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Original Article

NKX3.1 and PSMA are sensitive diagnostic markers for prostatic carcinoma in bone metastasis after decalcification of specimens

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Abstract: Background: Prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), and NKX3.1 are sensitive and prostate-specific markers frequently used for the diagnosis of metastatic prostate cancer (mPCa). International Society of Urological Pathology recommends use of PSA as the initial immunohistochemical (IHC) marker to identify PCa. If the tumor is equivocal/weak/negative for PSA, then P501S and NKX3.1 stains are suggested. However, no specific studies have attempted to compare the staining sensitivity of these markers post specimen decalcification of bone specimens. In this study, we analyze the staining sensitivity of PSA, PSMA and NKX3.1 in bone specimens with mPCa after decalcification. Design: We studied 24 cases of mPCa to the bone. All cases were decalcified for 24 to 48 hours prior to H&E staining and IHC workup. Eight cases were biopsies from vertebral bodies, five were from the femur, four came from the iliac bone, two were from ribs, three were from the pubic ramus, one was from the sacrum and one was from the skull. IHC staining pattern of PSA, PSMA and NKX3.1 was defined as follows: negative (no staining), focally positive (≤ 10%) and diffusely positive (≥ 10%). Focal and diffuse positivity are both considered positive. Results: PSA was positive in 64% (14/22) cases, while PSMA and NKX3.1 were positive in all cases (17/17 and 24/24, respectively). The frequency of positive PSMA staining in decalcified samples of prostatic carcinoma metastatic to the bone is statistically higher than that of PSA staining, when analyzed by the Chi-square test (Chi-square statistic: 5.389; P = 0.0203). The frequency of positive NKX3.1 staining in decalcified samples of prostatic carcinoma metastatic to the bone is also statistically higher than that of PSA staining, when analyzed by the Chi-square test (Chi-square statistic: 10.56; P = 0.0012). When comparing PSMA and NKX3.1 positive staining, NKX3.1 tended to be diffusely positive at a higher frequency. However, this difference was not statistically significant. Conclusion: This study demonstrates that PSMA and NKX3.1 are more sensitive markers than PSA for mPCa to the bone following decalcification. We recommend use of PSMA and NKX3.1, rather than PSA, as the IHC markers to confirm mPCa to the bone.

Keywords: NKX3.1, PSMA, prostatic carcinoma, bone metastasis

Introduction

Prostate specific antigen (PSA), prostate specific membrane antigen (PSMA) and NKX3.1 are sensitive and prostate-specific markers frequently used for the diagnosis of metastatic prostate cancer (mPCa). The International Society of Urological Pathology recommends the use of PSA, PAP, prostein and NKX3.1 as immunohistochemistry (IHC) markers to identify prostatic origin in metastatic carcinomas of unknown origins [1]. However, it also cautions that PSA and PAP can be reduced due to androgen deprivation therapy [1].

Although PSA is widely used for the identification of prostate cancer metastasis, some studies have demonstrated decreased sensitivity when compared to other prostate markers. PSMA IHC has been shown to be more sensitive than PSA for recognizing metastatic prostate cancer in cytology specimens [2]. The percentage of positive PSA staining metastatic cells varies greatly [3], which may hinder an accurate diagnosis when a limited sample of the tumor is provided in a biopsy. PSA expression tends to decrease in malignancy progression, with benign prostatic epithelium having the strongest immune reactivity [4, 5].

PSMA is weakly expressed in benign prostate epithelium, but highly expressed in adenocarcinoma of the prostate [6]. There seem to be conflicting reports on the homogeneity of PSMA expression. Some papers have described PSMA expression as homogenous in primary adenocarcinoma of the prostate [7, 8], as well as in lymph node metastasis [8], while others state its heterogenous character in both primary and metastatic prostate cancer [9]. Metastatic prostate cancer has been described as having a lower PSMA expression than primary prostate cancer [8]. Moreover, reports have also shown its positive expression in a variety of cancers in addition to prostatic adenocarcinoma [10].

