Differential Diagnosis of Azoospermia with Proteomic Biomarkers ECM1 and TEX101 Quantified in Seminal Plasma

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INTRODUCTION

Infertility affects about 15% of couples, and males are responsible for half of infertility cases (1). Fertility problems range from diminished production of sperm, or oligozoospermia, to nonmeasurable levels of sperm in semen, or azoospermia, which is diagnosed in nearly 2% of men in the general population (2). Assisted reproduction is used extensively to treat couples with male factor infertility and already accounts for as many as 5% of live births in some European countries (3).

Azoospermia has two major forms: obstructive azoospermia (OA) and nonobstructive azoospermia (NOA). OA, caused by a physical obstruction in the male reproductive tract, results from vassal or epididymal pathology and congenital anomalies (4). Physiological outcomes of OA are identical to those of vasectomy, a surgical severance of the vas deferens resulting in male sterilization. NOA, commonly referred to as testicular failure, is classified into subtypes of hypospermatogenesis (HS), maturation arrest (MA), and Sertoli cell–only syndrome (SCO) on the basis of histopathological examination of testicular tissue (5). Common management of men with OA and NOA includes retrieval of sperm from testis followed by assisted reproduction techniques (6).

Testicular histology with surgical exploration of the genital tract remains the only method for the differential diagnosis of azoospermia (7). Results of diagnostic testicular biopsy, usually a random sampling of the testis, may not accurately reflect the histopathology of NOA because of the spatial distribution of spermatogenesis. Extensive surgical dissection of the testis performed under general anesthesia is often the only way to determine whether men with NOA have any sperm within the testis. A noninvasive test for differential diagnosis of azoospermia forms and subtypes is thus an unmet need in urology. In OA patients, such a test could eliminate or reduce the need for a diagnostic testicular biopsy. In NOA patients, a good diagnostic test would provide more accurate assessment of histopathological subtypes, predict the success of testicular sperm extraction (TESE), and facilitate better planning for assisted reproduction. In men with normal spermatogenesis (NS) who have undergone vasectomy or vasovasostomy, the diagnostic test would confirm the completeness of vas deferens severance or ligation.

A set of studies evaluated prediction of azoospermia forms and subtypes using testicular volume or blood biomarkers, such as follicle-stimulating hormone (FSH), inhibin B, and anti-Müllerian hormone (8–10). The proposed markers, however, have relatively poor specificity and sensitivity. Proteins measured in seminal plasma (SP) were proposed as markers with better predictive value because SP also provides an opportunity for noninvasive diagnostics (11, 12).

We previously initiated the SP proteome project, aimed at discovering biomarkers of azoospermia. Using tandem mass spectrometry (MS), we identified more than 2000 proteins in SP of men with NS, men with NOA, and postvasectomy men (PV, simulated OA), and suggested a list of 79 biomarker candidates (13, 14). In the follow-up work, we verified by selected reaction monitoring (SRM) assay 30 potential biomarker proteins in 30 SP samples and eventually reduced the list to 18 biomarker candidates for differential diagnosis of azoospermia (12). Here, we aimed...
to confirm these 18 proteins in an independent set of clinical samples and select only a few markers suitable for developing a noninvasive clinical diagnostic assay for azoospermia.

RESULTS

Evaluation of biomarker candidates by SRM assay

Here, we focused on our previously identified 18 azoospermia biomarker candidates (12). To simultaneously evaluate multiple biomarker candidates, we opted to use an MS-based multiplex SRM assay. MS-based protein assays have recently matured to a point that allow for quantitative analysis of proteins in mammalian cells (15) and biological fluids (16) and facilitate translational proteomic research, such as verification and validation of biomarker candidates in large sets of clinical samples (17, 18).

To ensure high selectivity of our multiplex SRM assay (table S1), we first measured two unique proteotypic peptides per protein in SP samples obtained from two healthy fertile men before and after vasectomy. Unlike two negative control prostate-specific proteins, CD177 and KLK3, the relative abundance of the 18 biomarker candidates significantly decreased ($P < 0.01$) in the PV sample (fig. S1). A significant decrease observed for both monitored peptides in each protein also suggested the high specificity of the SRM assay. Second, we used serial dilutions of heavy isotope–labeled peptide internal standards to estimate the limits of detection (LODs), limits of quantification (LOQs), and the linear response ranges of all proteins (Table 1 and fig. S2). Third, we measured 18 candidate biomarker proteins and 2 prostate-specific control proteins in 119 SP samples from men with NS, NOA, and OA/PV (Fig. 1 and Table 1). Using the multiplex SRM assay, we reduced the list of initially selected 79 biomarker candidates down to 2 markers: ECM1 and TEX101. The SRM assays allowed us to quickly proceed through the biomarker development pipeline and successfully overcome its typical bottleneck, which is the lack of high-quality analytical assays to verify and validate multiple biomarker candidates and select only few biomarkers suitable for the development of clinical-grade assays.

