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The value of Tc-99m-tetrofosmin scintigraphy in the assessment of P-glycoprotein in patients with malignant bone and soft-tissue tumors

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P-glycoprotein (Pgp) overexpression has been shown to be correlated with resistance to chemotherapy in patients with malignant bone and soft-tissue tumors. The aim of our study was to investigate the role of ^{99m}Tc-tetrofosmin as a functional imaging agent reflecting Pgp expression in these tumors. ***Methods:*** Twenty eight patients with various malignant bone and soft-tissue tumors were studied. Radionuclide angiography with ^{99m}Tc-tetrofosmin was done first and planar images were acquired at 15 min and 90 min postinjection. Vascular phase was evaluated visually on dynamic images, metabolic state was evaluated both visually and quantitatively on planar images. Quantitative analysis was performed by the calculation of tetrofosmin uptake in the lesion against background and percent washout rate (WR%) of the tracer. Immunohistochemical analysis of Pgp was performed on biopsy specimens and the degree of expression was graded from 0 to 3. ***Results:*** There was a positive correlation between the Pgp score and the washout rate of tetrofosmin ($r = 0.73$, $p = 0.000$). The mean washout rate of tetrofosmin from the lesions with Pgp expression (31.81 ± 6.72) was found to be significantly higher than those of without Pgp expression (21 ± 3.49) ($p = 0.000$). No statistically significant correlation was found between 15 min and 90 min uptake ratios (UR) of tetrofosmin and Pgp score ($r = -0.10$, $p = 0.6$ and $r = -0.21$, $p = 0.2$, respectively). When the cut-off value of 24.5 (according to ROC-analysis) for the washout rate was used to discriminate the lesions with and without Pgp expression, the test yielded a sensitivity value of 87.5% with a specificity of 100%. ***Conclusions:*** In malignant bone and soft-tissue tumors, ^{99m}Tc-tetrofosmin uptake were not related to Pgp overexpression. Pgp overexpression was found to be correlated with the washout rate of the tracer. ^{99m}Tc-tetrofosmin scintigraphy with washout analysis may not only be a useful method for evaluating Pgp overexpression but also its function.

Key words: Tc-99m-tetrofosmin, P-glycoprotein, malignant bone and soft-tissue tumors

INTRODUCTION

ALTHOUGH the prognosis of primary malignant bone tumors has improved dramatically since the addition of chemotherapy to surgery, systemic relapses still occur in 40% to 50% of cases.[1] Multidrug resistance (MDR), a phenomenon in which malignant cells demonstrate resistance to multiple chemotherapeutic agents, is believed to be a factor contributing poor outcome for the majority of the patients.

MDR has been defined to be a multifactorial phenomenon which may include overexpression of drug resistance proteins, such as MDR1 P-glycoprotein (Pgp), MDR-associated protein (MRP1) and lung resistance protein (LRP).[2] Among these proteins, Pgp has been shown to be the most commonly expressed marker and Pgp expression correlates with resistance to chemotherapy in

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Table 1 Scintigraphic findings with ^{99m}Tc-tetrofosmin and P-glycoprotein expression

