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Original Article

Formation of vasculogenic mimicry in bone metastasis of prostate cancer: Correlation with cell apoptosis and senescence regulation pathways

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ABSTRACT

Vasculogenic mimicry (VM) has been found in prostate cancer (PCa) as an independent marker of poor prognosis. To investigate the correlation between VM and bone metastasis in PCa, a total of 80 cases were analyzed by CD31 and PAS dual-staining as well as the follow-up data. All cases were divided into two groups: VM-positive and VM-negative (VM-pos/VM-neg). Immunohistochemical staining for investigating the expression of Casepase-3, Bcl-2/Bax, and SA-β-gal was performed. 28 of the 80 PCa cases exhibited VM structure (35.0%). The incidence of bone metastasis in the VM-pos and VM-neg was 67.9% (19/28) and 38.5% (20/52), respectively. The positive rate of Casepase-3 and Bcl-2 expression was significantly different of the two groups (Caspases-3: VM-pos 71.4%, 20/28 vs VM-neg 42.3%, 22/52; Bcl-2: VM-pos 35.7%, 10/28 vs VM-neg 65.4%, 34/52). Bcl-2/Bax ratio of the VM-pos (0.71 ± 0.22) was lower than that of the VM-pos (0.89 ± 0.13). In addition, a higher frequency of SA-β-gal was detected in VM-pos (64.29 ± 86.42) than in VM-neg (25.37 ± 72.21). Taken together, our findings demonstrate that PCa with VM has the tendency to develop bone metastasis. Activations of cell apoptosis and senescence regulation pathways may play important roles in the formation process of VM structure.

Introduction

Prostate cancer (PCa) is the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths among men in western countries [1]. The incidence of PCa in Asian is significantly lower, but it has increased significantly in China over recent years [2]. Metastasis is more likely to occur in patients with advanced PCa, and the most frequent metastatic site is skeleton. Studies had shown that nearly one half of progressive castration-resistant, non-metastatic PCa would develop bone metastases within two years and more than 80% of patients who die of PCa had metastatic disease within the bone [3,4]. Patients with bone metastases have a higher risk of severe pain, fracture, nerve compression and even death. Virtually there is no highly effective treatment for metastatic disease. Therefore, it's urgent to identify relevant factors of PCa bone metastasis in order to predict prognosis and guide clinical therapy.

Traditional tumor blood supply consists of angiogenesis (the formation or sprouting of endothelium-lined vessels from pre-existing vessels) and vasculogenesis (the differentiation of precursor cells to endothelial cells that develop de novo vascular networks) [5,6]. However, accumulated evidence indicated that tumors not only secrete various angiogenic factors to promote tumor angiogenesis, but also directly form tumor vascular channels, which is known as vasculogenic mimicry (VM) [7].

As a new mode of tumor angiogenesis, VM is a pattern of vasculogenic-like networks lined exclusively by the highly aggressive tumor cells mimicking endothelial cells. Since Maniotis and Folberg [8] observed VM for the first time in highly invasive eye uveal melanoma micro-cycle in 1999, VM has been found in many tumors, including prostate cancer [9], breast cancer [10], hepatocellular carcinoma [6], osteosarcoma [11], and astrocytic tumor [12]. It has been demonstrated that VM provides convenience of tumor perfusion and dissemination functioning either independently of or simultaneously with angiogenesis [13–15]. However, we have no evidence to illustrate the role of VM in the process of PCa bone metastasis. Thus it makes sense to address the biological significance of VM in contributing to PCa bone metastasis. Besides, the mechanism of VM formation is not totally clear yet, and it is reported that apoptosis was involved in VM formation. So we also explored the possible molecular mechanism by evaluating levels of apoptosis-related proteins and SA-β-gal in the PCa patient

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specimens. Clarification of such association would help us develop better therapeutic strategies for PCa bone metastasis in the future.

Materials and methods

Patients

A total of 80 patients aged 54–72 (64.96 ± 4.07) years old, who underwent radical prostatectomy in the Second Hospital of Tianjin Medical University from January 1, 1995 to December 31, 2010. The preoperative PSA value ranged 5.1–76.7 (23.64 ± 13.04) ng/ml, and Gleason scores were within 6–10. Imaging diagnosis of all patients was clinical localized prostate cancer (T1-T2c), which was confirmed by preoperative prostate biopsy. All cases received surgery without radiotherapy and/or chemotherapy before operation and were diagnosed as prostate gland cancer by postoperative histopathology. Bone scan and MRI were performed regularly for patients, especially who had PSA failure or bone pain during follow-up. PSA failure and imaging suggestion were considered as the criterion of bone metastasis. The mean follow-up time was 82 months.

Reagents

Anti-CD31 monoclonal mouse anti-human primary antibody, anti-Bcl-2, Bax, Caspase-3 polyclonal rabbit anti-human primary antibody were purchased from Abcam Inc. Periodic acid and Schiff (PAS) solutions were obtained from Yili Fine Chemicals Biotechnology Co. Ltd, and senescence-associated β-galactosidase (SA-β-gal) staining kit from Genmed Scientifics Inc.

