

## ORIGINAL ARTICLE

# DNA cytometric proliferative index predicting organ confinement in clinical stage-B prostate cancer

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(Original manuscript submitted July 10, 1996; received in revised form Jan. 11, 1997; accepted Jan. 18, 1997)

*Clin Invest Med* 1997;20(2):119-26.

### Abstract

**Purpose:** To assess nuclear activity by DNA flow cytometry (FCM), Gleason score and serum prostate-specific antigen (PSA) levels in predicting extracapsular tumour involvement in patients with prostate cancer.

**Design:** Retrospective pathologic study.

**Patients:** Forty patients with clinical stage-B prostate cancer who underwent radical prostatectomy.

**Interventions:** The relationship between the pathologic state and each of the proliferative index (PI), Gleason score and PSA level was analysed retrospectively with the use of archival specimens. Preoperative serum PSA levels were measured by the Hybritech assay. Gleason score was determined by 2 of the authors. Tumours were classified as stage B (confined to prostate), C1 (focal capsular penetration) or C2 (involvement of seminal vesicles or capsular perforation). FCM PI measurements were performed on deparaffinized tumour specimens.

**Results:** All 40 specimens were diploid. There were 9 pathologic stage-B, 16 stage-C1 and 15 stage-C2 tumours. The serum PSA level was 20 ng/mL or less for all patients except 2, for whom the levels were 27.8 ng/mL and 45.9 ng/mL, respectively. A Gleason score lower than 7 had a 76.0% sensitivity and 53.5% specificity in predicting organ confinement. In contrast, a PI of 21 or lower had a 84.0% sensitivity and a 73.0% specificity in predicting organ confinement, with a positive predictive value of 84.0%. Of the 17 tumours with both "favourable" features (Gleason score lower than 7 and PI of 21 or lower), only 1 (5.9%) had extracapsular involvement (stage C2). Of the 6 tumours with both "unfavourable" features (Gleason score higher than 7 and PI of 21 or higher) 5 of 6 were stage C2 and 1 was stage C1.

**Conclusion:** The single most consistent predictor of organ confinement was PI alone.

### Résumé

**Objet :** Évaluer l'activité nucléaire par cytométrie de

flux (CMF) du DNA, l'indice de Gleason et les taux sériques d'antigène prostatique spécifique (APS) pour prédire une atteinte tumorale extracapsulaire chez les patients atteints d'un cancer de la prostate.

**Conception :** Étude pathologique rétrospective.

**Patients :** Quarante patients atteints d'un cancer de la prostate au stade clinique B et qui ont subi une prostatectomie radicale.

**Interventions :** On a analysé rétrospectivement à partir de spécimens d'archives le lien entre l'état pathologique et chacun des facteurs suivants, soit l'indice de prolifération (IP), l'indice de Gleason et le taux d'APS. On a mesuré les taux sériques préopératoires d'APS au moyen de la méthode de dosage Hybritech. Deux des auteurs ont établi l'indice de Gleason. Les tumeurs ont été classées dans les catégories suivantes : stade B (confinée à la prostate), C1 (pénétration capsulaire focale) ou C2 (atteinte des vésicules séminales ou perforation capsulaire). On a mesuré l'IP par CMF sur des spécimens de tissu déparaffinisé.

**Résultats :** Les 40 spécimens étaient tous diploïdes. Il y avait 9 tumeurs au stade B pathologique, 16 tumeurs au stade C1 et 15 tumeurs au stade C2. Le taux sérique d'APS s'est établi à 20 ng/mL ou moins chez les patients, sauf dans 2 cas où il a été de 27,8 ng/mL et de 45,9 ng/mL respectivement. Un indice de Gleason inférieur à 7 avait une sensibilité de 76,0 % et une spécificité de 53,5 % lorsqu'il s'agissait de prédire le confinement à l'organe. Par ailleurs, un IP de 21 ou moins avait une sensibilité de 84,0 % et une spécificité de 73,0 % lorsqu'il s'agissait de prédire le confinement à l'organe, et une valeur prédictive positive de 84,0 %. Sur les 17 tumeurs comportant les 2 caractéristiques «favorables» (indice de Gleason inférieur à 7 et IP de 21 ou moins), une seule (5,9 %) présentait une atteinte extracapsulaire (stade C2). Sur les 6 tumeurs comportant les deux caractéristiques «défavorables» (indice de Gleason de plus de 7 et IP de 21 ou plus), 5 sur 6 étaient au stade C2 et une était au stade C1.