Patient no.	Age/ sex	Diagnosis	Lesion site	Pgp score	Tc-99m tetrofosmin			
					Visual perfusion	Washout rate (%)	Uptake ratio/visual uptake	
							15 min	90 min
Patients without tetrofosmin uptake on 15-min image								
1.	18/F	Metastatic Ewing's sarcoma	Right scapula	0	0	—	—	—
2.	50/M	Metastatic indifferentiated carcinoma	Left femur	2	0	—	—	—
Patients with tetrofosmin uptake on 15-min image								
3.	20/M	Fibrosarcoma	Left thigh	0	2	18	1.73 (1)	1.41 (0)
4.	21/F	Ewing's sarcoma	Right calcaneus	0	2	24	8.92 (3)	6.77 (3)
5.	22/F	Synovial sarcoma	Right cruris	0	1	21	7.07 (3)	5.56 (3)
6.	51/M	Non-Hodgkin's lymphoma	Left calcaneus	0	2	25	4.01 (3)	3.0 (3)
7.	74/M	Lymphoma	Left humerus	0	2	14	2.59 (2)	2.22 (2)
8.	34/F	Osteosarcoma	Right femur	0	2	23	2.45 (3)	1.87 (2)
9.	50/M	Myxoid liposarcoma	Right buttock	1	2	20	1.93 (2)	1.54 (2)
10.	37/M	Metastatic undifferentiated carcinoma	Left femur	1	2	23	4.32 (3)	3.32 (3)
11.	2.5/M	Ewing's sarcoma	Left tibia	1	2	18	2.66 (3)	2.18 (3)
12.	20/M	Osteosarcoma	Left femur	1	2	24	3.35 (3)	2.54 (2)
13.	9/F	Osteosarcoma	Left femur	2	2	30	3.27 (3)	2.27 (2)
14.	13/M	Osteosarcoma	Left femur	2	2	33	2.29 (3)	1.52 (2)
15.	19/F	Fibrosarcoma	Right foot	2	2	14*	4.51 (3)	3.85 (3)
16.	19/F	Metastatic renal cell carcinoma	Right iliac	3	2	34	4.39 (3)	2.86 (2)
17.	50/F	Metastatic infiltrative ductal carcinoma (breast)	Left tibia	3	1	27	6.79 (3)	4.89 (2)
18.	38/F	Osteosarcoma	Right femur	3	1	26	1.53 (2)	1.13 (1)
19.	18/M	Osteosarcoma	Right tibia	3	2	30	5.56 (3)	3.88 (2)
20.	60/M	Renal cell sarcoma	Left femur	3	2	48	3.45 (3)	1.77 (1)
21.	27/M	Osteosarcoma	Right femur	3	1	37	1.38 (1)	0.86 (0)
22.	69/M	Metastatic transitional cell carcinoma (bladder)	Right femur	3	2	22*	3.34 (3)	2.61 (2)
23.	78/M	Round cell liposarcoma	Left cruris	3	2	35	4.0 (3)	2.61 (2)
24.	10/M	Ewing's sarcoma	Right tibia	3	2	28	3.84 (3)	2.75 (2)
25.	7/F	Malignant fibrous histiocytoma	Right femur	3	2	42	2.19 (3)	1.25 (1)
26.	18/M	Osteosarcoma	Right femur	3	1	35	2.08 (2)	1.34 (1)
27.	50/F	Malignant fibrous histiocytoma	Left femur	3	1	31	2.18 (2)	1.51 (1)
28.	45/F	Ewing's sarcoma	Right humerus	3	2	27	3.96 (3)	2.86 (3)

*Discordant washout ratios with respect to Pgp score when the cut-off value of 25.5 was used

osteosarcoma patients.[2–4] In soft-tissue sarcomas, Pgp expression has also been reported to be a prognostic indicator correlating with a poor outcome.[5,6]

Pgp is a 170-kD transmembrane glycoprotein which acts as an adenosine triphosphate-dependent efflux pump to reduce the intracellular accumulation of many chemotherapeutic drugs, including doxorubicin which is the most effective agent for the treatment of osteosarcoma.[7] ^{99m}Tc-tetrofosmin (^{99m}Tc-1,2-bis[bis(2-ethoxyethyl) phosphino]ethane) is an agent developed for myocardial perfusion imaging and has been shown to accumulate in viable tumor tissue.[8–14] The functional characteristics of this agent are similar to those of ^{99m}Tc-sestamibi.[15] Like sestamibi, tetrofosmin has also been suggested to be a potent agent for the prediction of multi drug resistance (MDR) in various tumour cell lines.[16–19]

The aim of our study was to examine the role of ^{99m}Tc-tetrofosmin as a functional imaging agent reflecting Pgp expression in malignant bone and soft-tissue tumors. For this purpose, the uptake and washout kinetics of ^{99m}Tc-tetrofosmin was compared with the degree of Pgp expression of the biopsy specimens.

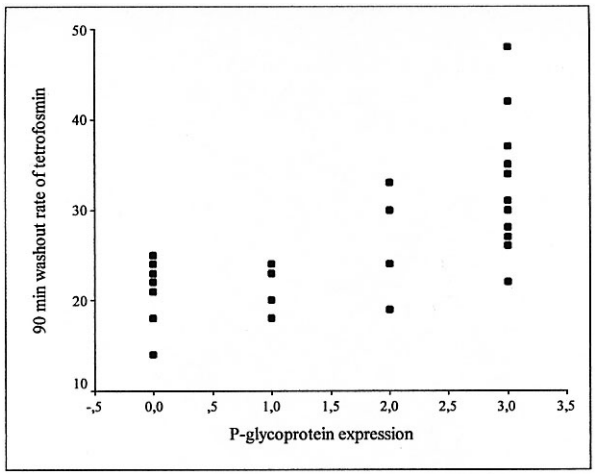

Fig. 1 Significant difference in tetrofosmin washout was observed between patients with Pgp scores 0–1 and 2–3 (p = 0.000). Tumors with high Pgp expression demonstrated higher tetrofosmin washout from lesions. A positive correlation was found between washout rate and Pgp expression (r = 0.73, p = 0.000).

