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CORRELATION BETWEEN FLUORESCENCE IN SITU HYBRIDIZATION AND TESTICULAR BIOPSY FOR THE PREDICTION OF SPERMATOGENESIS IN 37 PATIENTS WITH NONOBSTRUCTIVE AZOOSPERMIA

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ABSTRACT

Objectives. We applied interphase fluorescence in situ hybridization (FISH) to testis sections to examine the evidence of spermatogenesis in patients with nonobstructive azoospermia. This technique was evaluated and compared with conventional testicular histopathologic findings for the possibility of additional clinical applications.

Methods. Thirty-seven consecutive patients with nonobstructive azoospermia were carefully evaluated clinically. Testes were biopsied for both sperm extraction and histopathologic examination. FISH staining was performed with a CEP 18 SpectrumAqua/CEP X SpectrumGreen/CEP Y SpectrumOrange probe.

Results. Eight of 11 cases (sensitivity 73%) that were found to have spermatids on the histopathologic slides also were proven to produce haploid cells by FISH staining. On the other hand, 21 of the 26 cases (specificity 81%) for which no spermatids could be found on the histopathologic slides also had only diploid cells by FISH staining. On the basis of the good correlation between the FISH staining and conventional histopathologic findings, we could confirm the diagnosis of spermatogenesis using both methods.

Conclusions. FISH staining of testicular sections allows more reliable prediction of spermatogenesis and provides benefits for a patient's decision regarding fertility counseling. UROLOGY **60:** 1063–1068, 2002.

Intracytoplasmic sperm injection (ICSI) of the oocyte using testicular spermatozoa has enabled the treatment of patients with azoospermia due to primary gonad failure.[1] However, the successful testicular sperm recovery rate has a high variation of between 30% and 70% in patients with nonobstructive azoospermia (NOA).[2] Conventional parameters associated with spermatogenesis, including testicular size and serum follicle-stimulating hormone (FSH), are poor predictors. Only testicular histopathologic evaluation has been shown to be a strong predictor of whether testicular spermatozoa recovery will be successful; however, it only provides partial discrimination between successful and failed testicular sperm extraction.[2,3] In this study, we applied the interphase fluorescence in situ hybridization (FISH) technique, a powerful method to evaluate cellular ploidy and gene amplification,[4,5] to testicular biopsy to detect the presence of haploid cells (spermatids or spermatozoa), which indicate complete meiosis during spermatogenesis, in seminiferous tubules. The objective of our study was to examine the evidence of spermatogenesis that might not be detected with conventional histopathologic analysis, by interphase FISH in patients with NOA. We believed that the clinical application of this laboratory technique could offer compensatory information for the pathologic diagnosis in the future. The result of FISH staining combined with orthodox pathologic examination may become a good reference for future decisions of infertile couples with male factor of NOA.

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MATERIAL AND METHODS

PATIENTS

Thirty-seven consecutive male patients who had been diagnosed with NOA were included in this study after their con-

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sent. All patients underwent careful physical examination and laboratory tests, and the following clinical features were recorded: testicular size, measuring the long axis in centimeters; hormonal assay (plasma concentrations of FSH, luteinizing hormone, testosterone, and prolactin); chromosome study (karyotype examination of peripheral blood leukocytes); and Y chromosome microdeletion, including AZFa, AZFb, and AZFc regions (genomic DNA was extracted from peripheral leukocytes and amplified using a polymerase chain reaction-based assay with a combination of the oligonucleotide primers sY95, sY117, sY159, sY127, sY153, sY274, sY277, sY276, sY81, sY147, sY149, and Y6HP52).[6]

Specimens

The diagnostic testicular biopsy was made through a 0.5-cm incision of the scrotal wall and tunica albuginea under local anesthesia.[7] After the testicular tissue was exposed out of the tunica albuginea, 20 to 50 mg of tissue was excised. The size of the excised tissue was dependent on the size of the testis. Usually, only a tiny piece of tissue (less than 5 mg) was excised from an atrophic testis (long axis less than 1 cm). More tissue was obtained from the grossly normal testis (long axis more than 2 cm) through one incision. The removed testicular tissue was divided into three fragments of about equal size, and the fragments were individually placed in a four-well dish (Nunc, Denmark) containing HTF-HEPES solution (Irvine Scientific, Irvine, Calif) and immediately transported to an adjacent laboratory for examination.

