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Flow cytometric analysis of cellular DNA in human prostate cancer: relationship to 5α-reductase activity of the tissue

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Summary. Flow cytometry of 20 prostate cancer specimens was carried out and the percentage DNA content in each phase of the cell cycle was correlated to the 5α-reductase activity of the tumour. A group of 20 hyperplastic specimens was also entered into this study as control. Tumours were divided into 3 groups on the basis of their 5α-reductase activity. This classification made it possible to identify a group of tumours of which none formed metastases (diploid tumours with 5α-reductase activity greater than 20 pmol/mg protein/30 min and with less than 10% of the cells undergoing mitosis) and a second group of which 78% had metastasised at the time of presentation (diploid tumours with 5α-reductase activity less than 10 pmol/mg protein/30 min and with more than 15% of the cells undergoing mitosis). DNA content within each of the 5α-reductase groups appeared also to correlate with the histological grade of the cancer but no relationship was established with the stage of the disease. Our results suggest that a multi factorial discriminant combining DNA ploidy and 5α-reductase measurements offers a good prognostic index for the assessment of disease progression in prostate cancer and that this might form the basis of a new approach to the treatment of this disease.

Key words: Prostate cancer – DNA – 5α-reductase – Flow cytometry – Histology – Discriminant analysis

Introduction

A major problem associated with the management of prostate cancer concerns the absence of a single reliable marker for selecting the most appropriate form of therapy. Several prognostic factors have been identified including pathological staging [8], histological grading of the biopsy specimen [2] and intra-prostatic tumour distribution [14] but unfortunately none of these approaches have provided the ultimate discriminant.

One of the limitations of these prognostic factors is their inability to take full account of the heterogeneity of an individual prostate cancer. Recently a new method was developed whereby the classification and grading of tumours based on the DNA abnormality of the component cells of the tumour was made possible by the use of flow cytometry. Preliminary observations from our own laboratory [13] as well as those from a number of other centres [1, 3] have shown that flow cytometry not only quantifies the fraction of a cell population in each phase of the cell cycle but also demonstrates that diploid tumours have a better prognosis than aneuploid populations. Diploid tumours were manifested in all stages of the disease though the distribution of DNA in each phase of the cycle varied depending on the histological grade of the tumour [13].

Previously our studies have also shown that 5α-reductase activity in prostate cancer [10] could be correlated with the histological grade of the cancer, and as such 5α-reductase could be used as a biochemical marker for tumour differentiation.

The aim of the present study was to establish whether the assessment of DNA ploidy and 5α-reductase activity in concert in a series of prostatic carcinomas could provide a better prognostic index and form a suitable basis for the management of patients with protate cancer.

Patients and methods

5α-reductase activity and DNA content were measured in prostate tissues removed by transurethral resection from 20 patients with benign prostatic hyperplasia (BPH) and 20 patients with carcinoma of the prostate (CaP). The patients age ranged from 58–88 with a

Table 1. 5α-reductase and DNA content at various phases of the cell cycle in BPH and CaP

Tissue type	Gleason score	Mean 5α-reductase activity (pmol/mg protein/30 min) ± S.D.	Mean % of nuclei in G_1 phase ± S.D.	Mean % of nuclei in S phase ± S.D.	Mean % of nuclei in G_2+M phase ± S.D.
BPH ($N=20$)		24.43 ± 11.45	75.28 ± 4.15	17.19 ± 3.6	7.47 ± 1.29
CaP ($N=18$)	3–10	17.25 ± 17.54 n.s.	69.50 ± 10.42 $P<0.05$	17.18 ± 6.46 n.s.	13.29 ± 10.75 $P<0.05$
CaP ($N=5$)	≤4	39.01 ± 15.27 $P<0.05$	74.44 ± 2.71 n.s.	18.15 ± 2.90 n.s.	7.38 ± 2.07 n.s.
CaP ($N=8$)	5–7	11.68 ± 10.90 $P<0.02$	73.12 ± 5.48 n.s.	15.07 ± 6.60 n.s.	11.78 ± 3.90 $P<0.001$
CaP ($N=5$)	≥8	4.41 ± 2.47 $P<0.001$	58.76 ± 14.16 $P<0.001$	19.61 ± 8.68 n.s.	21.60 ± 18.04 $P<0.01$

n.s. = not significant

mean of 74 years. The resected tissue was stored at − 75° for periods up to 2 years pending analysis. From each patient a number of large prostate chippings were selected for biochemical (5α-reductase measurement) and flow cytometric analysis.

