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Prediction of Posttreatment Spermatogenesis in Patients With Testicular Cancer by Flow Cytometric Sperm Chromatin Structure Assay

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The hypothesis to be tested was that abnormal sperm chromatin structure is related to disturbed spermatogenesis in patients with testicular cancer. After orchiectomy but before further treatment ("pretreatment"), semen samples from 39 patients with testicular cancer were analyzed for sperm concentration by light microscopy and by the sperm chromatin structure assay (SCSA). In 28 patients assessment of sperm concentration was repeated 12–26 months after orchiectomy ("posttreatment"). The pretreatment SCSA results for the patients were compared to those from 18 healthy semen donors and assessed for correlation with the patients' posttreatment sperm concentration. Twenty-three patients displayed an abnormal chromatin structure in their pretreatment sample. For the nine evaluable patients on the surveillance program, the pretreatment SCSA results were not correlated with the posttreatment concentration. The results from 19 evaluable patients undergoing cytotoxic treatment (radiotherapy, 13; chemotherapy, 6) indicate that posttreatment recovery of spermatogenesis (recovery in 4 of 5 patients) is observed more often in patients with a normal pretreatment chromatin structure than in those with abnormal SCSA values before treatment (recovery in 2 of 14 patients; $P = 0.02$). The results of SCSA display sperm characteristics beyond those of light microscopically assessed sperm concentration. Pretreatment SCSA results might help clinicians to identify those testicular cancer patients with a high risk of long-lasting posttreatment disturbance of spermatogenesis. Cytometry 30:192–196, 1997. © 1997 Wiley-Liss, Inc.

Key terms: testicular cancer; spermatogenesis; sperm chromatin structure assay; SCSA

Testicular cancer is a curable malignancy in young men (11); unilateral orchiectomy is the primary treatment. Dependent on the tumor type, the extent of the disease, and the responsible hospital's treatment philosophy, the patient is either included into a surveillance protocol or undergoes surgery, abdominal radiotherapy, and/or cis-platin-based chemotherapy.

According to the Norwegian Radium Hospital's (NRH) observations, 15% of patients record infertility prior to the diagnosis of testicular cancer (Fosså, unpublished data). About 50% of patients with testicular cancer display considerable disturbances of their spermatogenesis when assessed 2–3 weeks after orchiectomy, before any further treatment (1,10). Studies in patients on surveillance protocols, however, have revealed that spermatogenesis improves in at least one-half of patients, if no cytotoxic treatment is given after orchiectomy (1).

Not unexpectedly, the majority of young patients with newly diagnosed testicular cancer are concerned about future fertility and want to be informed about the different treatment modalities' influence on spermatogenesis (12). Previous studies have shown that the initial serum follicle-stimulating hormone (FSH) and the age of the patient, but not the pretreatment sperm concentration or the type of standard treatment modality, are related to spermatogenesis 2–3 years after standard treatment (1). From univariate analysis, another important risk factor for impaired recovery was the finding of ≥10% noncondensed haploid cells in the ejaculate analyzed 2–3 weeks after orchiectomy but before further treatment (13).

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Evenson and Melamed (6) have recorded that the degree and the amount of denatured chromatin of sperm cells that have been exposed to heat or acid represent a measure of subfertility in general. These parameters were measured by flow cytometry (FCM) using the sperm chromatin structure assay (SCSA). In bulls, the percentage of sperm cells with abnormal chromatin structure was correlated with the animals' fertility index (3). Furthermore, 14 patients with testicular cancer, assessed after orchiectomy but before further treatment (7), displayed considerable variability in the SCSA results, most often revealing an increased percentage of sperm cells with abnormal chromatin structure. Given this clinical background, it was deemed reasonable to perform SCSA in a series of patients with testicular cancer to assess whether SCSA parameters of semen samples obtained after orchiectomy but before further treatment predict posttreatment sperm cell production mirrored by the demonstration of sperm cells in the seminal fluid obtained after therapy.

MATERIALS AND METHODS

Patients and Treatment

Each year, about 80 patients with newly diagnosed testicular cancer are referred to the NRH for further treatment 2–3 weeks after their diagnostic unilateral orchiectomy. Before further treatment is started (''pretreatment''), patients are asked to participate in a research protocol that aims to evaluate spermatogenesis before and 1 and 2–3 years after treatment (''posttreatment''). About 80% of all patients give their informed consent to be included in this research protocol. Eligible patients are offered sperm cell cryopreservation if it is desired.