The levels of TEX101, a protein with monospecific expression in testicular tissue, were found to be relatively high in NS samples (~2 μg/ml), but below the LOD of the SRM assay in OA/PV and NOA samples (<0.12 μg/ml). Likewise, the levels of ECM1 protein were high in NS (~40 μg/ml) and NOA (~20 μg/ml) samples, but notably decreased in OA/PV samples (~1 μg/ml) (figs. S3 and S4 and Table 1). Statistical analysis based on a cutoff derived from the current data suggested 100% specificity at >95% sensitivity for TEX101, LDHC, and PTGDS proteins (Table 2, OA/PV versus NS groups) and 94% specificity at >95% sensi-

<table>
<thead>
<tr>
<th>Protein</th>
<th>UniProt accession</th>
<th>Concentration in SP (μg/ml), median (IQR)</th>
<th>CV (%)</th>
<th>LOD (μg/ml)</th>
<th>LOQ (μg/ml)</th>
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<tr>
<td>NS (n = 42)</td>
<td>NOA (n = 25)</td>
<td>OA/PV (n = 52)</td>
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<tr>
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</table>

to detect the further increase in specificity would require more than 100 independent NOA samples. In addition, because the clinical applicability of biomarker combinations is technically and interpretatively more demanding, we decided to focus only on ECM1 and TEX101 proteins to distinguish between OA, NOA, and NS.

Establishing a clinically relevant diagnostic cutoff for ECM1 protein
After SRM analysis, we measured ECM1 protein by enzyme-linked immunosorbent assay (ELISA) in 159 samples. A data-derived cutoff value of 2.3 µg/ml provided 73% specificity at 100% sensitivity and an area under the curve (AUC) of the receiver operating characteristic curve (ROC) of 0.94 for distinguishing OA/PV from NOA, and 100% specificity at 100% sensitivity (AUC = 1.00) for distinguishing OA/PV from NS (Fig. 2). A sensitivity of 100% was chosen to ensure that there would be no OA patients misclassified as NOA. OA patients are typically fertile using TESE and assisted reproduction techniques. NOA patients who are misclassified as having OA (ECM1 <2.3 µg/ml, TEX101 <5 ng/ml) or patients with an inconclusive diagnosis (ECM1 <2.3 µg/ml, TEX101 >5 ng/ml) will still undergo TESE, which will confirm their fertility status. Thus, a cutoff level of 2.3 µg/ml will not exclude any patient who has a chance for sperm retrieval by TESE.

TEX101 in different histopathological subtypes of NOA
To investigate the role of TEX101 in NOA, we assessed TEX101 expression by immunohistochemistry in testicular tissues from men with NS (n = 5) and men with biopsy-confirmed HS (n = 5), MA (n = 5), and SCO (n = 5). Immunohistochemistry confirmed high levels of TEX101 expression in spermatocytes, spermatids, and spermatozoa, but not in basal cells, spermatogonia, Sertoli, or Leydig cells (Fig. 3, A to D, and table S2). We found low levels of TEX101 expression in tissues with HS and MA, whereas tissues with SCO showed no staining, because of the absence of germ cells.

Fig. 1. Evaluation of 18 biomarker candidates in 119 SP samples using MS-based SRM assay. Three groups of SP samples were analyzed: NS (n = 42), NOA (n = 25), and OA (n = 10) or PV (n = 42). Horizontal lines represent median concentrations of proteins in each sample set. ROC AUC values for OA/PV versus NS were used to rank proteins. For proteins with concentrations below the LOD and LOQ, background signal was used to calculate light-to-heavy ratios and estimate protein concentrations. Two prostate-specific proteins, KLK3 and CD177, were used as negative control proteins.

Fig. 2. Evaluation of 18 biomarker candidates in 119 SP samples using MS-based SRM assay.
Protein Ratio

Permatation by high specific enzymatic mechanisms during sperm maturation. In the epididymis (19, 20); and (iii) TEX101 is a soluble factor that mediates the acrosome reaction by triggering progesterone release in cumulus cells (21, 22). These facts explain why the physical obstruction of vas deferens and the absence of germ cells lead to the undetectable (theoretically zero) levels of TEX101 in SP of patients with OA, PV, and SCO. In MA and HS, TEX101 is expressed, but spermatocytes do not mature into sperm cells and thus never pass through the epididymis to allow for the cleavage of TEX101 from the surface of spermatocytes. However, TEX101 may be shed from the surface of spermatocytes inside the testis, which correlates with the number of germ cells in the testis and spermatids (23, 24), and after spermiisation in the epididymis (19, 20).

