Heat shock factor Y chromosome (HSFY) mRNA level predicts the presence of retrievable testicular sperm in men with nonobstructive azoospermia

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Objective: To evaluate heat shock factor Y chromosome (HSFY) mRNA as a biomarker for the presence of retrievable testicular sperm.

Design: Case-control study.

Setting: Academic medical center.

Patient(s): Men with nonobstructive azoospermia (NOA).

Intervention(s): Testicular tissue from men with successful or failed testicular sperm extraction was evaluated with quantitative real-time polymerase chain reaction (qRT-PCR) for expression of HSFY mRNA.

Main Outcome Measure(s): Area under the receiver operating characteristic curve (AUC), sensitivity, specificity, and probability of sperm retrieval based on HSFY testing.

Result(s): We found higher HSFY mRNA expression in testicular tissue from NOA patients in whom sperm were successfully retrieved compared with those in whom sperm were not found, with good discrimination between the groups in all histologic variants of NOA (AUC 0.89 overall, 0.98 for patients with Sertoli cell only [SCO] histology, 0.90 for patients with maturation arrest [MA] histology). Sensitivity and specificity were, respectively, 67% and 93% overall, 92% and 100% for SCO patients, and 67% and 92% for MA patients. The probabilities of sperm retrieval for HSFY-positive and -negative patients were, respectively, 93% and 31% overall, 100% and 7% for SCO patients, and 91% and 32% for MA patients.

Conclusion(s): Detection of HSFY mRNA expression by qRT-PCR has promising application in the evaluation and counseling of men with NOA before attempted sperm retrieval surgery. (Fertil Steril® 2011;96:303–8. ©2011 by American Society for Reproductive Medicine.)

Key Words: Nonobstructive azoospermia, sperm retrieval, heat shock factor Y chromosome (HSFY), testicular biopsy, male infertility

In our laboratory, we previously observed low testicular mRNA expression of the AZFb gene HSFY in patients with NOA who fail microdissection TESE, including patients with both early and late maturation arrest (MA), suggesting the potential role of HSFY as a marker of complete spermatogenesis (7). Our objective was to evaluate detection of HSFY mRNA expression by quantitative real-time polymerase chain reaction (qRT-PCR) on a random testicular tissue sample as a diagnostic test to predict the presence of retrievable testicular sperm in men with NOA.

MATERIALS AND METHODS
Patients
The Institutional Review Board approved this study. The study population included 54 men with NOA who underwent microdissection TESE. Nine men with obstructive azoospermia (OA) who underwent testicular biopsy during sperm retrieval surgery served as control subjects. Patients were selected based on availability of well preserved testicular biopsies for pathologic analysis and availability of testicular tissue for research. Preoperative evaluation included history, measurement of testis size with an orchidometer, semen analysis, serum FSH level, karyotyping, and Y chromosome microdeletion testing.

Sperm Retrieval
Azoospermia was confirmed on the day of sperm retrieval by microscopic analysis of ejaculated semen after centrifugation. Microdissection TESE was performed with the use of an operating microscope and a transverse incision in the tunica albuginea until sperm were found or the entire volume of...
testicular tissue was dissected (1). Extracted testicular tissue from each dis-sected region of the testis was immediately placed into a small volume of fluid and mechanically disrupted with sharp scissors and sequential passes through a 24-gauge angiocatheter. A small aliquot (10 microliters) of the testis tissue suspension was then placed on a slide and cytologically examined in the operating room for the presence of sperm by an experienced andrologist to direct the extent and duration of surgery. This slide was discarded after analysis. The remainder of each testis tissue suspension was subsequently ana-lyzed in the andrology laboratory for identification of sperm. Microndissec-tion TESE was considered to be successful if one or more sperm were found in the laboratory that were morphologically acceptable for ICSI.

**Tissue Acquisition for Histopathology and qRT-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 tubu-lar 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, imme-diately snap frozen in liquid nitrogen, and stored at −80°C.

**Pathologic Analysis**

Histopathologic analysis was performed as previously described (8). Sections were stained with hematoxylin and eosin and examined with a light mi-croscope under ×100–400 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), as MA when germ cells were identified anywhere in the biopsy specimen but elongated spermatids were completely absent, and as hypospermatogenesis (HS) when any con-densed oval sperm heads were identified.

