Fluorescence in situ hybridization analysis of matched primary tumour and lymph-node metastasis of D1 (pT2–3pN1M0) prostate cancer

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OBJECTIVE
To describe the chromosomal numerical changes present in primary prostate tumours and their matched lymph-node metastases, to identify a clonal cell migration process which could account for the metastatic behaviour.

MATERIALS AND METHODS
Twenty-eight cases of unsuspected stage D1 (pT2–3pN1M0) prostate cancer were detected among patients who had a radical prostatectomy for clinically localized prostate cancer. Fluorescence in situ hybridization (FISH), using centromeric probes to enumerate chromosomes 7, 8, 10 and 12, was used to assess numerical chromosomal changes. FISH analysis was used on isolated nuclei obtained from matched primary tumours and their lymph node metastases.

RESULTS
Of the 28 suitable cases it was possible to complete the study in 18 pairs of matched tissues; the remainder were excluded because of insufficient tissue or poor preservation of at least one of the tissues. There was cytogenetic change (aneuploidy) in 16 of the 18 primary tumours, the most common being monosomy 8, detected in 14, followed by trisomy 7, in 13 aneuploid tumours. All lymph node metastases were aneuploid by FISH. As in the primary tumours, monosomy 8 and trisomy 7 were the most common cytogenetic alterations, in 13 and 15 of the lymph node tissues. FISH analysis showed a high correlation (83%) in the cytogenetic pattern of changes between the primary tumours and their lymph node metastases. Moreover, a similar number of cells had the most common aneusomies when comparing prostate and the lymph node tissues.

CONCLUSIONS
These results show a similar pattern of cytogenetic alteration in the primary tumour and its lymph node metastasis, characterized by the frequent presence of trisomy 7 and monosomy 8, suggesting that clonal cell selection is not involved in the metastatic process.

KEYWORDS
FISH, prostate cancer, lymph node metastases, cytogenetics

INTRODUCTION
Prostate adenocarcinoma is the most common cancer in men, with ~85 000 cases diagnosed annually in the European Union, representing 13% of the malignancies in men and 9% of the mortality from cancer. An increase in the incidence in recent years may be attributed to the use of the PSA test and to the raised public awareness of the disease [1–3]. However, its optimum treatment remains controversial, part of the controversy stemming from an incomplete understanding of the natural course of the disease, as some tumours progress locally and metastasize rapidly, whereas others remain clinically latent for years. The Gleason histological grade and clinical stage roughly correlate with tumour potential to progress, some low-grade, low-stage tumours develop rapidly, whereas certain high-grade, high-stage tumours expand slowly. Moreover, many prostate adenocarcinomas are of intermediate Gleason grade and clinical stage, and have an indeterminate prognosis. It would be useful to find more accurate biological prognostic markers which could be used to differentiate between localized tumours and those with lymph node involvement.

Several studies have suggested that changes in DNA content, using flow-cytometry analysis, could provide independent prognostic information in addition to the Gleason grade and stage [4,5]. Subsequently, DNA ploidy analysis using fluorescence in situ hybridization (FISH) with centromere-specific probes, has shown greater sensitivity and specificity than flow cytometry for detecting aneuploid tumours [6–8], enabling the detection of specific chromosomal gains and losses within a tumour specimen. The technical aspects of FISH have developed, particularly in the preparation of nuclei for examination, and some studies have used FISH analysis on 5 μm thin sections of prostate tissue [8,9], with others using desegregated isolated intact nuclei [10,11], each technique having particular applications and advantages.

FISH analyses have already been used on samples from patients with pathological stages pT2N0M0 and pT3N0M0 [12,13], but stage D1 (pT2–3pN1M0) has not been widely evaluated [14–16]. To our knowledge only Gburek et al. [17] have used FISH analysis on thin sections of prostate adenocarcinoma primary tumours and their matched lymph-node metastases, describing an abnormal chromosome pattern after studying 12 matched cases.

We used FISH analysis on isolated nuclei, obtained from microdissection of paraffin-
embedded blocks which contained primary tumoral prostatic tissue, and their matched lymph node metastases, using a set of four centromeric probes for chromosomes 7, 8, 10 and 12, which have been shown to be the most frequently altered in prostate cancer. The specimens were obtained from patients selected from our archives who had stage D1 disease. We sought to describe the chromosomal numerical changes present in primary prostate tumours and their matched lymph-node metastases, to identify a clonal cell migration process which could account for the metastatic behaviour.