To examine TEX101 levels in SP of men with HS and MA, we developed a more sensitive immuno-SRM assay based on immunoenrichment of TEX101, followed by SRM analysis (tables S3 and S4). Immunoenrichment improved the LOD of TEX101 from 120 to 5 ng/ml. After this, we measured the relative abundance of TEX101 in SP from men with HS (n = 6), MA (n = 13), SCO (n = 8), OA (n = 5), and NS before (n = 5) and after (n = 5) vasectomy. With the improved LOD, TEX101 was observed in most of the samples with HS and MA (Fig. 3E). As expected, TEX101 was below the LOD in all SCO, OA, and PV samples.

Model of TEX101 expression and secretion into SP

Assessment of immunohistochemistry and immuno-SRM data, as well as literature review, allowed us to propose a possible model of TEX101 expression and secretion into SP in different forms and subtypes of azoospermia (Fig. 4). Such a model emerged from the following facts: (i) according to the Human Protein Atlas (http://www.proteinatlas.org), TEX101 is a membrane protein with monospecific expression in germ cells, but not in any other cell type or human tissue; (ii) GPI-anchored mouse TEX101 is expressed in testis but cleaved from the surface of spermatozoa by highly specific enzymatic mechanisms during sperm maturation in the epididymis (19, 20); and (iii) TEX101 is a soluble factor that mediates the acrosome reaction by triggering progesterone release in cumulus cells (21, 22). These facts explain why the physical obstruction of vas deferens and the absence of germ cells lead to the undetectable (theoretically zero) levels of TEX101 in SP of patients with OA, PV, and SCO. In MA and HS, TEX101 is expressed, but spermatocytes do not mature into sperm cells and thus never pass through the epididymis to allow for the cleavage of TEX101 from the surface of spermatocytes. However, TEX101 may be shed from the surface of spermatocytes inside the testis by nonspecific mechanisms and is thus detected in SP in low amounts (<120 ng/ml). As a result, SP concentration of TEX101 alone allows for the differentiation of histopathological NOA subtypes. A more sensitive TEX101 assay should facilitate the differentiation of men with NS (TEX101 >120 ng/ml) from patients with HS and MA (5 ng/ml < TEX101 < 120 ng/ml) and also from patients with SCO and OA (TEX101 <5 ng/ml, theoretically zero). Because the concentration of TEX101 in SP may correlate with the number of germ cells in the testis, TEX101 could be an informative biomarker for the whole spectrum of male fertility conditions, ranging from severe azoospermia to oligozoospermia and NS.
Fig. 2. Establishing a clinically relevant cutoff level of ECM1 protein in SP. As measured by ELISA in 159 SP samples, a cutoff value of 2.3 μg/ml (dotted line) provided 73% specificity at 100% sensitivity (AUC = 0.94) to distinguish OA/PV from NOA, and 100% specificity at 100% sensitivity (AUC = 1.00) to distinguish OA/PV from NS. The set of 159 samples included 119 samples analyzed by SRM and a set of SP samples from 40 additional patients (see table S6 for details).

**DISCUSSION**

Histopathological examination of testicular tissue after a diagnostic testicular biopsy is currently the only definitive way to differentiate OA from NOA and to distinguish between NOA subtypes, allowing prediction of the success of subsequent sperm retrieval by TESE. However, the results of the diagnostic testicular biopsy may not always accurately reflect the histopathology of NOA. Because the extent of spermatogenesis within the seminiferous tubules in the testis is not homogeneous and there may only be occasional pockets of sperm within the testis, a random testis biopsy does not provide a complete picture of the histology of the testis (23). In addition, a diagnostic testicular biopsy is an invasive procedure with possible complications such as bleeding, damage to testicles, vas deferens, and epididymis; chronic pain in the testicle; and possible loss of fertility (24). Noninvasive differential diagnosis of the categories of azoospermia is thus an unmet need in urology.