**Quantitative Analysis of HSFY mRNA Expression**

Frozen seminiferous tubular tissue for RNA extraction was thawed, weighed, and homogenized. We extracted RNA with Trizol LS Reagent (Invitrogen). To remove contamination with genomic DNA, extracted RNA was incubated with RNase-free DNase for 30 minutes (Qiagen) and purified with an RNA-binding spin column (RNeasy Mini Kit; Qiagen). We measured RNA concentra-tion spectrophotometrically at 260 nm, and purity was confirmed by measurement of the A260/A280 ratio. We synthesized cDNA from 1 μg pu-rified total RNA with random hexamer primers with the use of the Transcrip-tor First Strand cDNA Synthesis Kit (Roche Diagnostics). cDNA was stored at −20°C until use.

HSFY transcript variant 1 mRNA level was measured with the use of dual-color multiplex qRT-PCR with the Universal Probe Library (UPL) hydrolysis probe set on a Light Cycler 480 instrument (Roche Diagnostics). Porphobilinogen deaminase (PBGD) was used as the housekeeping gene for relative quantification based on observations in our laboratory of consistent PBGD expression in human testis regardless of histology (data not shown). The mul-tiplex assay was designed with the use of the UPL Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). HSFY tran-script variant 1 mRNA was detected with the forward and reverse primers 5′-GTCATGGGCTCATATCGT-3′ and 5’-GACGATTCCACCTTGGCA ACC-3’, respectively, and UPL Probe #40. PBGD mRNA was detected with a proprietary Human PBGD Gene Assay (Roche Diagnostics).

All qRT-PCR reactions were run in duplicate on 96-well plates. The 20-μL reaction mixture contained 5 μL 1:5 diluted cDNA and 200 mmol/L UPL probe, 200 mmol/L PBGD probe, 200 mmol/L forward and reverse primers for HSFY, 500 mmol/L forward and reverse primers for PBGD, and 1 × Light-cycler 480 Probes Master Mix. The cycle protocol was as follows: denatur-ation at 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds and 60°C for 30 seconds, and a cooling cycle to 55°C. HSFY/PBGD expression ratio was determined with Lightcycler 480 Relative Quantification software (Roche Diagnostics). Standard curves were generated during each PCR run for both HSFY and PBGD by running the multiplex reaction in triplicate with serially diluted cDNA from a patient with OA. Crossing points were determined by the second derivative maximum method. PCR efficiency correc-tions were applied by the software based on the standard curves and the calculated efficiencies for the HSFY (1.7) and PBGD (1.6) reactions. We confirmed assay validity by analysis of the HSFY/PBGD expression ratio in testicular tissue from a patient with an AZFc deletion that included loss of both copies of HSFY. HSFY/PBGD expression ratio in this patient was negligible.

**Statistical Analysis**

Statistical analysis was performed with Graphpad Prism Version 5.0c. Serum FSH, average testicular volume, age, and mean HSFY/PBGD ratio were ana-lyzed in relation to microdissection TESE outcome with the use of the Mann-Whitney test. The performance characteristics of HSFY/PBGD ex-pression ratio to predict the presence of retrievable sperm were determined by receiver operating characteristic (ROC) curve analysis. Positive and neg-ative likelihood ratios were calculated as sensitivity divided by (1 – specific-ity) and (1 – sensitivity) divided by specificity, respectively (9). The probability of sperm retrieval based on HSFY/PBGD testing was determined with Fagan’s nomogram for Bayes theorem (10) using the calculated likely-hood ratios and the overall and histology-specific sperm retrieval rates at our institution from 1999 to 2010.

**RESULTS**

The 54 patients in the study population included 44 men with unex-plained NOA, five with a history of cryptorchidism, three with prior chemotheraphy exposure, one with Klippel-F蕙rdrome, and one with an AZFc deletion. Sperm were retrieved in 27/54 cases (50%). Histopathology in the successful retrieval group was SCO, MA, and HS in 12, 6, and 9 patients, respectively. The failed-retrieval group included 15 patients with SCO histology and 12 with MA. There was no difference in mean age between the two groups [34.6 (95% confidence interval [CI] 31.4–37.8) years vs. 33.4 (95% CI 31.6–35.2) years; P = .910]. Patients in whom sperm retrieval was successful had higher mean FSH values [23.9 (95% CI 18.9–28.8) IU/L vs. 16.7 (95% CI 19.1–21.5) IU/L; P = .016] and lower mean testicular volumes [9.0 (95% CI 7.0–11.0) mL vs. 11.8 (95% CI 10.1–13.5) mL; P = .037] compared with patients in whom retrieval failed.

