Rapid Communication

Carcinoma In Situ of the Testis

Detection of Malignant Germ Cells in Seminal Fluid by Means of In Situ Hybridization

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Testicular germ cell tumors can be prevented if the neoplasia is diagnosed at the stage of carcinoma in situ (CIS). Hyperdiploid DNA content is one of the markers of CIS germ cells. We developed a noninvasive procedure for detection of CIS of the testis by means of a nonradioactive in situ hybridization assay with a probe for chromosome 1. Seminal cytospin smears from 2 men with isolated CIS changes, from 6 men in whom CIS was combined with a tumor, and from 16 control men without evidence of testicular neoplasia were tested. Ejaculates from men with CIS contained on average 2.6% hyperdiploid cells, whereas the corresponding percentage in the smears from controls was 0.2 (%P = 0.009). In a blind study we identified samples from both patients with isolated CIS changes and from 3 of the 6 men in whom CIS was accompanied by a tumor, based on the percentage of the hyperdiploid cells. No false-positive results were obtained. Thus this study confirmed findings of previous immunocytochemical and flow cytometric studies that indicated exfoliation of CIS germ cells into seminal fluid. For detection of aneuploid cells in semen, in situ hybridization may be a more sensitive technique than flow cytometry. Applied on seminal samples, in situ hybridization may become a valuable and fast tool for diagnosis of CIS and thereby for prevention of testicular cancer. Am J Pathol 1990, 136:497-502

In the Western World, testicular cancer is the most common neoplastic disease of young men. Despite the recent improvements in the outcome of this disease, it may still be a life-threatening condition for some of the patients. In addition, besides the large psychologic stress, chemotherapy, extensive radiotherapy, and lymphadenectomy used for treatment of these men pose serious side effects and hazards to the future quality of life for these young men. Efforts should therefore be made to diagnose testicular neoplasia at the preinvasive stage of carcinoma in situ (CIS).1,2 CIS of the testis precedes the development of all testicular germ cell tumors, except the spermatocytic seminoma.3,4 CIS can easily be cured by simple orchidectomy or localized irradiation without any adjuvant therapy.5 However, carcinoma in situ of the testis is an asymptomatic condition. Presently the diagnosis can only be established by means of surgical biopsy. Establishment of a reliable method for detection of CIS cells in seminal fluid is desirable because this would enable us to screen the male population and thereby start a campaign for prevention of invasive cancer of the testis.

Recently, exfoliation of CIS germ cells into seminal fluid has been demonstrated in men suffering from early testic-
ular neoplasia.® The CIS germ cells were identified by means of DNA flow cytometry, due to their hyperdiploid, aneuploid DNA content,® and by immunocytochemical methods with the monoclonal antibody M2A.® However, the sensitivity and specificity of these two methods are not yet sufficiently high for use in routine screening for CIS of the testis.

Using DNA flow cytometry, aneuploid cells were detected in ejaculates from only four of eight men with isolated CIS,® indicating that in some men with early testicular neoplasia the number of aneuploid cells in seminal fluid is less than the detection level of flow cytometry. In situ hybridization can be used to demonstrate numerical chromosome aberrations in interphase cells.® As a result it can be applied on routine paraffin sections and cell smears. Recently, in situ hybridization was shown to be a suitable method for detection of low-frequency aneuploid cells, even in cases in which such cells were not disclosed by flow cytometry.® The latter was achieved by means of in situ hybridization, an increased copy number of chromosome 1 has been demonstrated in cells from testicular germ cell tumors® and it was shown that this method can be used to determine the copy number of chromosome 1 in cells exfoliated in semen of infertile men.® Therefore we applied this technique to a retrospectively collected material of seminal smears to investigate whether in situ hybridization is a suitable method for identification of CIS germ cells in ejaculates.

