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Bone marrow micrometastases in patients with stage I–II localised prostate cancer

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Abstract

Prostate cancer commonly metastasises to the bones. Detection of bone marrow micrometastases (BMM) may give important information that helps define treatment strategies. This study was undertaken to analyse BMM in early prostate cancer patients and to determine the accuracy of immunohistochemical (IHC) and morphological methods in detecting cancerous cells. Preoperative core bone marrow biopsy (BMB) was performed in 103 patients with T1-2, N0, M0 prostate cancer after neoadjuvant androgen blockade. BMB were examined by IHC using monoclonal antibodies for cytokeratins (CK) (18, 19, PAN) and by cytomorphology of IHC-positive cells. In 103 patients, BMM were detected in 2 cases (2%) and an additional 3 cases (3%) were classified as suspicious. IHC alone revealed positive cells in 19 patients (18%). Cytomorphology disclosed IHC false-positive staining of some apparently normal bone marrow elements such as plasmocytes. The study shows a rather low rate of BMM in early prostate cancer. It also stresses the importance of cytomorphology as an adjunct to IHC as IHC alone may not be sufficient and appropriate for BMM detection. © 2001 Published by Elsevier Science Ltd.

Keywords: Localised prostate cancer; Micrometastasis; Bone marrow biopsy; Immunohistochemistry; Cytokeratins; Radical prostatectomy

1. Introduction

Prostate cancer often metastasises to the bone. Identification of bone marrow micrometastases (BMM) in patients with localised prostate cancer (LPC) might play an important role in clinical management, since there is no curative treatment for metastatic disease. In staging, based on currently available examinations, serum prostate-specific antigen (PSA), and bone scan (BSC) may underestimate the real extent of the disease. Approximately 30% of the patients with a negative BSC will develop osseous metastatic disease despite treatment of the primary [1–3]. A possible explanation is the presence of a potential metastatic tumour spread in the bone marrow at the time of the initial diagnosis undetectable by conventional methods, i.e. BSC and PSA. For this reason, neoadjuvant endocrine treatment prior to radical prostatectomy or radiotherapy with curative intention has been proposed to improve the therapeutic results [4,5].

Routine histopathology of the bone marrow can detect BMM only sporadically. Immunohistochemical staining using monoclonal antibodies against cytokeratins (CKs) improved the detection of micrometastatic epithelial cancers in bone marrow aspirates and/or core biopsy samples [6].

The aim of this study was to determine the frequency of BMM in patients with localised prostate cancer before the radical operation.

2. Patients and methods

The prospective study comprised 103 consecutive patients with LPC screened for elevated PSA from the Department of Urology, Sahlgrenska University Hospital, Göteborg, Sweden. The patients underwent radical prostatectomy between February 1999 and February

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2000. The study was performed under an University Ethical Committee approved protocol. The patients' age ranged from 43 to 75 years, median 62 years. All patients had a histopathologically-verified adenocarcinoma. Criteria for radical prostatectomy were PSA $\geqslant$ 3.0 μg/l, with increasing PSA-level recorded within 6 months, morphologically-verified primary, and clinical stage T1-2, N0, M0 International Union Against Cancer (UICC).

2.1. Clinical examination

The diagnostic work-up comprised a history, physical examination, and investigation of blood haemoglobin, white blood cells, platelets, (PSA), serum creatinine, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP) and bilirubin. Patients with a PSA value $>$60 μg/l were considered not to have a localised tumour and were not included in this study.

2.1.1. T-stage evaluation

Digital rectal examination was used according to the TNM classification system 1992. Two clinicians at the urological department were responsible for this evaluation. A suspected tumour extension into the extraprostatic urethra excluded the patient from the study. Biopsy was obtained with a transrectal ultrasound-guided tru-cut biopsy. A description or sketch of the areas of local changes if seen on the ultrasound were provided. The diameter of the largest hypoechoic lesion was recorded.

2.1.2. N-stage evaluation

A pelvic node dissection was performed as well as the prostatectomy. Bilateral lymph nodes from the obturator fossa were examined. Frozen section was only obtained if macroscopically-enlarged nodes were present, in order to exclude node-positive patients.

