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Evaluation of DNA Ploidy Combined with a Cytometric Proliferation Index of Imprints from Core Needle Biopsies in Prostate Cancer

Göran Ahlgren Ursula Falkmer Virgil Gadaleanu
Per-Anders Abrahamsson

Departments of Urology and Cytology/Pathology at the University of Lund, University Hospital, Malmö, Sweden, and Department of Oncology, University Hospital, Trondheim, Norway

Key Words

Prostate cancer · Ploidy · Proliferation index · Prognostic factors

Abstract

Objective: To evaluate if DNA ploidy analysis with a proliferation index (PI) derived from DNA cytometry of imprints from core needle biopsies predicts disease progression in patients with prostate cancer. ***Methods:*** Touch imprints were done on a consecutive series of core needle biopsies taken by the same urologist from 240 patients with suspected prostate cancer, 137 (46%) of whom were found to have prostate cancer and included in the study. Scattered cells to the right of the image cytometry (ICM) ploidy-establishing peak, the S-phase fraction, and those in the G2M area of the ICM DNA histograms, were counted in percent of the total number of tumor cells, this value being designated the ICM PI. Based on previous results in archival fine needle aspiration material, the following classification was used: DNA group I, diploid tumor with a low PI; DNA group II, diploid tumor with an intermediate PI and tetraploid tumor with a low or intermediate PI, and DNA group III, diploid or tetraploid tumor with high PI and all tumors with an aneuploid pattern. ***Results:*** Correlation was found to exist between DNA groups I–III and Gleason score (GS) ($p < 0.0001$), T-stage ($p = 0.006$), M-stage ($p = 0.009$) and disease progression ($p < 0.0001$). Among the 39 patients who had curative treatment and GS 5–7, the progression-free survival rate was 100% in DNA group I, as compared with only 38% in DNA group II and 55% in DNA group III within the follow-up period ($p = 0.008$). ***Conclusion:*** DNA ploidy combined with a PI derived from image cytometry of imprints from core needle biopsies yields additional prognostic information in patients with GS 5–7. Diploid tumors with a low PI (DNA group I) are associated with a low risk of disease progression.

Introduction

The cytometrically assessed DNA ploidy pattern of the nuclei of tumor cells in prostate carcinomas is correlating to tumor grade but its value as a prognostic factor is uncertain [1]. However, the clinical significance of the results of DNA ploidy analysis depends on the cytometry method used. As previously reviewed by one of us (U.F.), differences and even methodological errors occur, both when flow cytometry (FCM) and when image cytometry (ICM) are used [2]. ICM is known to manifest greater sensitivity than FCM in detecting nondiploid cell clones [3].

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G. Ahlgren, MD
Department of Urology, University Hospital in Malmö
SE–205 01 Malmö (Sweden)
Tel. +46 40 33 18 57, Fax +46 40 33 73 52
E-Mail fam.ahlgren@telia.com

We have previously used ICM DNA ploidy with a proliferation index (PI) determined on archival fine needle biopsy material to predict the disease-specific survival of patients with prostate cancer [4]. A prerequisite for obtaining an ICM DNA ploidy peak with a low coefficient of variation (CV) is to measure DNA content only in intact tumor cell nuclei. We therefore found it worthwhile to evaluate DNA ploidy combined with a PI derived from ICM of imprints from core needle biopsies in a consecutive series of patients in relation to disease progression after treatment with or without curative intent. Furthermore, a comparison of the results with those of disaggregated FCM and of ICM on fine needle aspirations was made in a small subgroup of cases.

