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Original Article

The Relationship between Urinary Pyridinoline, Deoxypyridinoline and Bone Metastasis in a Rat Breast Cancer Model

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Background: Bone metastasis from breast cancer is often recognized clinically, but there are nonetheless several difficulties in diagnosis. In this study we used an animal model of bone metastasis from breast cancer and clarified the relationship between the urinary Pyd/Cr and Dpd/Cr and the progression of bone metastasis, compared with other bone related markers: serum alkaline phosphatase bone isozyme (ALP-BI), osteocalcin, and calcium.

Methods: The evaluation of bone metastasis was assessed by histological examination of the thoracic and lumbar vertebrae. According to the histological findings 4 weeks after the tumor cell injection, 11 animals were retrospectively divided into 2 subgroups: (1) tumor-bearing rats with bone destruction due to bone metastasis (TBR-BD(+), n=5), (2) tumor-bearing rats without bone destruction (TBR-BD(−), n=6). These animals were compared to age-matched controls without tumor cell injection (n=6). An additional 5 animals were sacrificed at 2 weeks after the tumor cell injection to evaluate micrometastasis to bone.

Results: The values of other markers for bone metastasis in animals with micrometastatic foci in bone marrow did not differ significantly from those of the controls. Pyd/Cr and Dpd/Cr in the TBR-BD(+) group were significantly higher than those of the TBR-BD(−) and the control group (233±78.3 vs 93.8±6.5, 98.5±18.7, 123.1±35.9 vs 67.9±6.2, 60.6±9.8, $p<0.01$), while there were no significant differences between TBR-BD(−) and the control.

Conclusions: Both Pyd/Cr and Dpd/Cr are correlated significantly with the volume of bone metastasis, and are useful for the diagnosis and evaluation of progression of bone metastasis compared with other markers.

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Key words: Animal model, Bone metastasis, Breast cancer, Pyridinoline, Deoxypyridinoline

Bone metastasis of breast cancer is clinically recognized in 30% of patients showing a first recurrence[1,2] and in 70% of breast cancer patients subjected to autopsy[3]. Resultant symptoms such as pain, pathologic fractures and neuroparalysis seriously compromise the quality of life of these patients.

There are several difficulties involved in the diagnosis of bone metastasis and the evaluation of the therapeutic efficacy. Radiologic examination, which is widely applied, has some limitations. For instance, X-ray examination is convenient, but it is difficult to detect bone metastasis and evaluate the therapeutic efficacy since its sensitivity is low. Since bone scintigraphy is able to examine the whole skeletal system, it is suitable for use as a screening test for bone metastasis, but the false positive rate is relatively high[1]. CT and MRI are superior to X-ray and scintigrapy in the evaluation of local lesions, but these modalities cannot easily scan the whole skeleton, and repeated examinations are very expensive.

Serum or urinary bone metabolic markers reflect the activity of bone metastatic lesions to some extent, and can be measured repeatedly, but the relationship between the progression of bone metastasis and these markers has not been clarified.

Hydroxyproline, which has been widely used

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Abbreviations:
Pyd/Cr, Pyridinoline to creatinine ratio; Dpd/Cr, Deoxypyridinoline to creatinine ratio

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as a marker of bone resorption[4,5], is an abundant amino acid in all collagen molecules, and exsists in all molecules with a collagen-like structure such as complement factor C1q. About 80% of hydroxyproline derived from tissue degradation is metabolized to H_2O and CO_2 in the liver, and it is difficult to quantify the rate of collagen degradation by measuring urinary hydroxyproline levels[6-8].

Recently, pyridinoline (Pyd) and deoxypyridinoline (Dpd) have been used as new biochemical markers of bone resorption[9]. They participate in intermolecular cross-linking in collagen, and are mainly derived from bone and cartilage, but do not exsist in skin collagen. They also exist only in mature collagen, and not in newly synthesized molecules. Pyd and Dpd are excreted into the urine following collagen degradation associated with bone resorption, and are not affected by diet[10]. In a previous report, the value of urinary Pyd/Cr and Dpd/Cr were elevated in patients with disease of accelerated bone resorption such as Paget's disease or primary hyperparathyroidism[11,12].

These markers are expected to specifically correlate with bone metastasis and not with other organ metastasis because they originate from bone and cartilage.

In this study, using an experimental rat model of bone metastasis for breast cancer developed by innoculating c-SST-breast cancer cells[13,14], we have clarified the relationship between the urinary Pyd/Cr, Dpd/Cr and the progression of bone metastasis and compared it with other markers: serum alkaline phosphatase bone isozyme (ALP-BI), osteocalcin, and calcium.

Materials and Methods

Animals

Female spontaneously hypertensive rats (SHR) were purchased from Charles River Japan Inc and housed at the Laboratory Animal Center of Keio University in a pathogen-free environment for the duration of the experiment. All animals were maintained under the guidelines established by the Keio University School of Medicine.

Cell Line

c-SST-2 cells (breast cancer cells which spontaneously developed in an SHR rat) were donated by Dr Takeichi, Department of Pathology, Hokkaido University Medical School. Subcutaneous inoculation of this cell line produces local tumor and metastatic lesions in lymph nodes, lungs, heart, kidneys, and other organs[15].

Cell Culture

c-SST-2 cells were grown in Eagle's minimal essential medium containing 10% fetal bovine serum in a gas mixture of 5% CO_2 and 95% air at 37℃. Passage and medium exchange were performed once a week.

Surgical Procedure

The procedure has been described in detail elsewhere[13,14,16]. Briefly, after anesthetizing 6 week-old female SHR rats by intraperitoneal injection of pentobarbital 50 mg/kg, a small incision was made on the cervical skin under dissecting microscope. A polyethylene catheter PE-10, Becton Dickinsons) was inserted into the thoracic aorta from the left common carotid artery. Subsequently, after minor laparotomy, both hila of kidneys were exposed and clamped temporarily to prevent the inflow of tumor cells into the kidney. c-SST-2 cells (1×10^5 suspended in 0.2 ml of Hank's balanced salt solution) were injected into the thoracic aorta over 1 min. Then, the hila of the kidneys were declamped, the catheter was removed, and the left common carotid artery was ligated.

Evaluation of Bone Metastasis

Five animals were sacrificed 2 weeks after the tumor cell inoculation, and 11 animals 4 weeks after. Then, the thoracic and lumbar vertebrae, as well as the heart, lungs, kidneys, ovaries, uterus, liver and spleen were dissected for microscopical examination.

Bone Metabolic Markers

At 2 and 4 weeks after the tumor cell injection, blood and urine samples were collected from the animals that had fasted for 12 hours to measure serum alkaline phosphatase bone isozyme (ALP-BI) and serum calcium by routine techniques, serum osteocalcin by radioimmunoassay, and urinary Pyd/Cr and urinary Dpd/Cr by HPLC[17] at Mitsubishi Yuka Bio-Clinical Laboratories Inc. In order to evaluate the relationship between bone metastasis and bone metabolic markers, the animals were divided into two groups, (A) the tumor bearing rats (TBR), in which tumor cells were injected into the thoracic aorta ($n=11$) and

(B) the control group (control), which underwent sham operation and were given the same volume of saline into the thoracic aorta (n=6). Furthermore, TBR were divided into two subgroups according to the histological findings of bone metastasis; (1) tumor-bearing rats with bone destruction due to bone metastasis (TBR-BD(+), n=5), (2) TBR without bone destruction (TBR-BD(−), n=6), including 3 animals in which bone metastasis was not detected on histological examination.

Evaluation of Bone Metastasis[16]

Although some animals developed bone metastasis in the sternum and ribs, the most frequent sites were the thoracic and lumbar vertebrae. We could not detect bone metastasis in peripheral bones such as femur or skull. Therefore, we evaluated microscopic bone metastasis using thoracic and lumbar vertebrae. In order to quantify spinal bone metastasis, the severity of bone metastasis in each vertebra was graded by a score from 0 to 2 according to histological findings as described previously[16]; 0 indicating no metastasis, 1 fine metastasis in the bone marrow without bone destruction, and 2 metastasis with bone destruction. The total score from all thoracic and lumbar vertebrae in each animal was calculated and corresponded to the severity of bone metastasis. The score per animal was given by the formula: (no. of vertebrae, score 1)×1+(no. of vertebrae, score 2)×2.

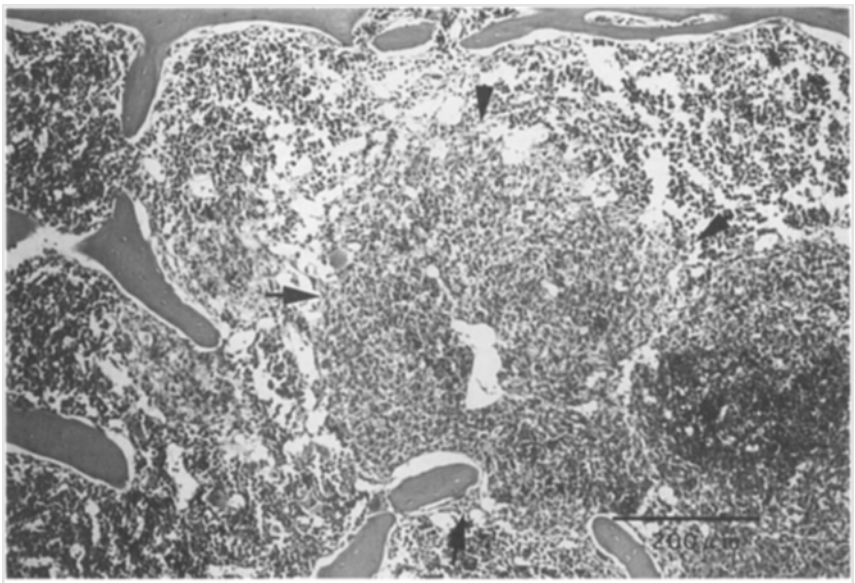

Fig 1. Microscopic metastasis in the bone marrow (H.E. stain). Black arrow shows a tumor nest.

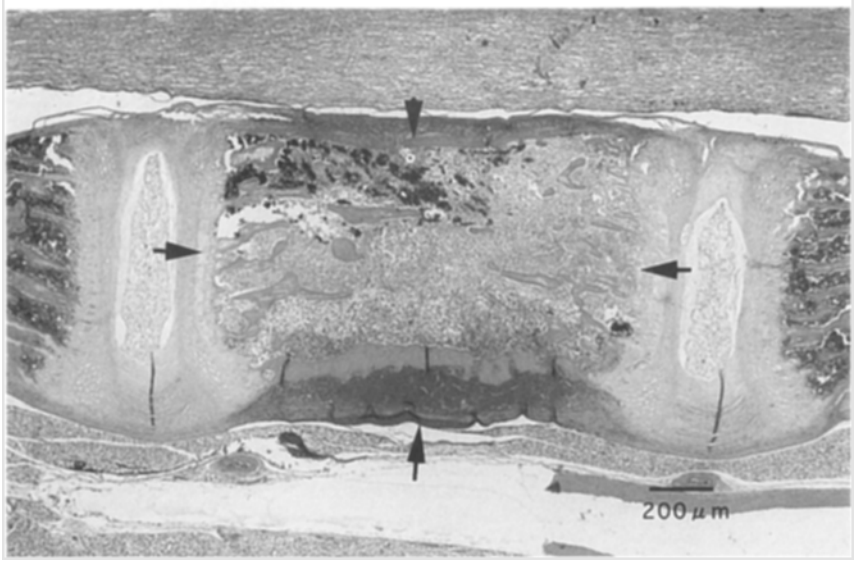

Fig 2. Bone metastasis with overt bone destruction (H.E. stain). Bone matrix is destroyed with necrosis in the column. Black arrow shows tumor.

Statistical Analysis

Results obtained from biochemical markers were presented as mean±SD and analysed by Student's *t*-test. $p<0.05$ was considered significant.

Results

Two weeks after the tumor cell injection, 3 of 5 rats (60%) showed microscopic metastasis in bone marrow without bone destruction (Fig 1). Levels of bone metabolic markers did not differ from those of the control. Four weeks after the tumor cell injection, 8 of 11 rats (73%) developed bone metastasis. Five of eight (63%) developed bone metastasis with overt bone destruction (Fig 2) (TBR-BD(+)), and 3 (37%) had bone metastasis without bone destruction (TBR-BD(−)). Al-

Table 1. Metastases to Organs Other than Bone in the Tumor-Bearing Rats

Organs	Group	
	TBR-BD(+) (n=5)	TBR-BD(−) (n=6)[a]
Heart	2 (40)	1 (17)
Lungs	2 (40)	3 (50)
Liver	1 (20)	0 (0)
Kidneys	3 (60)	6 (100)
Ovaries	4 (80)	5 (83)
Uterus	0 (0)	0 (0)

TBR-BD(+); Tumor bearing rats with bone destruction; TBR-BD(−); Tumor bearing rats without bone destruction.
[a] Including micrometastasis in the bone marrow without bone destruction.
Figures in the parenthesis indicates percentage.

Table 2. Change in Bone Related Markers 2 and 4 Weeks after Tumor Cell Injection

	Weeks after the tumor cell injection			
	2 weeks		4 weeks	
ALP-BI				
TBR-BD(+)	428±111.2		346±135.5	*
TBR-BD(−)	410±54.6	N.S.	204.3±64.6	
Control	345.7±42.2		221.2±29.1	
Osteocalcin				
TBR-BD(+)	61.9±9.0		67.8±9.1	
TBR-BD(−)	56.1±11.5	N.S.	88.4±25.2	N.S
Control	74.0±15.7		65.2±7.7	
Calcium				
TBR-BD(+)	9.4±1.0		11.7±1.5	*
TBR-BD(−)	8.8±1.3	N.S.	11.0±1.1	*
Control	9.8±1.0		9.8±0.4	
Pyd/Cr				
TBR-BD(+)	129.3±28.4		233±78.3	**
TBR-BD(−)	118.6±15.9	N.S.	98.5±18.7	**
Control	117.8±25.6		93.8±6.5	
Dpd/Cr				
TBR-BD(+)	91.5±19.4		123.1±35.9	**
TBR-BD(−)	73.5±12.9	N.S.	60.6±9.8	**
Control	98.0±19.5		67.9±6.2	

*$p<0.05$; **$p<0.01$.

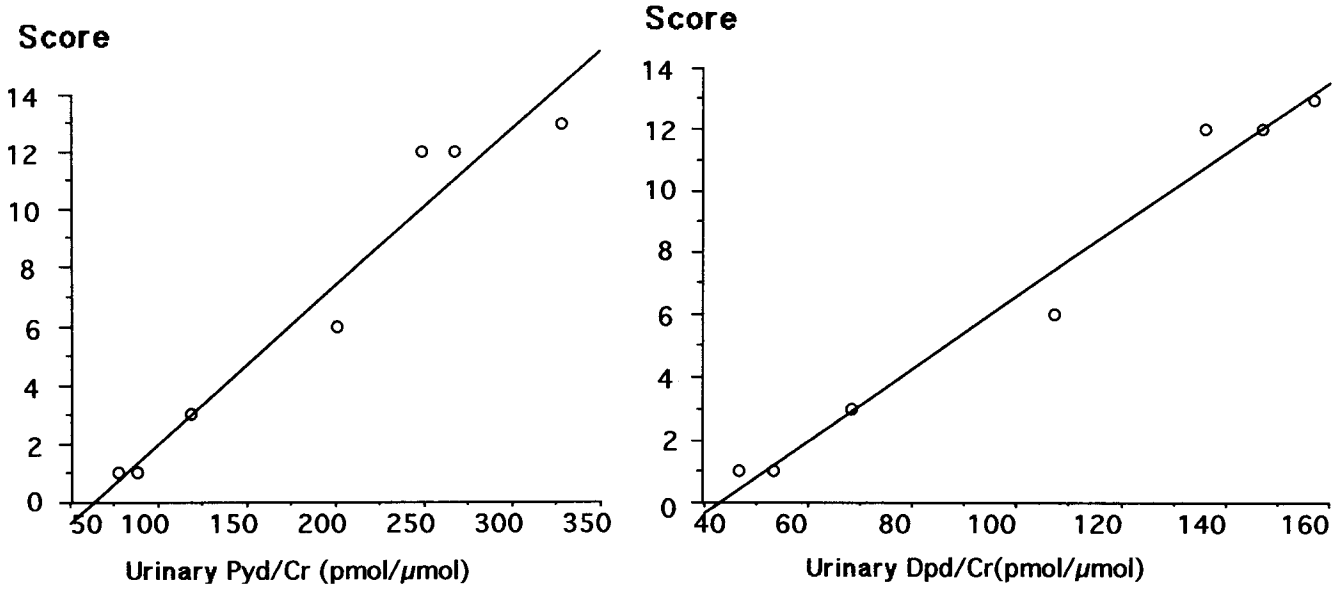

Fig 3. The relationship between urinary Pyd/Cr (left), Dpd/Cr (right) and the degree of bone metastasis (score).
Score=−3.43+0.054×Pyd/Cr, $r=0.975$, $p=0.0002$ (left).
Score=−4.953+0.116×Dpd/Cr, $r=0.989$, $p=0.0001$ (right).

