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FROM THE DEPARTMENTS OF CLINICAL PATHOLOGY, UROLOGY, AND SURGERY, CENTRAL HOSPITAL, VÄSTERÅS, THE DEPARTMENT OF ONCOLOGY, AKADEMISKA HOSPITAL, UNIVERSITY OF UPPSALA, UPPSALA, THE DEPARTMENT OF MEDICAL RADIATION BIOLOGY, KAROLINSKA INSTITUTE, STOCKHOLM, AND THE DEPARTMENT OF STATISTICS, UNIVERSITY OF UPPSALA, UPPSALA, SWEDEN.

MODAL DNA VALUES AND ESTRAMUSTINE-BINDING PROTEIN (EMBP) AS PROGNOSTIC MARKERS IN PROSTATIC CANCER

H. NORDGREN, S. NILSSON, S. EKLÖV, P. STATTIN, W. NAINING, T. HEIDEN, L. BERGKVIST, L. KARLBERG, C. LUNDHOLM, B. HARVIG, P. STRANG, R. BERGSTRÖM and B. TRIBUKAIT

Abstract

In this study, we have investigated the modal DNA values and the expression of estramustine-binding protein (EMBP) in formalin-fixed and paraffin-embedded TUR specimens from 76 untreated patients with prostatic cancer. In addition, specimens from 13 patients were analyzed for tumour EMBP expression only. Ploidy was measured as diploid, tetraploid and non-tetraploid aneuploid or aneuploid in the near-diploid region. All patients had been referred during 1978–1981, and were subjected to TUR due to urinary obstruction. Survival data were obtained for all patients through March 1988. Statistical analyses were performed using a Cox's regression model with respect to survival and cause specific survival and correlated to the DNA pattern and the expression of EMBP. The existence of a near-diploid aneuploid cell population as well as poor differentiation grade were both statistically significantly correlated with poor survival. Near-diploid aneuploid cell lines were seen in 9/76 (12%) of the patients and were also seen in well differentiated cancers (4/17). The expression of EMBP was most abundant in the moderately differentiated cancers. However, all prostatic cancer specimens investigated were positive for the antigen. Patients with poorly differentiated carcinomas and high EMBP expression showed a tendency towards better prognosis than those with poorly differentiated carcinomas and low EMBP expression. The present patient material was, however, too small to show a statistically significant correlation between EMBP and survival.

Key words: Prostatic cancer, estramustine-binding protein, EMBP, DNA, flow cytometry, prognostic marker.

Prostatic carcinoma displays great variations in its biological behaviour. The median age at diagnosis is close to 70 years, i.e. an age when other pathological conditions often affect the patient. Present classification systems are not satisfactory since there are patients among 'good risk' groups, such as well differentiated cancer, that exhibit a rapid progress in their disease and have a short survival. There is thus an urgent need for better prognostic factors and treatment selection criteria.

DNA analysis has become a useful tool in the characterization of malignant tumours and the assessment of the clinical aggressiveness of genito-urinary neoplasms such as urinary bladder carcinoma (1). In prostatic cancer a correlation has been observed between ploidy and survival, with the best survival among patients with diploid tumours and the worst prognosis in patients with aneuploid tumours with several cell lines (1, 2).

Estramustine-binding protein (EMBP) is an intracellular protein that binds estramustine, the main metabolite of estramustine phosphate (Estracyt), with high affinity (3). This protein is present in large amounts in prostatic cancer, especially in moderately differentiated tumours, and also in other neoplasms such as pulmonary cancer (4, 5). Since is it present in larger amounts in neoplastic tissues than in normal prostatic tissue and benign hyperplasias (BPH) (4), we considered this protein interesting to investigate as a possible prognostic marker.

In the present study, we have investigated model DNA and EMBP content in TUR specimens from untreated patients with prostatic cancer. The tissue specimens were obtained from TUR operations on patients who presented with obstructive symptoms.

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An improved DNA preparation technique was used to ensure a high resolution in the histograms. To reduce normal surrounding tissue as much as possible, the portions of the TUR specimens that contained cancer were cut out and re-embedded before sectioning. Before preparation of DNA, the re-embedded specimens were analyzed histopathologically and also immuno-histochemically with respect to EMBP.

Material and Methods

Patients. Eighty-nine patients with untreated, biopsy-verified prostatic cancer, T0-4 Nx, M0-1 were included in the study. All patients had been referred to the department during 1978–1981, and were subjected to TUR due to urinary obstruction. The mean age of the patients at diagnosis was 73.2 years with a range from 53 to 95 years. The main treatment for localized disease was radiation therapy. In some patients with localized disease and in patients with disseminated disease, the treatment offered was castration or estrogen/Estracyt therapy.