After a diagnostic testicular biopsy, extensive dissections of the testis under a microscope are used in attempts to retrieve sperm for assisted reproduction. Success rates of sperm retrieval by TESE correlate with histopathological subtypes and are estimated to be 79% for HS, 47% for MA, and 24% for SCO (23). Theoretically, the success rate for pure SCO subtype should be 0%. However, histopathological subtyping of NOA is based on the most predominant pattern, and mixed patterns such as HS with SCO and MA with SCO may be present in some cases. Because SP concentration of testis-specific proteins such as TEX101 may reflect the cumulative yield of spermatogenesis, such proteins may facilitate the prediction of not only pure HS, MA, and SCO patterns but also the proportion of mixed patterns.

Serum concentrations of FSH, inhibin B, and luteinizing hormone have been proposed for NOA diagnosis and prediction of sperm retrieval (24) but eventually were found to be not very specific or sensitive markers. Our group previously proposed PTGDS protein as an SP biomarker for diagnosis of OA. PTGDS, however, could not differentiate between men with NOA and OA (25). Results of the present study confirmed the utility of PTGDS to detect OA, but its sensitivity for distinguishing between NOA and OA was low.

Several reports proposed a possibility of diagnosis of azoospermia based on analysis of proteins in SP (25, 26). Recent proteomic studies also indicated that the SP proteome is as complex as the blood plasma proteome and has a dynamic range of around nine orders of magnitude (13, 27). In our previous work, we detected a total of 3200 proteins, which constitute the largest SP proteome ever identified (13, 14, 28). A large number of SP proteins have high tissue specificity and thus may indicate a pathological process in their corresponding gland with high specificity. Analysis of Tissue-specific Gene Expression and Regulation database reveals 855 testis-specific proteins (29) and shows that the number of tissue-specific proteins expressed in the testis is greater than the number of tissue-specific proteins expressed in any other human tissue.

Our present work revealed that germ cell–specific proteins may be able to serve as biomarkers of azoospermia. Our experimental data also allowed us to propose a simple decision tree for the sensitive and specific differential diagnosis of azoospermia forms and subtypes based on ECM1 and TEX101 levels in SP (Fig. 5; see also Figs. 2 and 3E for data). When azoospermia is diagnosed by semen analysis, low SP levels of ECM1 (<2.3 μg/ml in our data set) suggest an obstruction of the vas deferens (OA), whereas high SP levels of ECM1 (>2.3 μg/ml) suggest NOA. SP levels of TEX101 can distinguish between SCO (<3 ng/ml, theoretically zero) and HS or MA (5 to 120 ng/ml in our data set). Men with biomarker evidence of OA, HS, and MA are thus recommended for TESE and have high chances of successful sperm retrieval, whereas for men with SCO, sperm retrieval is unlikely and TESE could be avoided. The proposed simple two-marker decision tree could eliminate most of the diagnostic testicular biopsies performed before TESE and intracytoplasmic sperm injection. Some patients with HS and MA may still have TEX101 below 5 ng/ml (Fig. 3E), but our sample size is too small to draw conclusions on the success of TESE to retrieve sperm in these patients. In addition, future studies will be required to address some limitations of our present work. First, cutoff points established with the present data set and associated sensitivity and specificity estimates should be validated in a separate set of samples. Second, prospective validation of the marker combination with larger numbers of samples would be necessary before the test reaches the clinic. Third, there may be possible confounding factors because of age differences between groups (the population of NS and PV men is, on average, 6 years older than that of OA and NOA men). Finally, additional studies may be required to rule out changes in the levels of ECM1 and TEX101 proteins in SP samples over time.