Sperm Extraction

Seminiferous tubules were dissected and separated into a single, long tubule about 1 to 2 mm in length from a piece of biopsy tissue (usually about 10 to 20 mg in size, but only 0.1 × 0.1 × 0.1 cm in size if the testis was atrophic) and placed in HTF-HEPES solution with two 26-gauge needles. After the transfer, the single seminiferous tubule in each HTF medium drop was over layered with buffered paraffin oil (Embryo tested, Sigma). The width and thickness of the tubules were measured under a microscope. After the measurement, the seminiferous tubule was minced into fine pieces and immediately examined for the presence of any testicular spermatozoa. If no spermatozoon was found after extraction, the testicular tissue was then incubated at 37°C in an atmosphere of 5% carbon dioxide, 20% oxygen, and 75% nitrogen for 2 days. The incubated testicular tissue was repeatedly examined during these 2 days to detect the presence of any testicular spermatozoa after the culture.

Histologic Analysis

A piece of testicular tissue (usually about 10 to 20 mg in size, but only 0.1 × 0.1 × 0.1 cm in size if the testis was atrophic) was preserved immediately in Bouin's solution. After fixation, more than six paraffin-embedded sections (5 μm in thickness) were stained and examined under a light microscope at 400× magnification by the same pathologist. The spermatogenesis pattern of the seminiferous tissue was histopathologically divided into four categories: Sertoli cell only syndrome, maturation arrest, hypospermatogenesis, and focal spermatogenesis.[8] The definition and the degree of spermatogenesis impairment of these four categories is as follows:

1. Sertoli cell only syndrome: no evidence of spermatogenesis could be detected in all the histologic sections. Only Sertoli cells were found in some of the atrophic or partially atrophic seminiferous tubules.

2. Maturation arrest: different stages of immature spermatogenic cells were present in some of the seminiferous tubules, including spermatogonium, primary spermatocytes, and secondary spermatocytes. However, basically no mature sperm or round spermatid was noted in all the seminiferous tubules.

3. Hypospermatogenesis: no spermatozoa noted in the seminiferous tubules, although round spermatid appeared in some tubules, considered "post-meiotic maturation arrest" according to our pathologist.

4. Focal spermatogenesis: testicular biopsies demonstrating focal spermatogenesis containing two populations of tubules. The smaller tubules exhibit Sertoli cell only and tubules of increased diameter show spermatogenesis that is usually reduced.

Fluorescence In Situ Hybridization

A piece of the testicular biopsy (usually about 10 to 20 mg in size but only 0.1 × 0.1 × 0.1 cm in size if the testis was atrophic) was fixed immediately in 4% paraformaldehyde. Each paraffin-embedded section was 5 μm in thickness and was processed for FISH analysis. Each testicular section was deparaffinized and allowed to air dry. Protein was digested by 30% pretreatment solution (Oncor, Gaithersburg, Md) at 43°C for 40 minutes and proteinase K at 43°C for 30 minutes. These sections were dehydrated in ethanol and allowed to air dry. A CEP 18 SpectrumAqua/CEP X SpectrumGreen/CEP Y SpectrumOrange probe (Vysis) was used for the FISH study in separated reactions. CEP X SpectrumGreen was the chromosome X centromere region Xp11.1-q11.1 (DXZ1) directly labeled with SpectrumGreen; CEP Y SpectrumOrange was the chromosome Y centromere region Yp11.1-q11.1 (DYZ3) directly labeled with SpectrumOrange; and CEP 18 SpectrumAqua was the chromosome 18 centromere region 18p11.1-q11.1 (D18Z1) directly labeled with SpectrumAqua. The tissues and probes were co-denatured at 90°C for 10 minutes and incubated at 37°C overnight. FISH detection was performed according to the manufacturer's instructions. Analyses were done using an Olympus BX60 epifluorescence microscope equipped with filter sets for DAPI, FITC, Texas Red, and Aqua.

