INTRATUMORAL NUCLEAR MORPHOLOGIC HETEROGENEITY IN PROSTATE CANCER

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ABSTRACT

Objectives. Tumor heterogeneity can be measured by quantifying variance of nuclear characteristics by image analysis. Heterogeneity of cell nuclear features correlated with increased local progression in prostate cancer. In the present study, the influence of tumor heterogeneity on prostate-specific antigen (PSA) recurrence after radical retropubic prostatectomy was analyzed and tumor heterogeneity was compared in patients with and without neoadjuvant hormonal therapy.

Methods. Retrospectively, radical prostatectomy material of 44 patients without and 12 patients with neoadjuvant hormonal treatment with a postoperative follow-up of at least 4 years was studied. Each prostatectomy specimen was systematically embedded in paraffin, and each tumor area within the prostate was marked and analyzed by an image analysis system for 32 nuclear features comprising nuclear shape, size, DNA content, and chromatin pattern. Several clinical features were available: preoperative serum PSA, hemoglobin concentration, Karnofsky score, tumor stage, and Gleason score.

Results. Increased tumor heterogeneity, as expressed by differences in karyometric values between tumor areas in nuclear shape and chromatin pattern within the tumor, was significantly correlated with earlier PSA recurrence rate. As compared with nonpretreated patients, hormonally pretreated specimens showed smaller and less heterogeneous tumors. In particular, chromatin pattern heterogeneity was decreased in patients who underwent preoperative hormonal treatment compared with patients who were not pretreated. However, decreased heterogeneity was accompanied by a higher percentage of aneuploid areas per tumor in the pretreated patients. Cox regression analysis showed that karyometric determination of nuclear shape heterogeneity in combination with preoperative PSA level could predict time to PSA recurrence after radical prostatectomy in patients without hormonal pretreatment.

Conclusions. Increase in karyometric tumor heterogeneity in nuclear shape and chromatin pattern was correlated with a shorter PSA recurrence-free interval after radical prostatectomy. Preoperative PSA and karyometric tumor heterogeneity were the best predictors of PSA recurrence in a multivariate analysis. Intratumoral heterogeneity was decreased in patients with prostate cancer who underwent neoadjuvant hormonal therapy.

Image analysis methods enable objective determination of nuclear and cellular features in light microscopy. Several studies showed the value of morphometric grading in prostate adenocarcinoma. Nuclear shape correlated with prognosis in patients with low-stage prostate carcinoma. Other studies, however, could not confirm this correlation with prognosis.

Tumor heterogeneity may have led to the inconsistent findings of morphometric analysis as a prognostic factor. Heterogeneity in ploidy pattern in the tumor was observed in 40% to 50% of cases. Nuclear shape and chromatin variation in the tumor were of higher predictive value than were absolute data obtained in radical prostatectomy material.

To reduce tumor size preoperatively, hormonal pretreatment has been suggested. Neoadjuvant hormonal treatment was shown to reduce the number of positive resection margins and lymph node metastases. The influence on long-term
prognosis remains to be established. Hormonal treatment may change histomorphology of the tumor. In the present study, tumor heterogeneity in nuclear morphology and ploidy pattern were evaluated to answer two questions: (1) Is morphologic intratumoral heterogeneity correlated with prognosis? and (2) Does neoadjuvant treatment influence tumor heterogeneity?

MATERIAL AND METHODS

From 1986 to 1992, 56 consecutive radical prostatectomy specimens were obtained. Forty-four patients underwent surgery without prior hormonal intervention, whereas 12 patients were treated with hormonal agents before surgery.

Patient age ranged from 42 to 73 years (median 65). For each patient, preoperative clinical stage was available and was obtained by digital rectal examination (DRE), transrectal ultrasound (TRUS), and sextant prostate biopsies. Moreover, serum prostate-specific antigen (PSA), hemoglobin, and acid phosphatase levels and performance status were documented. Four- to 10-year follow-up data (median 5.1 years) were available. PSA recurrence was taken as the end point, and was performed by the Hybritech Tandem-E singlepoint assay; analysis after February 1, 1995 was performed with the multipoint assay. All patients reached nadir of PSA value several months after surgery. PSA recurrence was defined as an increase in serum PSA level of more than 1 ng/mL and 0.04 ng/mL for the singlepoint and multipoint Hybritech Tandem-E assay, respectively. Hormonal treatment consisted of maximal hormonal blockade with a luteinizing hormone-releasing hormone (LHRH)-agonist (mostly goserelin) and an antiandrogen (flutamide) for at least 2 months. Radical prostatectomy was performed within 8 weeks after hormonal manipulation.