The mean weight of testis tissue used for RNA extraction was 78.1 (range 10–240) mg. Sufficient RNA for analysis was extracted in all cases. The mean HSFY/PBGD expression ratio in the control patients with OA was 0.673 (standard error 2.56 × 10−2, range 0.145–0.946). The HSFY/PBGD expression ratios in NOA patients in relation to microdissection TESE outcome are presented in Figure 1 and Table 1. We observed higher HSFY/PBGD expression ratios in patients with successful microdissection TESE compared with those with failed microdissection TESE within the overall study population, as well as within the SCO and MA subgroups. Comparison was not possible for the HS subgroup, because sperm were retrieved in all cases. However, HSFY/PBGD expression ratios were high or very high in 8/9 patients with HS histology (Fig. 1). The one patient with HS who had a low expression ratio was a patient with an AZFc deletion, in whom diagnostic biopsy revealed that 98% of tubules were SCO pattern and 2% of tubules contained very rare mature sperm. The areas under the ROC curves (AUCs) for the entire cohort, the SCO subgroup, and the MA subgroup were 0.89, 0.98, and 0.90, re-spectively (Fig. 2). The optimal cutoff values for a positive HSFY/ PBGD test were >4.48 × 10−3 for the entire cohort, >1.20 × 10−3 for the SCO subgroup, and >7.40 × 10−3 for the MA subgroup. Performance characteristics of HSFY/PBGD expression ratio to predict the presence of retrievable testicular sperm are presented in Table 2.
DISCUSSION

Patients and physicians accept the high failure rates of testicular sperm retrieval in NOA for two reasons. First, genetic parenthood is such a critical quality of life issue that affected couples are often willing to assume the risks and costs of TESE despite the possibility of failure of sperm retrieval. Second, the performance characteristics of available clinical tests to predict TESE outcome are insufficient in almost all cases to preclude an attempt at testicular sperm retrieval. Neither serum hormone assays, such as FSH and inhibin B, nor noninvasive assessments, such as testicular volume, alter the probability of sperm retrieval sufficiently to direct clinical management (5).

The only noninvasive method that is helpful in selecting patients for microdissection TESE is Y microdeletion testing. Y microdeletions that involve loss of the complete AZFa or AZFb regions are
incompatible with sperm production and are found in up to 6% of American men with NOA (11). We do not recommend microdissection TESE to these patients.

Open or percutaneous testicular biopsy for histologic assessment is more informative than noninvasive testing and allows for therapeutic sperm retrieval in some cases. Despite having a relationship with microdissection TESE outcome (8, 12), testicular histology does not change the probability of sperm retrieval enough to affect clinical management for men with NOA. Reported SRRs in men with SCO histology, the least favorable histologic diagnosis, are 24%–48% (4). In our experience, nearly all of these patients elect to proceed with sperm retrieval, given the possibility of successful

### TABLE 1

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<th>HSFY/PBGD expression ratios in NOA patients with respect to mTESE outcome.</th>
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<td><strong>NOA, sperm retrieved</strong> (n = 27)</td>
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<tr>
<td>All patients</td>
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<tr>
<td>SCO</td>
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Note: Values are given as mean ± SEM. HS = hypospermatogenesis; MA = maturation arrest; NOA = nonobstructive azoospermia; SCO = Sertoli cells only. * Mann-Whitney test.


### FIGURE 2

Receiver operating characteristic curves for HSFY mRNA detection by quantitative real-time polymerase chain reaction to predict the presence of retrievable testicular sperm among the (A) overall study population and the (B) Sertoli cell–only (SCO) and (C) maturation arrest (MA) subgroups.

In recognition of the minimal clinical impact of testicular histology among men with NOA, our and other centers have abandoned the routine use of preoperative testicular biopsies in these men. However, routine use of preoperative testicular biopsy may be worth reconsidering in the context of new molecular diagnostic tests, such as HSFY mRNA detection with qRT-PCR, that significantly improve the prognostic utility of testicular biopsy.

