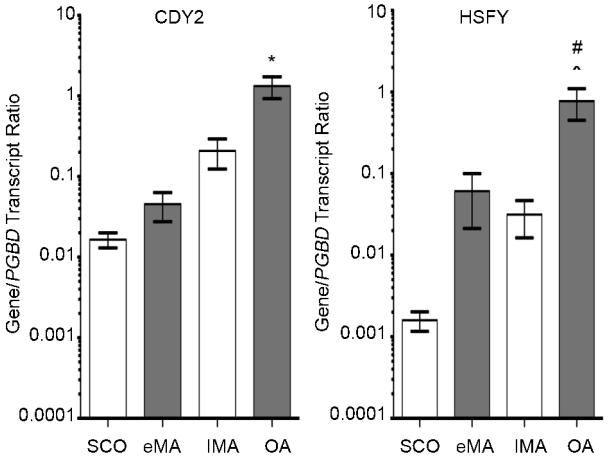

Figure 5 Relative testicular RNA transcript expression of CDY2 and HSFY in patients with histological variants of NOA (SCO, eMA and IMA) and OA. Gene expression is normalized to expression of the housekeeping gene *PBGD*. Statistical analysis between cohorts was performed with one-way ANOVA with Bonferroni *post hoc* analysis. *$P<0.0001$ compared with SCO, eMA and IMA. #$P<0.0001$ compared with SCO and ^$P<0.001$ compared with eMA and IMA. eMA, early maturation arrest; IMA, late maturation arrest; NOA, nonobstructive azoospermia; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase; SCO, Sertoli cell only.

HSFY was the other RNA transcript associated with MA that was underexpressed in testicular tissue derived from men with idiopathic NOA due to MA. Two copies of *HSFY* are present within palindrome P4 of the AZFb region of the Y chromosome.[26] These genes encode three different mRNA transcripts that are expressed in human testis. The protein translated from mRNA transcript variant 1 contains a heat-shock factor-like DNA-binding domain,[27] suggesting that this mRNA is the critical *HSFY* transcript.

Though the function of *HSFY* is not presently understood, it is expressed in human germ cells and Sertoli cells[28] and likely acts by moderating expression of heat-shock proteins, which serve as important transcription factors. The DNA binding capacity of HSFY protein suggests a regulatory role for this gene during spermiogenesis. Decreased expression of HSFY protein has been associated with MA in humans,[29] and expression analysis of a mouse orthologue demonstrated predominant expression in round spermatids, supporting a role for *HSFY* in the later stages of spermatogenesis.[30] Our data suggest that *HSFY* RNA expression may play a critical role in germ cell maturation in the American population.

Interestingly, three oligozoospermic patients with identical partial AZFb deletions leading to isolated loss of *HSFY* have recently been reported.[31] This unique Y-chromosome microdeletion was detected in 3/1186 infertile men and 0/1179 control men, confirming that *HSFY* deletions are associated with infertility, but contradicting our findings that *HSFY* expression is essential for germ cell development. One plausible explanation for the different effects of *HSFY* loss in different populations is that X-linked or autosomal compensatory mechanisms may exist in some populations that can partially compensate for absence of *HSFY*. Indeed, all three of the reported oligozoospermic men with *HSFY* deletions belonged to the same specific Y-chromosome haplotype (R1b1b1a1b).

Although our data establish an association between underexpression of *CDY2* and *HSFY* and idiopathic NOA associated with MA, it would be premature to conclude that these genes play a causal role. Differential testicular expression of candidate gene mRNA in infertile patients with MA could reflect either a true pathogenic mechanism, or simply the absence of cell types to which candidate gene expression is normally localized. Our study (or any evaluation that compares gene expression with histology) cannot distinguish between these two competing explanations, which represents a significant limitation. Nonetheless, the observations reported in this study contribute to the present understanding of the function and expression of Y-chromosome genes in NOA associated with MA. These data have the potential to empower researchers and clinicians to improve the clinical care of patients with NOA. Even testicular microdissection, which is widely considered to be the most effective sperm retrieval

procedure available today, fails in 37%–57% of cases.[32] Further elucidation of *CDY2* and *HSFY* function and expression may lead to novel therapeutic or diagnostic approaches that could benefit patients with NOA.

AUTHOR CONTRIBUTIONS

PJS, PNS and DAP conceived and designed the study. PJS and ANM collected the data. PJS, CEB and DAP performed the statistical analysis. PJS, ANM, CEB, PNS and DAP drafted and revised the manuscript. All authors read and approved the final version.

COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests.

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