Material of radical prostatectomy was cut for evaluation of the entire prostate in quarter-prostate sections. Material was routinely processed by fixation in formaldehyde. For embedding, paraffin was used. After embedding, 4-μm sections were cut. From each block (at least 10 per patient), a hematoxylin-eosin (HE) and adjacent Feulgen-stained slide was obtained. On the HE-stained slide, all tumor areas were marked by the pathologist. An experienced uropathologist reviewed all HE-stained slides and marked each tumor area. Different tumor areas were discriminated based on Gleason grade, morphology, and individual location. When large tumor areas were present with clear variation in Gleason grade, those areas were separately marked and analyzed. In 56 specimens, 349 different tumor areas were detected. For each separate tumor location, we estimated volume by delineating the tumor area and multiplying it by 4 μm (section thickness). Subsequent sections were analyzed, and data were summed per tumor area. The marked tumor areas were drawn on the adjacent Feulgen-stained slide, and each tumor area was analyzed by an image analysis system as described previously.

The system automatically measured all nuclei present in the image. Overlapping nuclei were automatically rejected on the basis of abnormalities in shape. Because segmentation problems could occur if debris or degenerated nuclei were present in the image, a technician judged every segmented nucleus and rejected incorrectly segmented and degenerated nuclei. Autologous leukocytes present on the slide were selected for reference of 2 c for DNA ploidy analysis. Overall analysis of one tumor area required approximately 60 minutes.

Within each tumor, the selected tumor areas were compared. For each tumor, we were able to calculate the highest and lowest value for each nuclear feature per tumor area. Therefore, we defined as a measure for intratumoral heterogeneity the difference between the highest and lowest value for nuclear shape, size, and chromatin pattern. When differences between tumor areas within a specimen were high, tumor heterogeneity was considered high, whereas homogeneity was defined when only slight differences in nuclear features among different tumor areas within one prostatectomy specimen were detected. Moreover, ploidy analysis was performed for each tumor area. Aneuploidy was defined when a peak in the DNA histogram was present over 2.5 c, which means that no discrimination was made between triploidy and tetraploidy. For each tumor, the percentage of aneuploid tumor foci was calculated.

The morphometric and clinical data were compared with PSA recurrence by multivariate Cox regression analysis using SPSS/PC+ software. The forward-conditional method was applied with a P value of 0.05 for entry and of 0.10 for removal. To evaluate the influence of neoadjuvant hormonal treatment, we applied the nonparametric Mann-Whitney U test.

RESULTS

Distribution of clinical stage and grade is shown in Table I. Pathologic staging findings are shown in Table II.

The overall PSA recurrence rate was 39.3%. All patients developed pathologic proven recurrence, with increase in serum PSA values. Pathologic stage tended to correlate with PSA recurrence in patients both with and without neoadjuvant treatment, but this was not statistically significant (Table II).

In a multivariate analysis of clinical and karyometric features, heterogeneity in nuclear shape was the best predictor of pathologic tumor stage. The number of tumor foci per specimen was correlated with the total tumor volume (Pearson, r = 0.69, P = 0.001). Karyometric tumor heterogeneity increased with the number of tumor foci and total tumor volume.

### Table I. Clinical stage and PSA recurrences *

<table>
<thead>
<tr>
<th>Stage</th>
<th>cT1a</th>
<th>cT1b</th>
<th>cT1c</th>
<th>cT2a</th>
<th>cT2b</th>
<th>cT2c</th>
<th>cT3a</th>
<th>cT3b</th>
<th>cT3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neoadjuvant</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>2(1)</td>
<td>7(2)</td>
<td>2(2)</td>
<td>12(1)</td>
<td>8(2)</td>
<td>5(1)</td>
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<td>1(1)</td>
<td>1(1)</td>
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<tr>
<td>Yes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2(1)</td>
<td>0</td>
<td>2(1)</td>
<td>7(6)</td>
<td>1(1)</td>
<td>0</td>
</tr>
</tbody>
</table>

In cases of neoadjuvant treatment, clinical stage before hormonal manipulations were instituted is shown.