MATERIALS AND METHODS

Seventy-five patients with prostate adenocarcinoma stage D1 were selected from those diagnosed and treated in our department from 1991 to 2000. Fifty patients were diagnosed with D1 (T1–3N1M0) stage by imaging such as CT or MRI, with the result that no surgical procedure was used and no lymphatic tissue available for the study. The remaining 25 patients had negative imaging studies but with lymphatic metastases, confirmed by histopathological examination after bilateral pelvic lymphadenectomy. Ten of these 25 were excluded from the study because of insufficient tissue for analysis or bad preservation (lymph node or primary tumour). Three additional cases were obtained from the Clínica San José (Barcelona). These tissue blocks were also reviewed by the same pathologist who had already reviewed the 15 cases that were suitable. Thus, 18 cases were available for the study, with samples of both the primary tumour and its matched lymph node metastases.

After pathological confirmation that at least half the tissue was tumour or lymph node metastasis (and using additional 5 µm haematoxylin-eosin stained sections), three adjacent 50 µm pathological tissue sections were used for FISH analysis. FISH was then applied using the technique of Brown et al. [7] with some modifications. The tissue was deparaffinised as follows; 50 µm unstained middle tissue sections were washed with xylene five times for 15 min. The tissue was then dehydrated in 100% ethanol (twice, 5 min) and then rinsed in water (twice, 5 min). Tissues were digested in a pepsin solution at 37 ºC for 2.5 h. After filtering, isolated nuclei were washed twice with PBS and vortexed. The resultant nuclear suspension was applied to slides previously treated with special adhesive 3-aminopropyltriethoxysilane (Sigma-Aldrich, St Louis, MO), and the slides oven-dried at 65 ºC for 10 min. α-satellite centromeric DNA enumeration probes for chromosomes 7 and 8 (spectrum orange, Vysis, Inc., Downers Grove, IL, USA) and chromosomes 10 and 12 (spectrum green) were used. A dual-colour probe solution (7 µL of hybridization mix and 2 µL of each probe) was denatured at 75 ºC for 6 min and kept on ice until it was added to the slides. Simultaneously, DNA was denatured by incubating the slides in 70% formamide/2 × standard sodium citrate at 75 ºC for 5 min, followed by dehydration in ice-cold ethanol series. After sealing, the slides were then incubated overnight at 37 ºC. After hybridization the slides were washed in 50% formamide/2 × sodium citrate three times for 10 s, 2 × sodium citrate for 10 s, and 2 × sodium citrate NP40 for 10 s at 37 ºC. The counterstain 4,6-diamino-2-phenyleindole was added before analysis.

FISH probes labelled with spectrum-orange and spectrum-green, specific for the centromere of chromosomes 7, 8, 10 and 12, were used as numerical changes on chromosomes 7 and 8 are the most frequent in prostate cancer, whereas 10 and 12 were used as controls.

The centromeric signals were assessed using a microscope equipped with the appropriate filters, and the hybridization signals in 300 interphase nuclei counted. To minimize sampling error and the possible influence of tissue heterogeneity, one observer evaluated 150 nuclei in one half of the hybridization area, while another evaluated 150 nuclei in the other half. All intact nuclei (except for overlapping nuclei) within the analysed area were assessed. The hybridization was repeated and recounted if the slide was unsuitable or there was a counting discrepancy.

The criteria established at the Mayo Clinic [7], 16 of 18 primary tumours showed some kind of aneuploidy in the chromosomes assessed and only two showed no numerical change in these chromosomes. The distribution of centromeric signals in the cells corresponding to the prostatic tumour tissue in each case is listed in Table 1. As expected, the most common chromosomal changes were detected in chromosomes 7 and 8, occurring in 15 of the 16 aneuploid tumours, whereas chromosomes 10 and 12 were aneuploid in nine and eight of the aneuploid tumour specimens, respectively. Monosomy of chromosome 8 (Fig. 1) and trisomy of chromosome 7 were the most frequent alterations in 14 and 13 of the 16 aneuploid tumours, respectively.

Of the 18 lymph node samples assessed all had chromosomal changes, also detailed in
detected, in 13 and 15 lymph tissues.

In the case of primary tumours, monosomy of chromosomes 7 and 8 were altered in six and seven, respectively. As expected, after FISH analysis, there was no relationship in three (nos 1, 12 and 14).

There was no statistical difference (Wilcoxon test) in the percentage of cells with trisomy of chromosome 7 and monosomy of chromosome 8 in primary tumours and lymph nodes, with mean (SD) rates of nuclei with trisomy 7 of 12 (11)% vs 9 (7)%, and of monosomy 8 of 15 (12)% vs 14 (9)%.