To detect haploid and diploid chromosomes in interphase nuclei, we applied two sets of probes. One was a dual color probe for chromosome X and Y centromere regions, the other a centromeric probe for chromosome 18. Because the incidence of sperm chromosomal abnormalities in men with poor sperm quality is higher than in normal men, we always applied two sets of probes to detect different chromosomes for the differentiation of haploid or diploid nuclei. We usually examined more than two slides and counted 20 to 50 cells for the positive findings of the haploid cells. The slides were screened by a technician and finally checked by us.

Statistical Analysis

The median values between the groups were compared with the *t* test. The results for the comparisons between the groups are presented as two-sided *P* values, with 95% confidence intervals. $P < 0.05$ was judged as statistically significant.

RESULTS

In this study of 37 patients with NOA, 14 patients (37.8%) had known causative factors before the diagnostic testicular biopsy. The incidence of chromosomal abnormalities (21.6%) and gene deletions in the Y chromosome (10.8%) was higher in this series than in reported cases of NOA.[6] Two patients (5.4%) had a remarkable varicocele in the

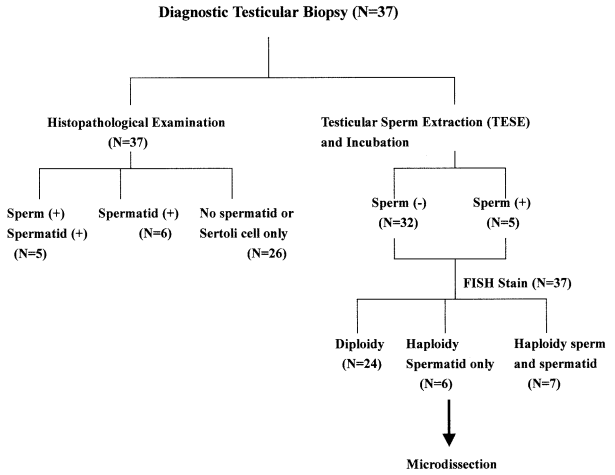

FIGURE 1. *Screening protocol and result of the testicular tissue examinations for the predictive diagnosis of spermatogenesis.*

left scrotum. A discrepancy in size between the two testes, with the left testis definitely smaller than the right, also existed. On physical examinations, the average size of the testis in these 37 patients was generally smaller (at less than 2 cm on the long axis) than normal. An elevated FSH value has been recognized as a sign of testicular atrophy on the hormonal assay.[9] It was detected in 11 of our patients when we set our standard FSH level at less than 15 mIU/mL for the adult male with normal fertilization.

A total of 37 patients underwent diagnostic testicular biopsy for sperm extraction, histopathologic examination, and FISH study. We did immediate testicular sperm extraction, as well as incubation, and found that motile sperm existed in 5 of 37 patients during the incubation period of 2 days. On histopathologic examination, 5 cases had focal spermatogenesis with sperm present in some seminiferous tubules, 19 had Sertoli cell only, and another 7 cases with maturation arrest had spermatocytes and spermatogonia only. The other 6 cases were diagnosed as hypospermatogenesis with round spermatids but no spermatozoa identified in the sections. Additional FISH stain examination was then done to look for any evidence of haploid cell existence. Twenty-four cases only had diploid cells on the slide with FISH staining. Seven cases definitely had haploid sperm, and in the other 6 cases, only haploid, round spermatid cells could be detected (Figs. 1 and 2).

When we correlated the findings between the histopathologic examination and the diagnosis with FISH staining, 8 (73%) of 11 cases that were found to have spermatids on the histopathologic slides also proved to have haploid cells with FISH staining. On the other hand, 21 (81%) of 26 cases in which no spermatids were found on the histopathologic slides also had only diploid cells with