Materials and Methods

We studied seminal smears from two men (ages 23 and 27 years) with isolated CIS of the testis. The diagnosis of CIS was established by testicular biopsy. The two patients had the biopsies performed as a screening for CIS due to an extragonadal germ cell tumor and an unilateral cancer of the testis, respectively. In addition, ejaculates from six patients (age, 26 to 37 years) who had CIS associated with invasive cancer of the testis (four seminomas and two nonseminomas) were tested. Controls were 15 men (age, 20 to 40 years) without any evidence of testicular neoplasia who were attending our infertility clinic and one man (age, 29 years) who 3 years earlier was treated for CIS by localized irradiation® (Table 1). One sample was obtained from each patient and control. The sperm density in samples from patients with CIS and from controls is shown in Table 1.

Processing of Smears

The smears were made from fresh ejaculates on uncoated glass slides by cytocentrifugation as previously described® and stored at −20°C for up to 6 months. After thawing the cells were fixed in 4% formaldehyde (15 minutes, 4°C). Fixation was followed by digestion with porcine pepsin (Sigma Chemical Co., St Louis, MO) in 0.01 N HCl (5 μg/ml, 12 minutes, 37°C), dehydration in alcohol and air drying.

DNA Probe

We used the DNA probe specific for chromosome 1 previously described by Cooke and Hindley.® It recognizes the (per)centromeric region in which several 10,000 copies of a 1.77-kb sequence are present in a tandem repeat. The probe was biotinylated using Bio-11-dUTP in a nick translation reaction. By variation of the DNA concentration during the polymerase reaction the fragment length of the labeled probes was 200 to 400 bases.

In Situ Hybridization

The hybridization procedure was performed as described before.® The probe was diluted to 1 ng/μl in a buffer containing 60% formamide (v/v), 2X SSC of pH 7.0 (=0.3 mol NaCl, 30 mmol Na-citrate), 10% dextran sulfate (w/v), and salmon sperm DNA (1 μg/μl) as carrier DNA was added to dry smears (5 μl per slide) and was covered by a coverslip. The probe was denaturated together with the target DNAs for 3 minutes at 70°C and hybridization was performed overnight at 37°C. Posthybridization washings were done twice in 60% formamide, 2X SSC of pH 7.0 for 5 minutes at 42°C. The probe was detected immunocytochemically by subsequent incubation with monoclonal mouse antibiotin antibody (DAKO, Copenhagen, Denmark) diluted 1:100 followed by peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:80. The color reaction was developed in 3,3′-diaminobenzidine-H2O2 and the smears were counterstained with hematoxylin. By this technique each copy of chromosome 1 is visualized as a distinct brownish spot in the nucleus of the cell. Haploid cell thus appear with one spot (Figure 1a) and a diploid cell shows two spots for chromosome 1 (Figure 1b).

Light Microscopic Examination

All smears were evaluated blindly by the same investigator, who did not know the origins of individual samples. Cells for analysis were randomly selected. For each smear an attempt was made to score 200 cells if a sufficient number of nuclei with well-preserved morphology and positive in situ hybridization reaction was present in
Table 1. Clinical Data and Results of In Situ Hybridization for Chromosome 1 in Patients with CIS as Compared to Controls

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Age (years)</th>
<th>Sperm density (x10^6/μl)</th>
<th>Total number of cells counted</th>
<th>Percentage of cells with</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIS, other testis previously removed because of cancer</td>
<td>27</td>
<td>0</td>
<td>129</td>
<td>26.3</td>
</tr>
<tr>
<td>2</td>
<td>CIS*</td>
<td>23</td>
<td>8</td>
<td>123</td>
<td>37.4</td>
</tr>
<tr>
<td>3</td>
<td>CIS + seminoma*</td>
<td>34</td>
<td>39</td>
<td>60</td>
<td>71.7</td>
</tr>
<tr>
<td>4</td>
<td>CIS + seminoma*</td>
<td>37</td>
<td>11</td>
<td>116</td>
<td>69.1</td>
</tr>
<tr>
<td>5</td>
<td>CIS + nonseminoma*</td>
<td>29</td>
<td>4</td>
<td>57</td>
<td>75.4</td>
</tr>
<tr>
<td>6</td>
<td>CIS + nonseminoma*</td>
<td>26</td>
<td>14</td>
<td>121</td>
<td>61.2</td>
</tr>
<tr>
<td>7</td>
<td>CIS + seminoma*</td>
<td>26</td>
<td>13</td>
<td>196</td>
<td>45.9</td>
</tr>
<tr>
<td>8</td>
<td>CIS + seminoma*</td>
<td>32</td>
<td>0</td>
<td>180</td>
<td>20.0</td>
</tr>
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</table>