To propose possible mechanisms leading to decreased levels of presented biomarkers in azoospermia, we reviewed the literature on functional roles of ECM1 and TEX101 proteins. ECM1 is a ubiquitous extracellular matrix protein that maintains the integrity of tissue through interaction with a variety of structural and extracellular proteins (30, 31). Within the male reproductive tract, epididymal tissue has a high level of ECM1 expression, which explains the marked decrease of ECM1 in OA, but not in NOA. TEX101 is a GPI-anchored (32) membrane glycoprotein present at the cell surface of mouse germ cells (33–35). Recent studies on spermatogenesis in mice revealed that TEX101 is cleaved from the sperm...
cell surface by a specific enzyme during post-testicular sperm maturation in the epididymis (19, 21). Recently, such an enzyme was identified as a testis-specific angiotensin-converting enzyme (20). The specific function of TEX101 remains unknown, although recent studies in mice suggest its roles as an ADAM3-specific molecular chaperone (20) and as a soluble factor to trigger acrosome reaction after its cleavage from the cell surface (21). Coimmunoprecipitation experiments showed that mouse TEX101 may modulate urokinase signaling through binding to urokinase-type plasminogen activator (uPA)/uPA receptor (uPAR) complexes (36, 37). Upon release from the sperm cell surface, TEX101 interacts with female cumulus cells and triggers calcium-mediated progesterone release, leading to degradation of the extracellular matrix surrounding the cumulus cells (21, 22). According to the Human Protein Atlas (http://www.proteinatlas.org), TEX101 is a protein with monospecific expression in germ cells, but not in any other human cell or tissue (fig. S6). The role of human TEX101 in fertilization is still to be elucidated through identifying TEX101-interacting partners and potential receptors on female cells. Because of its monospecific expression and the direct involvement in fertilization, TEX101 is a potential target for developing nonhormonal male contraceptives and a candidate compound biomarker to evaluate novel male contraceptives (38, 39). Indeed, anti-TEX101 antibody treatment as well as TEX101 immunization was shown to significantly decrease the rates of in vitro and in vivo fertilization in mice (20, 21).
To conclude, we propose a simple two-marker decision tree for the noninvasive differential diagnosis of OA and NOA and, in addition, for the differential diagnosis of NOA subtypes. Clinical immunoassays of ECM1 and TEX101 have the potential to eliminate most of the diagnostic testicular biopsies and TESE procedures for patients with pure SCO, improve the confidence of NOA diagnosis, facilitate prediction of the outcome of sperm retrieval procedures used for assisted reproduction, and reduce the total cost of azoospermia diagnosis. Because of their promising performance, ECM1 and TEX101 may emerge among the clinically useful biomarkers discovered by proteomics including TEX101, are not detectable in SP because of the physical obstruction or surgical severance of the vas deferens. In NOA with HS and MA, spermatocytes do not mature into spermatozoa and thus never pass through the epididymis to allow for specific TEX101 cleavage from the cell surface. However, nonspecific shedding of TEX101 from spermatocytes inside the seminiferous tubules results in its low levels in SP (<120 ng/ml). In NOA with SCO, germ cells are absent, so TEX101 is not expressed and is not detected in SP.

Fig. 4. Model of TEX101 expression and secretion into SP in men with NS and azoospermia. In tissues with NS, TEX101 is expressed in spermatocytes, which mature into sperm cells and move to epididymis for post-testicular sperm maturation. In epididymis, glycosylphosphatidylinositol (GPI)-anchored TEX101 is cleaved from the sperm cell surface by a specific enzymatic mechanism and is released into SP at a concentration of about 2 μg/ml. In men with OA and PV, spermatogenesis occurs, but testicular proteins, including TEX101, are not detectable in SP because of the physical obstruction or surgical severance of the vas deferens. In NOA with HS and MA, spermatocytes do not mature into spermatozoa and thus never pass through the epididymis to allow for specific TEX101 cleavage from the cell surface. However, nonspecific shedding of TEX101 from spermatocytes inside the seminiferous tubules results in its low levels in SP (<120 ng/ml). In NOA with SCO, germ cells are absent, so TEX101 is not expressed and is not detected in SP.

Fig. 5. Two-marker decision tree for differential diagnosis of azoospermia (OA versus NOA) and prediction of NOA subtypes. The presented decision tree is based on the results of ECM1 ELISA, SRM, and immuno-SRM assays and the evidence from TEX101 immunohistochemistry experiments. When azoospermia is diagnosed by semen analysis, low SP levels of both markers ECM1 (<2.3 μg/ml) and TEX101 (<5 ng/ml) suggest obstruction of vas deferens, whereas high SP level of ECM1 (>2.3 μg/ml) suggests NOA. SP level of TEX101 distinguishes between SCO (<5 ng/ml) and HS or MA (5 to 120 ng/ml). Men with OA, HS, and MA are good candidates for TESE and have high chances of successful sperm retrieval, whereas for men with SCO, sperm retrieval is unlikely and TESE could be avoided. Note that the cutoffs for each marker are derived from our data set and will require independent validation. *TEX101 levels in OA and pure NOA-SCO should be theoretically zero. **In some NOA-HS and NOA-MA samples, TEX101 levels may be lower than 5 ng/ml, but immunohistochemical data suggest that TEX101 is still expressed in such tissues and thus may be detected by ultrasensitive analytical assays.

Azoospermia is diagnosed by semen analysis

2-marker analysis

ECM1 < 2.3 μg/ml
TEX101 < 5 ng/ml*

Obstructive azoospermia

ECM1 > 2.3 μg/ml
TEX101 < 120 ng/ml

Nonobstructive azoospermia

TEX101 5-120 ng/ml**

Hypospermatogenesis

Maturation arrest

Sertoli cell-only syndrome

High chances of sperm retrieval by TESE

Low chances of sperm retrieval by TESE. TESE could be avoided
and stimulate further searches for biomarkers of other urogenital diseases. Further studies on SP proteins may provide panels of markers to assess individual stages of spermatogenesis as well as additional targets for developing effective male contraceptives. This will facilitate developing diagnostic and therapeutic tools to manage the opposite sides of the same coin: male infertility and fertility.

