SPANDIDOS PUBLICATIONS

Presence of prostate cells in bone marrow biopsies as a sign of micrometastasis in cancer patients

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Abstract. The presence of prostate cancer cells in bone marrow of patients with clinically localized disease is associated with increased chance of disease recurrence. The presence of prostate specific antigen (PSA) in bone marrow aspirates has been used to indicate the presence of micrometastasis. The aim of this study was to present a prospective study of prostate cancer patients to determine the presence of cells that express PSA in aspirates taken from bone marrow and to compare with bone marrow biopsy samples. Results indicated a significant difference between the frequency of cells detected in bone marrow aspirate and biopsy samples ($P<0.0002$) when all patients were considered. There was no difference between the frequencies of cells detected in bone marrow aspirate and biopsy of patients analyzed before treatment. However, there was a significant ($P<0.003$) difference between them after treatment. There was also a significant difference in the frequency of PSA positive cells detected ($P<0.005$) in Stages I to IV as well as in the frequency of cells detected ($P<0.0002$) when analyzed according to Gleason score. The present results explain the lack of correlation between positive aspirates and prognosis in numerous clinical cases.

Introduction

Prostate cancer is the most common type of cancer in men and the second leading cause of cancer death in Chile with a high incidence of bone metastasis (1). The presence of prostate cancer cells in the bone marrow of patients with clinically localized disease is associated with an increased chance of disease recurrence. However, not all patients develop recurrence. It has been reported that the presence of prostate cells in the bone marrow is associated with disease-free survival in patients undergoing radical prostate- ctomy (2). These authors evaluated the bone marrow of 86 patients with clinically localized prostate cancer treated by radical prostatectomy for the presence of prostate cells using molecular biology studies.

Prostate cancer cells frequently metastasize to the skeleton, and it has been hypothesized that this environment selectively supports the growth of these tumours. Specifically there is strong evidence that interactions between tumour cells and bone marrow stromal cells play a major role in supporting prostate cancer growth and survival in bone (3). Bone metastasis is incurable and contributes significantly to disease-specific morbidity and mortality. The molecular and cellular mechanisms leading to the development of bone metastasis in prostate cancer remain unclear, but are currently under intensive investigation (4,5). There is a genetic predisposition that makes prostate cancer cells more prone to spread to bone. Bone-derived factors are involved in the development of bone metastasis at the level of the local microenvironment, suggesting interactions between metastatic prostate cancer cells and endothelial cells, osteoblasts and osteoclasts from bone marrow.

The presence of prostate specific antigen (PSA) and cytokeratin positive cells in bone marrow aspirates has been used to indicate the presence of micrometastasis. There is an association with tumor stage, Gleason score and time of biochemical failure in cancer survival (2,6-8). The cells showed the same cytogenetic abnormalities of the original tumor (9). The aim of this study was to present a prospective study of prostate cancer patients to determine the presence of cells that express PSA in bone marrow aspirates and to compare with bone marrow biopsy samples.

Materials and methods

Prostate cancer patients. A prospective study of prostate cancer patients was performed. The inclusion criteria was a confirmed diagnosis of prostate cancer and a written consent to allow bone marrow aspiration and biopsy with sedation and local anesthesia, without medical contra-indications for the procedure. Bone marrow samples of 71 men with prostate cancer with a mean age of 74.3 (SD ±11.54, range 47-91) years were analyzed from the Institute of Radio-Oncology,

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Table I. PSA protein expression in cells present in bone marrow aspirate (BMA) and biopsy of prostate cancer patients.

A, Total number of patients distributed by stage TNM before and after treatment (n=71)

Stage TNM	Total no. of patients before treatment	Total no. of patients after treatment	Total no. of patients
I	10	9	19
II	17	8	25
III	19	6	25
IV	1	1	2
Total	47	24	71

NS, non-significant.