MATERIALS AND METHODS

Patients

Twenty eight patients (12 F, mean age: 27.66 ± 15.19; 16 M, mean age: 37.34 ± 24.53; age range: 2.5 to 78 y) with various malignant bone and soft tissue tumors were included in the study. All patients were examined scintigraphically before biopsy. The diagnoses were proven pathologically in specimens obtained by biopsy and/or surgery, Eight patients had osteosarcoma, 5 Ewing's sarcoma, 2 malignant fibrous histiocytoma, 2 fibrosarcoma, 2 liposarcoma, 2 lymphoma, 1 synovial sarcoma, and 6 bone metastatic tumors (Table 1).

Each patient underwent ^{99m}Tc methylene diphosphonate (MDP) three-phase bone scanning as a routine procedure. At least two days later, dynamic and static ^{99m}Tc-tetrofosmin scans were obtained.

^{99m}Tc-Tetrofosmin Imaging

The adults received intravenous injection of 370–600 MBq ^{99m}Tc-tetrofosmin. This activity was reduced in the children. The limbs in question were examined with a large field of view gamma camera (Camstar, GE Medical Systems) equipped with a low-energy all-purpose collimator at most adequate projection in each patient. Data were obtained every 2 sec for 60 sec for radionuclide angiography (128 × 128 matrix). Then, planar 5-min ^{99m}Tc-tetrofosmin images (256 × 256 matrix) were obtained 15 and 90 min after radionuclide administration.

Data Analysis

Radionuclide angiography was visually evaluated by two blinded observers and the degree of perfusion increase was classified into four grades: a) 0 = no increase; b) 1 = mild increase; c) 2 = moderate increase; d) 3 = marked increase. Static ^{99m}Tc-tetrofosmin images were evaluated visually and quantitatively. In visual analysis, two blinded observers evaluated the degree of radionuclide uptake using a four-grade scoring system: a) 0 = background activity; b) 1 = slight increase in uptake; c) 2 = moderate uptake; d) 3 = strong uptake.

In quantitative analysis, a manual region of interest (ROI) was set on the lesion (L) and a symmetrical ROI was set on the contralateral normal area (B) on 15 min images. Identical ROIs were applied to 90 min images. The uptake ratio (UR) of both 15 and 90 min images was calculated by dividing the count density of the lesion by that of the background ROI. After decay correction of the mean counts in the ROIs drawan on the 90 min images, the washout rate (WR%) of ^{99m}Tc-tetrofosmin from the lesion was determined using the following formula:

$$\text{WR\%/90 min} = \frac{\text{(L/B) 15 min} - \text{(L/B) 90 min}}{\text{(L/B) 15 min}} \times 100$$

Detection of P-Glycoprotein Expression

The lesions were resected by open biopsy to obtain histopathological diagnosis. Immunohistochemical (IHC) staining was performed according to the standard streptoavidin-biotin method. The primary antibody applied for P-glycoprotein was "monoclonal antibody PGP (MDR)" (Immunotech company, France, Ready for use, No. 2142).

Sections of tissue were fixed in formaldehyde solution and embedded in paraffin, then were cut out and mounted on silane-coated glass slides. The sections were deparaffinized with xylene for 30 min and washed in 90% ethanol twice. After inhibition of endogenous peroxidase with 30% H_2O_2/methanol and nonspecific binding of antibody by "ultratech HRP (AEC) kit, protein blocking agent." Ultratech kit is suitable for use with all primary antibodies derived from rabbit, mouse, rat or guinea pig.