The homeobox protein NKX3.1 is a transcription factor and tumor suppressor. Loss of heterozygosity (LOH) of its gene, *NKX3.1*, is frequently encountered in prostate adenocarcinoma and the frequency of LOH increases with grade and stage [11]. NKX3.1 has been shown to be highly specific for prostatic origin. Prostate adenocarcinoma and lobular carcinoma of the breast are the only cancers that have been shown to express it [12]. Earlier studies seemed to show that NKX3.1 expression was lost in tumor progression and metastatic lesions of prostatic origin [12, 13]. However, this may have been due to the use of older anti-NKX3.1 antibodies. Chuang et al. [14], using a more recently developed anti-NKX3.1 antibody, demonstrated that NKX3.1 was expressed from 92-97% of high grade prostatic adenocarcinomas. NKX3.1 proved to be specific and sensitive when differentiating high-grade prostatic adenocarcinomas from poorly differentiated urothelial carcinomas [14]. Gurel *et al.* [15], using the more recently developed NKX3.1 antibody, demonstrated that, although with a somewhat weaker staining pattern when compared to primary adenocarcinoma and primary prostate epithelium, NKX3.1 sensitivity to lymph node metastasis of prostate adenocarcinoma was 100%. This was compared to a PSA sensitivity of 98%.

A probable pitfall that may affect bone metastasis diagnosis may be the process of decalcification. Strong acid decalcification on bone and bone marrow tends to have a negative effect on the expression of certain antigens, particularly CD markers [16]. Tissue decalcification procedures with hydrochloric acid seem to negatively affect the IHC staining pattern of TTF-1, CK5/6, CK7, p63, ER, S100 protein, CD3, leukocyte common antigen, and synaptophysin, while decalcification with formic acid negatively affects CK8/18, CK7, and TTF-1 staining [17]. Some studies have reported that decalcification with EDTA does not show any significant changes [16]. However, another study has reported that decalcification with EDTA, as well as decalcification with formic acid, has a modest negative effect on ER, PR and HER2 receptor IHC in breast cancer [18]. To our knowledge, only one study from 1985 has attempted to determine the sensitivity of prostatic carcinoma markers post-decalcification in bone metastasis [19]. Shan *et al.* [19] concluded superiority of PSA versus PAP in determining prostatic origin of bone metastasis. However, PSA sensitivity was only 86% [19]. The effect of decalcification on more modern prostatic adenocarcinoma markers and their comparison with PSA has not been researched. In this study, we attempt to determine the effect on IHC of two of these markers, NKX3.1 and PSMA after sample decalcification. We also compare the sensitivity of these markers to PSA in recognizing prostatic origin in bone metastases.

Materials and methods

Hematoxylin and eosin stain

Tissue hematoxylin and eosin stains were performed by the histology laboratory of the Department of Surgical Pathology at Tisch Hospital of the NYU Langone Medical Center.

Immunohistochemistry

Immunohistochemistry was performed by the histology laboratory of the Department of Surgical Pathology at Tisch Hospital of the NYU Langone Medical Center. Rabbit polyclonal anti- human NKX3.1 antibody (Biocare Medical, Concord, California), mouse monoclonal anti-human PSA antibody (Clone ER-PR8; Ventana Medical Systems, Inc., Tucson, Arizona) and mouse monoclonal anti-human PMSA antibody (Clone 3E6; Agilent Technologies, Santa Clara, California) were used. The Ultraview Universal DAB Detection Kit (Ventana Medical Systems, Inc.) was used for identification.

Decalcification

Decalcification of bone biopsies were performed in the gross room of the Department of