B, Comparison between PSA protein expression in cells of bone marrow aspirate (BMA) and biopsy in the total number of prostate cancer patients (n=71)

	Positive bone marrow biopsy	Negative bone marrow biopsy	Total
BMA			
Positive	30	3	33
Negative	19	19	38
Total	49	22	71

P<0.0002, Chi-square.

C, Comparison between PSA protein expression in cells of bone marrow aspirate (BMA) and biopsy of total number in prostate cancer patients before treatment (n=47)

	Positive bone marrow biopsy	Negative bone marrow biopsy	Total
BMA			
Positive	24	2	26
Negative	16	5	21
Total	40	7	47

NS, non-significant (Two-tail Fisher's test).

D, Comparison between PSA protein expression in cells of bone marrow aspirate (BMA) and biopsy of total number of prostate cancer patients after treatment (n=24)

	Positive prostate biopsy	Negative prostate biopsy	Total
BMA			
Positive	6	1	7
Negative	3	14	17
Total	9	15	24

P<0.003, Two-tail Fisher's test.

Table I. Continued

E, Comparison between PSA protein expression in cells of bone marrow aspirate (BMA) and biopsy of total number of prostate cancer patients considering stages (TI-TIV) (n=72)

Stage	Positive BMA	Positive bone marrow biopsy	Total
TI	5	8	13
TII	9	16	25
TIII	17	13	30
TIV	2	1	3
Total	33	38	71

P<0.005, Chi-square.

F, Comparison between PSA protein expression in cells of bone marrow aspirate (BMA) and biopsy of total number of prostate cancer patients considering Gleason score (≤5-8) (n=72)

Gleason	Positive BMA	Positive bone marrow biopsy	Total
≤5	16	12	28
6	10	15	25
7	5	9	14
≥8	2	2	4
Total	33	38	71

P<0.002, Chi-square.

Santiago, Chile and samples used were from January 2005 to August 2007. Forty-seven samples were taken before treatment and 24 samples after treatment. The removal of the samples was prior to definitive treatment or between 6 months and 1 year after definitive treatment, to avoid contamination by cells released at surgery. The exclusion criterion were androgen blockage or systemic therapy. The disease stage and Gleason score were considered. Twenty women undergoing bone marrow aspiration and biopsy for hematological disorders were used as controls. Bone marrow aspirates and biopsies were obtained. We performed two types of exams: i) aspiration of bone marrow (4 ml) from the posterior superior iliac crests collected in EDTA, and removal of blood to obtain mononuclear cells that were separated using Histopaque® 1,077 and differential centrifugation according to instruction (Sigma-Aldrich, St. Louis, MO) and ii) removal of fresh biopsy sample smeared onto 2 silanized slides, and the bone core placed into 40% formalin.

Immunocytochemical studies. Gel differential centrifugation was used to concentrate the sample to remove any fragments of the original sample, allowing only free floating cells or small clumps. The cells were washed in PBS, pH 7.4 and suspended in concentrated autologous plasma for a suspension of two million cells per ml and 25 μl to prepare slides. All slides were air dried for 24 h and then fixed in