Methods

Patient Characteristics and Treatment

From November 1992 to July 1995, touch imprints were made on all core needle biopsies in a consecutive series of 240 patients with suspected prostate cancer examined by the same urologist (G.A.) at the Department of Urology, Malmö, Sweden. Of these patients, 144 were found to have prostate cancer and 137 of them were included in the study. None of these patients had previously received any treatment for prostate cancer. Seven patients were excluded due to missing or poor quality imprints. This cohort constitutes 22% of all patients in whom prostate cancer was diagnosed in Malmö during this time period. The median age at diagnosis was 70 years (range 49–88). Treatment for cure was offered to all patients with an expected minimum survival of 10 years and without evidence of metastasis at bone scan. Of the 53 patients who accepted such treatment, 26 underwent radical prostatectomy and 27 underwent irradiation treatment. Of the 84 patients treated palliatively, 27 had deferred treatment (i.e. active surveillance), and 57 (including 15 patients with metastasis) had early hormonal treatment. Median age was 65 years (range 50–78) for patients receiving treatment for cure and 73 years (range 49–87) for those receiving palliative treatment. All patients were followed clinically at regular intervals until death (22 patients) or for a median of 39 months (115 patients). Primary failure was defined as biochemical failure, local progression and/or metastasis in patients treated for cure and in patients on active surveillance. Biochemical failure was defined as a serum PSA level >0.5 ng/l following radical prostatectomy, a doubling of the serum PSA nadir to a value >4.0 ng/ml after radiation treatment, and a doubling of the serum PSA level in patients on deferred treatment. Clinical progression in patients on hormonal treatment was regarded as secondary failure. Chart reviews were done in January 1998.

Biopsy Specimens, Tumor Grade and Clinical Stage

All slides were reviewed and graded according to Gleason by the same pathologist (V.G.) [5]. The two most common Gleason grades in the biopsies were determined the Gleason score (GS). Clinical T-stage according to the TNM classification [6] was assessed by the same urologist (G.A.). Node dissection was done in all 53 patients receiving treatment for cure except 3 who had radiation treatment. Of these 50 patients, 49 had node-negative disease, and 1 had node-positive disease. Of the 127 patients who underwent a bone scan, 15 (11.8%) had metastasis.

In 56 patients, one ultrasound-guided fine needle aspiration (FNA) biopsy was taken before the core needle biopsies and was subsequently Feulgen-stained. Unfortunately, only 28 of these FNA specimens had >100 tumor cells allowing representative DNA ploidy analysis. In 61 cases, the biopsy yielded enough tumor tissue to allow FCM analysis, either from the remaining specimen in the paraffin block of the core biopsy (42 cases) or from two central 40 µm-thick sections (19 cases).

DNA Cytometry Techniques: Calculation of PI

Image Cytometry. The imprints were air-dried and then Feulgen-stained as previously described [7]. A commercial image analysis system (ACAS System, Bargteheide, Germany) was used for DNA densitometry. In each staining bath, the reference cells (both for quality control and as 'external 2c standard') were derived from cerebellar cortex autopsy specimens. Random tumor cells from all over the specimen were analyzed. A minimum of 100 neoplastic cells were counted (mean 175 ± 4). Lymphocytes or granulocytes were used as 'internal 2c standard' cells. Previously described software and criteria were used for computer-assisted evaluation and classification of DNA histograms [3]. Tumors with a DNA index (DI) of 0.9–1.1 were classified as diploid, those with a DI of 1.9–2.1 and containing ≥ 15% of the total cell count as tetraploid and those yielding a histogram with a DI outside the diploid and tetraploid ranges and >10% of the cell count as aneuploid. The mean CV for the G1 peak was 6.0% (± 0.2%). Scattered cells to the right of the ploidy establishing peak on the x-axis, including cells within the G2M range, were considered to represent the PI, and were calculated automatically by the software in percent of the respective total tumor cell count [3]. The tumors were classified according to the histograms as having a low, intermediate or high PI (<5, 5–10 and >10%, respectively), and were divided into three prognostic DNA groups as previously described [4]: DNA group I, diploid tumors with a low PI; DNA group II, diploid tumors with an intermediate PI or tetraploid tumors with a low or intermediate PI and DNA group III, diploid or tetraploid tumors with a high PI and all aneuploid.