**DISCUSSION**

The present study contains the most samples reported to date analysed with FISH in matched primary prostate tumours and lymph node metastases. There was an abnormal DNA content in 16 of 18 primary tumours (aneuploid), whereas all of the lymph nodes were aneuploid. These results agree with those obtained by Jenkins et al. [18], who detected chromosome anomalies in 68% of primary tumours and in 96% of nodal metastases after FISH analysis. Gburek et al. [17] also reported 84% aneuploid tumours and 94% aneuploid lymph nodes. Although these aneuploid rates are similar in the three studies, there were slight differences which could be attributed to the genetic discrepancy in the different foci of the same gland [9]. Similar reasoning can be applied to case no. 14, the third case in which the cytogenetic pattern differed in the primary tumour and its lymph node metastasis. The multifocal origin and intratumoral heterogeneity of prostate cancer could account for the differences.

Only two of 18 tumours did not share the same ploidy pattern with the matched lymph node. These tumours showed no chromosome alteration and were classified as diploid, which could be attributed to the unsuitable sampling of nondominant tumour foci leading to lymph node metastasis. The multiform origin and intratumoral heterogeneity of prostate cancer could account for the genetic discrepancy in the different foci of the same gland [9]. Similar reasoning can be applied to case no. 14, the third case in which the cytogenetic pattern differed in the primary tumour and its lymph node metastasis.

As expected, after FISH analysis, chromosomes 7 and 8 were the most frequently changed, both in primary prostate tumours (17/18) and lymph nodes (13 for chromosome 7 and 15 for chromosome 8). These changes were practically constant and

**Table 1**: Specific chromosomal changes detected with FISH for individual cases in primary tumours and matched lymph nodes, and the percentage (in brackets) of aneusomy cells to the total nuclei counted.

<table>
<thead>
<tr>
<th>Case</th>
<th>Cytogenetic pattern</th>
<th>Tumour</th>
<th>Lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Diploid</td>
<td>–7 (15)–8 (16)–12 (12)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>–7 (16)+7 (10)–8 (16)+8 (9)</td>
<td>+7 (9)–8 (14)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>–7 (12)+7 (8)–8 (14)+8 (7)</td>
<td>–8 (12)–7 (14)+7 (14)–10 (14)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+7 (37)</td>
<td>–7 (37)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>–7 (16)+7 (7)HT7 (6)</td>
<td>–7 (49)–8 (25)–10 (43)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>–7 (13)+7 (16)T7 (29)HT7 (8)</td>
<td>+7 (24)T7 (31)HT7 (10)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>–7 (14)+8 (13)T8 (24)HT8 (10)</td>
<td>+8 (22)T8 (27)HT8 (28)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>+7 (10)–8 (21)–12 (17)</td>
<td>+7 (8)–8 (25)–12 (21)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>+7 (9)HT7 (6)</td>
<td>–7 (12)–8 (13)+8 (10)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>–8 (12)+8 (7)</td>
<td>–7 (7)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>–8 (57)–10 (18)–12 (18)</td>
<td>–7 (12)+7 (8)–8 (13)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Diploid</td>
<td>–7 (12)HT7 (7)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>–9 (14)+8 (11)HT8 (7)HT10 (7)</td>
<td>–8 (14)HT8 (6)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>–8 (14)+8 (7)HT8 (6)</td>
<td>+7 (9)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>+7 (12)–8 (23)+8 (11)</td>
<td>–7 (13)+7 (13)T7 (12)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>–7 (13)+7 (11)HT7 (6)</td>
<td>+7 (22)T7 (7)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>–9 (14)–8 (16)</td>
<td>–7 (23)–8 (29)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>–9 (14)+7 (8)HT8 (7)</td>
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</tr>
<tr>
<td></td>
<td>–10 (12)+10 (7)T10 (12)</td>
<td>–10 (12)+10 (13)</td>
<td></td>
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<tr>
<td></td>
<td>+12 (8)T12 (13)</td>
<td>–12 (14)+12 (7)</td>
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(+) gain of a chromosome copy (trisomy); (−) loss of a chromosome copy (monosomy); (T) tetrasomy; (HT) more than four chromosome copies (hypertetrasomy).