At the NRH, the principal postorchiectomy treatment of testicular cancer patients (''further treatment'') is as follows: seminoma, no metastases: abdominal radiotherapy 30 Gy; metastases: cisplatin-based chemotherapy (four cycles); non-seminoma, no metastases: surveillance or two cycles of adjuvant chemotherapy; metastases: four to six cycles of cisplatin-based chemotherapy dependent on the extent of the disease.

It has previously been shown that four cycles of cisplatin-based chemotherapy and abdominal radiotherapy (30 Gy) have a similar impact on the 1- and 2/3-year spermatogenesis in testicular cancer patients (1). Based on this experience and in the view of the limited number of patients in the present series, only two principal subgroups were formed among 28 patients in whom posttreatment light microscopic follow-up sperm counts were available 1–2 years after standard treatment (surveillance, abdominal radiotherapy, or four cycles cisplatin-based chemotherapy): surveillance, 9 patients; standard cytotoxic treatment, 19 patients (radiotherapy, 13; chemotherapy, 6) (Table 1).

Control Group

Ejaculate samples from healthy voluntary semen donors from the infertility clinic served as the control group for the SCSA results. These males displayed normal values for sperm concentration, morphology and motility.

Table 1
Patient Characteristics

Characteristic	Pretreatment	Posttreatment	
		Surveillance	Cytotoxic therapy
Number of patients	39	9	19[a]
Median age in years (range)	32 (20–47)	29 (22–32)	34 (21–47)
Seminoma/non-seminoma	18/21	0	13/6
Metastatic disease	7	0	6
Radiotherapy	17	0	13
Median interval between pretreatment and follow-up sperm analyses in months (range)		19 (13–26)	15 (12–26)
Pretreatment fertility			
Paternity recorded	24	5	11
Infertility problems	2	0	2
History of maldescent	5	0	4

[a]Radiotherapy, 13 patients; chemotherapy, 6 patients.

Light microscopic sperm cell analysis was performed in the ejaculate obtained from 41 patients after at least 3 days of sexual abstinence in connection with the initial referral to the NRH. The sperm concentration per milliliter and the sperm cell motility were assessed at a single laboratory according to the WHO recommendations (14). In view of the close relation between sperm concentration and sperm cell motility in the present study, only the sperm concentration was assessed as the principal parameter of light microscopic sperm analysis. The following definitions were applied for the sperm concentration ($\times 10^6$/ml), the categorization based on the light microscopic observations: 0, azoospermia; 1–10, oligospermia; >10, normospermia. If possible, the light microscopic sperm cell count was repeated at least once, 1–2 years after discontinuation of all treatment.

Flow cytometric DNA content analyses were performed for the pretreatment ejaculates as previously described (13). Two samples containing diploid cells only were excluded from further analysis.

Sperm Chromatin Structure Assay

SCSA was done only for the pretreatment ejaculates. Thirty-nine semen samples from patients and 28 control samples were handled in the same manner upon arrival in the laboratory. Aliquots of 100 µl of semen were diluted tenfold with TNE buffer (0.15 M NaCl, 0.01 Tris, 0.001 M EDTA, pH 7.4) containing 10% glycerol and frozen at −80°C until flow cytometric analysis.

The SCSA and the two-step acridine orange staining method it employs have been described extensively (5). Briefly, individual semen samples were thawed in a 37°C

water bath, placed on ice, and diluted to a final concentration of approximately 2×10^6 cells/ml with TNE buffer. To 200 µl of semen was added 400 µl of low-pH detergent solution (0.15 M NaCl, 0.08 N HCl, 0.1% Triton X-100, pH 1.4) for 30 s, and then 1.2 ml of acridine orange staining solution (6 µg/ml acridine orange [AO] in 0.2 M Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate, pH 6.0) was added and the samples were analyzed by flow cytometry. AO is a metachromatic fluorochrome, which is used to stain differentially double- vs. single-stranded nucleic acids (11). The SCSA is based on the principle that abnormal sperm chromatin is more susceptible to physical induction of partial DNA denaturation in situ by acid or heat (4,9) than normal sperm DNA. AO intercalates into native double-stranded DNA and fluoresces green when excited in blue light, whereas AO associated with single-stranded DNA (or RNA if present) fluoresces red. The percentage of cells with DNA denaturation can thus be quantified for each sample using this assay. The parameter is expressed as %COMP (COMP: cells outside the main population).