Flow Cytometry. FCM was performed on paraffin-embedded material using a conventional technique [8]. Thus, two sections, about 40 µm thick, were cut from the formalin-fixed, paraffin-embedded specimens, deparaffinized in xylene, and rehydrated. After enzymatic disaggregation with Subtilisin Carlsberg (Sigma Protease type 24, St. Louis, Mo., USA), the cellular suspension was washed in phosphate-buffered saline (PBS), and stained with DAPI (Partec, Münster, Germany) at a final concentration of 5 µmol. The DNA fluorescence analyses were performed with a Partec cytophotometer (PAS 2, Münster, Germany). The number of cells being analyzed was mean 20,469 (1,297–40,000) and the CV value of the G1 peak was mean 6.2 ± 2.6.

Statistics

The StatView® software was used for all calculations. Correlation between nominal variables was tested with the χ^2 test. Survival was estimated with the Kaplan-Meier method. Mean values with standard error of the mean (SEM) or median values with range are given for continuous variables. Differences were tested with the Mann-Whitney U test or the Kruskal-Wallis test. p values <0.05 were considered statistically significant.

Table 1. ICM vs. FCM DNA ploidy in 61 core needle biopsies from 59 patients

ICM DNA ploidy	FCM DNA ploidy			Total
	diploid	tetraploid	aneuploid	
Diploid	27	0	2	29
Tetraploid	10	12	2	24
Aneuploid	2	3	3	8
Total	39	15	7	61

63.9%. 24.5%. 11.5%.

Results

ICM vs. Disaggregated FCM

In the 137 patients, ICM DNA ploidy could be analyzed on imprints from 287 biopsies with cancer (mean 2.1 biopsies/patient). In patients from whom two or more biopsies with cancer were obtained, a difference in ICM DNA ploidy was seen in 27/112 cases (24%).

ICM of core biopsy imprints and FCM of deparaffinized, disaggregated core needle specimens yielded concordant results for DNA ploidy analysis in 69% (42/61) of the cases, 34 core biopsies being nondiploid with any of the two methods (table 1). Sensitivity in detecting non-diploid tumors was 94% (32/34) for ICM and 65% (22/34) for FCM. Of the two that were diploid according to ICM but aneuploid according to FCM, one was classified as ICM DNA group II with an intermediate PI, the other as DNA group III with a high PI.

ICM Results in Imprints vs. FNA Material

ICM yielded concordant results for imprints and for FNA material in 57% (16/28) of cases where FNA biopsies were also obtained, 23 of them being nondiploid irrespective of the type of biopsy (table 2A). The sensitivity of ICM in detecting nondiploid tumors was 91% for imprint material and 78% for FNA material. Concordant DNA grouping was obtained in imprint and in FNA material in 64% (18/28) of cases (table 2B).

PI vs. Ploidy in Imprints, Tumor Grade and Clinical Stage

Of the 137 tumors, 48 (35%) were classified as diploid, 62 (45%) as tetraploid and 27 (20%) as aneuploid. Aneuploid tumors differed significantly in median PI both from diploid and tetraploid tumors (8 vs. 3% ($p = 0.008$) and 2% ($p = 0.0004$), respectively). GS 8–10 tumors differed significantly in median PI from GS 5–7 tumors (3.0 vs. 6.5% ($p = 0.01$)) and from GS 2–4 tumors (1.9% ($p = 0.001$)). Stage T3–4 differed significantly in median PI from T1–2 tumors (6.3 vs. 2.3% ($p = 0.006$)). Metastatic tumors differed in median PI from nonmetastatic tumors (5.4 vs. 3.0%, respectively), though the difference was not significant ($p = 0.13$).

Table 2. DNA ploidy (**A**) and DNA groups I–III (**B**) on FNA specimens obtained from 28 patients vs. imprints from core needle biopsies

A

FNA: ICM DNA ploidy	Imprints: ICM DNA ploidy			Total
	diploid	tetraploid	aneuploid	
Diploid	5	5	0	10
Tetraploid	2	8	3	13
Aneuploid	0	2	3	5
Total	7	15	6	28

B

FNA: DNA groups I–III	Imprints: DNA groups I–III			Total
	I	II	III	
I	4	3	0	7
II	0	10	5	15
III	0	2	4	6
Total	4	15	9	28

DNA Grouping in Imprints vs. WHO Grade, TNM Classification and Serum PSA Level

Correlation was found to exist between DNA grouping (I–III) and GS ($p < 0.0001$), T-stage ($p = 0.006$) and M-stage ($p < 0.009$). However, there was no significant difference in median serum PSA levels between DNA groups I, II and III (16.4, 18.2 and 19.0 ng/ml, respectively, $p = 0.65$). The proportion of patients receiving treatment for cure was 31% (14/45) in DNA group III, as compared with 42% (39/92) in DNA groups I and II (table 3).