The sections were then incubated with primary antibody 5–10 min at room temperature which binds to specific tissue antigens. Color was developed with AEC system, and nuclear staining was done with Meyer's hematoxylin. Tissue sections from a normal kidney were used for control staining because the reactivity of proximal tubules have been shown to be different from that of glomeruli, so that results of Pgp immunostaining were positive in proximal tubules and negative in glomeruli. In the sections of the lesions, the degree of Pgp staining was scored from 0–3 based on the distribution of positivity of immunostaining of the plasma membrane and the Golgi region as follows: a) Score 0 when no stain was observed; b) Score 1 when less than 10% of the cells were stained; c) Score 2 when 10% to 49% of the cells were stained; and d) Score 3 when 50% or more of the cells were stained. The scores of 0 and 1 were considered as negative, 2 and

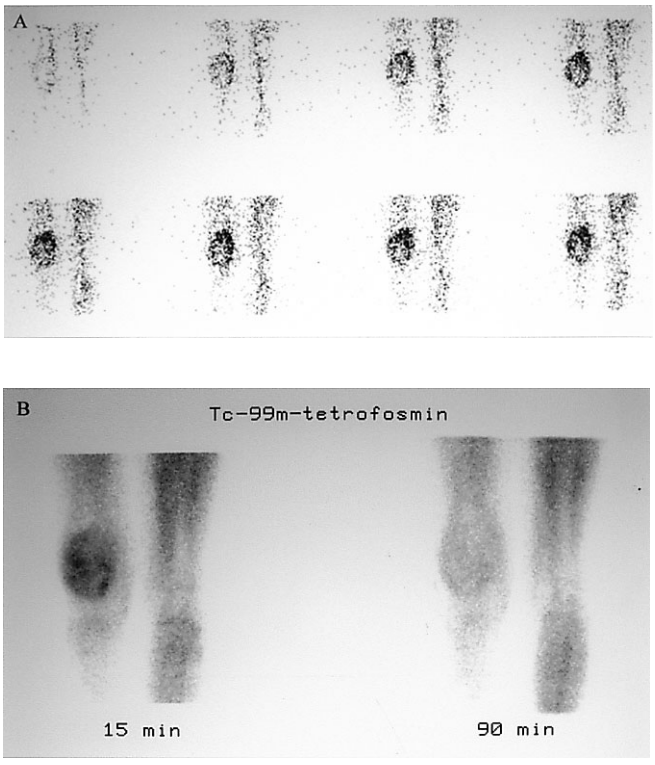

Fig. 2 A 7-yr-old girl with malignant fibrous histiocytoma on right femur (Patient 25). A: Radionuclide angiography shows a highly vascular lesion. B: Significant tetrofosmin uptake is seen on 15 min image; 90 min image shows significant washout of the tracer. C: Diffuse positive reaction was seen immunohistochemically (IHC × 200). Arrows indicate strong positivity in multinuclear giant cells (Score 3).