* Number of recurrences is shown in parentheses.
TABLE II. Pathologic stage and PSA recurrence*

<table>
<thead>
<tr>
<th>Stage</th>
<th>pT2a</th>
<th>pT2b</th>
<th>pT2c</th>
<th>pT3a</th>
<th>pT3b</th>
<th>pT3c</th>
<th>pT4a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neoadjuvant</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Number of recurrences in parentheses.

Whereas clinical staging was not significantly different between patients with and without hormonal pretreatment, pathologic stages were significantly higher in patients with no previous treatment. Several nuclear features were different between hormonally pretreated and nonpretreated tumors. In particular, heterogeneity in chromatin pattern (Fig. 1) and nuclear shape (MPASS, Fig. 2) was lower in patients with neoadjuvant hormonal treatment. The maximal and minimal values per group are shown in Fig-
ures 1b and 2b. The length of the bar corresponds to the difference values as shown in Figures 1a and 2a. The percentage of aneuploid tumor foci within the tumor was higher in the neoadjuvant group (Fig. 3).

In a univariate Cox regression analysis, several clinical features correlated with PSA recurrence in the group without pretreatment (Table III). Of the nuclear features describing heterogeneity, that is, differences within the tumor, heterogeneity in chromatin pattern (DL2) and nuclear shape (DPASS) were negatively correlated with the PSA recurrence-free interval. In the neoadjuvantly treated patients, as for those without pretreatment, the PSA recurrence-free interval also tended to decrease with an increase in tumor heterogeneity, as assessed by karyometric analysis (Figs. 1, 2). Because only 12 patients were included in this group, no statistical analysis could be performed.

The multivariate Cox regression analysis for the prediction of PSA recurrence-free interval in the group without pretreatment (n = 44) showed pre-operative PSA level and heterogeneity in nuclear shape to be the best predictors (Table IV).

**COMMENT**

Prognosis after radical surgery for prostate cancer is dependent on pathologic grade and the status of the resection margins. Local extension of disease decreases recurrence and progression-free intervals.

Nuclear shape, quantitated by image analysis methods, was shown by some investigators to provide prognostic information in addition to tumor stage in low-stage prostate carcinoma. Others, however, could not confirm the prognostic value of nuclear shape and propagated variation of nuclear size within the tumor. A possible explanation for this discrepancy is that prostate cancers occur multifocally in most cases. Although little is known about the way in which tumors progress and become invasive or metastasize, genetic instability is assumed to play an important role in the process. Selecting only the morphologically most atypical tumor area for grading may not provide a reflection of the spectrum of malignant genetic changes that occur in the prostate.

For renal carcinoma, nuclear shape and chromatin changes within the tumor are highly correlated with tumor progression in localized cancer. In metastasized renal tumors, heterogeneity of the primary tumor was of no predictive value in prognosis, putatively because in disseminated disease genetic instability has already resulted in a highly malignant advanced tumor cell line determining prognosis. This was confirmed by the finding that

**FIGURE 3.** Comparison by PSA recurrence of percentage of aneuploid tumor cell foci within the tumor in pretreated patients and in patients not pretreated. Recurrence: No (hatched columns); yes (open columns).

in advanced disease prognosis was correlated with the absolute values of aberrant nuclear shape and DNA content rather than with intratumoral heterogeneity.

In prostate cancer, intratumoral heterogeneity was detected for Gleason grade, DNA ploidy, p53 mutations, and androgen receptor expression. Heterogeneity in nuclear shape and chromatin pattern was shown to correlate with local tumor progression. Moreover, the coefficient of variation in nuclear size, rather than the absolute mean value, correlated with survival after radical prostatectomy. In the present study, nuclear features correlated with PSA recurrence after radical prostatectomy. Absolute morphology nuclear values were less important for predicting progression than was variation in nuclear characteristics within the tumor. An increase in nuclear polymorphism and heterogeneity in chromatin pattern was inversely correlated with the PSA recurrence-free interval. We assume that genetic instability causes the intratumor differences.