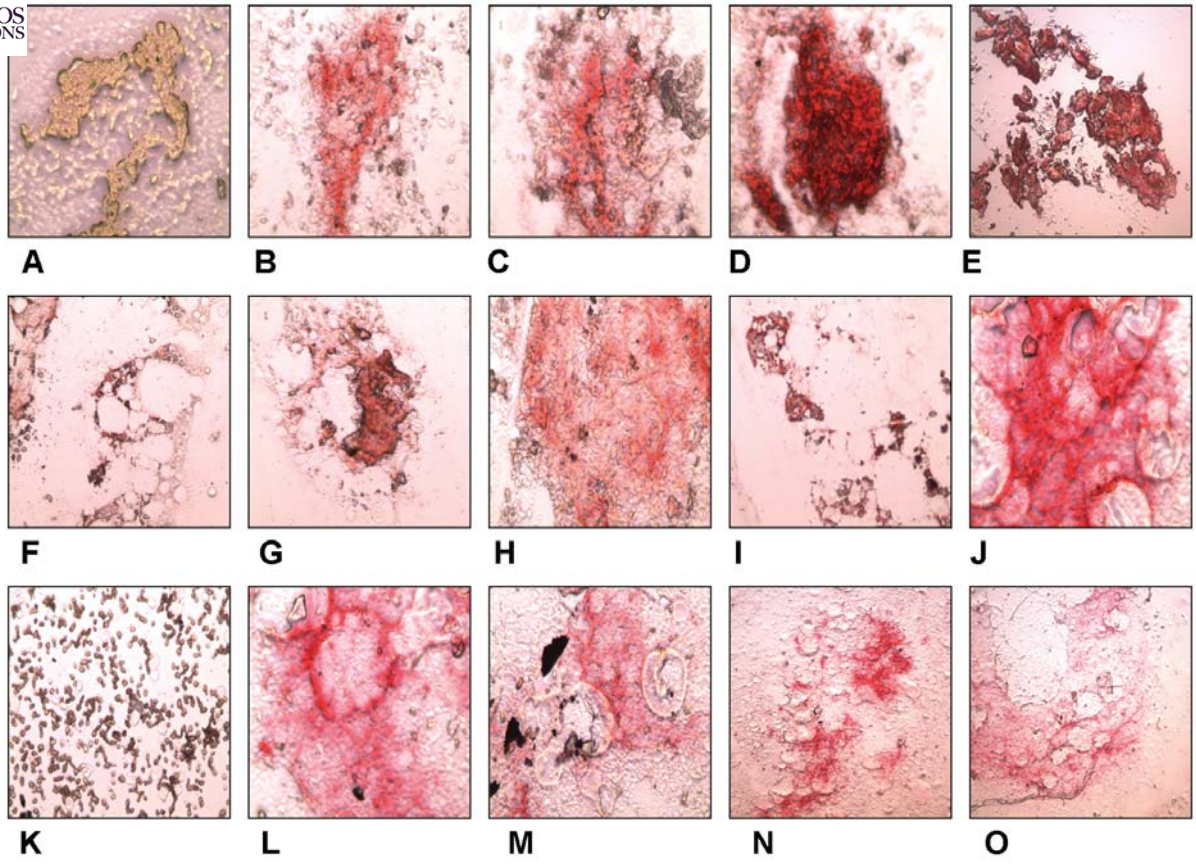

Figure 1. Representative images of PSA protein expression in bone marrow biopsies: (A) negative and (B-O) positive cases.

70% ethanol, 5% formalin and 25% PBS pH 7.4 for 3 min and finally washed in PBS. Monoclonal antibodies against PSA Clone 28A4 at a concentration of 2.5 μg/ml (Novacastra, UK) were incubated at room temperature for 1 h. Prostate cells were identified in the samples by using the LSAB2 kit according the manufacturer's instructions (Dako, USA) based on alkaline phosphatase-antialkaline phosphatase with neofuschin as chromogen. The definition of a prostate cell was a cell with a nucleus staining positive for PSA, based on the criteria of other authors (10). Levamisole (Dako) was used as an endogenous alkaline phosphatase inhibitor. Prostate cells were identified using ocular x10 and objective of x10 and then confirmed with objective x80 of light microscopy. Photographs were taken using a digital camera (Nikon, Japan).

Statistical analysis. Descriptive statistics were used to analyze demographic features, Chi-squared to compare frequencies and ANOVA for differences in means. An α error of 0.05, a ß error of 0.20 and $p<0.05$ was considered statistically significant. For ethical considerations the study was performed in strict accordance with the principles of Helsinki and the modifications of Hong Kong and Venice, and the Chilean Health Ministry's guidelines.