Table 1. In the primary tumour tissues, the most common chromosomal alterations were in chromosomes 7 and 8, in all and 16 cases, respectively, whereas chromosomes 10 and 12 were altered in six and seven, respectively. As in the case of primary tumours, monosomy of chromosome 8 and trisomy of chromosome 7 were also the most frequent changes detected, in 13 and 15 lymph tissues.

There was no statistically significant difference between the frequencies of presentation of these aneuploidies when comparing primary tumours and metastatic lymph nodes. Fifteen of 18 cases showed concordance in the aneusomic pattern, with total agreement in seven (nos 3, 6, 7, 10, 11, 13 and 16) and moderate concordance in eight (nos 2, 4, 5, 8, 9, 15, 17 and 18). There was no relationship in three (nos 1, 12 and 14).
could be regarded as specific. Chromosomal changes are known to be an early event in the development of prostate cancer, with a confirmed presence in high-grade PIN lesions at a similar rate to that in prostate cancer lesions [19]. Gburek et al. [17] found aneuploidy in chromosome 7 and 8 in 87% and 69%, respectively, whereas Jenkins et al. [18] detected 50% and 78% aneuploid rates for each chromosome.

The presence of multiple aneumies, hypertetrasomic cell populations and trisomy of chromosome 7 is already regarded as a marker of poor prognosis in prostate cancer [12]. At least one of these poor prognosis criteria was present in all the patients except one with aneuploid primary tumours (case no. 8). All the matched lymph nodes also had at least one of these criteria, and 17 of 18 had at least two.

There was a good correlation between primary tumours and the matched lymph node metastasis (15 cases), showing the same aneumies in seven. These data show that a very similar cell population was present in the nodal metastasis and in the primary tumour; it may therefore be assumed that there are no cell clones with specific aneumies that favour their presence in the metastasis. Moreover, supporting the idea that there is no cell clone selection during the process of lymphmatic metastasis, there were similar numbers of cells carrying the cytogenetic aneumies in the primary tumour and its lymph node metastasis.

Earlier studies detected deletions affecting a specific region of the short arm of chromosome 8 (8p21.3–21.1) [20] and have found them in all nodal metastasis and in 75% of primary tumours, which is consistent with the present results. Subsequent studies also suggested the presence of a tumour suppressor gene in 8p22, indicating that the loss of heterozygosity in 8p22 has a cytogenetic role in prostate cancer progression. Furthermore, trisomy of chromosome 8 detected by FISH has not only been regarded as an overall gain in genomic material, but also as a loss of some regions of 8p and a gain in the centromeric region. This gives rise to the concept of the acquisition of multiple 8p isochromosomes in tumoral cells [20,26,27]. Isochromosomes can be identified by FISH analysis as false monosomies with a high presence of trisomies. Amplification of c-myc seems to be related to gains in the long arm of chromosome 8 (8q24). This has also been detected in lymph node metastasis of prostate cancer [8,9,15,18,28] and associated with high-grade PIN progressing to prostate cancer [8,11,19]. Thus, the numerical cytogenetic aberrations could represent the loss of a tumour suppressor gene in 8p22, which is important in the tumorigenic process.

Earlier FISH studies already showed that aneumies of chromosome 7 is frequent in prostate cancer, and is associated with an advanced pathological stage, higher tumour grade and poor prognosis [6,8,9,12]. Furthermore, cytogenetic studies showed that gains, deletions and translocations of 7q22 and 7q31 were common in prostate cancer [29,30]. Other independent studies indicated the presence of relevant genes in 7q31, like c-met proto-oncogene [31–33]. Several studies [6,8,12,29,30,34,35] showed that the long arm of chromosome 7 is genetically unstable in prostate cancer and associated with a poor prognosis [12]. Moreover, Greene et al. [36] detected gains of the 7p region in 10% of localized cancers and in 56% of recurrent and hormone-resistant prostate tumours, using comparative genomic hybridization.

In conclusion, we confirm the usefulness of FISH analysis of isolated nuclei for detecting cytogenetic alterations. Our results show a high incidence of aneuploidy and aneumies in stage D1 (pT2–3N1M0) prostate cancer affecting chromosomes 7 and 8, monosomy 8 being the most frequent aneumie, followed by trisomy of chromosome 7. The good correlation in the cytogenetic pattern found in the primary tumours and their lymph node metastases, together with the similar number of aneuploid cells in both lesions, suggest that clonal cell selection is not involved in the metastatic process.

CONFLICT OF INTEREST
None declared.

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Abbreviations: FISH, fluorescence in situ hybridization; PIN, prostatic intraepithelial neoplasia.