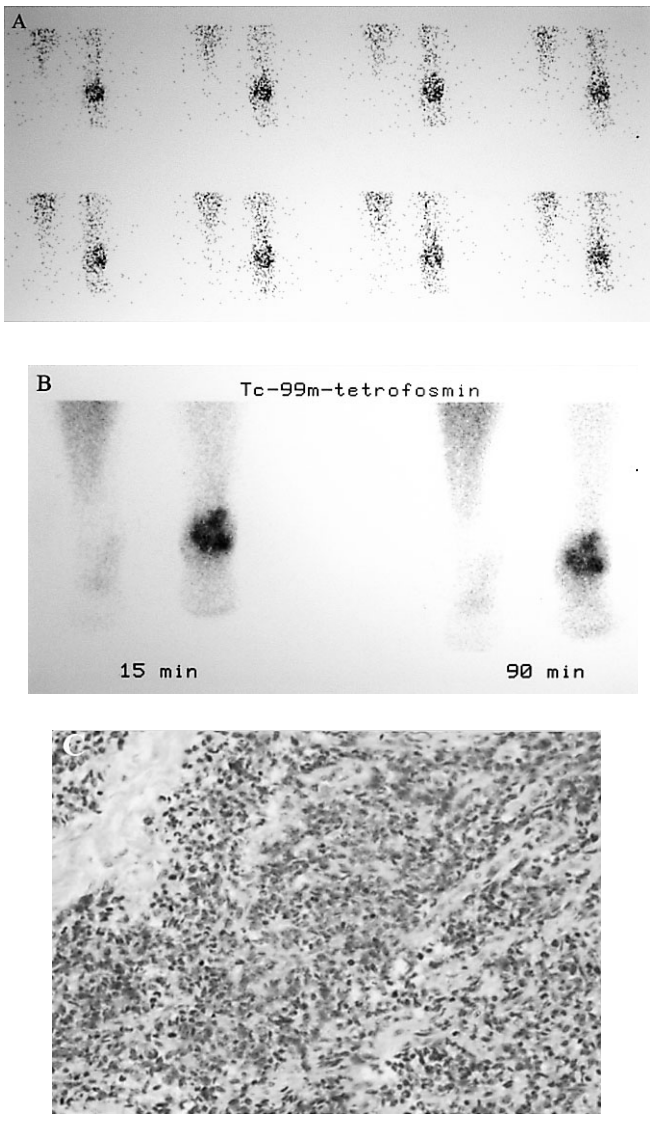

Fig. 3 A 21-yr-old female patient with Ewing's sarcoma of the right calcaneus (Patient 4). A: Radionuclide angiography shows a hypervascular lesion. B: 15 min image shows intense tetrofosmin uptake in the lesion without significant washout on 90 min image. C: In the section of the case, no staining was observed with Pgp (IHC × 200).

3 were considered as positive for Pgp expression.

Statistical Analysis

The values were presented as mean ± SD. The Mann-Whitney test was used to determine the differences between the tumors with and without Pgp. $P < 0.05$ was considered as significant. The correlation between Pgp levels and tetrofosmin results were analyzed by Spearman's rank correlation coefficient . For determining the sensitivity and specificity values of tetrofosmin washout ratio in assessing Pgp positivity, the cut-off value of 24.50 was determined according to the ROC-analysis.

RESULTS

All patients' data are listed in Table 1. When the scores of 0–1 were considered as negative, and 2–3 were considered as positive for Pgp expression, Pgp positivity was detected in 17 patients (Pgp scores were 3 and 2 in 13 and 4 patients, respectively). In 11 patients, Pgp expression was not significant (Score 1 in 4, score 0 in 7 patients).

There were only two lesions (2/28, 7%) without tetrofosmin uptake. Visually, 26 out of 28 lesions (93%),

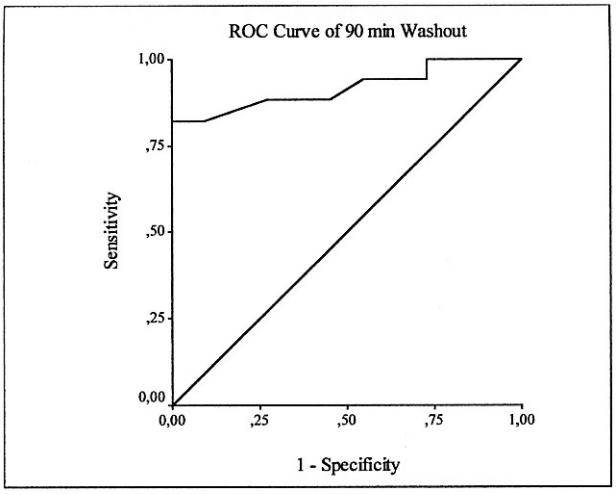

Fig. 4 According to ROC analysis, cut-off value for washout rate was determined as 24.5.
Area under curve: 0.90 (ci: 0.79–1.02)
Sensitivity: 82.4%
Specificity: 99%

with or without Pgp expression showed tetrofosmin accumulation. Among them, nineteen lesions (19/26) showed strongly (grade 3), five lesions (5/26) showed moderately (grade 2) and two lesions (2/26) showed slightly (grade 1) increased tetrofosmin uptakes on 15 min images. On 90 min images, 17 lesions (17/26, 65%) showed tetrofosmin washout in any degree, while tetrofosmin uptake grade persisted in 9 lesions (9/26, 35%). Washout group (17 lesions) consisted of 14 lesions with Pgp expression (82%) and 3 lesions without Pgp expression (17%). Persistence group (9 lesions) consisted of 7 (77%) and 2 (23%) lesions without and with Pgp expression, respectively. In other words, washout group mostly included lesions with Pgp expression (82%), while the lesions without Pgp expression mostly showed persistent activity (77%).

Except two lesions which did not accumulate tetrofosmin, all lesions showed increased perfusion on radionuclide angiography. Both Pgp-positive and Pgp-negative tumors showed hypervascularization.

In quantitative analysis, positive correlation between the Pgp score and the washout rate of tetrofosmin was detected ($r = 0.73$, $p = 0.000$). The relationship between the Pgp score and the tetrofosmin washout rate is shown on Figure 1. No statistically significant correlations were found between 15 min and 90 min uptake ratios (UR) of tetrofosmin and Pgp score ($r = -0.10$, $p = 0.6$ and $r = -0.21$, $p = 0.2$).