Controls (n = 12)

<table>
<thead>
<tr>
<th>mean range</th>
<th>31-30</th>
<th>22-40</th>
<th>0-127</th>
<th>76-200</th>
<th>30.0-68.1</th>
<th>31.5-70.0</th>
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<tbody>
<tr>
<td>1 spot</td>
<td>46.8</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>2 spots</td>
<td>54.0</td>
<td>2.8</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>≥3 spots</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Biopsy from the contralateral testis showed no sign of malignancy.

the preparation. In each smear the numbers of cells with 1, 2, 3, 4, or more spots (Figure 1), respectively, were counted and the percentage of hyperdiploid cells (3 or more spots) was estimated. The criteria for counting spots were previously described. Thus small spots originating from so called minor binding sites were not counted.

A pilot study, prior to the present study, indicated that smears from controls contain no more than 1 to 2 cells with 3 or more spots for chromosome 1 and the percentage of these cells in control smears is less than 1. Therefore, to imitate a screening procedure, an arbitrary threshold at the level of 1% was defined. Only samples in which the blind evaluation revealed more than 1% hyperdiploid cells were designated as originating from patients with testicular neoplasia.

Statistical Analysis

For statistical evaluation two-sample t-test and Fisher's exact test were used.

Results

Successful preparations were obtained for smears of all 8 patients and 12 of the 16 controls. Smears from four of the controls were excluded because the number of cells suitable for quantification was too low (less than 30).

The mean number of nuclei scored was 123 in the smears from men with CIS and 146 in the controls. The number of cells counted and distribution of cells with 1, 2, and ≥3 spots, respectively, is shown in Table 1.

With the preparation procedure applied in this study no hybridization spots were found in spermatozoa (Figure 1a), which was probably due to the very condensed DNA. Also the uroepithelial cells, recognized by their large cytoplasm-to-nucleus ratio, were negative. In these two cell types, hybridization patterns showing a haploid and diploid DNA content, respectively, could be achieved by increasing the concentration of papain. However, at these concentrations of the proteolytic enzyme, most of the CIS cells were lost from the glass slides or the morphology of the cells was almost completely destroyed.

Clear examples of hyperdiploid cells were seen in smears from both men with isolated CIS changes and in three of the six men with CIS associated with a tumor (Figure 1 c to e), although the number of these cells was rather low (range, 2 to 10). Smears from three controls contained one cell that was scored as having three or more spots. However, in these cases cellular morphology was rather poorly preserved and it could not be excluded that these spots were originating from two or three nuclei that overlapped. No hyperdiploid cells were found in samples from the remaining controls.

The average percentage of cells with 3 or more spots was 0.2 in the controls and was significantly higher, 2.6 (two-sample t-test: P = 0.009), in the group of 8 men with CIS that was isolated or combined with invasive cancer.

In both smears from men with isolated CIS and in three of the six men with CIS combined with an invasive cancer the percentage of these cells was higher than 1% (Table 1). All smears from controls contained less than 1% of cells with three or more spots. To evaluate the potential of our method as a screening procedure, the number of samples with proportion of hyperdiploid cells exceeding 1% was compared for the group of men with CIS (n = 5)
and for the controls \((n = 0)\). The difference was statistically significant (Fisher's exact test: \(P = 0.007\)).