Histological examination of the prostate chippings confirmed diffuse carcinomatous replacement throughout the surgically removed specimens and the grading of the tissue had been carried out by a single pathologist according to the Gleason system [7]. The differentiation of the tumour was based on 3 categories: Well differentiated (summed Gleason < 3), moderately well differentiated (summed Gleason 4–7) and poorly differentiated specimens (summed Gleason < 7). None of the cancer patients had received any therapy – endocrine or otherwise – before entering into this study. Furthermore, at the time of diagnosis and before the transurethral resection, all malignant tumours were clinically staged using the TNM system [11]. Outpatient follow-up was carried out at 3 monthly intervals with a mean time to death or current follow-up of 10.6 month (1–23 month).

Flow cytometric analysis

Cryostat sections (2 × 30 μM) were taken up in centrifuge tubes, then thawed, minced and digested in 3 ml 0.5% pepsin (Sigma) in 0.01 M phosphate buffered saline (PBS; pH = 1.5) for 45 min at 37°C.The resultant suspension was filtered through a 45 μ nylon mesh and the supernatant was subsequently centrifuged at 2,000 rpm for 10 min. The pellet was washed in 0.01 M phosphate buffer solution pH 7.2, 3 times and the final nuclear suspension was stained with 1 ml solution containing 50 μg/ml propidium iodide in 0.01 M Tris buffer (pH 7.0) containing 5 mM $MgCl_2$, 0.1% Triton X-100 and 15 μg/ml ribonuclease (Sigma). After 60 min incubation at 4°C, this was centrifuged at 2,000 g for 10 min and resuspüended in PBS in preparation for the flow cytometry analysis.

The flow cytometer used in this study was an EPICS "C" (Coulter Electronics) equipped with a 5 watt argon ion laser emitting light at 488 nm wavelength and 150 mW. A total of 2,000 nuclei were analysed from each specimen at a flow rate of 100–150/s. Nuclei of chicken erythrocytes were used with each sample as an internal control. The proportion of nuclei in each phase of the cell cycle was determined by the parametric analysis of the flow cytometric data as detailed in our earlier paper [13].

Measurement of 5α-reductase activity

The assay had previously been described in detail [9, 11]. Briefly prostatic chips (150 mg) were chopped, minced and homogenised in 3 ml of 10 mM Tris/HCl, pH 7.4, containing 1.5 mM EDTA and 1.0 mM dithiothreitol. The homogenates were filtered through one layer of nylon gauze to remove all debris and centrifuged for 15 min to obtain the 800 g supernatant. Aliquots (40 μl) of the resultant supernatant were subsequently transferred to incubation tubes containing 50 nM of radiolabelled testosterone and 5×10^{-4} nm of NADPH generating system. Incubation proceeded for 30 min with constant shaking at 37°C and the reaction was stopped by the addition of 1 ml diethylether followed by 100 μl ethanol containing approximately 500 cpm of tracer labelled hormone and 25 μg of unlabelled steroid as carries. The ether extracted steroids were subsequently separated and identified by thin layer chromatography. The measurement of 5α-reductase activity was based on the formation of 5α-dihydrotestosterone and 3α(β) androstanediol from testosterone. The results of 5α-reductase activity were expressed as pmol/mg protein/30 min.