**Conclusion :** L'IP était à lui seul le prédicteur le plus constant du confinement de la tumeur à l'organe.

## Introduction

Prostate cancer is the most common type of cancer in North American men. Surgery is one of the main modalities in the treatment of patients with localized carcinoma. Accurate prediction of organ confinement before radical prostatectomy remains problematic. Currently, high serum prostate-specific antigen (PSA) levels and high pathologic grade (Gleason score) from biopsy specimens are associated with more advanced tumour stages. However, they have been relatively inadequate in reliably identifying patients with extra-capsular tumour involvement.<sup>1,2</sup> Several studies have shown DNA content (ploidy) to be an independent prognostic factor in patients with low-grade prostate cancer<sup>3-14</sup> as well as in those with stage-D1 prostatic adenocarcinoma.<sup>15</sup> In addition, an increase of S-phase fraction has proven to be of prognostic value in many types of cancer, such as breast, colon, ovarian and lung cancer.<sup>16-19</sup> To date, few studies have addressed this issue in prostatic carcinoma.<sup>20,21</sup>

The purpose of this study is to assess cellular proliferation as a prognostic indicator of prostatic carcinoma through: (1) determination of the proliferative index (PI), which is the total percentage of cells in the S, G<sub>2</sub>, and M phase of the cell cycle, by flow cytometry; (2) analysis of the relationship between the pathologic stage and each of the PI, the Gleason score and the preoperative PSA level.

## Materials and methods

### Patients

Forty patients with clinical stage-B prostate cancer who underwent radical prostatectomy at London Health Sciences Centre, University Campus, London, Ont., were included in this study.

### Serum PSA determination

Preoperative serum PSA levels were determined for all patients with the use of the Hybritech radioimmunoassay (Hybritech Inc., San Diego, Calif.).

### Tumour grading and pathologic staging

Paraffin blocks from the patients' prostatectomy

specimens were retrieved from archive files of the Pathology Department, London Health Sciences Centre, University Campus, London, Ont. Tissue sections were reviewed, and the diagnosis of prostate carcinoma was confirmed. Each tumour was graded with the use of the Gleason scheme<sup>22</sup> by 2 of the authors (M.M. and G.P.) working independently. Tumours with a Gleason score of 6 or lower were considered low grade, and tumours with a score of 7 or higher were listed as high-grade tumours.

Tumours were staged as: B = confined to prostate, C1 = having focal capsular penetration, C2 = involving seminal vesicles or capsular perforation.

### DNA analysis and PI determination

After review of the hematoxylin- and eosin-stained slides, areas of invasive carcinoma were outlined with a marking pen on the corresponding paraffin blocks. Paraffin blocks that contained tumours were selected for each case for flow cytometric analysis. 50- $\mu$ m-thick sections from all of the tumour foci in each prostate were processed according to a previously described modification of the method of Hedley and colleagues<sup>23,24</sup> and Frei and Martinez.<sup>25</sup> The sections were deparaffinized and rehydrated; the tissue was disaggregated through a series of enzymatic digestion. Portions of the nuclear suspensions were filtered with RNase (Sigma, Mississauga, Ont.) and stained with propidium iodide (Sigma) at 4°C in the dark over night.

### Staining for cytokeratin

The rest of the suspensions were stained over night at 4°C with cytokeratin (CK) antibody (CAM 5.2, Becton Dickinson, Mississauga, Ont.) diluted 1:5 or with nonimmune mouse IgG (MIG, Coulter Corp., Mississauga, Ont.) diluted 1:10 000. The suspensions were then stained with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (goat-anti-mouse Ig FITC, Dimension Labs, Mississauga, Ont.) diluted 1:20 and incubated in the dark at room temperature for 30 minutes.

### Data acquisition

Listmode data from each suspension were obtained

from approximately 20 000 cells or nuclei passed through an Epics Profile Flow Cytometer (Coulter).