**Discussion**

Using the \textit{in situ} hybridization technique with a probe for chromosome 1, we demonstrated a significantly increased number of aneuploid cells in ejaculates from men with CIS of the testis as compared to controls. Although the number of these cells, even in smears from men with CIS, was rather low, it corresponded well to the number of cells reacting with the monoclonal antibody M2A, as found in a previously published immunocytochemical study. In addition, we could, blindly, identify smears from both patients with isolated CIS changes and from three of the six men in whom CIS was adjacent to a tumor, whereas the percentage of aneuploid cells did not exceed the arbitrary threshold of 1\% in smears from any of the 12 controls. Thus our study demonstrated the potential of \textit{in situ} hybridization as a screening method for CIS tests. We have not directly compared the sensitivity of \textit{in situ} hybridization to that of DNA flow cytometry in detection of CIS cells in semen. However, we believe that the former
method may be the most sensitive of these two techniques. Thus in a previous study we found that several hundred aneuploid cells must be present in the ejaculate to allow their detection. The immunocytochemical study indicated, however, that in some men with CIS the number of malignant cells shed in semen is less than 100. This number is less than the detection level of flow cytometry. Such very low numbers of CIS cells are typically found in ejaculates from men in whom the CIS is accompanied by testicular tumor. Men with CIS adjacent to a tumor are supposed to exfoliate a lower number of malignant germ cells into seminal fluid than men with isolated CIS changes. In the former group the presence of tumor implies that the volume occupied by CIS may be rather small. Unlike the CIS germ cells, which are located inside the seminiferous tubules, tumor cells are not expected to be exfoliated into semen because the tumor usually has no connection to the excurrent seminal ducts. Accordingly, using DNA flow cytometry we detected aneuploid cells in semen of some men with isolated CIS changes, but not in ejaculates from three men with CIS combined with a tumor (Giwercman and Clausen; unpublished data). In present study, using in situ hybridization, aneuploid cells were detected in samples from both men with isolated CIS changes and from three of the six men with combined CIS and a tumor.

The number of aneuploid cells found in cytospin smears corresponded well to the number of M2A-positive cells detected in a previous immunocytochemical study. However, the disadvantage of the immunocytochemical approach is presence of aberrant positive staining in smears from controls. Such unexpected positive reaction is a well-known phenomenon in immunocytochemistry applied on smears. It may, for instance, be due to presence of nonviable cells, which are also expected to be found in semen. Thus in situ hybridization may offer a higher specificity than immunocytochemistry in detection of malignant cells in semen.

Routine cytologic techniques are unsuitable for diagnosis of CIS because the morphology of seminal cells other than sperm is very poorly preserved.

We have no data on chromosome 1 ploidy in testicular tumors of the six men in whom CIS was accompanied by an invasive cancer. For reasons mentioned above, the cells of the tumor are not expected to be shed in semen. The aneuploid cells found in the seminal fluid are therefore rather cells from the CIS tissue adjacent to the tumor. Recently Waet al. using the same probe for chromosome 1, have shown an increased copy number of this chromosome as a constantly occurring phenomenon in CIS tissue.

An occasional hyperdiploid cell was also found in smears from three controls. This can be expected because nonmalignant germ cells, such as primary spermatocytes and dividing spermatogonia, may have a hyperdiploid DNA content due to meiotic and mitotic division, respectively. Cells with only one in situ hybridization spot, detected in seminal fluid, are mainly the haploid spermatids. Such cells were, however, also found in ejaculates of men with very low sperm counts or azoospermia. This might be due to maturation arrest at the stage of spermatids. The diploid cells found in semen are characterized as Sertoli cells, spermatogonias, secondary spermatocytes, cells of the lining epithelium of the excurrent ducts, or inflammatory cells.

In conclusion, this study confirmed that men with CIS exfoliate aneuploid cells into semen. Although the method will need some modifications to increase the yield of aneuploid cells, in situ hybridization with chromosome-specific probes may become a suitable method for population screening of CIS tests.

References

11. Hopman AHN, Poddigehe PJ, Smeets WGP, Moesker O, Beck JLM, Vooijs P, Ramaekers FOS: Detection of numeri-