Data analysis

All incubations were performed in triplicate and the data has been presented as mean ± S.D. Differences in enzyme activities, DNA analysis and histological grading were tested for statistical significance by student T-test and the Mann-Whitney U-test. Correlation of the degree of association for any two parameters was determined by calculation of the correlation co-efficient; r values presenting a probability of less than 0.05 were considered to be statistically significant.

Results

5α-reductase activity

Table 1 summarises the results obtained from the various BPH and cancer groups. Although the mean

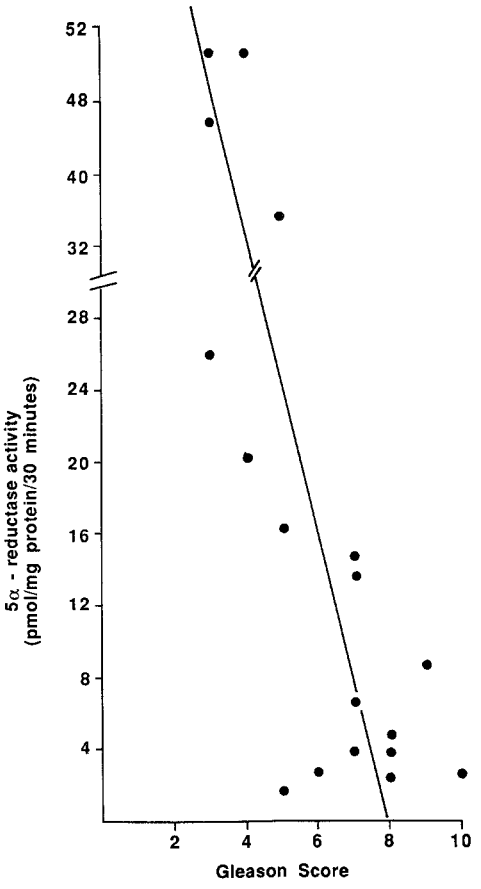

Fig. 1. The correlation between histological grading expressed as the combined Gleason Score (primary plus secondary pattern) and 5α-reductase activity in 18 CaP specimens. $N = 18$; $r = 0.727$; $P < 0.01$

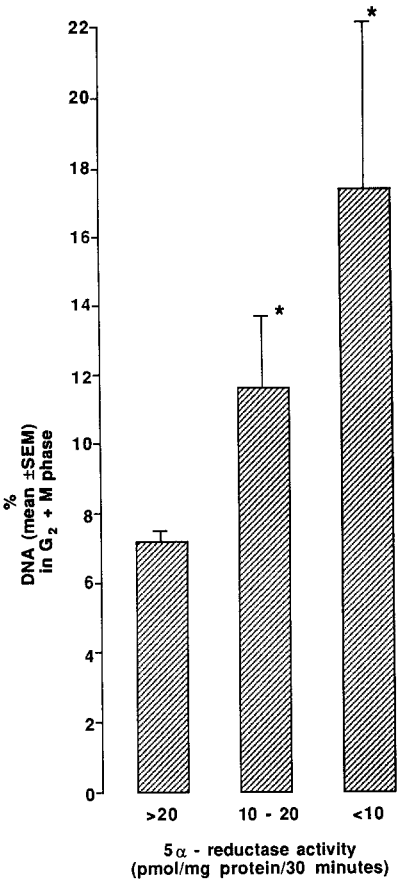

Fig. 2. The relationship between DNA content in the $G_2 + M$ phase of the cell cycle and 5α-reductase activity in 18 CaP specimens. * $P < 0.01$

5α-reductase activity in the cancer population (17.25 ± 17.54 pmol/mg protein/30 min) was lower than that measured in the BPH specimens (24.43 ± 11.45 pmol/mg protein/30 min) a wide spread in the activities of cancerous specimens was noted and this accounted for the lack of statistical difference between the 2 groups ($P > 0.1$). There was however a very strong correlation between 5α-reductase activity and the histological grade of the tumour (Fig. 1; $r = 0.727$, $P < 0.001$) and if the cancer specimens were grouped according to their degree of differentiation, the moderately well differentiated tumours (Gleason Score: 5–7) and the poorly differentiated tumours (Gleason Score: 8–10) demonstrated significantly lower activity than that measured in either BPH or the well differentiated cancerous specimens ($P < 0.02$).