Tissue specimens. The TUR specimens were fixed in formalin and paraffin-embedded. Before analysis, the portions containing cancer were marked and cut out. Thereafter, these portions were re-embedded in paraffin. For the DNA analyses, two 50 μm sections were cut, dewaxed using two changes of xylene, and rehydrated in a sequence of 100, 95, 70, and 50% ethanol overnight with a tissue processor (Shandon) at room temperature. The tissue was then washed in distilled water, incubated in 0.1% protease XXIV (Sigma) in Tris buffer pH 7.2 for 30 min at 37°C while shaking. Staining was performed with 5 μmol/l DAPI in 400 μmol/l $HPO4^{2-}$. The samples were filtered through 90 μm pore nylon gauze before analysis. Sections were also obtained for morphology and for immuno-histochemistry.

Immuno-histochemistry. The tissue specimens were deparaffinated in xylol and rehydrated in graded alcohols. A mouse monoclonal antibody raised against rat EMBP and cross-reacting with human EMBP employed, utilizing the ABC technique (Vectastain, Burlingame) (6). In the negative controls, the EMBP antibody was replaced by normal mouse serum or PBS. The depelopment was performed with 3-amino-9-ethylcarbazole and the sections were thereafter counterstained with hematoxylin. The intensity in the staining was graded as ++, +, and − respectively.

DNA analysis. Samples were analyzed with a Partec (Muenster, BRD) PAS II flow cytometer powered by a mercury arc lamp. The fluorochrome DAPI was excited in the ultraviolet (350–400 nm), and the fluorescence was measured in the blue region (>435 nm). Usually about 40 000 cells were analyzed from each sample. The tumours were judged from the DNA histograms as described earlier (7) as: 1) diploid, 2) tetraploid, 3) non-tetraploid aneuploid, 4) aneuploid, multiple cell lines, 5) aneuploid in the near diploid region.

Statistics. All patients were followed through March 1988 and the data and cause of death as well as the values on acidic phosphatase, alkaline phosphatase, positive or negative bone scan and treatment were obtained from the patient records. Survival and cause of specific survival were studied using a Cox's proportional hazards model for univariate and multivariate analysis.

Results

Preliminary data presented below are focused on the correlation between DNA profile, EMBP expression and deaths due to prostatic cancer. Forty-seven of 89 (53%) TUR specimens were classified as moderately differentiated prostatic carcinomas, whereas 20/89 (22%) were well differentiated and 22/89 (25%) poorly differentiated.

DNA was prepared from TUR specimens from 76 of the 89 patients and subjected to analysis using flow cytometry. A representative DNA histogram from a diploid tumour is shown in Fig. 1A. as can be seen from Fig. 1B, showing a well differentiated tumour from another patient, the technique also allowed the identification of near-diploid aneuploid cell populations. Most of the well differentiated tumours (11/17) as well as the moderately differentiated tumours (23/41), were diploid whereas less than half (7/18) of the poorly differentiated tumours were diploid. A comparatively high proportion of the well differentiated carcinomas (4/17) exhibited near-diploid aneuploid cell populations. The corresponding fraction for the moderately and poorly differentiated carcinomas was 5/41 and 0/18 respectively. The results from the DNA analyses are summarized in Table 1.

All cancers were postively immuno-stained with the EMBP antibody. Fifty-three (60%) of the tissue specimens were intensely stained (++) and the rest (40%) moderately positive (+). The majority of the moderately differ-

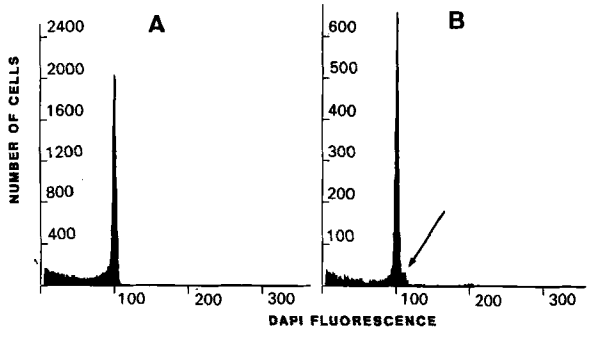

Fig. 1. A) Flow-cytometric DNA analysis of a diploid well-differentiated prostatic cancer TUR specimen. B) Flow-cytometric DNA analysis of a well-differentiated prostatic cancer TUR specimen. The arrow shows a peak corresponding to a near diploid aneuploid cell population.