Neoadjuvant hormonal treatment influenced tumor heterogeneity. Comparison of material after neoadjuvant treatment with material from patients treated only with surgery showed that the tumors were much smaller and less heterogeneous in the former group. With regard to neoadjuvant treatment, the present study was performed on material from nonrandomized studies. Clinical tumor stage in the neoadjuvant and non-neoadjuvant group, however, was comparable, whereas pathologic stage was significantly lower in the patients treated neoadjuvantly, suggesting that the hormonal treatment caused so-called "downstaging." Although only
TABLE III. Univariate Cox regression analysis of clinical, pathologic, and karyometric features for the prediction of the PSA recurrence-free interval in patients without neoadjuvant treatment (n = 44)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Beta</th>
<th>P Value</th>
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<tbody>
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<td>Age</td>
<td>0.0592</td>
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</tr>
<tr>
<td>Karnofsky score</td>
<td>0.0362</td>
<td>0.4826</td>
</tr>
<tr>
<td>Preoperative PSA level</td>
<td>0.0415</td>
<td>0.0612</td>
</tr>
<tr>
<td>Clinical stage</td>
<td>0.0200</td>
<td>0.1887</td>
</tr>
<tr>
<td>Pathologic stage</td>
<td>0.3060</td>
<td>0.0777</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.1518</td>
<td>0.2477</td>
</tr>
<tr>
<td>Gleason score</td>
<td>0.1592</td>
<td>0.7363</td>
</tr>
</tbody>
</table>

Karyometry*

- DPASS = 0.2889, 0.0275
- DFPE = 21.1101, 0.1880
- DFELL = 13.9092, 0.0403
- DPPR = 0.0472, 0.0205
- DL2 = 5.5422, 0.2896
- DAREA = 0.0092, 0.6398
- Mean FPE = 2.6307, 0.8914
- Mean PASS = 0.1195, 0.5928
- Mean AREA = 0.0259, 0.4899
- Mean PPR = 0.0968, 0.3398
- Mean FELL = 2.0199, 0.8248

Key: PPE = nuclear roundness factor; FELL = form ellipse (major axis divided by minor axis); PASS = nuclear shape feature based on smoothed Freeman difference chain code; AREA = nuclear size; DL2 = Markovian chromatin texture feature—entrophy; PPR = polyploidy rate; DPASS = the difference between the minimal and maximal MPASS value of tumor areas within the tumor.

D indicates difference between highest and lowest value between tumor areas within one tumor.

* A selection was made of the karyometric features. All features not presented concerning nuclear size, shape, DNA content, and ploidy pattern did not show correlation with PSA recurrence rate.

TABLE IV. Multivariate Cox regression analysis of clinical, pathologic, and karyometric features for the prediction of the PSA recurrence-free interval

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Beta</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPASS</td>
<td>0.3674</td>
<td>0.0115</td>
</tr>
<tr>
<td>Preoperative PSA level</td>
<td>0.0571</td>
<td>0.0171</td>
</tr>
</tbody>
</table>

Key: DPASS = the difference between the minimal and maximal MPASS value of tumor areas within the tumor.

12 patients were included in the neoadjuvant group, patients with more heterogeneous tumors, like the patients who were not pretreated, had a shorter PSA recurrence-free interval. The trend toward an increased percentage of aneuploid cell populations in patients treated neoadjuvantly, despite the decrease in tumor size and nuclear heterogeneity as compared with tumors not pretreated, needs further analysis. This finding may partly explain the higher PSA recurrence rate of the patients with hormonal pretreatment. Patient groups are too small to allow us to draw any conclusions regarding the long-term effects of neoadjuvant treatment on prognosis.

Nuclear appearance discloses information regarding tumor malignancy in prostate cancer. To obtain adequate information on prognosis, one should analyze different tumor areas to estimate intratumoral heterogeneity. Because nuclear morphometric analysis is a time-consuming procedure, it cannot yet replace visual tumor grading. Neoadjuvant hormonal treatment appears to reduce intratumoral heterogeneity. In this small population, such reduction was not correlated with improved prognosis. The finding of increased aneuploidy in patients treated neoadjuvantly is also of interest.

REFERENCES