Samples were stained and measured sequentially on a Cytofluorograf 50H laser flow cytometer (Ortho Diagnostic Systems, Raritan, NJ; modified for use of AO by one of the authors [D.E.]) equipped with a 5 W argon ion laser tuned to 488 nm excitation wavelength. A semen sample from a normal donor was used as a reference sample for instrument calibration, and reference samples were measured periodically throughout the analysis of patient samples in order to monitor instrument stability. Five thousand cells were analyzed for each sample and the following parameters stored as list mode data: green fluorescence (emission 515–530 nm), red fluorescence (emission >610 nm), and forward light scatter.

Statistical Analysis

The percentage of cells within and outside the main population (%COMP) on the green vs. red fluorescence cytogram was calculated by setting a software gate around all cells (100%) and another gate around the cells outside the main population. Normal sperm samples were used to determine (set up) these regions, and patient samples were then analyzed for %COMP using the same region settings (Fig. 1).

The normal range of %COMP was defined as the mean value %COMP ±2 standard deviation (S.D.) observed in the control samples. Values above this range were considered to characterize an ''abnormal'' semen sample. Standard tests for statistical significance were applied. (Student's *t*-test, Fisher's exact probability test, Spearman rank test). A *P* value of <0.05 was considered to be statistically significant.

RESULTS

Control Group

The mean value of %COMP in the control group was 9.4, and the upper limit of the normal range was 16.

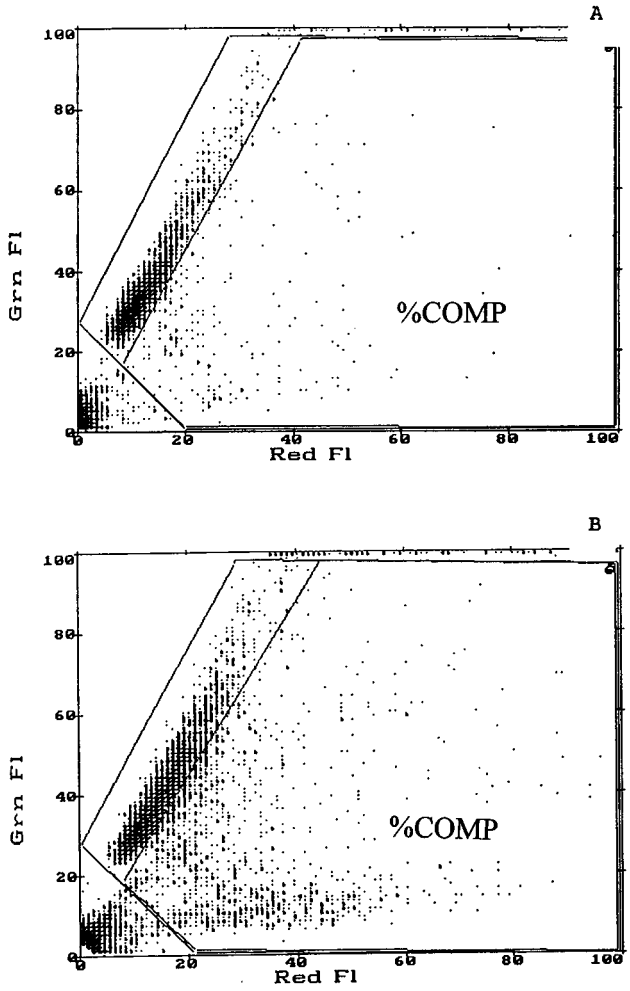

FIG. 1. Flow cytometric cytograms of two-parameter (green/dsDNA vs. red/ssDNA fluorescence signal) distribution of sperm from a normal human control (**A**) and a patient with testicular cancer (**B**). The box marked %COMP shows the cells outside the main population with an abnormal chromatin structure.

Patients

One of the thirty-nine patients with haploid cells in the pretreatment ejaculate was azoospermic by light microscopy after orchiectomy before further treatment (surveillance, radiotherapy, or chemotherapy). Eleven patients displayed oligospermia, whereas 27 patients presented with normospermia. There was no statistically significant correlation between the pretreatment sperm concentration and the individual values of %COMP ($r = -0.310$, $P = 0.058$).