### Analysis

All analyses were performed using Elite (Coulter) and Multicycle (Phoenix Flow System, San Diego, Calif.) software. The Elite software converted the profile data into DNA histograms. The ploidy status and the PI were determined from the DNA histograms with the use of 2 types of analysis: (1) "consensus" analysis (Elite and Multicycle software), which gives results for all of the nuclei in the sample, whether they are tumour, stromal or inflammatory cells. The software permits the user to subtract debris, sliced nuclei and clumps from the total raw count. Thereafter, it separates cell-cycle phases (Fig. 1A), and (2) CK stain analysis (Multi2D software), which is used to calculate the percentage of CK-positive cells (epithelial cells) only in each specimen or in each subpopulation in the specimen (Fig. 1B). The proportion of cells in each phase of the cell cycle among the CK-stained cells in these phases was then compared with the proportion among all of the cells in each specimen. When more than one cell population is present, the software gives the percentage of cells in each population and the DNA index and the DNA content ratio of the aneuploid G1 peak to the diploid G1 peak (Fig. 1B).

### Data evaluation

From the computer analysis, the number of cells in each sample after subtraction of clumps and debris was calculated. The percentage of CK-positive cells analysed was then calculated as the ratio of the cell number in the CK analysis to that in the corresponding "consensus" analysis of the same listmode data. This figure should be at least 10%. Ploidy was classified as diploid if there was a single G1 peak in the "consensus" analyses, corresponding to stromal, inflammatory and neoplastic cells. A second peak was considered aneuploid, and the DNA index (DI) was given by the software. Samples with more than 15% of cells in the G2/M peak and in their own G2 (octoploid) peak were considered tetraploid.

### Quality control

Histograms were considered suitable for analysis if the coefficient of variation of the diploid G0/G1 peak was less than 8%, the debris content was less than 30%, the clumps were less than 10%, the number of cells in the analysed cell population was more than 10 000, and the CK-positive cells were more than 10% of the sample in CK analyses.

### Results

Of the 40 patients with prostatic carcinoma, 9 had pathologic stage-B, 16 stage-C1 and 15 stage-C2 tumours.

The serum PSA level was 20 ng/mL or lower for all patients (median 8.6 ng/mL) except for 2 patients, in whom the level was 27.8 ng/mL and 45.9 ng/mL, respectively. There was no significant correlation between PSA serum levels and the pathologic stage.

Twenty-six patients (65%) had low-grade tumours (Gleason score 6 or lower) and 14 (35%) had high-grade tumours (Gleason score 7 or higher). Nineteen of the 26 patients with low-grade tumours (73.1%) had stage-B or -C1 tumours (tumour confined to the prostate) and 7 had stage-C2 tumours. Of the 14 patients with high-grade tumours, 8 patients (57.1%) had stage-C2 tumours and 6 had stage-B or -C1 tumours. A Gleason score lower than 7 had a 76% sensitivity and a 53.5% specificity in predicting organ confinement.

All 40 specimens were diploid. The PI was 21 or lower for 25 patients and higher than 21 for 15 patients. Of the 25 patients with a PI of 21 or lower, 21 (84%) had stage-B or -C1 tumours and 4 had stage-C2 tumours. Of the 15 patients with a PI higher than 21, 11 (73.3%) had stage-C2 tumours and 4 had stage-B or -C1 tumours. A PI of 21 or lower had an 84% sensitivity and a 73% specificity in predicting organ confinement, with a positive predictive value of 84%.

Of the 40 patients included in the study, 17 (42.5%) had combined "favourable" features (a Gleason score lower than 7 and a PI of 21 or lower). Only 1 patient (5.9%) in this group had extracapsular involvement (stage-C2 tumour). A total of 6 patients had both "unfavourable" features (a Gleason

score of 7 or higher and a PI higher than 21). In this group, 5 patients (83.3%) had stage-C2 tumours and 1 had a stage-C1 tumour (Fig. 2).