2.1.3. M-stage evaluation

If the PSA value was $>$10 μg/l, a technetium bone scan was performed. When indicated, complementary skeletal X-ray was done.

2.1.4. Treatment

All patients received total androgen blockade with the gonadotrophin-releasing hormone (GnRH) antagonist leuprolerine (Enanton® depot) 3.75 mg subcutaneous (s.c.) every 4th week plus anti-androgen flutamide (Eulexin®) 250 mg three times daily (t.i.d.) orally for 3 months prior to radical prostatectomy and bone marrow biopsy (BMB).

2.1.5. Bone marrow biopsy and immunostaining

Before admission to the study, each patient gave full oral and written information. After a signed informed consent, unilateral BMB was obtained from the posterior iliac creast with an 8 gauge trephine (MD TECH Medical Device Technologies, Gainesville, FL, USA) under general anaesthesia immediately prior to the radical prostatectomy. Two core specimens were taken from each patient. BMB columns were fixed in 4% neutral formaldehyde and decalcified in 15% nitric acid with 1.5% chromium trioxide for 8 h. After embedding in paraffin, the tissues were cut into 7 μm thick sections. The sections were stained with haematoxylin-eosin (HE) and for CK expression. Prior to immunohistochemistry (IHC), all sections were processed in a Micromed Basic microwave unit (Milestone, Sorisole, Italy) at 800 W for 15 min and at 300 W for 25 min afterwards, to revitalise the antigens. For CK detection, the sections were incubated for 2 h with anti-CK monoclonal antibodies (MAbs) DC-10 (CK18-specific), BA-17 (CK19 specific), C-11 (PAN-CK) that were developed and characterised at the Masaryk Memorial Cancer Institute [7] and with the AE1/AE3 CK cocktail (Biogenex, San Ramon, CA, USA). To detect specific binding, the alkaline phosphatase technique was used employing EnVision TM + kit (Dako, Copenhagen, Denmark) with the New Fuchsin Substrate Pack (Biogenex). Endogenous phosphatase activity was blocked with 2.5 mM solution of levamisole. As a negative control, an unrelated primary antibody was used. Two sections of each specimen were stained with each monocloncal antibody (MAb) and examined by two experienced pathologists. Discrepant results were resolved by consensus.

Immunohistochemical procedure was complemented by careful morphological analysis of the stained cells using the LUCIA G (LIM, Prague, Czech Republic) image analysis system. Suspicious or positive structures were captured by 3-CCD (3-cold charged device) TV camera (HITACHI, Tokyo, Japan) and stored in a computer. Some image preprocess or transform functions of the LUCIA system, e.g. contrast enhancement, sharpening, gamma or red, green, blue (RGB) corrections, were used to show selected details of cellular structure in several unclear cases. The cytomorphological criteria, e.g. shape and size of the cell, cytoplasmic–nuclear ratio, number and feature of the nucleoli and chromatin structure were evaluated to determine the malignant origin of the positively labelled cell.

2.1.6. Classification system

The sample was considered positive for BMM when at least five dissociated cells or one aggregate of cells was positively labelled with any of the MAbs and malignant cytological features could be identified.

Samples with 1–4 cells showing CK positivity in at least one immunoreaction, but with or without clear morphological criteria suggesting malignancy, were considered as suspicious for BMM [8]. If only one positive cell was found, but without evident blastic or

Table 1
Patient characteristics of the 103 men with prostate cancer clinical stage I–II

Age at diagnosis (years)	Patients *n* (%)
<60	30 (29)
⩾60	73 (71)
S-PSA (μg/l)	
3.0–9.9	77 (75)
⩾10	26 (25)
Clinical stage	
T1a	6 (6)
b	1 (1)
c	47 (46)
2a	32 (31)
b	17 (17)
Biopsy Gleason score	
4	2 (2)
5	8 (8)
6	55 (53)
7	30 (29)
8	7 (7)
9	1 (1)
Total no. of patients	103 (100)

S-PSA, serum-prostate specific antigen.

malignant cytology, e.g. nucleoli or chromatin details not visible or chromatin details not preserved, such cases were classified as negative or not suspicious. Normal bone marrow cells were occasionally non-specifically immunostained. These cells were not taken into consideration.