A total of 23 patients had pretreatment semen samples with an abnormal sperm chromatin structure (Table 2). The values of pretreatment sperm concentration categorized as azoo-, oligo-, or normospermia were not significantly related to the presence of abnormal chromatin ($P = 011$). One interesting discrepancy was observed: The haploid cells in the ejaculate of an azoospermic patient as judged by light microscopy displayed a normal chromatin

Table 2
Pretreatment Sperm Concentration (Assessed by light microscopy) and Chromatin Structure (SCSA)

SCSA	Azoospermia (0)[a]	Oligospermia (1–10)[a]	Normospermia (>10)[a]	Total
Abnormal[b]	0	9	16	25
Normal	1	2	11	14
Total	1	11	27	39

[a]$\times 10^6$/ml.
[b]Elevated value of %COMP.

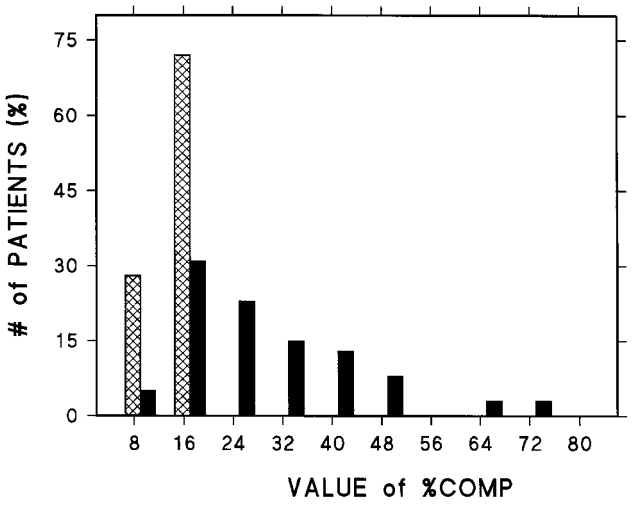

FIG. 2. Distribution of individual %COMP values. Cross-hatched bars, 18 samples from healthy semen donors; solid bars, 39 semen samples from patients with testicular cancer after unilateral orchiectomy before further treatment.

structure assessed by SCSA. This patient was not truly azoospermic, but rather highly oligo-azoospermic. This finding was also in accordance with the fertility outcome during this patient's surveillance period. He fathered a child 15 months after the orchiectomy.

The patients' mean value of %COMP (±SD) was 24.8 (±14.6), with significant differences compared to the respective mean values from the control group ($P < 0.001$). The distribution of %COMP in the 39 patients was significantly different from that of the 18 samples from the controls (Fig. 2). Fourteen of the thirty-nine patients had %COMP values above the normal range.

Tables 3 and 4 show the pre- and posttreatment sperm concentrations in the 28 evaluable patients and pretreatment SCSA separately for the two principal therapeutic subgroups. The results from SCSA did not predict the posttreatment sperm concentration in the 9 surveillance patients. Only 2 of the 14 patients with abnormal chromatin structure before cytostatic treatment regained normospermia after treatment ($>10 \times 10^6$/ml sperm cells), whereas posttreatment semen samples from 4 of 5 patients with initially normal SCSA values after treatment revealed a sperm concentration of $>10 \times 10^6$/ml ($P = 0.02$).

Table 3
Pre- and Posttreatment Sperm Analysis in Nine Patients on Surveillance

Patient no.	Interval (months)	%COMP[a]	Sperm concentration ($\times 10^6$/ml) Pretreatment	Posttreatment
1183	26	28	11	13
1247	24	5	46	67
1250	25	25	7	24
1254	15	20	30	56
1257	23	24	50	66
1275	13	17	0	3
1280	15	21	90	13
1288	13	40	1	0
1291	17	14	5	0

[a]Upper limit of the normal range for %COMP is 16.

Table 4
Pretreatment and Follow-Up Sperm Analysis in 19 Patients Receiving Standard Cytotoxic Treatment

Patient no.	Interval (months)	%COMP[a]	Sperm concentration ($\times 10^6$/ml) Pretreatment	Posttreatment
A. Abdominal radiotherapy (30 Gy)				
1188	26	15	15	21
1220	12	10	61	34
1240	26	35	1	10
1242	12	68	1	3
1244	25	43	78	10
1248	23	10	34	22
1259	13	11	23	20
1268	17	19	15	6
1274	15	43	3	2
1290	14	16	5	5
1293	12	46	76	45
1298	12	19	9	2
1299	15	27	16	4
B. Cisplatin-based standard chemotherapy				
775	23	38	36	1
1210	23	21	19	0
1238	20	25	37	18
1263	14	29	25	0
1266	14	36	2	3
1271	12	62	28	0

[a]Upper limit of the normal range for %COMP is 16.