Results

The presence of circulating prostate cells in bone marrow aspirates as well as in bone marrow of prostate cancer patients was analyzed by determining PSA protein expression. Table I, A-F corresponds to PSA protein expressions in cells present in bone marrow aspirate (BMA) and biopsy of cancer patients.

Table I, A corresponds to total number of patients distributed by stage TNM before (n=47) and after (n=24) treatment. There was no significant difference between the frequencies of cells detected in bone marrow aspirate and biopsy of patients analyzed before treatment. Table I, B shows a comparison between positive or negative PSA protein expression in bone marrow aspirates and biopsies in the total number of prostate cancer patients. Results indicated a significant difference between the frequency of cells detected in bone marrow aspirates and biopsy samples ($P<0.0002$, Chi-square) when all patients were considered (Table I, B). There was no difference between the frequencies of cells detected in bone marrow aspirates and biopsy of patients analyzed before treatment (Table I, C). However, there was a significant ($P<0.003$) difference after treatment (Table I, D) and in the frequency of PSA positive cells detected when Stages from I to IV ($P<0.005$) were considered (Table I, E) and also according to Gleason score ($P<0.0002$) (Table I, F).

Fig. 1 shows images of PSA protein expression in bone marrow biopsies both negative (A) and positive (B-O) cases. Fig. 2 corresponds to images of PSA protein expression in bone marrow aspirate of prostate cancer patients. Negative cases were observed (A to C), positive smear (D), positive aspirates (E-H) and bone marrow fragments (I-L).