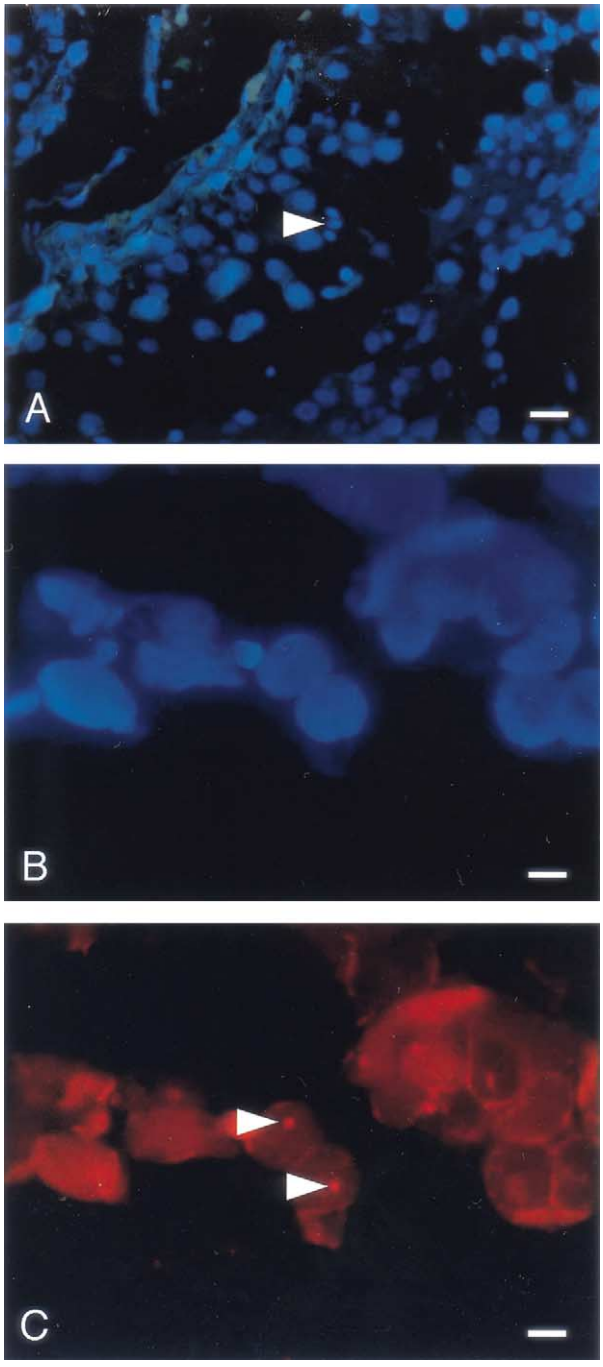

FIGURE 2. *(A,B) DAPI staining of testicular tissue with spermatogenesis. White arrowhead indicates nucleus of testicular sperm. (A) Lower magnification; scale bar equal to 25 μm. (B) Higher magnification; scale bar equal to 10 μm. (C) FISH staining of testicular tissue with spermatogenesis. White arrowhead indicates labeling of CEP Y SpectrumOrange probe in nuclei of haploid spermatids. Scale bar indicates 10 μm. (B, C) Same area of same section at same magnification.*

FISH staining. If we analyzed the predictive value of our FISH technique, 8 (61.5%) of 13 cases with haploid cells were found to have spermatids with histopathologic examination. It seems more accurate in negative prediction when we noted that no

TABLE I. *Correlation of results between histopathologic findings of testicular biopsy and FISH staining*

	Histopathologic Findings		
FISH Stain	Round Spermatid or Sperm	No Spermatid or Sperm	Total
Haploidy	8	5	13
Diploidy	3	21	24
Total	11	26	37

KEY: FISH = fluorescence in situ hybridization.
Sensitivity 73% (8 of 11); specificity 81% (21 of 26); positive predictive value 61.5% (8 of 13); negative predictive value 87.5% (21 of 24).

spermatids could be detected on the pathologic slides for 21 (87.5%) of 24 cases with diploid cells only on FISH staining (Table I).

Additional confirmation of the strong correlation between the histopathologic diagnosis and FISH staining was clearly demonstrated by analysis of the result of FISH staining according to the detailed examinations concerning the degree of spermatogenesis. In patients with focal spermatogenesis found on the histopathologic examinations, sperm could either be extracted in the wet preparation or be detected with FISH staining; in patients with hypospermatogenesis, sperm could only be found with FISH staining. When the spermatogenesis of the testicular histopathologic specimen became worse, haploid sperm did not appear with FISH staining; however, haploid spermatid cells were still present in some cases with a histopathologic diagnosis of maturation arrest and Sertoli cell only syndrome (Table II).