Detection of candidate spermatogenesis-specific mRNA in testis tissue has been used previously with some success to detect occult foci of spermatogenesis in men with NOA (13–15). These studies evaluated detection of CDY1 (13), protamine, and cyclin (14, 15) mRNA transcripts to predict TESE outcome, but they were all limited by the inability to discriminate between patients with SCO histology who did and did not harbor retrievable testicular sperm. In contrast, in the present pilot study we demonstrated that the level of HSFY transcript variant 1 mRNA is highly predictive of microdissection TESE outcome in men with all histologic variants of NOA, particularly in men with SCO histology.

Two copies of the HSFY gene are present within palindrome P4 of the AZFb region of the Y chromosome (16). These genes encode three different mRNA transcripts that are expressed in human testis. Only the protein translated from transcript variant 1 contains a heat shock factor–like DNA-binding domain (17), suggesting that this mRNA is the critical HSFY transcript. Although the function of HSFY is not presently understood, it is expressed in human germ cells and Sertoli cells (18) and likely acts by moderating expression of heat shock proteins, which serve as important transcription factors.

It is interesting that HSFY mRNA expression seems to specifically reflect the presence of mature germ cells in the testis, despite the fact that expression is present within Sertoli cells and is not germ cell specific. Several explanations for this observation are feasible. Sertoli cell expression of HSFY mRNA may depend on cellular crosstalk with mature germ cells. Alternatively, expression in Sertoli cells may be a driver of meiosis or spermiogenesis. The latter hypothesis is supported by the observation that men with AZFb deletions, in which both copies of HSFY are deleted, invariably present with nonobstructive azoospermia and diffuse maturational arrest.

The HSFY/PBGD expression ratio measured by qRT-PCR may be used in combination with testicular histopathology and historical SRRs from the treating center to counsel individual patients about their chances of sperm retrieval. The benefit of HSFY testing is illustrated by considering the case of a patient with idiopathic NOA whose diagnostic biopsy shows SCO pattern. In the absence of HSFY testing, we would counsel this patient that his chance of successful sperm retrieval is 35%–40% (19). Nearly all such men elect to proceed with microdissection TESE, given the reasonable chance of success. However, if such a patient tested positive for HSFY expression, he could be counseled that the chance of sperm retrieval is close to 100%. Conversely, if he were to test negative the estimated chance of sperm retrieval would be 7%. Considering the 29%–40% ongoing pregnancy rates reported in IVF-ICSI cycles using testicular sperm from men with NOA (20), the chance of achieving an ongoing pregnancy in such a patient would be 2%–3%. Though some men might still elect to proceed in this scenario, the medical risks and financial expenses may not be justified.

Despite its promising performance in this study, the clinical applicability of HSFY testing remains to be determined. First, it is important to recognize that the calculated posttest probabilities of sperm retrieval based on HSFY testing were derived from our institutional SRRs and may differ considerably at institutions with different
SRRs. The efficacy of microdissection TESE depends on many factors, including degree of microdissection, experience of the operating surgeon, and expertise of the andrology laboratory. Therefore, the clinical utility of HSFY testing needs to be evaluated at other centers. Furthermore, we obtained testicular tissue for RNA extraction by microsurgical open testicular biopsy under general anesthesia. This approach may be overly invasive and expensive to be a practical cost-effective method for assessment of the prognosis for sperm retrieval. Because the tissue obtained in this study was obtained from random sites with limited tissue, it is likely that samples could be obtained percutaneously in the outpatient setting under local anesthesia. Patients could undergo one simple office-based procedure during which limited testicular tissue could be procured for simultaneous histologic assessment and HSFY testing, and during which a limited attempt could be made at therapeutic sperm retrieval.

Large-needle percutaneous aspiration biopsy is simple and on average yields 385 mg of testicular tissue (21) (far more tissue than was required for the present study). Fine-needle aspiration (FNA) is performed with a smaller-bore needle but is a more complex procedure which typically requires multiple passes with the needle in multiple directions. These procedures are best performed by an experienced urologist, owing to the risk of disrupting centripetal arterioles within the testicular parenchyma, which may lead to clinically significant bleeding and subsequent development of intratesticular fibrosis. Further studies are needed to evaluate the adequacy of large-needle aspiration biopsy and FNA for detection of testis-specific RNA transcripts.

Despite the potential limitations of the present analysis, the results suggest that measurement of HSFY mRNA in testicular tissue may significantly improve our ability to counsel patients with NOA and to select patients most likely to have sperm found with microdissection TESE. We hope that this research will eventually help to minimize the number of futile attempts at testicular sperm retrieval and to increase the SRR in NOA.

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REFERENCES