Using IHC alone, and not considering the morphology, the sample was determined as positive when any cell showed a medium or high intensity of red cytoplasmic immunostaining with any of the CKs. Weakly stained cells were not determined as positive.

3. Results

Patient characteristics are listed in Table 1. The median age was 62 years, range 43–75 years. Prior to radical prostatectomy, the serum PSA level ranged up to 9.9 μg/l in 77 patients (75%). 26 patients (25%) had continuously increased S-PSA values equalling or exceeding 10 μg/l. The majority of cases were classified as T1c and T2a, (46 and 31%, respectively). Patients had a biopsy Gleason score of 6 in 55 cases (53%) and a score of 7 in 30 cases (29%), respectively.

3.1. Detection of metastatic cells

Table 2 summarises the results expressed as positive, suspicious and negative biopsies. The table comprises separate results obtained with the combined IHC and morphological approach and those based on the IHC data only. Although the overall frequency of metastatic cells in the specimens is rather low, there is a striking difference in the number of positive cases between these two methods. Combined IHC and morphological analysis revealed 2 cases positive for BMM (cell clusters, Fig. 1a and b), and 3 suspicious cases (Fig. 1c), whereas IHC identified another 14 positive cases. Non-specifically labelled, apparently normal elements exemplified in Fig. 1e–g account for this discrepancy. The most frequent falsely stained cells represented plasmocytes, often showing a strong plasmatic CK-positivity (Fig. 1g). Moreover, occasional CK-positive histiocytes (macrophages or siderophages) were identified, although the staining intensity was low or moderate (Fig. 1e and f). In 2 cases only, one CK-positive cell was found without malignant cytomorphological appearance (Fig. 1d). The above cases were all classified as negative. Routine HE-staining did not reveal any positive finding in the whole patient set.

4. Discussion

Our study identified BMM in 2 (2%) patients. In addition, 3 (3%) cases were suspicious. In the previous studies, the prevalence of IHC-positive cells in BMB, classified as BMM in untreated LPC patients, varied between 23.7 and 54.5% [9,10]. The selection of patients may account for some of the differences; in our group, there were patients with early LPC diagnosed by screening for elevated S-PSA who received neoadjuvant

Table 2
Results of bone marrow biopsy in 103 patients with prostate cancer stage I-II according to the mode of evaluation

	Bone marrow biopsy			
	No of patients			
Mode of evaluation	Positive *n* (%)	Suspicious *n* (%)	Negative *n* (%)	Total *n* (%)
Immunohistochemistry	19 (18)	–	84 (82)	103 (100)
Immunohistochemistry + morphology (BMM)	2 (2)	3 (3)	98 (95)	103 (100)

BMM, bone marrow micrometastasis.