MATERIALS AND METHODS

Study design

The objectives of this study were to evaluate the previously proposed 18 biomarker candidates, select the minimal number of markers necessary for differential diagnosis of azoospermia, confirm these markers by ELISA and immunohistochemistry, consider potential molecular mechanisms involved, and propose a simple decision tree for further validation in the clinic. Power calculations were performed to estimate the number of independent samples required to validate the previously measured performance of biomarker candidates (12). A power of 80% can be achieved using 11 NS, 15 NOA, and 15 OA/SP samples (table S5) for the top markers previously identified for each of the three group comparisons (α = 0.05, two-tailed Wilcoxon-Mann-Whitney test, means and SDs from our previous work (12)). Although the minimal required sample sizes were relatively small, we decided to measure our markers in all 119 independent samples available in our laboratory (42 NS, 25 NOA, and 52 OA/SP) and establish the sensitivity and specificity of each marker with higher accuracy.

Patients

Men with NS were confirmed fertile men with normal sperm count by semen analysis (>15 million/ml according to the World Health Organization reference values). These men were referred for vasectomy, and SP samples were obtained before vasectomy. The PV group included SP samples obtained from fertile men 3 to 6 months after vasectomy, and zero sperm count was confirmed by at least two semen analyses. The OA group included men with biopsy-confirmed OA, obstruction at the epididymis, normal testicular volume, and normal FSH (1 to 18 IU/liter). Four men in the OA group had congenital bilateral absence of the vas deferens. The NOA group included men with azoospermia by semen analysis and elevated FSH (>18 IU/liter) or NOA confirmed by testicular biopsy. Y-chromosome deletion status was known for a limited number of NOA cases and was not used as an independent parameter. None of the men were taking any medications related to genitourinary tract disorders. The patient groups had the following median age and interquartile age range: NS, 39 years (37 to 43 years); NOA, 34 years (32 to 40 years); PV, 40 years (36 to 43 years); and OA, 33 years (32 to 37 years). Additional details on patients, sample, and clinical data are presented in tables S6 and S7. NOA subtypes were determined on the basis of histopathological examination of hematoxylin and eosin (H&E)–stained testicular tissues obtained from patients by diagnostic testicular biopsies. H&E staining, histopathological subtyping, and TEX101 immunohistochemical staining were provided and reviewed by a staff pathologist at Mount Sinai Hospital (B.M.).

SP samples

Semen samples from men with NS before and after vasectomy and men with azoospermia were collected after a minimum of 3 days of sexual abstinence with informed consent and Institutional Review Board approval from Mount Sinai Hospital, Toronto. The sample collection procedure was identical for all groups of samples. Only SP samples with known clinical information were included in our study. Seminal fluid was left to liquefy for 1 hour, aliquoted in 1-ml portions, and centrifuged at 16,000 rpm for 30 min at 4°C three times to separate SP from cells and cellular components. Aliquots of SP samples were stored at −80°C until further use.

Chemicals and reagents

The following chemicals were used: sequencing-grade modified trypsin (Promega Corp.); iodoacetamide, DL-dithiothreitol, and l-methionine (Sigma-Aldrich Co.); and RapiGest SF surfactant (Waters Corp.). Heavy isotope–labeled peptides were provided by Thermo Fisher Scientific Inc.