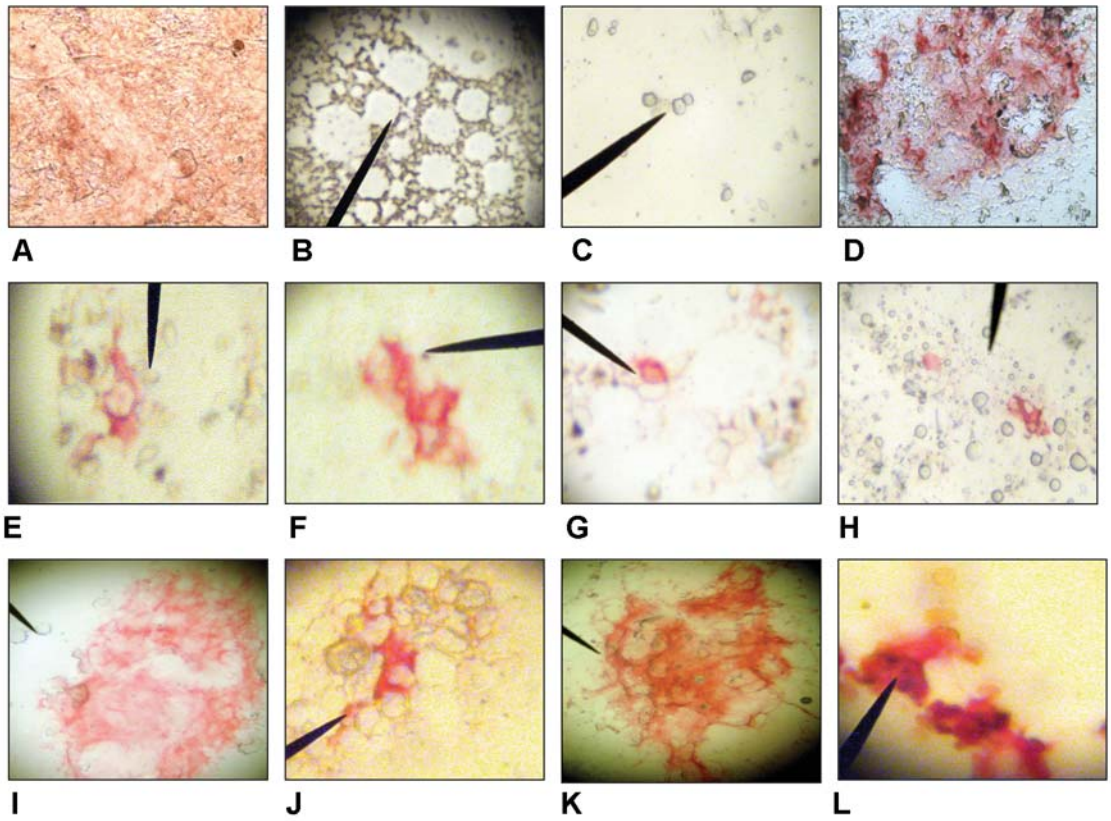

Figure 2. Representative images of PSA protein expression in bone marrow aspirate of cancer prostate patients: (A-C) negative cases, (D) positive smear, (E-H) positive aspirates and (I-L) positive bone marrow fragments.

Discussion

These studies analyzed the presence of prostate cells in bone marrow aspirates and biopsy samples in order to determine prevalence of micrometastatic cells in the bone marrow of men with histological confirmed prostate cancer. The patients were analyzed by the TNM stage. There was a significant difference in the frequency of detection of PSA expressing cells in aspirate and biopsy when stages were considered. Similar findings have been previously reported for stage TI and TII (2,6,8,11). There was also a significant difference between the frequency of PSA positive cells detected in aspirate and biopsy when analyzed according to Gleason score. There was no significant difference in patients with higher Gleason score. Previous studies have shown a frequency between 23 and 38% for Gleason score of ≤6 (6,8,11).

To improve cell sample numbers before immunocytochemical detection, samples were concentrated by using gel-based differential centrifugation. This system removes any fragment from the original sample and thus only floating cells or small clumps were analyzed. Bone marrow biopsies are not routinely used for immunochemical detection since the use of standard pathology methods of decalcification destroys many epitopes, thus rendering an immunochemical analysis doubtful as to the presence or absence of specific cell markers. However, the use of 'touch preps' from fresh samples, and analysis of the cellular imprint has been widely used in hematology to obtain a more rapid result, while awaiting formal histology. The use of such touch preps could be important to determine the presence of micrometastasis in bone marrow since it permits the analysis of micro-fragments with immunochemical techniques.

Other studies (7) have shown that prostate micrometastasis have a low proliferating index thus it would be expected that the cells remain adherent to the endostium and not in the intertrabecular spaces, that is negative aspirate and biopsy positive. This suggests that bone marrow aspirates may not truly represent micrometastatic disease due to bone marrow cell kinetics. This may help to explain why patients have a relapse of the disease with negative bone marrow aspirate. It is postulated that a bone marrow aspirate, for its nature, allow to get either a sample of cells that are in transit or a sample that are removed from the endostium. The cells in transit could be circulating from the original tumor, or have detached from proliferating micrometastasis.

There are no studies of *in vivo* tumor cell rheology in the bone marrow. However, there are *in vivo* optical imaging studies in laboratory animals to demonstrate the mechanisms of tumor cell attachment to the endostium that are similar to stem cell engraftment (12,13). Topological and chronological patterns of stem cell seeding have shown that most cells drift within the bone marrow space, then are gradually found close to the endosteal surface. The center of the bone marrow space seems to be the site of proliferation of transplanted cells and not the endosteal surface (14). Further studies have shown that the adherent cells are viable, whereas cells in transit contain a percentage of dead or dying cells (15).

ntal data suggest that adhesion is the rate limiting int of homing and early seeding and a crucial event that preserves the viability of cells towards successful engraftment (15). Since cells attached to the endosteal surface are not usually detected it may explain why not all patients with positive bone marrow aspirate develop metastasis. Conversely, patients with negative bone marrow aspirate may have cancer cells attached to the endosteal surface that later may develop into metastasis. This may explain in part the lack of correlation between positive aspirates and prognosis in some studies (16,17). Results of this study are consistent with this theory. Based on the present studies larger studies are suggested to redefine the concept of bone marrow micrometastasis for only those patients with positive fragments.

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