To establish the data of spermatogenetic function, we measured testicular size, determined serum FSH, and calculated the width and thickness of the seminiferous tubules during sperm extraction in all 37 patients. When we divided the patients into haploidy (n = 13) and diploidy (n = 24) groups according to the results of the FISH stain analysis, some correlations with other parameters of spermatogenesis could be identified. In the haploidy group, testicular size was larger, the serum FSH was lower, and the average thickness of the seminiferous tubules was less than those values of the diploidy group, with statistical significance. No relation was found between the detection of haploid cells by FISH staining and the diameter of the seminiferous tubules measured with testicular sperm extraction (Table III).

COMMENT

With recent advances in ICSI for the treatment of NOA, successful sperm or round spermatid[10] retrieval and pregnancy have been reported even in patients with cryptorchism and chromosomal disorders.[11,12] However, methods to predict the existence of spermatozoa or spermatids in an atrophic testis, as well as ways to harvest spermatozoa with the microdissection technique in a small testis, remain a great challenge in the management of NOA. Conventional testicular biopsy was thought to be the final diagnosis for the existence of spermatogenesis. Actually, scant testicular sperm might not be found in the testicular section, and furthermore, round spermatids cannot be definitely differentiated in seminiferous tubules on histologic slides. Visualization of testicular spermatids correlating with the probability of successful testicular sperm extraction is consistent with many reported findings.[6,13] To date, some laboratory techniques for spermatid identification exist, such as transmission electron microscopy, flow cytometry, and computer-guided morphometric analysis; however, none of them can be practically and accurately applied in clinical diagnosis.[14–16] Some pioneer works are enabling the visualization of the testicular spermatids in histologic sections; however, they require more experience from well-trained cytopathologists.[16–18] Recently, germ cell-specific messenger RNA detected by Northern blotting or reverse transcriptase-polymerase chain reaction was used as a molecular marker for the histologic diagnosis of Sertoli cell only; however, the investigators commented that elaborated molecular screening seems to provide no immediate clinical benefit.[19] Although seminal anti-müllerian hormone was also mentioned as a noninvasive marker of persistent hypospermatogenesis in cases of NOA, it is an indirect marker for the success of testicular sperm recovery, and its relationship with spermatogenesis requires further evaluation.[20]

In this study, we used the FISH technique to identify haploid cells (testicular sperm and round spermatids) as a screening procedure for the existence of spermatogenesis. Our results demonstrated a strong correlation between FISH and conventional histopathologic diagnosis (Table I; sensitivity 73%, specificity 81%, positive predictive value 61.5%, and negative predictive value 87.5%). In 5 cases, testicular spermatids were detected by FISH but not identified by histopathologic examination. In another 3 cases, no haploid cells were detected by FISH; however, testicular spermatids were present on the histopathologic sections. They may be due to only some rare foci existing in the seminiferous tubules that present with active spermatogenesis and because the production of spermatids was so low in these poorly functioning gonads.[21,22] The existence of scant spermatogenesis could only partly be diagnosed on conventional histopathologic slides, as well as be detected by the FISH technique with the presence of haploid cells. Recently, we divided the histopathologic diagnosis

TABLE II. *Variable results of FISH staining as related to testicular histopathologic findings with decreasing spermatogenesis*

Testicular Histopathologic Findings	TESE Sperm (+)	FISH Sperm (+)	FISH Spermatid (+)	FISH Spermatid (−)
Focal spermatogenesis (n = 5)	5	5	5	0
Hypospermatogenesis (n = 6)	0	2	3	3
Maturation arrest (n = 7)	0	0	3	4
Sertoli cell only (n = 19)	0	0	2	17

KEY: FISH = fluorescence in situ hybridization; TESE = testicular sperm extraction.