Disease Progression vs. DNA Grouping, Tumor Stage and WHO Grade

Correlation was found to exist between disease progression (primary or secondary) and GS ($p < 0.0001$), tumor stage ($p = 0.006$) and DNA grouping ($p < 0.0001$) (fig. 1a, b). Of the 28 patients with DNA group I tumors, only 2 manifested evidence of primary progression, and

Table 3. GS, TNM stage, DNA group, serum PSA and treatment in relation to DNA groups I–III

	Imprint DNA group			Total count
	I	II	III	
GS 2–4	6	6	1	13
GS 5–7	22	45	21	88
GS 8–10	0	13	23	36
T-stage 1–2	23	41	21	85
T-stage 3–4	5	23	24	52
N-stage N0	12	25	15	52
N-stage N1–2	2	2	1	5
N-stage Nx	14	37	29	80
M-stage M0	28	54	30	112
M-stage M1	0	6	9	15
M-stage Mx	0	4	6	10
s-PSA, ng/ml				
<10	9	20	13	42
11–20	9	17	10	36
21–100	10	23	11	44
>100	0	4	10	14
Curative treatment	12	27	14	53
Palliative treatment	16	37	31	84
Total	28	64	45	137

none of hormone refractory disease. DNA groups I–III also yielded significant correlation to disease progression in the 88 patients with GS score 5–7 tumors ($p = 0.008$). In these patients, the pretherapeutic serum PSA level was median 17.6, 16.0 and 7.1 in DNA groups I, II and III, respectively ($p = 0.48$).

Discussion

The study population was heterogeneous and representative of all tumor stages, but consecutive patients were found to have prostate cancer by one and the same urologist (G.A.) during a 2.5-year period and constituting 22% of all patients diagnosed as having prostate cancer in Malmö during the period. Only 11% of the patients had metastasis, thus explaining the low mortality from prostate cancer (8%) in the series during the follow-up. However, the aim of the study was to ascertain whether reliable ICM DNA ploidy analysis results could be obtained from imprints of core needle biopsies, as we believe image cytometry of intact cell nuclei to be necessary to obtain a

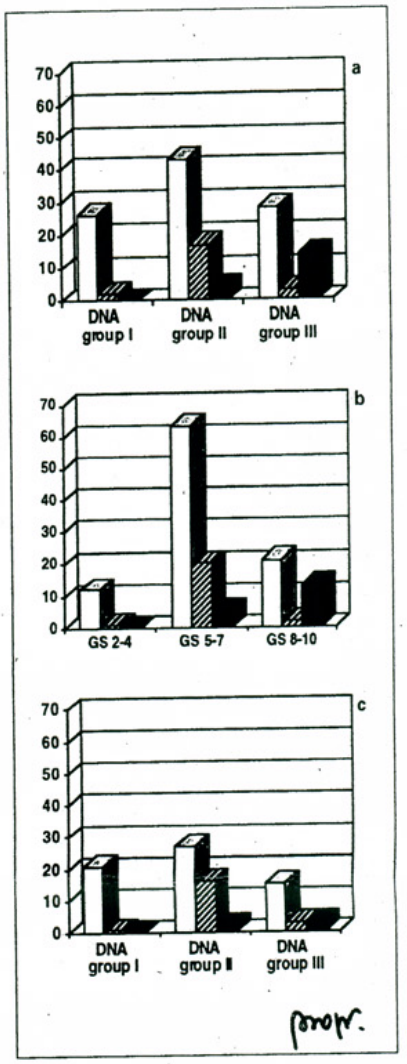

Fig. 1. Number of patients with no progression (white bars), primary progression (gray bars) and hormone refractory disease or death from prostate cancer (black bars) in relation to DNA groups I–III (**a**) and GS (**b**) in 137 patients receiving curative or palliative treatment for prostate cancer. Disease progression (**c**) in relation to DNA groups I–III in 88 tumors with GS 5–7 is given.

high sensitivity for detecting nondiploid cell clones and proliferating cells in prostate cancer specimens.