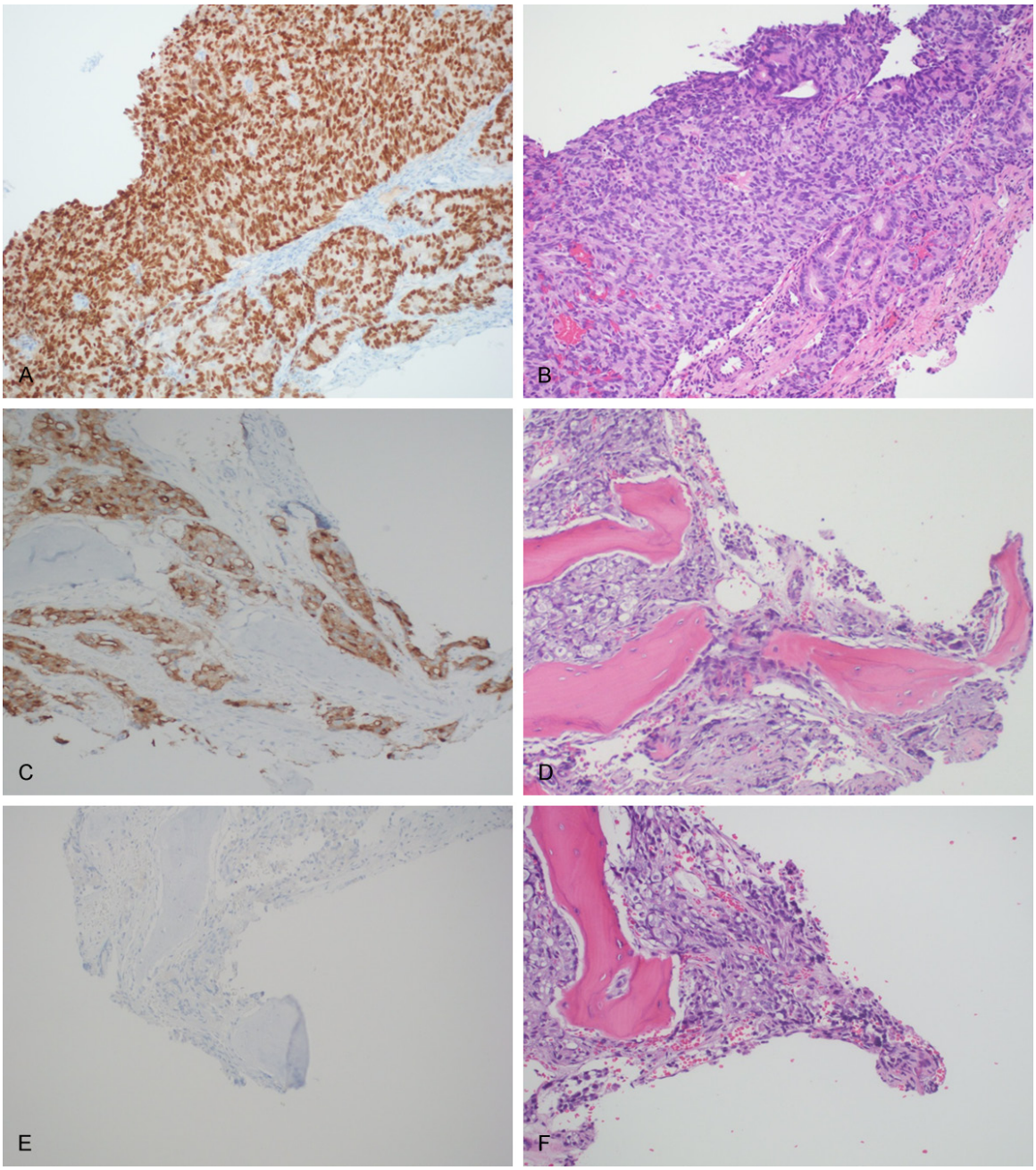

Figure 1. Representative Sections and Stains for Prostatic Adenocarcinoma Metastasis to the Bone. A, B. Section with diffuse NKX3.1 staining with corresponding H&E. C, D. Section with diffuse PSMA staining with corresponding H&E. E, F. Section with negative PSA staining with corresponding H&E.

Surgical Pathology at Tisch Hospital of the NYU Langone Medical Center. As per protocol, the specimens are fixed in 10% neutral buffered formalin (Avantik Biogroup, Springfield, New Jersey) and then placed in Decal® (decal Chemical Corporation, Tallman, New York), which has a pH of 2.1 and is a mixture of deionized water, hydrochloric acid and ethylenediaminetetraacetic acid, for 4 hours. The specimens are subsequently washed in running water and returned to 10% neutral buffered formalin prior to processing.