Table 1

Flow-cytometric DNA analyses and histopathological characterization of prostatic cancer TUR specimens

	DNA pattern				
	1*	2	3	4	5
Differentiation grade**					
WD	11	0	2	0	4
MD	23	9	4	0	5
PD	7	6	5	0	0
Total	41	15	11	0	9

*The numbers denote: 1 = diploid; 2 = tetraploid; 3 = aneuploid; 4 = aneuploid with multiple cell lines; 5 = aneuploid with cell populations in the near diploid region.

**HD, MD, and PD denotes well, moderately and poorly differentiated carcinoma respectively.

entiated carcinomas expressed an intense staining whereas essentially all of the well differentiated carcinomas expressed a moderate staining. The poorly differentiated carcinomas formed an intermediate group with about half of the specimens expressing intense staining and the other half moderate staining for the antigen.

The results from the immuno-histochemical analyses and the histo-pathological classification are summarized in Table 2.

When death due to prostatic cancer was studied, a significantly worse prognosis was found for patients with a low differentiation grade and those with a positive bone scan at the time of diagnosis in the univariate model. Age at diagnosis, alkaline phosphatase or EMBP content in this material was not significantly associated with survival.

Patients with tumours exhibiting near-diploid aneuploid cells did worse than those with diploid tumours, although the difference was not statistically significant in the univariate model ($p = 0.065$). However, when all the explanatory variables were put together in the model, tumours exhibiting near-diploid aneuploid cells showed a clearly worse course than diploid tumours ($p = 0.0065$), as did those with low differentiation ($p = 0.0028$).

Table 2

Immuno-histochemical staining with respect to EMBP

	Differentiation grade		
	WD*	MD*	PD*
EMBP			
+	19	3	14
++	1	44	8
Total	20	47	22

*WD, MD and PD denotes well, moderately, and poorly differentiated carcinoma respectively.

EMBP content alone did not seem to be correlated with survival. However, when a stratified analysis was performed using the strata in Table 2, there was a tendency towards worse prognosis for patients with poorly differentiated tumours and a low EMBP content than for those with poorly differentiated tumours and a high EMBP content.

Discussion

The statistically significant correlation between a near-diploid aneuploid DNA pattern and poor survival is of interest. Almost 50% of the patients already had evidence of spread disease at the time of diagnosis and, interestingly, almost half of these had well-differentiated cancers whereas the rest of the group had moderately differentiated cancers. None of the patients with a poor prognosis belonged to the poorly differentiated carcinoma group. A prerequisite for the detection of these near-diploid aneuploid cell lines is the tissue preparation technique used and the high resolution of flow-cytometric analysis. This is an advantage of this technique, compared with single cell analysis on slides. Selection of tumour cells by cutting out normal tissue from the TUR specimens minimized gross 'contamination' by non-cancerous diploid cells.

The biological function of EMBP is still obscure. The protein is expressed in higher amounts in prostatic carcinomas than in benign prostatic tissue and it is most abundantly expressed in moderately differentiated carcinoma. This finding, together with the detection of the antigen in lung cancer cells (5), leads to the suggestion that EMBP synthesis in malignant cells might be due to derepression of a normally silent gene. This could in part explain the uneven distribution of the antigen in tumours with different degree of differentiation. Patients in the present study with poorly differentiated carcinoma and low amounts of EMBP measured immuno-histochemically, tended to have shorter survival than patients with poorly differentiated 'EMBP-rich' cancer. Some strata, however, were very small and an expanded study is under way.

One factor that has to be borne in mind is that tumour stages at the time of diagnosis differed between patients, as did the treatment. However, there are so far no data showing a gross benefit with respect to survival derived from early treatment in patients with disseminated disease. In fact, when patients who had received treatment in the form of hormones or Estracyt were compared with those not receiving systemic treatment, the treated group fared worse. This is maybe not surprising since the treatment was not given on a regular basis or in an adjuvant situation, but to those with the most extensive disease at diagnosis. Taking treatment into account in the multivariate model the finding of poorer survival for those with near-diploid aneuploid tumours was not changed. Studies are currently under way in our laboratory on an extended

patient material investigating the possible prognostic significance of various ploidy patterns and the expression of EMBP.

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Corresponding author: Dr Sten Nilsson, Department of Oncology, Akademiska Hospital, University of Uppsala, S-751 85 Uppsala, Sweden.

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