DISCUSSION

As was described previously (13) and confirmed in the present study, haploid cells can be observed in semen samples from light microscopically azoospermic patients as judged by light microscopy. This finding indicates an ongoing, though probably highly reduced, spermatogenesis. Patients with azoospermia by light microscopy may prove to be oligo-azoospermic if their semen sample is analyzed by flow cytometry. Given the background of modern microinjection techniques for in vitro fertilization, this finding might be of particular significance for patients who are trying to father a child by assisted fertilization, especially if the sperm cells display normal chromatin structure, as was the case in one of our patients.

This investigation focused primarily on the question of whether SCSA predicted ongoing spermatogenesis ≥1

year after treatment discontinuation or not. This is one of the justifications for using sperm concentration as the only parameter in this limited study, neglecting possible maturation disturbances that would be mirrored by decreased motility and disturbed morphology. Furthermore, a close relationship has been shown between low sperm concentration and decreased motility (2). Third, given today's possibilities for assisted fertilization by microinjection techniques, sperm cell motility might play a less significant role than previously in achievement of posttreatment fatherhood.

Based on the findings in the ''normal'' male population, the WHO has defined oligospermia as a sperm concentration $<20 \times 10^6$/ml. In the present study, we have used a cutoff point of 10×10^6/ml; because the majority of testicular cancer patients have permanently reduced sperm counts (mean sperm count in 585 testicular cancer patients with 1,700 observations: 27×10^6/ml, with only 40% $>20 \times 10^6$/ml; Fosså, unpublished results).

In a previous series of pretreatment serum samples from 14 patients with testicular cancer, normal SCSA parameters were found in only one case, confirming the high frequency of disturbed spermatogenesis in these patients (9). The present SCSA study revealed 23 of 39 patients with abnormal sperm cell chromatin. Fourteen of these twenty-three samples were normospermic. On the other hand, normal chromatin structure was observed in the sperm cells of 3 of 12 azo- or oligospermic patients. SCSA thus seems to provide clinicians with information beyond that achieved by light microscopic sperm cell analysis.

In a prospective study of healthy males, Evenson et al. have shown that SCSA parameters remain relatively unchanged over a period of several months (9). At present, we have not examined the patients' posttreatment semen samples by SCSA. This would be of interest, especially for patients on the surveillance program. Light microscopic results of sperm analysis have revealed considerable improvement of spermatogenesis in these patients 1–3 years after orchiectomy. Future studies should assess whether this recovery is mirrored by normalization of SCSA parameters.

The SCSA reveals chromatin structure abnormalities, which may be due, for example, to disturbed cell maturation caused by toxicants such as cytotoxic drugs (8). At present, no definitive explanation can be given for the high incidence of abnormal SCSA values in the pretreatment semen samples of our patients. Influences that may be responsible for the disturbed spermatogenesis in testicular cancer patients before cytostatic treatment and the high frequency of abnormal chromatin structure are stress factors, abnormal hormone production (estrogens, human chorionic gonadotropin), or other factors linked to the development of testicular atrophy and dysplasia, including premalignant changes.

Because of the small number of patients included in this preliminary report and the lack of long-term follow-up, the clinical significance of our results cannot be definitively determined. In particular, the limited size of the present series representing patients with various kinds of treatment prohibits the performance of a multivariate analysis. Such analysis would have to include the most important and easily available clinical parameters such as patient age and initial serum FSH level (1). Our observations, however, indicate that posttreatment recovery of spermatogenesis seems to be delayed in cytotoxically treated testicular cancer patients who display an abnormal chromatin structure in the pretreatment semen sample. In patients with normal SCSA values after orchiectomy but before further treatment, a more rapid and complete recovery of sperm cell production seems to take place after standard chemotherapy or radiotherapy. If this hypothesis can be confirmed in future studies, clinicians would have a method to identify those patients who should be offered pretreatment sperm cell cryopreservation and whose treatment should be as minimally intensive as possible in order to preserve spermatogenesis.

In conclusion, the results of SCSA display sperm cell characteristics beyond those of sperm concentration assessed by light microscopy. Testicular cancer patients with abnormal chromatin structure after orchiectomy but before further treatment seem to be more vulnerable to cytotoxic treatment than similar patients with normal SCSA parameters.

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