Paraffin sections of prostate cancer metastasis to bone

Pathologically confirmed prostate carcinoma bone metastasis specimens were obtained

Table 1. Summary of results

Case	Location	PSA staining	PSMA staining	NKX3.1 staining
1	Vertebral body, L2	1	2	2
2	Vertebral body, T7	2	2	2
3	Iliac bone	---	---	2
4	Femoral head	0	2	1
5	Femoral head	0	---	2
6	Vertebral body, L2	0	---	2
7	Sacrum	0	2	2
8	Iliac bone	0	2	2
9	Iliac bone	1	---	2
10	Rib	2	1	2
11	Pubic Ramus	2	2	2
12	Rib	2	1	2
13	Epidural/skull	2	2	2
14	Femur	0	2	2
15	Vertebral body, T3	1	2	2
16	Femur	2	2	2
17	Pubic ramus	1	1	2
18	Femur	0	2	1
19	Vertebral body, T11	0	2	2
20	Vertebral body, T12	2	2	2
21	Iliac bone	2	---	2
22	Pubic ramus	---	---	2
23	Vertebral body, T9	2	---	2
24	Vertebral body, L1	2	2	2

from patients that had undergone treatment at the New York University (NYU) Langone Medical Center. Medical record reviews were approved by the institutional review board. A retrospective study was performed on paraffin-embedded prostate adenocarcinoma bone metastasis samples from twenty patients. Eight cases were biopsies from vertebral bodies, five were from the femur, four came from the iliac bone, two were from ribs, three were from the pubic ramus, one was from the sacrum and one was from the skull.

Evaluation of PSA, NKX3.1, and PSMA expression

Immunohistochemical staining pattern of PSA, PSMA and NKX3.1 was defined as follows: negative (no staining), focally positive (≤ 10%) and diffusely positive (≥ 10%). Focal and diffuse positivity are both considered positive (**Figure 1**).

Statistical analysis

Statistical analysis comparing the expression of PSA, PSMA and NKX3.1 was done using the Chi-square test. *P* values < 0.5 were considered statistically significant.

Microscopic images

All images were obtained using the Olympus Microscope Camera, DP26®, and the CellSens image software program (Olympus Corporation©, Tokyo, Japan).

NKX3.1 and PSMA expression in prostate adenocarcinoma metastasis to the bone exceeds PSA expression

PSMA and NKX3.1 were expressed in all twenty specimens analyzed (**Figure 1**) (17/17 and 24/24, respectively). PSMA expression was focal in 18% (3/17) of cases and diffuse in 82% (14/17) of cases. NKX3.1 expression was focal in 8% (2/24) of cases and diffuse in 92% (22/24) of cases. When comparing PSMA and NKX3.1 positive staining, although not a statistically significant difference, there was a trend observed that NKX3.1 tended to be diffusely positive at a higher frequency.

PSA was expressed in 64% (14/22) of all cases. The frequency of positive PSMA staining in decalcified samples of prostatic carcinoma metastatic to the bone is statistically higher than that of PSA staining, when analyzed by the Chi-square test (Chi-square statistic: 5.389; *P* = 0.0203). The frequency of positive NKX3.1 staining in decalcified samples of prostatic carcinoma metastatic to the bone is also statistically higher than that of PSA staining, when analyzed by the Chi-square test (Chi-square statistic: 10.56; *P* = 0.0012). Results are summarized in **Table 1**.

To evaluate the specificity of NKX3.1, we look at staining status of NKX3.1 in the bone metastatic carcinoma of non-prostate origin after decalcification. NKX3.1 were not detected in all 18 cases (100%) of non-prostate origin bone metastatic carcinoma after the specimen been decalcification. The primary origin of these cases are lung (4), gastrointestinal (4), liver and pancrease (3), kidney (RCC, 2), urinary bladder (1), thymus (1) and unknown primary (3).