Immunohistochemical CD31-PAS and SA-β-gal staining

Formalin-fixed, paraffin-embedded tissues were cut into serial 5-μm sections. Heat-induced epitope retrieval in citrate buffer (0.01 mol/L; pH 6.0) was applied to all slides before immunohistochemical staining.

First, CD31, Bcl-2, Caspase-3 and Bax staining was performed according to the two-step immunohistochemical detection kit; then dual staining was applied as follows: sections were treated with 0.5% periodate for 10 min, followed by tap water for 2 min (rinsing), Schiff solution for 15 min in a dark chamber, after another tap water for 2 min (rinsing), the sections were soaked in distilled water for 10 min. Sections were counter-stained with hematoxylin. PAS staining in stomach mucinous adenocarcinoma was used as positive control. All procedures were performed under the same conditions.

Finally the slides were observed under the light microscope to analyze the morphological characteristics and distribution of VM in tumor. The dual staining sections were viewed at 400×. VM was defined as channels lined by PAS-positive material with red blood cells (RBCs) in the center, but not lined by CD31-positive endothelial cells.

The scoring of Bcl-2, Caspase-3 and Bax immunohistochemical staining were performed on the basis of percent positivity. The scope of staining was classified as 0 for negative, 1 for <10%, 2 for 10–25%, and 3 for >25% staining. The intensity of staining was graded as 0 for absent, 1 for weak, 2 for moderate, and 3 for intense. The staining index was determined by the product of the two scores (0–12). Tissues scored ≤4 were in negative group in contrast to tissues >4 in positive group [16].

SA-β-gal staining was performed according to the manufacturer protocol with cryosections (6 μm). SA-β-gal positive cells exhibited a blue color. Total 500 cells from five different fields per section were counted at 400× at least twice. All of the stained sections were examined by two independent observers who were blinded to the histopathologic features.

Statistics processing

Statistical analysis was subjected to SPSS19.0. The chi-square (χ^2) test and T test were used to evaluate differences between the categorical variables. Significance level was set at $P < 0.05$.

Results

Correlation between VM and PCa bone metastasis

The presence of CD31/PAS double-staining of the VM was observed. The wall of the VM channel was positive for PAS staining, while tumor cells lining the external wall were negative for CD31 staining. Our results suggest that VM constructs like a lacunar architecture which is formed by tumor cells, while without endothelial cells lining cover. RBCs can be seen in those channels (Fig. 1).

28 out of 80 PCa cases (35.0%) were considered VM positive. The incidence of postoperative bone metastasis in VM-positive group (VM-pos) was much higher than that in VM-negative group (VM-neg) (67.9%, 19/28 vs 38.5%, 20/52), the difference is statistically significant ($P = 0.019$).

Expression of apoptosis-related proteins

To elucidate the relationship between VM and apoptosis, we compared the expression levels of apoptosis-related proteins caspses-3, Bcl-2 and Bax in two groups. VM-pos tumors expressed significantly higher level of caspses-3 protein (20/28) than VM-neg ones (22/52) (71.4% vs 42.3%, $P = 0.013$) (Fig. 2). In contrast, VM-neg specimens (34/52) had more Bcl-2 expression than VM-pos (10/28), the incidence rate was 65.4% vs 35.7% ($P = 0.011$) (Fig. 2). No significant difference was found in the expression of Bax between the two groups, although both had widespread positive signal (VM-pos 24/28, 85.7% vs VM-neg 41/52, 78.8%, $P = 0.453$) (Fig. 2). The ratio of Bcl-2/Bax was derived from the scores of the two proteins. Bcl-2/Bax ratios in the VM-pos and VM-neg were 0.71 ± 0.22 and 0.89 ± 0.13, respectively. The difference was statistically significant ($P = 0.000$). The Statistical data of apoptosis-related proteins expressions was showed in Table 1.

Senescence-related β-galactosidase staining

Blue particle precipitate was formed in senescent cells by SA-β-gal staining analysis (Fig. 3). The number of senescent cells in every 500 cells observed from VM-pos specimens was significantly higher than that from the VM-neg ones (VM-pos 64.29 ± 86.42 vs VM-neg 25.37 ± 72.21) ($P = 0.048$) (Table 1).

Discussion

VM has been found in many malignancies by the characteristic lacunar architecture formed by tumor cells which RBCs can be seen in those channels. However, a large number of smooth muscle cells in PCa produce confounding effects on visualizing VM in PCa channels, in fact these cells are normally covered by a delicate basement membrane-like material making the diagnosis of VM difficult, which is not present in uveal melanoma, cerebral astrocytoma or even breast carcinoma [17]. Therefore, the best way of confirming the presence of VM appears to visualize them within the tumor or to demonstrate the presence of RBCs within these channels, which is widely accepted.