SRM assay development

Two unique proteotypic peptides per protein (table S1) were selected using SRM atlas (http://www.srmatlas.org) or our in-house two-dimensional liquid chromatography (LC)–MS/MS identification data (12, 15) and measured in triplicate in three digests of pre- and postvasectomy SP samples. The uniqueness of each proteotypic peptide was confirmed with protein Basic Local Alignment Search Tool (table S8). Possible post-translational modifications, protein isoforms, or single-nucleotide variants that could affect SRM measurements were investigated using the neXtProt database (http://www.nextprot.org) and summarized in tables S9 and S10. The impact of peptide chemical modifications, such as cysteine alkylation, methionine oxidation, glutamine and asparagine deamidation, and formation of pyroglutamate and pyro-carbamidomethyl cyclization at N-terminal cysteine, was assessed by SRM during method development. To suppress the oxidation of methionine residues of internal standard peptides, storage buffer was supplemented with a large excess of l-methionine (5 mM), which limited oxidation to 10% or less, as measured by SRM. Likewise, storage conditions (~80°C) and addition of 5 mM methionine to the solution of trypsin-digested peptides limited the oxidation of methionine residues in endogenous peptides. In general, the magnitude of peptide chemical modifications did not exceed 20% and was similar across different SP samples processed simultaneously. To determine LOD and LOQ of SRM analysis, a dilution series of a mixture of 20 heavy peptide internal standards (0.01 to 300 fmol per injection) were added to the digest of one normal SP sample (11.2 μg of total protein, an equivalent of 0.25 μl of SP). Three digests were analyzed per each concentration point, and each digest was analyzed in duplicate. Because the amounts of light endogenous peptides were constant, the heavy-to-light peptide ratio was used to build calibration curves and determine variability of analysis. LOD was calculated using the minimal amount of a heavy peptide measured with CV <30% for the heavy-to-light peptide ratio. LOQ was estimated using the minimal amount of a heavy peptide measured within the linear response range of heavy-to-light peptide ratio of the calibration curve.

Biomarker verification by SRM assay

Ten microliters of SP was diluted 10-fold with 50 mM ammonium bicarbonate (pH 7.8), and an aliquot equivalent to 0.5 μl of SP was subjected to proteomic sample preparation in 96-well plates. To avoid possible contamination of PV samples with NS samples due to LC carryover, a restricted randomized block design was used for sample randomization. Samples were split into NS, NOA, and OA/SP blocks of 10 to 12 samples, and blocks were randomized between five 96-well plates. Proteins were denatured at 60°C with 0.1% RapiGest SF surfactant, and the disulfide bonds were reduced with 10 mM dithiothreitol. After reduction,
the samples were alkylated with 20 mM iodoacetamide. Samples were then trypsin-digested overnight at 37°C. One hundred eighty femtomoles of 20 heavy C13- and N15-labeled peptide internal standards was added to each digest. RapiGest was cleaved with 1% trifluoroacetic acid, and 96-well plates were centrifuged at 2000 rpm for 20 min. Each digest was subjected to microextraction with 10-µl OMIX C18 tips (Varian Inc.). After sample preparation, plates were stored at −20°C. Each plate was thawed right before LC-SRM analysis, and each sample was analyzed in duplicate. One to four MS quality control samples were run every 12 injections, after each block of samples, and after each plate. The LC EASY-nLC 1000 (Thermo Fisher Scientific Inc.) was coupled online to TSQ Vantage triple-quadrupole mass spectrometer (Thermo Fisher Scientific Inc.) using a nanoelectrospray ionization source. Peptides were separated on a 2-cm trap column (150-μm inside diameter (ID), 5-μm C18) and eluted onto a 5-cm resolving column (75-μm ID, 3-μm C18). Forty peptides and 120 transitions representing 20 proteins were scheduled within 1.5-min intervals during a 30-min LC gradient. The SRM method had the following parameters: optimized collision energy (CE) values; mass/charge ratio (m/z) scan width, 0.010; scan time, 0.015 to 0.040 s; FWHM resolution of the first quadrupole (Q1), 0.4; FWHM resolution of the third quadrupole (Q3), 0.7; pressure of the second quadrupole, 1.5 mtorr; tuned-s-lens values; declustering voltage, +1 V. Raw files recorded for each sample were analyzed with the Pinpoint software, and peptide areas were used to calculate light-to-heavy peptide ratios and protein concentrations in each sample. Results of SRM analysis were open to all investigators.

ECM1 ELISA
Immunoassay was performed according to the manufacturer’s protocol (SEK10362, Sino Biological Inc.). OA and PV SP samples were diluted 1000-fold with 4% bovine serum albumin, whereas NOA and normal samples were diluted 100,000-fold to match the linear response range of ELISA (23 to 1500 pg/ml). Upon dilution, duplicates of 159 samples were divided between six 96-well plates using a completely randomized design and measured according to the manufacturer’s protocol.

TEX101 immunohistochemistry
Rabbit polyclonal anti-human TEX101 antibody HPA041915 (Atlas Antibodies AB) was used to stain 12 testicular tissue samples fixed in 10% buffered formalin. Dilutions of 1:2000 and 60-min incubation at room temperature were used. Heat-induced epitope retrieval was performed in the citrate buffer at pH 6.0. Vectastain Elite ABC Kit (Vector Laboratories Inc.), 3′-diaminobenzidine substrate (Sigma-Aldrich Corp.), and Lab Vision 720 autostainer (Thermo Fisher Scientific Inc.) were used for detection.