Discussion

Our results demonstrate that the use of NKX3.1 and PSMA as markers for prostatic origin in metastatic lesions to the bone after decalcification is more reliable than the use of PSA. PSA was expressed only in 55% of the cases of prostatic adenocarcinoma metastatic to the bone. In contrast, all cases expressed NKX3.1 and PSMA. Previous reports had demonstrated loss of NKX3.1 expression in prostate cancer metastasis [11-13]. However, recent investigations have demonstrated high NKX3.1 IHC reactivity in metastatic prostate adenocarcinoma [15]. This may be due to the use of more recently developed anti-NKX3.1 antibodies. Similar to our results, Gurel et al. [15], demonstrated that 100% of cases of metastatic prostate adenocarcinoma to the lymph node were positive for NKX3.1. Additionally, our PSMA results are consistent with other investigations. Queisser et al. [5] demonstrated PSMA positivity for prostate cancer in 98% of lymph node metastases and 100% for distant metastases. Similarly, Sweat et al. [8] showed 98% positivity in lymph node metastases. Ananias et al. [20], demonstrated PSMA positive in 100% of bone metastases. Moreover, PSMA seems to be a more sensitive marker than PSA for prostate cancer metastasis in cytology specimens [2].

The decrease in PSA expression in malignancy progression and metastasis has been reported by other investigators [4, 5]. Additionally, we report that only 55% of metastasis to the bone we tested were positive for PSA. Though lower when compared to other prostatic markers, the majority of other investigations report higher sensitivity using PSA in metastatic surgical pathology specimens than we found. PSA seems to be more sensitive for diagnosing metastatic prostate carcinoma in lymph nodes than in distant metastasis. Reports of PSA sensitivity in metastasis in lymph nodes range from 89% to 98% [5, 15, 21, 22], while PSA sensitivity in distant metastasis ranges from 70% to 97% [5, 15, 21]. However, these reports do not state which number of these are bone metastasis nor whether or not these specimens were decalcified. Bernacki et al. [2] reported a PSA sensitivity to prostate metastasis similar to our findings at 58%. However these were processed as cell block preparations from cytology specimens. One of explanations for this discrepancy may be explained by decalcification. Papers have reported that decalcification with hydrochloric acid, formic acid and EDTA all have negative effects on the detection of CK8/18, CK7, TTF-1, CK5/6, CK7, p63, ER, S100 protein, CD3, leukocyte common antigen, synaptophysin, p53, Ki67, ER, PR and Her2 expressions in tissue specimens [17, 18, 23]. Shah et al. [19], reports a PSA sensitivity of 85% in biopsies of decalcified bone specimens with prostate cancer metastasis. Roudier *et al.* [3] reported a study of 14 autopsies of patients who died from metastatic prostate cancer. They analyzed PSA expression from samples taken from bone metastasis after decalcification in a 10% formic acid solution. They report that, although all of the cases expressed PSA in bone metastasis, the PSA expression was very heterogeneous and variable. The percent of tumor cells expressing PSA ranged from 90% in some patients to less than 50% in other patients [3]. This heterogeneity, which can be due to either true lack of PSA expression in bone metastatic prostate cancer or due to decalcification effects, can explain the lack of PSA staining in 45% of our decalcified bone metastasis specimens.

The ability to detect expression of NKX3.1 and PSMA was maintained in all of our biopsy specimens after decalcification. It seems that NKX3.1 and PSMA are more reliable markers for detecting prostatic origin in bone metastasis. However, although PSMA is highly sensitive to prostatic origin, it is less specific. Positive PSMA expression can be seen in some GI adenocarcinomas, urothelial carcinomas, renal carcinomas and skin cancers [24, 25]. It is also expressed in normal bladder, testes, adrenal gland and brain tissue, as well as in Barrett's Esophagus [25]. NKX3.1 is expressed in benign prostate tissue and normal tests. However, the only other carcinoma that expresses NKX3.1, besides prostate carcinoma, is in infiltrating ductal and infiltrating lobular carcinoma of the breast [12]. This makes NKX3.1 much more specific than PSMA. Gelmann et al. [12], reports that NKX3.1 is more tissue specific than PSA.

Our results demonstrate that NKX3.1 is more sensitive to prostatic origin than PSA and that decalcification does not seem to affect this sensitivity as a marker. Other reports also show that NKX3.1 is significantly more specific than PSMA. Together, we conclude that NKX3.1 is the superior expression marker for identifying

prostate origin in bone metastases and every immunohistochemical panel used for analyzing bone metastases for possible prostatic origin should include NKX3.1 expression.

Disclosure of conflict of interest

None.

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