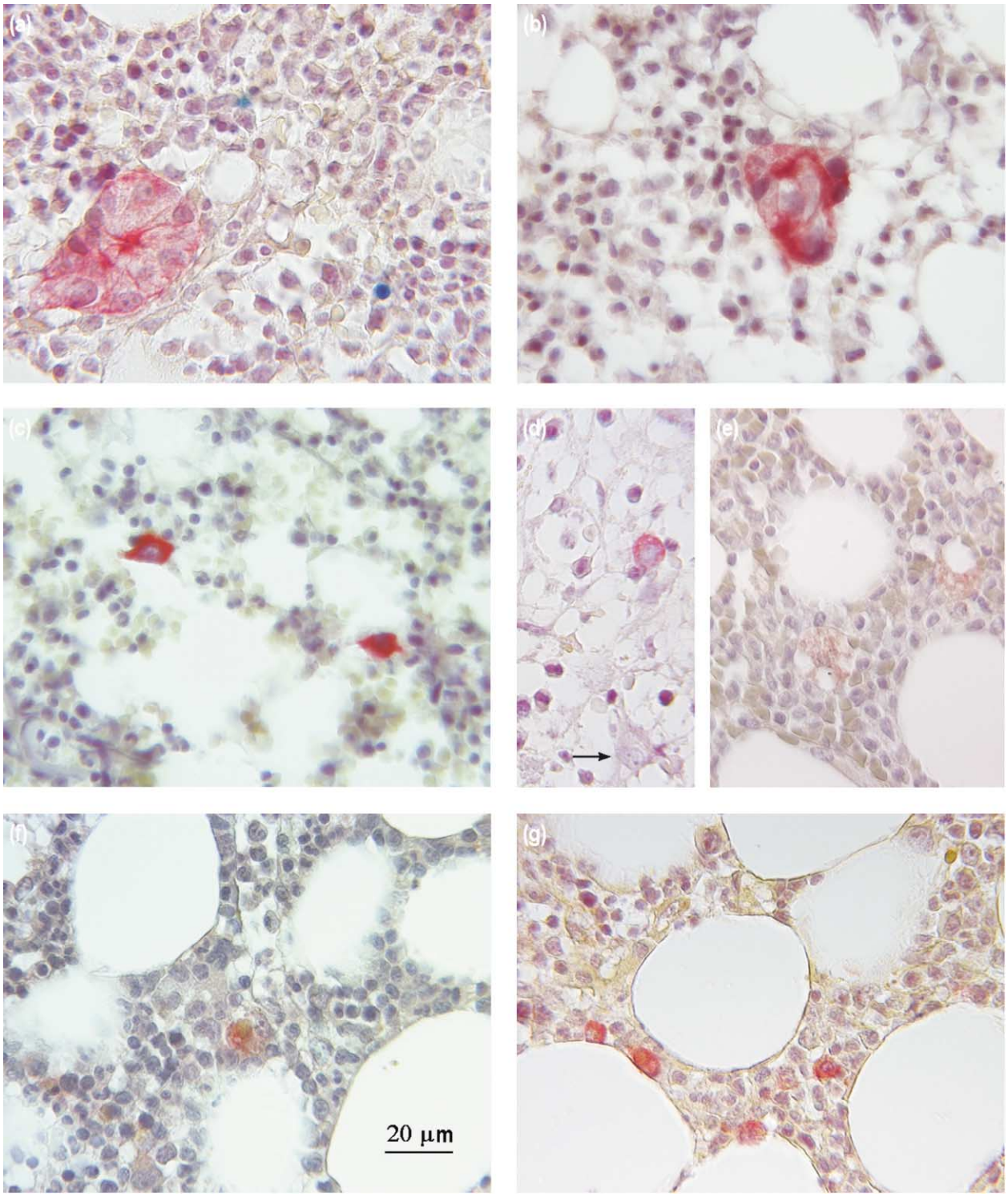

Fig. 1. Cytokeratin 18 immunohistochemistry: (a) Acinar micrometastasis. Blue-green sideroblast is also evident. Pearls reaction, in addition to immunohistochemistry. (b) Irregular micrometastatic aggregate. Heterogeneous positivity for cytokeratin. (c) Suspicious case: Two strongly positive cells with malignant morphology. (d) Negative case: Only one positive cell not reaching morphological criteria of malignancy. The nucleus is analogous to the nucleus of a negative histiocyte (arrow). (e) Non-specific immunostaining—macrophages with plump cytoplasm, weakly positive. (f) Non-specific staining of a siderophage. Yellow-brown granule of haemosiderin is evident in continuity with red immunoproduct. (g)Non-specific immunostaining of several plasmocytes. Plasmocyte nuclei are small and have typical excentrical location.

total androgen blockade prior to BMB. Furthermore, Solomayer and colleagues [11] demonstrated in patients with breast cancer a lower sensibility for detecting BMM using a core BMB compared with an aspiration technique.

Different IHC results in BMB could also be explained by the use of different methodologies, including variations in the definition of BMM. A BMM occurs when there has been implantation of tumour cells in the bone marrow, and often a reaction of the surrounding tissue, assuming that the diagnosis of BMM is correct only by histopathological means as has currently been proposed by Hermanek and colleagues [12]. Based on our experience with BMM detection in breast cancer patients with a 20-year follow-up, we defined BMM when at least five single or an aggregate/cluster of clearly MoAb-labelled cells were detected and could be morphologically classified as malignant [8].

In our study, we strived to reach a high sensitivity of BMM detection simultaneously with high specificity. Cytomorphological approach allowed the elimination of non-specific immunostainings that occasionally occur in some plasmocytes, histiocytes, megakaryocytes, macrophages or siderophages. Moreover, cases with only one redly-immunostained cell, but without evident malignant cytology, were considered as BMM-negative. In some rare cases, the very intensively stained immunoproduct can obscure the nuclear details. In these cases, we analysed an additional set of slides of the particular case with a diluted anti-CK antibody.