TEX101 immuno-SRM assay
SRM assay was used to assess TEX101 enrichment from SP by two antibodies: rabbit polyclonal HPA041915 (developed using the recombinant protein epitope signature tag; Atlas Antibodies AB) and mouse polyclonal ab69522 (developed against the full-length human protein, protein G-purified; Abcam PLC). To ensure high assay selectivity, three unique proteotypic peptides of TEX101 were used for qualitative assessment, whereas the fourth peptide and its heavy isotope-labeled peptide internal standard were used for quantification (table S3). Only ab69522 antibody could reproducibly enrich TEX101 from SP and was used to develop the immuno-SRM assay. ELISA plate was coated with 500 ng of ab69522 antibody, and 10 µl of SP was used for the enrichment. Upon stringent washing, proteins in each well were digested with trypsin as described above and quantified using SRM assay. Relative abundance of TEX101 in each sample was estimated as a ratio to the spiked-in heavy isotope-labeled peptide internal standard. To estimate the LOD of immuno-SRM assay, serial dilutions of an NS SP sample with known TEX101 concentration (as measured by SRM assay) were subjected to immunoenrichment and remeasured by SRM assay.

Statistical analysis
A nonparametric Kruskal-Wallis test was used to evaluate the significance of difference in protein concentrations in the three groups of samples (NS, NOA, and OA/PV). A two-tailed Mann-Whitney U test with Bonferroni correction was used for pairwise comparisons between groups, and adjusted P values of <0.05 were considered significant. Immuno-SRM data were analyzed with a Kruskal-Wallis test followed by a two-tailed Mann-Whitney U test for pairwise comparisons between groups. Statistical analysis was performed with IBM SPSS Statistics (version 20), and GraphPad Prism (version 5.03) was used for calculation of ROC AUC area, sensitivity, and specificity. Power calculations were done with G*Power software (version 3.1.7, Heinrich Heine University Dusseldorf).

SUPPLEMENTARY MATERIALS
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Fig. S1. Two-peptide SRM assay for biomarker candidates in SP.
Fig. S2. Calibration curves used to establish LODs and LOQs of SRM assays.
Fig. S3. LC-SRM chromatograms of TEX101 and ECM1 proteins in three patients’ SP samples.
Fig. S4. Heat map of log2-transformed mean-centered protein concentrations measured by SRM.
Table S1. Parameters of a multiplex SRM assay.
Table S2. TEX101 immunohistochemistry analysis.
Table S3. Parameters of immuno-SRM assay for TEX101 protein.
Table S4. TEX101 protein sequence and proteotypic peptides analyzed by immuno-SRM assay.
Table S5. Power calculations.
Table S6. Summary of SP samples analyzed in the present study.
Table S7. Clinical information for 119 patients analyzed by SRM.
Table S8. Bioinformatic approach to verify the uniqueness of proteotypic peptides.
Table S9. Proteins and protein isoforms quantified by SRM assay.
Table S10. Single-nucleotide variants and posttranslational modifications that affect quantitative SRM data.

REFERENCES AND NOTES

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Editor's Summary

To Retrieve, or Not to Retrieve, May No Longer Be the Question

Infertility is a very common medical problem, and male partners are responsible for about half the cases. However, male infertility is not a homogeneous disorder, but a collection of diagnoses with different causes and different potential treatments. One type of male infertility, obstructive azoospermia (OA), is caused by physical obstruction to the movement of sperm, whereas the sperm cells themselves are normal. Nonobstructive azoospermia (NOA), on the other hand, is caused by abnormalities in the production of sperm. Hypospermatogenesis (HS) and maturation arrest (MA) are types of NOA where some sperm cells are still present, but their number is decreased or they do not fully mature. Meanwhile, in Sertoli cell–only syndrome (SCO), the patients do not make sperm cells at all.

Although all of these diagnoses lead to an infertile phenotype in unaided reproduction, they differ as to whether the patient’s testes contain any fertile sperm. In cases of OA, and often even HS and MA, sperm can be retrieved from the testis and used for assisted reproduction. In SCO, however, no sperm cells are available for retrieval. With current technology, the only way to distinguish between these scenarios is to search for sperm within the testis, which often requires a full surgical procedure. Now, Drabovich and coauthors have identified two protein biomarkers in seminal plasma that should help facilitate the differential diagnosis of azoospermia. Using these markers, physicians may be able to distinguish patients with OA and NOA, and SCO versus other types of NOA, and thus avoid subjecting patients with SCO to ineffective surgical interventions.

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