DNA analysis

Table 1 also demonstrates the percentage of DNA measured in the G_0/G_1 phase (the pre-synthetic gap), the S phase (DNA synthesis) and in the G_2 (the post-synthetic gap) and M (mitotic) phases of the cell cycles for BPH and CaP tissues.

All BPH specimens produced identical patterns and these consisted of a major narrow G_1 peak containing on average 75.28% ± 4.15 of the DNA in the tissue whilst a smaller proportion of the nucleic acid (24.66%) was associated with the S and $G_2 + M$ phases of the cycle.

In cancer, two distinct flow cytometric profiles emerged. The first, a diploid pattern which accounted for approximately 90% of the specimens so far analysed, and this was essentially a repeat of the patterns seen in BPH though the relative DNA content in each phase of the cycle varied and was dependent upon the histologic grade of the tumour (Table 1). We found that in the well differentiated cancer (Gleason score ≤ 4) the % DNA in the G_0/G_1 phase was approximately of the same order of magnitude as that measured in BPH specimens whereas the DNA content of the poorly differentiated tumour (Gleason score ≥ 8) was significantly depleted ($P < 0.001$). This contrasted with the

Table 2. The relationship between 5α-reductase activity and flow cytometric data for cancer of the prostate patients

Name	5α-reductase (pmol/mg protein/30 min)	G_1	$G_2 + M$	TNM staging	Gleason Score	Length of follow-up
1. J. McK.	52.49	70.86	8.45	T_3M_0	2 + 2	1 DOOC
2. W. D.	52.17	73.60	4.68	T_0M_0	1 + 2	5
3. W. T.	44.87	75.20	6.56	T_0M_0	2 + 1	18
4. J. H.	34.86	74.92	9.45	T_2M_0	2 + 3	8
5. A. K.	25.67	78.36	5.65	T_2M_0	2 + 1	9
Mean ± S.D.	42.01 ± 11.61	74.59 ± 2.71	6.96 ± 1.96		3.6 ± 0.89	
6. W. W.	19.88	74.19	7.59	T_3M_0	2 + 2	20
7. W. S.	16.01	68.60	15.91	T_3M_1	3 + 2	6
8. T. W.	14.38	73.23	7.78	-	3 + 4	-
9. J. M.	13.38	75.24	10.41	T_0M_0	4 + 3	18
Mean ± S.D.	15.91 ± 2.85	73.57 ± 4.44	10.42 ± 3.88		5.75 ± 1.5	
10. K. M.	8.49	63.53	18.00	T_2M_1	5 + 4	6 DOOC
11. J. D.	6.46	79.99	15.90	T_4M_1	4 + 3	20
12. J. T.	4.74	60.38	10.88	T_4M_1	4 + 4	5
13. R. C.	3.90	68.17	15.90	T_2M_0	3 + 4	5
14. J. B.	3.89	57.30	15.90	T_3M_1	4 + 4	22
15. P. G.	2.69	64.81	8.00	T_2M_1	3 + 3	23
16. W. S.	2.67	36.66	53.34	T_3M_1	5 + 5	7
17. G. B.	2.29	75.57	10.45	T_2M_1	4 + 4	6
18. P. D.	1.78	76.07	14.29	T_2M_0	2 + 3	9
Mean ± S.D.	4.10 ± 2.18	64.76 ± 13.01	18.01 ± 13.6		7.55 ± 1.5	

DOOC = died of other causes

marked increase ($P < 0.01$) in DNA content of the G_2+M phase of the poorly differentiated tumours entered in this study. Details of the distribution of DNA in each phase of the cell cycle for the 3 histologic grading categories of prostate cancer are summarised in Table 1.