When the 15 and 90 min mean URs of lesions with Pgp expression (3.42 ± 1.47 and 2.37 ± 1.14) were compared with those of lesions without Pgp expression (3.90 ± 2.34 and 3.04 ± 1.77), the differences between the groups were not significant ($p = 0.7$ and $p = 0.3$, respectively). The mean washout rate of tetrofosmin from the lesions with Pgp expression (31.81 ± 6.72) was found to be significantly higher than those of without Pgp expression (21 ± 3.49) ($p = 0.000$). Representative cases are shown on Figures 2 and 3.

When the cut-off value of 24.5 (according to ROC-analysis) for the washout rate was used to discriminate the lesions with and without Pgp expression, the test yielded a sensitivity value of 87.5% with a specificity of 100% (Fig. 4). There were two cases with discordant results (Table 1, patients with 15 and 22 numbers).

DISCUSSION

The results of the present study demonstrated that the washout of ^{99m}Tc-tetrofosmin in malignant bone and soft-tissue tumors was correlated with Pgp overexpression. Although the 15 and 90 min mean URs of the lesions without Pgp expression was slightly higher than those of the lesions with Pgp expression the difference was not significant. For this reason, washout analysis and delayed imaging in addition to the early imaging seems to be necessary when tetrofosmin is used in the evaluation of Pgp expression in malignant bone and soft-tissue tumors.

Preoperative chemotherapy has changed the prognosis of bone tumors dramatically.[20] It can eliminate micrometastases, induce total necrosis in the primary tumor, reduce the tumor's size and vascularity, and thus facilitates the surgical excision of the margins safely. Clinical trials revealed that Pgp overexpression correlates with a poor chemotherapeutic response and poor prognosis in bone and soft-tissue sarcomas.[1,5,6] Thus, there is a real challenge for the detection of Pgp in these tumors.

Noninvasive detection of Pgp uses lipophilic cationic compounds characterized as substrates for MDR1 Pgp. Among these compounds, ^{99m}Tc-sestamibi and ^{99m}Tc-tetrofosmin are nonmetabolizable radiopharmaceuticals with monocationic charges, like most chemotherapeutic agents in the MDR phenotype.[21,22] Cellular uptake of these tracers depends on series negative transmembrane and mitochondrial potentials,[23] and they can accumulate to a much higher degree in malignant cells due to differences in mitochondrial density and membrane polarization.

^{99m}Tc-tetrofosmin has been validated as a transport substrate for Pgp in a variety of MDR human cells.[16,18,24] Also *in vivo* studies have shown that ^{99m}Tc-tetrofosmin imaging has been a potential tool for understanding Pgp expression in lung cancer,[25] malignant lymphoma[26] and breast cance.[27] From a review of the literature, no reports have been published previously concerning the relationship between Pgp expression and cellular kinetics of tetrofosmin in musculoskeletal tumors. Previous experience has focused on ^{99m}Tc-sestamibi and proved that this agent has been a substrate for Pgp in these tumors.[28,29] According to our results, ^{99m}Tc-tetrofosmin imaging may be useful in bone and soft-tissue sarcomas as well, when

efflux rate of the tracer is used as an indicator of Pgp expression. Prior data have shown an inverse relationship between levels of Pgp and the magnitude of tetrofosmin uptake in malignant lymphoma, breast and lung cancers.[26,27,30] Contrasting with prior data, tetrofosmin accumulated in most musculoskeletal sarcomas even with high Pgp expression (Table 1) in our study. Such contrasting points for sestamibi were also raised in the published data and attributed to the presence of subsets of cells simultaneously expressing varying and multiple resistance mechanisms, or poor penetration of tracer because of reduced blood flow in tumors undergoing necrosis.[2] In accordance with our findings, Taki et al.[28] and Burak et al.[29] did not find significant correlations between tumor to background URs and Pgp expression using ^{99m}Tc-sestamibi, and washout analysis was recommended as a conclusion.

The method has failed to detect Pgp expression in two lesions (patient Nos. 15 and 22) when the cut-off value of tetrofosmin was considered as 25.5%. Although the immunostaining of Pgp was strong, the washout of tetrofosmin from these lesions was slower than the threshold level, suggesting a negative result. Suboptimal functional capacity of the Pgp efflux pump was thought to be the possible mechanism to explain the discordant findings for these two lesions. