Research Article

Study of urine Hydroxyproline:creatinine ratio as a bone turnover marker of Bone Metastases along with some routinely used biochemical parameters in patients with Prostate Cancer in comparison with Bone Scintigraphy

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Abstract: Skeletal metastases are a most common event in prostate cancer patients with advanced cancer disease and a significant problem. Now a days imaging techniques such as bone scintigraphy with Technetium 99mMDP (methylene diphosphonate) is a frequently used method for detection of bone metastases. Without the aid of radiological tools it is difficult to diagnose, treat or follow bone metastases cases clinically. This study was designed to evaluate the utility of the bone resorption marker, hydroxyproline: creatinine ratio (OHPr: Cr ratio) in spot urine sample of the patients, along with the estimation of commonly used bone formation marker such as serum total alkaline phosphatase (ALP), serum calcium and serumphosphorous in comparison with whole body skeletal scintigraphy with Technetium99mMDP, for the diagnosis of bone metastases (BM) in prostate cancer (PC) patients. *Experimental design:* Four groups of samples were analysed. 1st group consist 52 normal males (cancer free men), 2nd group consists 38 PC patients without BM, 3rd group consists 27 PC patients with limited BM (3 or less than 3 skeletal lesions) and 4th group consists 34 PC patients with extensive BM(4 or more than 4 skeletal lesions), confirmed by whole body skeletal scintigraphy with Technetium99m MDP. *Results:* One way ANOVA was used to compare urine hydroxyproline: creatinine ratio (OHPr: Cr ratio), serum ALP, serum calcium and serum phosphorus among these groups. Both urine OHPr: Cr ratio and serum ALP are strongly elevated in extensive bone metastasescases, thus can be used as a bone turnover marker (P<0.0001). Serum calcium and serum phosphorus will give additional background values.

Keywords: Prostate Cancer (PC), Bone Metastasis (BM), limited bone metastases (Lim.BM), extensive bone metastases (Ext.BM), Scintigraphy, Technetium99m MDP (methylene diphosphonate), hydroxyproline: creatinine ratio, total alkaline phosphatase (ALP).

INTRODUCTION

One of the most common types of cancer is prostate cancer representing 19% of all diagnosed cancers in the western world in the year 2002 with 679000 new cases[1]. At autopsy, bonemetastases are found in 60% to 85% of men with prostate cancer [2]. Bone metastases are a major cause of pain, disability, and death.Almost all patients who die of prostate cancer have skeletal involvement [3]. When the primary tumor metastasizes to the bone - causing a lesion of high bone remodelling and destroying the bone structure. This results in severe bone pain, pathological bone fractures, spinal cord compression, hypocalcaemia and increased mortality [4,5]. Skeletal metastases in PC are predominantly sclerotic and pathological fractures occur more commonly in PC than in other cancers metastasizing to bone [6]. Bone fractures predict poor survival in prostate cancer patients [7].Early detection of bone metastases is crucial to initiate successful therapy with bisphosphonates, targeting the skeleton in prostate cancer patients with bone metastases.

Bone scintigraphy is considered as gold standard and is traditionally used method for initial evaluation to detect and monitor the metastatic bone involvement. Since scintigraphy is expensive and is not particularly suitable for all the follow-up patients and also may cause fear of radiation due to repeated use, bone turnover markers can be used as indicators for bone metastases.

Iminoacid hydroxyproline is one of the constituents of bone collagen, excreted in the urine, is a reliable indicator of bone matrix turnover.It became known as early as 1960s that BM of malignant tumours are associated with an increased urinary excretion of hydroxyproline [8,9].Hydroxyproline, derived from post-translational hydroxylation of proline, represents about13% of the amino acid content of the various forms of collagen. As hydroxyproline released during collagen degradation is not used again in collagen synthesis, this can function as a marker of collagen

metabolism or osteoclastic bone resorption. Hydroxyproline is present in urine in three forms. 1) Free hydroxyproline of which nearly all is reabsorbed by tubules and degraded by the liver. 2) Small hydroxyproline-containing peptides that are dialyzable and represent over 90% of urinary hydroxyproline excretion and 3) Nondialyzable polypeptides containing hydroxyproline which are formed from the breakdown of newly synthesised collagen, a marker of bone formation. So hydroxyproline excretion is considered to be bone collagen turnover. As most of the endogenous urinary hydroxyproline is derived from the breakdown of bone, the urinary excretion of hydroxyproline of a patient on collagen free diet, can be used to assess collagen metabolism and in particular, the breakdown of bone[10]. As it is not convenient to collect a 24 hour urine specimen, Nobbs et al and Gasser et al., [11,12] proposed a single early morning (spot) urine specimen of the subject on a gelatine free diet for the determination of hydroxyproline:creatinine ratio, thus making this parameter as the measurement of choice for bone metastases. Fasting urine OHPr:Cr ratio is a sensitive marker than estimation of hydroxyproline alone [13].

In this study we measured fasting spot urine hydroxyproline: creatinine ratio as resorption marker and total alkaline phosphatase as bone formation marker along withserum calcium and serum phosphorus estimation among groups of normal men and prostate cancer patients with and without bone metastases, based on whole body skeletal scintigraphy.

MATERIALS AND METHODS:

The present study was conducted in the Department of Biochemistry, Gandhi Hospital, Secunderabad and in the Department of Nuclear Medicine, MNJ Institute of Oncology and Regional Cancer Centre, Red hills, Hyderabad. The study population comprised of 151 males and they are classified into four groups.

Group1 consists of 52 normal cancer free males aged 20- 80 years.
Groups 2, 3 and 4 consists of patients who are histo-pathologically and radiologically diagnosed with Prostate Cancer and are thus categorised based on the absence or presence of skeletal lesions and their number, found in the whole body skeletal scintigraphy with Technetium99mMDP.
Group2 consists of 38 prostate cancer patients without bone metastases.
Group3consists of 27 prostate cancer patients with limited bone metastases (3 or less than 3 hot spots).
Group4 consists of34 prostate cancer patients with extensive bone metastases (more than4 hot spots).

Patients and control group were recruited after informed consent was obtained. The patients and control group were advised to have collagen free diet for 48 hrs. and an overnight's fasting. Then venous blood samples were drawn, allowed toclot at room temperature. Spot urine samples were collected.Serum alkaline phosphatase, urine hydroxyproline and creatinine were estimated. A dose of 20 mCi Technetium99mMDP is administered intravenously to the patients. After 2 – 4 hours, whole body bone scintigraphy was conducted on these patients using Duel Headed Gama Camera.

Subjects with fractures, primary and secondary hyperparathyroidism, and all bone related problems are considered as exclusion criteria for normal group.Patients treated with aromatase inhibitors and bisphosphonates and patients with extensive BM for various times prior to enrolment are the exclusion criteria for carcinoma prostate samples in this study.

Determination of Urinary Hydroxyproline:
Method:Modified Neuman and Logan [14].
Principle: Hydroxyproline is treated with $CuSO_4$ and H_2O_2 in an alkaline solution, this results in the formation of pyrroline-4-carboxylic acid, which upon acidification is converted to pyrrole-2-carboxylic acid. The latter condenses with p-dimethylaminobenzaldehyde to give a coloured complex which is measured at 540nm.
Reagents:
(i) Copper sulphate (0.01M): Dissolve 0.159 g of CuSO4 in 100 ml distilled water.
(ii) Sodium hydroxide (2.5 N): Dissolve 10 g of NaOH in 100 ml distilled water.
(iii) 6% hydrogen peroxide.
(iv) Sulphuric acid (3N).
(v) p-dimethyl aminobenzaldehyde (5% solution in n-propanol).
(vi) Hydroxyproline standard: A series of standards corresponding to 5, 10, 15, 20, 25, 30, 35, 40, 45 micro grams of hydroxyproline are prepared.

Procedure:(Table1)

S.No.	REAGENT/SAMPLE	BLANK	STANDARD	TEST
1.	Urine		-	1 ml
2.	Standard	-	1ml	-
3.	Distilled water	1ml	-	-
4.	$CuSO_4$(0.01M)	1ml	1ml	1ml
5.	NaOH (2.5N)	1ml	1ml	1ml
6.	6.6%H_2O_2	1ml	1ml	1ml

The solutions are mixed and shaken occasionally during a period of 5minutes, and then placed in water bath at 80^0 C for 5 minutes with frequent vigorous shaking. The heating and shaking destroys the excess of peroxide. Traces of peroxide which remain will decrease colour formation and produce an orange-red hue. The tubes are chilled in an ice water bath and then added.

7.	3N H$_2$SO$_4$(with agitation)	4ml	4ml	4ml
8.	5% p-dimethylamino benzaldehyde	2ml	2ml	2ml

The tubes are placed in water bath at 70^{0}C for 16 minutes and then cooled in tap water. Read the colour at 540nm.

1) Hydroxyproline is estimated in μg per 100 ml of urine and creatinine is estimated in mg per 100 ml of urine. Values are expressed as milligrams of hydroxyproline to milligrams of creatinine excreted.

2) Urinecreatinine is estimated in a semi auto analyser by Jaffe's reaction.
3) Serum Alkaline phosphatase is estimated by pNPP-AMP (IFCC) Kinetic Assay method.
4) Serum Calcium is estimated by O-Cresolphthalein Complexone, End point Assay method.
5) Serum phosphorus is estimated by UV Molybdate, End point Assay method.

RESULTS:

One way ANOVA was used to compare spot urine OHPr: Cr ratio, serum ALP, serum calcium and serum phosphorous among groups.

(Table 2).

	GP1 normal N=52		GP2 PC without BM N=38		GP3 PC with limited BM N=27		GP4 PC with Extensive BM N=34		P value
	Mean	SD	mean	SD	mean	SD	mean	SD	
OHPr:Cr ratio	0.01	0.004	0.011	0.003	0.013	0.003	0.078	0.034	0.0001
ALP	78.63	29.98	81.89	25.88	148.51	86.22	733.79	277.72	0.0001
Calcium	9.752	0.454	9.63	0.478	9.219	0.873	7.806	1.758	0.0001
Phosphorous	3.787	0.538	3.589	0.498	3.263	0.661	2.374	0.970	0.0001

Mean values of urine OHPr: Cr ratio, serum ALP, serum calcium and serum phosphorous, in different groups basedon wholebody skeletal scintigraphy in prostate cancer patients.

The mean value of fasting (spot) urine hydroxyproline: creatinine ratio in 52 healthy, cancer-free males aged 20 to 80 years was 0.010 ± 0.004 and the values range from 0.003 to 0.016. In 38 patients with PC without BM the mean value of fasting OHPr: Cr ratio was 0.011 ± 0.003, the values range from 0.004 to 0.016. In 27 patients with PC with limited BM the mean value of fasting OHPr: Cr ratio was 0.013 ± 0.003, the values rangefrom 0.006 to 0.018 ($p < 0.001$) and in 32 patients with extensive BM the mean value of fasting OHPr:Cr ratio was 0.078 ± 0.034, and the values ranges from 0.034 to 0.168 ($p < 0.0001$)(Fig. 1).

The mean value of serum ALP in 52 healthy, cancer-free males aged 20 to 80 years was 78.6 ± 30 and the values range from 42 IU/L to 140 IU/L. In 38 patients with PC without BM the mean value of serum ALP was 81.9 ± 25.9, the values range from 49 to 140 IU/L. In 27 patients with PC with limited BM the mean value of serum ALP was 148.5 ± 86.2, the values range from 58 to 367 IU/L ($p < 0.0001$) and in 32 patients with extensive BM the mean value of serum ALP was 277.7 ± 183.1, and the values range from 231 to 1403 IU/L ($p < 0.0001$)(Fig. 2).

The mean value of serum calcium in 52 healthy, cancer-free males aged 20 to 80 years was 9.75 ± 0.45 andthe values range from 8.7mg/dl to 10.6mg/dl. In 38 patients with PC without BM the mean value of serum ALP was 9.63 ± 0.48, the values rangefrom 8.6mg/dl to 10.4mg/dl. In 27 patients with PC with limited BM the mean value of serum calcium was 9.22 ± 0.87, the values range from 7.6mg/dl to 10.8mg/dl($p < 0.003$) and in 32 patients with extensive BM the mean value of serum calcium was 7.81 ± 1.76, and the values range from 5.4mg/dl to 11.9mg/dl ($p < 0.0001$)(Fig. 3).

The mean value of serum phosphorus in 52 healthy, cancer-free males aged 20 to 80 years was 3.79 ± 0.54 and the values ranges from2.6 to4.8mg/dl. In 38 patients with PC without BM the mean value of serum phosphorus was 3.59 ± 0.50, the values range from2.4 to4.5mg/dl. In 27 patients with PC with limited BM the mean value of serum phosphorus was 3.26 ± 0.66, the values range from 2.3 to 4.4mg/dl($p < 0.001$) and in 32 patients with extensive BM the mean value of serum phosphorous was 2.37 ± 0.97, and the values range from 1.0 to 4.8mg/dl($p < 0.0001$)(Fig. 4).
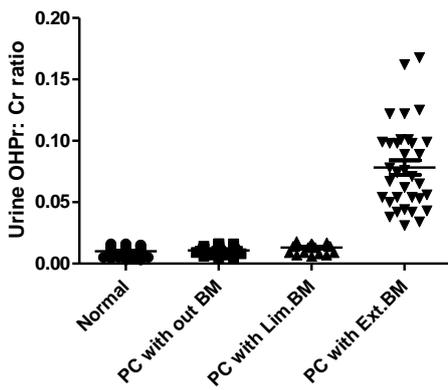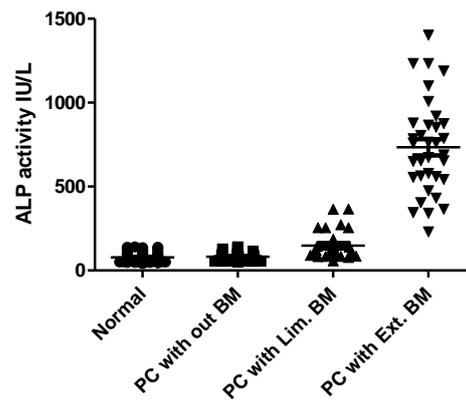
Fig-1

Fig-2

Fig1: Urine hydroxyproline: creatinine values in different groups based on whole body Scintigraphy in Prostate Cancer patients.

Fig2: Serum ALPactivity in different groups based on whole body Scintigraphy in Prostate Cancer patients.
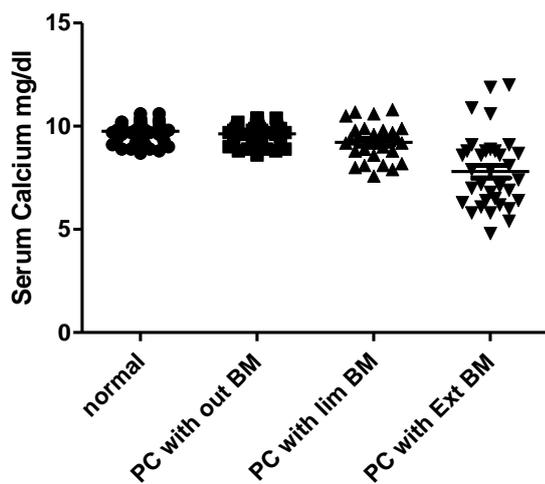
Fig-4

Fig-3

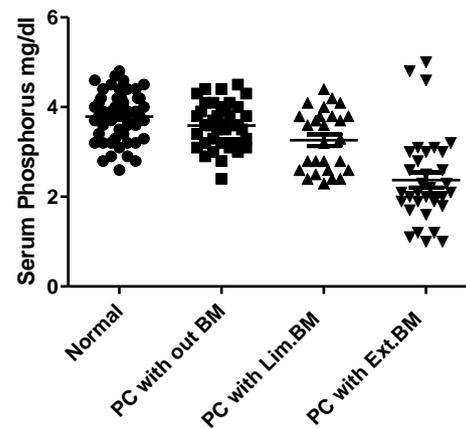Fig3: Serum calcium values in different groups based onwhole body Scintigraphy in Prostate Cancer patients.

Fig4: Serum Phosphorus values in different groups basedon whole body Scintigraphy in Prostate Cancer patients.

DISCUSSION:

Prostate cancer is the most frequently diagnosed non-cutaneous cancer and the second leading cause of cancer deaths among men in the United States[15]. In prostate cancer patients, the incidence of bone metastases is observed to be very high at about 70–80% [16,17,18]. When treating patients with prostate cancer, identification of bone metastases is an important issue. Image studies such as plain radiography, bone scintigraphy, computerized tomography and magnetic resonance play a major role in detection and follow-up of bone metastases of prostate cancer patients. But each image measure has its own limitation. The advantage of bone biochemical markers over image studies are, non-invasive, cost-effective, no fear of radiation, fast and easy to perform repeatedly and also show rapid response to treatment, differentiate healing lesions from progressive lesions and provides more information on the mechanisms and cellular dynamics of bone destruction[19,20,21,22,23,24].Metastases in prostate cancer are characterized by excess of abnormally dense bone showing increased bone turnover. This is due to increased activity of both osteoblasts and osteoclasts. The relative amount of osteoblastic activity exceeds that of the osteoclasts, resulting in excess bone formation.

We have compared fasting urine OHPr: Cr ratio along with total alkaline phosphatase,serum calcium and serum phosphorous with whole body skeletal scintigraphy and are found to be significantly correlated with bone metastases. Fasting spot urine OHPr: Cr ratio along with serum total alkaline phosphatase higher in prostate cancer patients with bone metastases than the normal subjects and both the markers are correlated to each other. The patients with limited bone metastases has also showed significant values(p <0.001), but some patients showed normal excretion of hydroxyproline in their urine,but in extensive bone metastases all the patients have increased hydroxyproline in their urine and increased hydroxyproline creatinine values (p <0.0001). Urine OHPr: Cr ratio is not significantly different in PC patients with limited bone metastases,butincreased values are found in patients with extensive BM with four or more skeletal lesions in image studies(Fig.5). Patients without bone metastases had normal hydroxyproline: creatinine ratio.
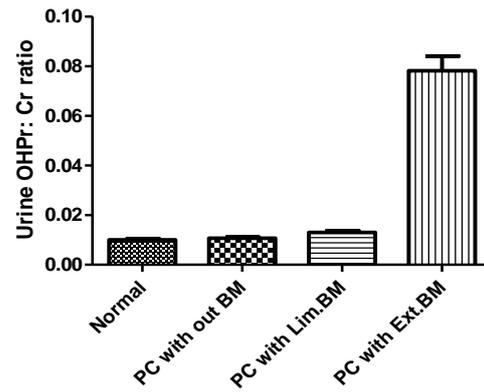

(Fig.5)
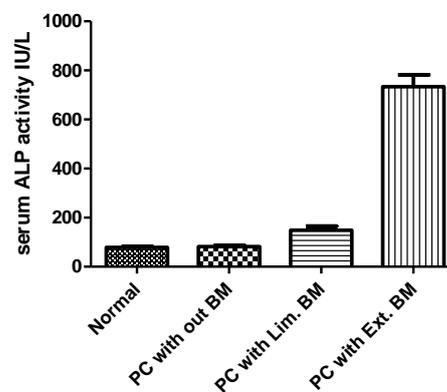
(Fig.6)

ALP is alsoshowedsignificant correlation as bone formation marker (p< 0.0001). The elevated levels of ALP in bone metastases are difficult to interpret due to the possibility of the liver metastases. Serum ALP is increased significantly in limited bone metastases (p <0.0001),may be due to liver metastases as lung and liver are the most frequent sites of distant prostate cancer metastases. This can be overcome by identifying the patient, whose increased levels should be due to bone disease and not due to liver disease. ALP is also showed significant correlation in extensive bone metastases with extension of bone metastases(p <0.0001) (Fig.6).Bone specific ALP is more specific osteoblastic marker for bone metastases along with fasting urine OHPr: Cr ratio.

Serum calcium also showed significant correlation (p <0.0001)(Fig.7). Among patients with limited BM, 11% of cases showed hypercalcemia and 14.8% cases showed hypocalcemia and in patients with Ext. BM 13% cases showed hypercalcemia and 53% showed hypocalcemia. Prostate cancer patients tend to have more hypocalcemia than hypercalcemia and this could be due to the metastases that is predominantly osteoblastic [25].Intense osteoblastic response seen in prostate cancer is preceded at a cellular level by osteoclast activation. Avid uptake of calcium by

osteoblastic metastases of prostate is the main cause of hypocalcemia [26].
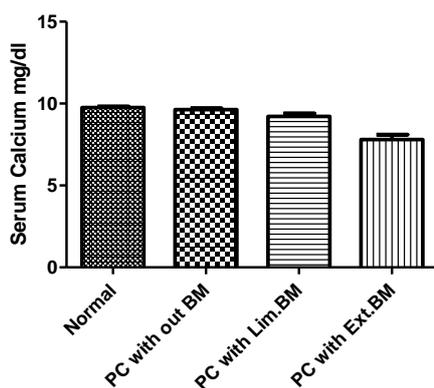
(Fig.7)
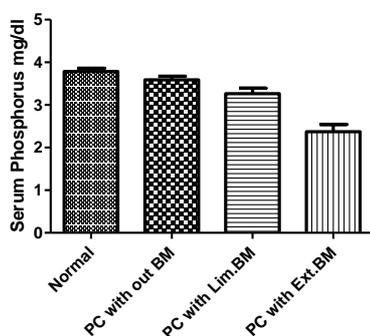
(Fig.8)

Serum phosphorus alsoshowedsignificant correlation (p <0.0001). Among patients with limited BM, 14.7% of cases showed hyperphosphatemiaand 11.8% cases showedhypophosphatemia and in patients with Ext. BM 8.8% cases showed hyperphosphatemia and 61.8% cases showedhypophosphatemia(Fig.8).

CONCLUSION:

The Biochemical marker, fasting spot urine OHPr: Cr ratio,can beused alone as a bone turnover marker or in combination with routinely used bone formation marker ALP to detect bone metastatic spread. Fasting spot urine OHPr: Cr ratio is not markedly elevated in limited bone metastases but is strongly elevated in extensive bone metastases. The usefulness of this marker is not only in detection of BM but also in detecting the extent of metastases, and can be used for early detection of extensive bone metastases.In this study both ALP and OHPr: Cr correlated with each other and both these markers are increasing consistently, with the presence of bone metastases and the extent of skeletal involvement. Serum calcium and serum phosphorus will give additional background values for clinical guidance.

References:

1. Parkin D M, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin, 2005;55:74108.

2. Galasko CSB. Skeletal metastases. London: Butterworth; 1986.

3. Landis S H, Murray T, Bolden S, Wingo PA. Cancer statistics. C A Cancer J Clin,1999;49:8-29.

4. Oefelein M G, Ricchiuti V, Conrad W, Resnick M I. Skeletal fractures overall survival in men with prostate negatively correlate with cancer. J Urol, 2002; 168: 1005-7

5. Coleman R E. Metastatic bone disease: clinical features, pathophysiology and treatment strategies. Cancer Treat Rev, 2001; 27: 165-76.

6. Coleman RE. Skeletal complications of malignancy. Cancer 1997;/80:/1588/94.

7. Oefelein MG, Ricchiuti V, Conrad W, et al. Skeletal fractures negatively correlate with overall survival in men with prostate cancer. J Urol, 2002;168:1005–7.

8. Kontturi, M. J., Sotaniemi, E. A., Larmi, T. K. I.: Hydroxyproline in the early diagnosis of bone metastases in prostatic cancer.Scand. J. Urol., 1974; 8, 91.

9. Laitinen, O.,Nikkila, E. A., Kivirikko, K. I.: Hydroxyproline in the serum and urine.Acta med. scand., 1966;179, 275.

10. A. Hodgkinson, T. Thompson Measurement of the fasting urinary hydroxyproline: creatinine ratio in normal adults and its variation with age and sex. J Clin Pathol; 1982; 35:807-811.

11. Nobbs BT, Walker AW, Davies TJ. A simplified method for the estimation of urinary total hydroxyproline. Clin Chim Acta;1975; 64:219-21.

12. A. B.Gasser, D. Depierre and B. Courvoisier Total urinary and free serum hydroxyproline in metastatic bone disease. Br. J. Cancer, 1979;39, 280.

13. T.J.Powles, C.L.Leese, P.K.Bondy. Hydroxyproline excretion in patients with breast cancer and response to treatment. Br Med J; 1975; 2: 164–166.

14. Robert E. Neumanand MilanK Lo The determination of hydroxyprolinegan.The journal of biological chemistry1949; 22.

15. Altekruse S F, Kosary C L, Krapcho M, Neyman N, Aminou R, Waldron W et al. (eds) SEER Cancer Statistics Review, 1975–2007 http://seer.cancer.gov/csr/1975_2007. Based on November 2009 Seer data submission, posted to the Seer web site, 2010.

16. Carlin BI, Andriole G L. The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma. Cancer 2000; 88: 2989–2994.

17. Bridwell K H. Treatment of metastatic prostate cancer of the spine. Urol Clin North Am 1991; 18: 153–159.

18. Keller E T, Zhang J, Cooper CR, Smith PC, McCauley L K, Pienta K J et al. Prostate

carcinoma skeletal metastases: cross-talk between tumor and bone. Cancer Metastasis Rev, 2001; 20: 333–349.

19. Coleman R E. Biochemical markers of malignant bone disease. London (UK): Martin Dunitz, Ltd.; 2000.

20. Galasko C S. The significance of occult skeletal metastases, detected by skeletal scintigraphy, in patients with otherwise apparently ''early'' mammary carcinoma. Br J Surg ,1975;62:694 – 6.

21. Sanal S M, Flickinger F W, Caudell M J, et al. Detection of bone marrow involvement in breast cancer with magnetic resonance imaging. J Clin Oncol, 1994; 12:1415 – 21.

22. Yeh K A, Fortunato L, Ridge J A, et al. Routine bone scanning in patients with T1 and T2 breast cancer: a waste of money. Ann Surg Oncol, 1995;2:319 – 24.

23. Coleman R E, Mashiter G, Whitaker K B, et al. Bone scan flare predicts successful systemic therapy for bone metastases. J Nucl Med, 1988; 29: 1354 –9.

24. Vogel C L, Schoenfelder J, Shemano I, et al. Worsening bone scan in the evaluation of antitumor response during hormonal therapy of breast cancer. J Clin Oncol, 1995;13:1123 – 8.

25. Roodman G D. Biology of osteoclast activation in cancer. Clin Oncol. 2001; 19(15): 3562-3571.

26. Rabbani S A, Gladu J, Harakidas P, Jamison B, Goltzman D. Over-production of parathyroid hormone-related peptide results in increased osteolytic skeletal metastasis by prostate cancer cells in vivo. Int J Cancer. 1999; 80(2): 257-264