Omitting the cytological evaluation of the immunostained cells, the number of positive cases was increased to 19 (18%) which is an increase of 14 cases when compared with the total of 5 patients (5%) confirmed by cytological morphology. As expected, the number of positive stains was different among the anti-CK MAbs used in this study, but these differences were not statistically analysed because of the low number of cases.

It is suggested that the non-cytomorphological procedures on bone marrow aspirates can lead to a great number of false-positive findings, leaving non-specific labelling undetected [13]. Plasmocytes, in particular, have been shown to exhibit quite strong non-specific cytoplasmic staining [14] and our results were in agreement with this observation. This is probably caused by hydrophobic interactions between the primary antibodies and immunoglobulins that can be present in large amounts in the cytoplasm of a particular plasmocyte. Different blocking sera and detergents may diminish this non-specific hydrophobic binding. In fact, we did try different protocols, but without success. As shown in our material (Fig. 1a), the iron staining reaction may be used following the IHC for identification of siderophages which in some cases show non-specific immunostaining. Furthermore, the problem of false-positives can be overcome using a double staining analysis of CKs combined with CD45 as demonstrated in flow cytophotometry (FCM) [15] or immunocytochemistry [16].

Polymerase chain reaction (PCR), another non-morphological method, has also been shown to exhibit a high percentage of false-positive results that are often difficult to interpret [17]. Using PCR, Lange and Vessella [18] have found that the high rate of bone marrow positivity before radical prostatectomy did not correlate with the clinical results; approximately 15–30% of the patients developed clinical recurrence and not all of these progressed to osseous metastases. In the follow-up of these patients by PCR, it was found that a few remained PCR-positive after surgery, but that the majority reverted to a PCR-negative status within 6–9 months.

In our experience, various anti-CK antibodies—CK18, CK19, PAN-CK—did not seem to differ in terms of sensitivity of BMM detection. These MAbs showed comparable positive results in our sample of cases that were used as positive controls. However, cells of particular micrometastasis may be heterogeneous in the CK staining intensity. A MAb cocktail was developed to label 100% of carcinoma cells to identify BMM in breast cancer [19]. Nevertheless, sampling error may be important as BMM appear in only some of the serial sections.

Further studies comparing these different techniques, as well as establishing their prognostic significance and therapeutic value are needed.

The clinical significance of detected micrometastatic tumour cells in BMB from a clinical perspective has been reviewed by Braun and Pantel [20]. Weckermann and colleagues [21] observed in patients with LPC that only individuals with three or more CK18-positive cells in BMB tended to have a poor prognosis, but this was not statistically significant. As demonstrated by Cher and colleagues [22] in patients with clinically LPC, there is a wide variability in the prevalence of micrometastatic cells in BMB and the cell proportion that is proliferating. For further evaluation of the clinical significance of IHC-positive cells in BMB, the results should be documented according to uniform criteria and isolated IHC-positive cells should be distinguished from BMM [12].

After 1 year of follow-up, 3 patients have died of intercurrent disease without cancer. There were no clinical relapses or sign of metastatic disease in the remaining patient group of 100 patients. All 103 patients were symptom-free. In the follow-up, we defined laboratory relapse when S-PSA exceeded 0.4 μg/l in two serum samples. In the two BMM-positive patients, PSA was unmeasurable, and only 1 of the 3 BMM suspicious patients had a S-PSA of 0.74 μg/l. PSA-relapse has occurred in 10 of the 98 BMM-negative patients, varying within 0.7–10.4 μg/l. A long-term observation of the whole patient group will be informative, although our

results indicate that BMB is not required as a staging procedure for early localised prostate cancer (stage I–II). In conclusion, we found 98 patients out of 103 were negative for BMM. Long-term follow-up is necessary to determine the clinical relevance of the positive and suspicious findings in the remaining 5 patients. We stress that quality assurance must be maintained and that, attention must be given to the risk of false interpretation when the BMM detection rate relies solely on immunohistochemistry staining for CK and neglects the cytological morphology.

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