The second distinct profile associated with prostate tumours was characterised by the presence of 3 major peaks, the third being situated between G_1 and G_2+M peaks (histogram not shown; a profile indicative of aneuploidy [12]. In the present study there were only 2 aneuploid tumours identified and these were not incorporated in the parametric analysis detailed in Table 1.

The relationship between DNA content and 5α-reductase activity

Although no direct correlation had been established between DNA ploidy and the capacity of the prostate cancer to metabolise testosterone, attempts were made to establish whether a combination of these 2 parameters would be of any prognostic value. Tumours were therefore divided into 3 groups on the basis of their 5α-reductase activity, the first group had an activity greater than 20 pmol/mg protein/30 min, the second showing activities in the region between 10 and 20 pmol/mg protein/30 min and the third group exhibiting activities less than 10 pmol/mg protein/30 min (Fig. 2). Enzyme activities were related in turn to the DNA content of each phase of the cell cycle, to the grade and stage of the tumour, and to the length of survival following treatment (Table 2). The results outlined in Table 2 suggest that this classification made it possible to identify a group of diploid tumours with a high 5α-reductase activity (> 20 pmol/mg protein/30 min) and a low G_2+M DNA content (≤ 10) of which no tumour formed metastasis. This group had a significantly ($P < 0.01$) lower summed Gleason score (3.6 ± 0.89) than that associated with a second group (mean Gleason score = 7.55 ± 1.5) which maintained a significantly depleted 5α-reductase activity (< 10 pmol/mg protein/30 min) and a relatively raised G_2+M DNA content (mean ± S.D. = 18.01 ± 13.6). This latter group manifested metastasis in 90% of its tumours at the time of presentation. It is also worth noting that the entire group of patients with high 5α-reductase activity was selected for deferred treatment whereas 6 out of 9 patients within the low 5α-reductase group showed

progression and were receiving some form of treatment (details shown in Table 2). No correlation with survival was derived from the classification detailed in Table 2.

Discussion

This is the first time that the measurement of 5α-reductase activity and flow cytometry had been undertaken together on prostatic tissue from the same individuals. A survey of the literature confirms that indeed a number of earlier studies had been carried out to evaluate separately the potential of either parameter as a tumour marker in the management of prostate cancer [5, 6, 10, 15]. To date, however, the prognostic value of flow cytometry had been limited to the selection of aneuploid tumours who had a significantly worse prognosis and who seemed to die within a few years of diagnosis whatever the treatment [4, 13]. However, as aneuploid tumours account for only 10% of the prostate cancer population [12, 13] such an application of flow cytometry would be somewhat restrictive to the managment of the larger proportion of prostatic cancer.

Recently we have demonstrated that the biochemical activity of the androgen-metabilising enzyme, 5α-reductase, correlates well with the histopathological grading of prostate cancer [10]. The results detailed in the present paper confirm our earlier findings. It was therefore suggested that by jointly considering the activity of this enzyme in conjunction with flow cytometry, it would be possible to single out the aneuploid tumours and also to sub-classify the diploid tumours selecting out those carcinomas which are most likely to show an aggressive behaviour and the development of blood-borne metastases.

The combination of the two assays on the same tumour produces a prognostic index of greater significance than either investigation taken singly. Indeed the use of both parameters together provides a precise index for distinguishing those diploid tumour patients presenting with metastases (5α-reductase activity less than 10 and with more than 15% of the cells undergoing mitoses) from those who have not and are likely to benefit from a deferred treatment. Furthermore, in view of the minute quantities of tissues required for both methods, this new approach has also the added advantage of sampling the tumour at different sites, thereby catering for the heterogeneous histology of the tumour.

It should however be stressed from the outset that the measurement of 5α-reductase and indeed DNA ploidy requires considerable expertise and should only be undertaken by the specialist in the field. Clearly this is a facility which could only be offered by a few centres and perhaps it might be necessary to rationalise these tests to a multi-regional reference centre.

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