TABLE III. *Results of FISH staining correlated with other parameters of spermatogenesis in 37 patients with nonobstructive azoospermia*

Parameters of Testicular Failure (Range)	FISH Results		*P* Value
	Haploidy (n = 13)	Diploidy (n = 24)	
Testicular size (cm)	1.7 (0.8–2.2)	1.2 (0.5–2.5)	0.023*
Serum FSH (mIU/mL)	17 (2–44.2)	29.22 (11.5–71.5)	0.017*
Tubular diameter (μm)	168 (125–190)	143.6 (75–200)	0.094
Tubular thickness (μm)	12.1 (8.5–17)	20.9 (7–27)	0.01*

KEY: FISH = fluorescence in situ hybridization.
** P value <0.05.*

of testicular biopsy for patients with NOA into four categories in accordance with the degree of spermatogenesis. The results of FISH staining in this study were very compatible with this histopathologic classification. The haploid sperm could only be found in the testicular section with hypospermatogenesis, and the haploid spermatid cells could be detected in any kind of spermatogenetic defect in the histopathologic pictures. The percentage of positive findings of haploid spermatid cells by FISH staining was significantly reduced in parallel to decreasing spermatogenesis in the histopathologic diagnosis (Table II). On the basis of the good correlation of these two examinations, we confirmed the diagnosis of spermatogenesis by both methods for patients with NOA. Routine preparation and staining for FISH examination of some testicular sections will allow the prediction of spermatogenesis to be more reliable and will be of benefit for a patient's decision concerning further procedures in fertility counseling.

The accuracy of the FISH technique could also be validated by the correlation with other parameters examined in this study. Thirteen patients found to have haploid cells on FISH staining also had a larger testicular size and lower FSH level than did the other 24 patients in whom only diploid cells were found. A larger testicular size and lower FSH level were the favorable signs of possible spermatogenesis in patients with NOA. They correlated with the existence of haploid cells that indicated meiosis of diploid spermatogonium did occur, and possibly, viable sperm could be detected in the process of spermatogenesis. Clinically, details of the seminiferous tubule can be observed under inverted microscopy. We measured the thickness and width of the tubules in every patient. The mean of tubule thickness was significantly higher in patients with only diploid cells detected by FISH. This may have been due to the sclerotic change of the seminiferous tubules. On the other hand, the mean of the tubule width showed no significant difference in our patients regardless of whether haploid cells were present by FISH staining. However, seminiferous tubules containing many developing germ cells, rather than Sertoli cells alone, were likely to be wider than tubules without spermatogenesis inside. The large volume of intratubular germ cells within those tubules having spermatogenesis causes those tubules to enlarge.[23] In this study, the minimal spermatogenesis of the tubules with haploid cells may not have produced a significant increase in tubule width compared with that of tubules with no spermatogenesis.

For patients with haploid cells in FISH staining, additional confirmation should be performed using the microdissection technique on testicular tissue. The microdissection technique can be scheduled before the ICSI procedure, with a possible backup of donor's sperm. It was thought to be the best way of sperm retrieval for patients with NOA.[17] In this series, 8 patients had only spermatid haploid cells found in FISH staining without any sperm found in the diagnostic testicular biopsy tissue. Three of them had decided to undergo ad-

ditional microdissection (two with following ICSI and donor sperm backup), and 1 of the 3 patients experienced successful sperm retrieval. Although we cannot predict the possible additional sperm retrieval in the patients with only spermatid haploid cells found on FISH staining, we believe it is worthwhile to provide confirmatory and even further information other than that found by histopathologic examination with only extra tiny pieces of tissue for FISH staining. The FISH assay became our routine laboratory examination; even now we use the technique of one session diagnosis with microdissection for the seminiferous tubules of testes. The additional results of FISH staining are beneficial in continuing counseling, especially of couples who failed in the ICSI cycles. The information of haploid cells on FISH stain may also suggest a greater chance for further ICSI technology such as spermatid injection or fusion with donor sperm backup.

CONCLUSIONS

The results of this study suggest that haploid cells detected by FISH as a measurement of germ cell status are a reliable predictive factor for evaluating spermatogenesis in a diagnostic testicular biopsy. It also provides evidence of spermatogenesis that can be used to decide whether to proceed further with sperm retrieval by the microdissection technique. We expect that FISH staining of testicular sections can be widely used clinically as an informative reference and may improve the testicular spermatozoa recovery rate before the ICSI procedure.

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