A major problem in obtaining reliable prognostic information from biopsies is tumor heterogeneity. The outcome of DNA ploidy analysis of pieces from different parts of radical prostatectomy specimens is discordant inasmuch as 40% of cases are measured by FCM [9]. Still, correlation between DNA ploidy findings in core needle biopsies and radical prostatectomy specimens has been found previously using ICM on tissue sections [10]. However, a method where DNA ploidy can easily be evaluated on every single core biopsy manifesting tumor growth will improve the sensitivity of the method for nondiploid cell clones. Sextant biopsies were not done routinely at our department by the time of this study and the mean number of cores with cancer per patient was 2.1 in our series. Nonetheless, ploidy patterns differed from one core to another in 24% of all tumors, while a difference in tumor grade was registered in only 11% of the tumors.

A possible explanation of why a cytometric PI improves the prognostic value of DNA ploidy is that proliferating cells as well as small nondiploid cell clones in mainly diploid tumors will be classified as DNA group II or III because of an increased PI. In this study, PI was higher in aneuploid, grade 3 and locally advanced (T3–T4) tumors. Correlation has been found to exist between cytometric S-phase values and tumor cell proliferation as determined with immunohistochemistry (IH) in breast carcinoma [11]. Several IH studies with antibodies against Ki-67 have shown tumor cell proliferation in prostate cancer to be a significant determinant of disease progression [12]. The increase in DNA content in tumor cell nuclei with DNA ploidy assessments corresponds to a frequent gain of chromosomes in the primary tumor that has been found in node-positive disease as detected by fluorescence in situ hybridization, a method manifesting significantly greater sensitivity than FCM in detecting aneuploidy [13]. In the present study, ICM of imprints had a sensitivity of 95% of nondiploid tumors, as compared to 64% for FCM of deparaffinized specimens. Similar results have been found in other studies [14] supporting that ICM has a higher sensitivity for nondiploid cell clones than FCM.

The median duration of follow-up in this study was short, and only preliminary conclusions can be drawn about the prognostic value of the three DNA groups at this point. We have defined biochemical progression after radical prostatectomy as a repeat serum PSA value >0.5 ng/ml after radical prostatectomy, as we have noticed elevations to PSA 0.2–0.3 ng/ml without progression during years. A previously used definition of recurrence, a doubling of serum PSA to a level >4.0 ng/ml, was used for patients who received radiation therapy [15]. GS is a recognized prognostic factor of disease-specific survival in prostate cancer in series of conservative treatment [16] as well as after radical prostatectomy [17]. In these papers, a consistent finding is that patients with GS 2–4 tumors have a low risk of dying from prostate cancer and that patients with GS 8–10 tumors have a poor prognosis, which corresponds with the early data in our study. We also found correlation to exist between DNA grouping (I–III) and disease progression (primary or secondary). However, the number of tumors with intermediate GS is high and the new DNA groups I–III correlated significantly to disease progression when only the 88 patients with GS 5–7 tumors were included in the present study. Pretreatment serum PSA levels in these patients did not differ significantly between the three DNA groups. These results suggest that GS 5–7 with a single diploid peak in the DNA profile and a low PI (DNA group I), analyzed by ICM measurements on imprints from core needle biopsies, are characterized by a low risk of progression. This is consistent with our earlier results in a retrospective study done on archival FNA material [4]. In the present study of unselected consecutive patients, tumors classified as DNA group I were found in 20% of the series as a whole, and in 23% of the subgroup treated for cure. Deferred treatment may be an alternative option for these patients, especially for the elderly male.

In conclusion, ICM DNA ploidy with a cytometric PI can be used on imprints from core needle biopsies and add prognostic information to that obtained from tumor grade.

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