though metastases to other organs were also observed, most were microscopic, and there was no difference in the distribution of metastatic sites between TBR-BD(+) and TBR-BD(−) (Table 1). The bone metabolic markers were measured at 2 and 4 weeks in each animal (Table 2). Two weeks after the tumor cell injection, there were no differences among the 3 groups with regard to bone metabolic markers. Four weeks after the tumor cell injection, serum ALP-BI in TBR-BD(+) was significantly higher than that of TBR-BD(−) (346.8±135.5 [mU/ml] vs 204.3±64.6, $p<0.05$). There were no significant differences in serum osteocalcin levels among 3 groups. Serum calcium in both TBR-BD(+) and TBR-BD(−) was significantly higher than that of control (11.7±1.5, 11.0±1.1 vs 9.8±0.4, $p<0.05$). Pyd/Cr and Dpd/Cr (pmol/μ mol) in TBR-BD(+) were significantly higher than those in TBR-BD(−) and control animals (233±78.3 vs 93.8±6.5, 98.5±18.7, 123.1±35.9 vs 67.9±6.2, 60.6±9.8, $p<0.01$). There were no significant differences between TBR-BD(−) and control (Table 2). Pyd/Cr significantly correlated with Dpd/Cr, and both markers significantly correlated with the bone metastasis score ($p<0.01$, Fig 3).

Discussion

In this study we used an animal model of bone metastasis to investigate the relationship between bone metastasis and bone metabolic markers. Various methodologies used to develop experimental bone metastasis models have been reported[18-21]. Our model requires surgery, but operative death was practically non-existent. It has already been reported that in our model bone metastases occur at higher rates when compared with other models[13,14]. Furthermore, in a rat model, monitoring of bone metabolic markers is possible in the same animals during the experiment. Two weeks after the tumor cell injection, 60% of rats developed micrometastasis in the bone marrow without either osteoclastic or osteoblastic reaction.

Four weeks after the tumor cell injection, 8 of 11 rats (73%) developed bone metastasis, including 5 animals with overt bone destruction. Osteoclastic bone resorption or fibrous osteogenesis were also seen. These findings mimic bone metastasis from breast cancer in patients. It has been reported that tumor cells first produce micometastatic foci in red bone marrow, followed by osteoclastic bone resorption activated by humoral factors released from tumor or stromal cells, and ultimately tumor cells themselves cause bone destruction[22-24]. When collagen is degraded by bone resorption caused by bone metastasis, pyridinoline and deoxypyridinoline are excreted in the urine freely or bound to peptide[25].

As pyridinoline and deoxypyridinoline are stable substances that exist specifically in the collagen of bone and cartilage, the amount excreted in the urine may accurately reflect the degree of bone resorption. In this study, we quantified bone metastasis using a scoring system described previously[16]. In the animals with larger bone metastasis and more active bone resorption at the time of urine sampling, the value of urinary Pyd/Cr and Dpd/Cr were high. However, 2 weeks after tumor cell injection, there was no difference in Pyd/Cr and Dpd/Cr levels among TBR-BD(+), TBR-BD(−) and control rats. In the animals with only micrometastatic foci in the bone marrow without bone destruction, Pyd/Cr and Dpd/Cr did not increase. Thus, it was considered that the elevation of urinary Pyd/Cr and Dpd/Cr required a certain degree of bone destruction. On the other hand, 4 weeks after the tumor cell injection, there were no differences in Pyd/Cr and Dpd/Cr levels between TBR-BD(−) and control, although these markers were significantly higher than both groups in the TBR-BD(+) group. This suggests that increases in Pyd/Cr and Dpd/Cr are only due to bone metastasis with bone destruction, and are not influenced by metastasis to other organs.

There were no significant differences among the 3 groups with regard to the other bone related markers (serum ALP-BI, osteocalcin, Ca). Even 4 weeks after the tumor cell injection, differences in these markers were not as obvious as differences in Pyd/Cr and Dpd/Cr.

In an animal model the influence of aging on these markers should be considered. In our preliminary study, both urinary Pyd/Cr and Dpd/Cr in rats were monitored from an age of 6 to 13 weeks. Levels of these markers gradually decreased and reached a plateau at 8 weeks (data not shown). At 4 weeks after the tumor cell injection the levels of AlP-BI in each group were lower than levels 2 weeks after the tumor cell injection, which may reflect a decrease of bone turnover owing to aging. We have already reported that the urinary Pyd/Cr and Dpd/Cr in the pamidronate

treated group were significantly lower when compared with the control group. These ratios also correlate with the bone metastasis score[16]. It is suggested that urinary Pyd/Cr and Dpd/Cr accurately reflect bone resorption associated with metastasis, and are useful in the diagnosis of bone metastasis as well as the evaluation of therapeutic efficacy against bone metastasis. Pyd/Cr significantly correlated with Dpd/Cr, so the two have similar power as a marker of bone metastasis. The measurement of Pyd/Cr or Dpd/Cr will be clinically useful for the diagnosis and follow-up of bone metastasis from breast cancer.

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