Records of clinical follow-up (digital rectal examination and PSA level) for all 40 patients were available for periods ranging from 3 to 7 (mean 3.58) years after radical prostatectomy. Eight patients had received immediate adjuvant therapy (4 received ra-

diotherapy and 4 hormonal blockade). All of these patients had tumours of at least pathologic stage C2. Two of the patients who received radiotherapy had a biochemical relapse (with a PSA level of 0.5 ng/mL) after 3 years; they had a PI of 34.15 and 26.8 and a Gleason score of 7 and 8, respectively. The remaining 6 patients continue to have no biochemical or clinical evidence of disease. Of the 32 patients who

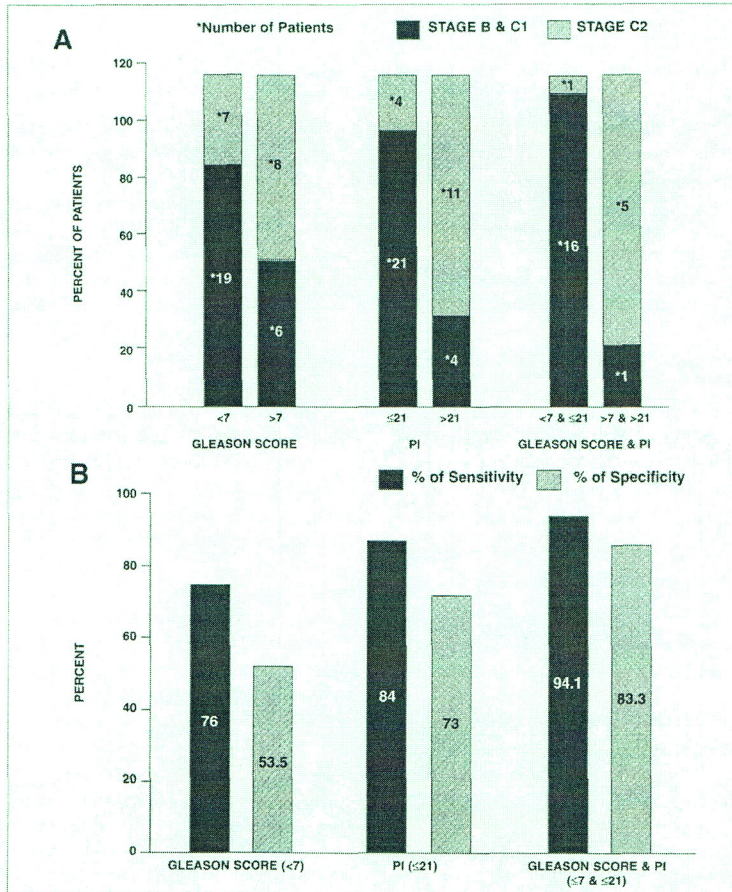


Fig. 1: DNA histograms obtained from prostate adenocarcinoma. The top graph (A) shows the result of "consensus" analysis (Multicycle software), which gives results for all the nuclei in the sample. The bottom graph (B) shows the result of cytokeratin-stain analysis (Multi2D software), which gives results for stained epithelial cells only from the sample.

did not receive immediate adjuvant therapy, 8 (25%) had early biochemical evidence of prostatic activity (a PSA level higher than 0.2 ng/mL). One patient re-

ceived delayed hormonal blockade, 1 patient received delayed radiotherapy, 1 patient refused further therapy and the remaining 5 patients (those with

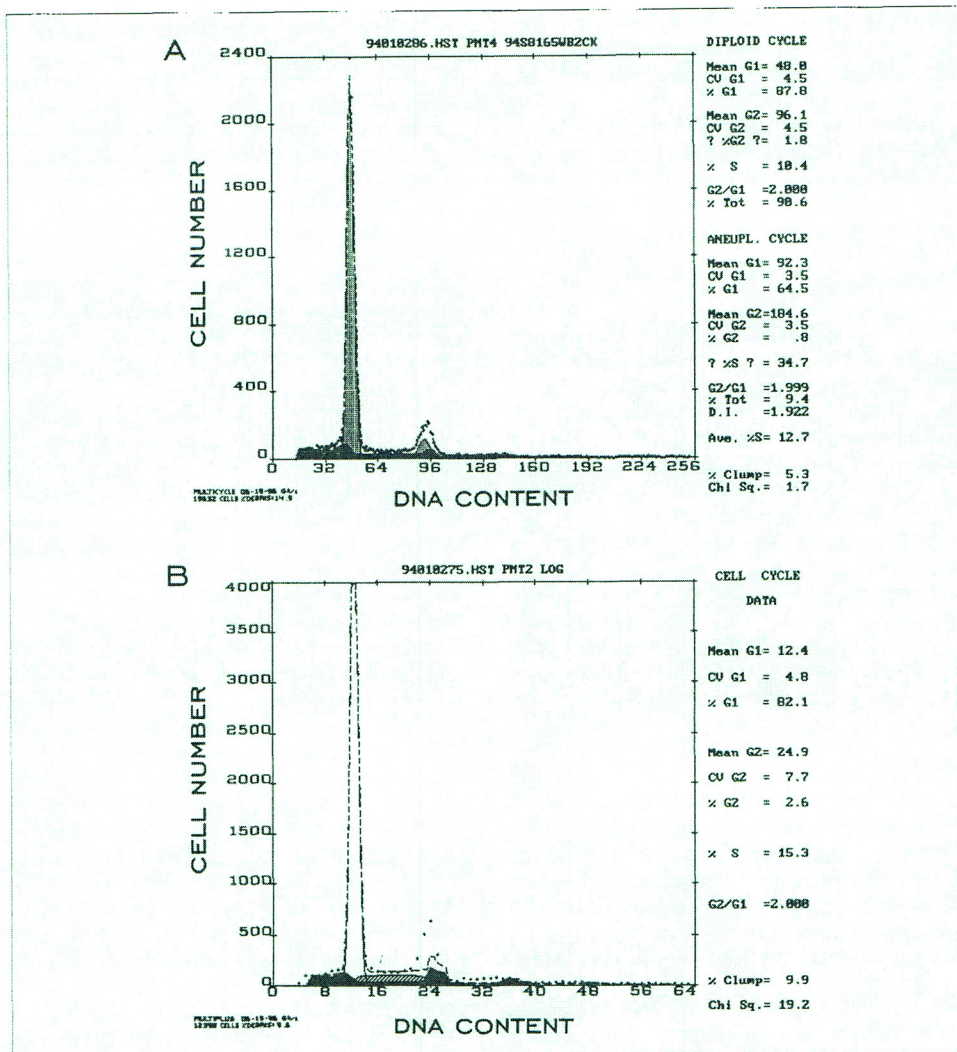


Fig. 2: The top graph (A) shows the number and percentage of patients in the sample of 40 with stage-B or -C1 (black portion of bars) and stage-C2 (shaded portion of bars) prostate tumours, according to Gleason score, proliferative index (PI) or both. The bottom graph (B) shows the sensitivity (black bars) and specificity (shaded bars) of a low Gleason score, PI or both in predicting organ-confined tumours.

PSA levels of less than 1.0 ng/mL) were under "watchful waiting." Each of these 8 patients had significant pathologic extracapsular extension. Preoperative PSA levels and Gleason scores were not statistically significant in predicting recurrence. The remaining 24 patients (75%) who did not receive adjuvant therapy currently have no evidence of disease. Of these, 20 (81%) had tumours of pathologic stage C1 or lower. In this group, the preoperative PSA level tended to be lower than 10 ng/mL (among 75% of the patients), but neither the PSA level nor the Gleason score could predict the absence of recurrence with statistical significance.

## Discussion

Although radical prostatectomy is still regarded as the treatment of choice for most younger patients with localized prostate cancer, the problems of accurate prediction of organ confinement before surgery and of proper patient selection remain. High microscopic grading of the prostate biopsy and high preoperative serum PSA levels are associated with higher stages, but these measures lack specificity.<sup>1-3</sup> More recently, studies of the DNA content of prostate cancer by flow cytometry and image analysis have generally concluded that DNA aneuploidy is associated with tumour progression and poor prognosis.<sup>5-11</sup> In addition, measurements of cellular proliferation have proven to be of considerable interest in many solid tumours,<sup>26,27</sup> surpassing DNA content as a prognostic indicator in some types of cancer, such as node-negative breast cancer.<sup>28,29</sup> Flow cytometric analysis of prostate cancer S-phase has been reported in a limited number of studies.<sup>20,21,30</sup> However, these studies lacked consistency in the predictive significance of this type of analysis.<sup>7</sup> None of these studies has assessed the PI, an indicator of nuclear activity, as a prognostic indicator in prostatic carcinoma. A recent study showed that the ploidy index was not a good prognostic indicator for prostatic cancer; however, cytokinetic parameters, such as the PI of the tumours, appeared to be more sensitive.<sup>30</sup> In that study, a significant correlation was observed between the PI and tumour stage. Our study revealed similar results; there was no difference in the ploidy status between the low- and high-grade tumours and there was no correlation between

DNA ploidy and the pathologic stages of the tumours. All of the tumours were diploid, and no aneuploid or tetraploid tumours were observed, although 40% of the analysed tumours were stage C2 (involvement of seminal vesicles or capsular perforation). Intratumoural heterogeneity of DNA content in solid tumours such as those found in renal-cell carcinoma,<sup>31</sup> gastric cancer,<sup>32</sup> colon cancer,<sup>33</sup> breast cancer<sup>34</sup> and lung cancer<sup>35</sup> as well as in prostate cancer<sup>5,36,37</sup> have been examined by several investigators. These studies have indicated the importance of sample size in detection of all DNA stemlines present in tumours. In our study, all of the tumour foci from each prostate were submitted for DNA analysis. Therefore, sampling size was not a major contributor in our results. Our findings are in keeping with those of Jones and associates.<sup>38</sup> Some investigators have proposed that the sensitivity of the flow cytometry device or the nature of fresh tissue versus archival paraffin-embedded material<sup>14,39</sup> may be factors in detecting aneuploid or tetraploid populations in prostate cancer.

None of the previous studies have used CK staining to label epithelial cells in the tissue submitted for DNA analysis. In our study we used CK as a second parameter for DNA flow cytometry analyses. The advantage of this procedure is that it identifies epithelial cells selectively in the samples submitted from the tumour foci for DNA analysis.<sup>25</sup>

In our study, there was no significant correlation between the preoperative serum PSA levels and the pathologic stage, possibly because a PSA level higher than 20 ng/mL was generally considered a contraindication for radical prostatectomy. However, our results, in keeping with those of de Leval and associates,<sup>30</sup> illustrated a significant correlation between the PI and the pathologic stage of the tumour. A Gleason score lower than 7 alone had a 76.0% sensitivity and a 53.5% specificity in predicting organ-confined tumours. In contrast, a PI of 21 or lower alone had an 84.0% sensitivity and a 73.0% specificity in predicting organ confinement.

The most reliable predictor of organ confinement or extracapsular involvement was combination of "favourable" features (a Gleason score lower than 7 and a PI of 21 or lower). Of the 17 tumours with both "favourable" features, the vast majority (94.1%) were organ-confined.

The single most consistent predictor of organ confinement was PI alone (21 or lower v. more than 21), with 21 of 25 patients with stage-B and -C1 tumours (84%) having a PI of 21 or lower and 11 of 15 patients with stage-C2 tumours (73.3%) having a PI higher than 21.

The clinical follow-up of the patients for 3 to 7 (mean 3.58) years suggests that significant extracapsular extension is associated with early recurrence of prostate cancer, and that the PSA level and the Gleason score are not strongly predictive. However, the small size of this study and the early follow-up do not provide adequate statistical power to determine the significance of this observation, which requires validation in a future study.

### Conclusion

Cellular proliferation, as measured by PI analysis, is a significant predictor of organ confinement. Assessment of tumour proliferative activity by flow cytometric analysis and determination of PI from needle biopsy specimens, along with Gleason scoring, may complement clinical data and may assist in the selection of surgical candidates.

### Acknowledgements

The authors wish to acknowledge Mrs. Violetta J. Martinez and Ms. Kris Milne of the Department of Pathology, London Health Sciences Center, University Campus and University of Western Ontario, London, Ont., for technical assistance.

This work was supported in part by the University Hospital Foundation. Preliminary results were presented at the combined Royal College of Physicians and Canadian Association of Pathologists meeting, held in Montreal in September 1995.

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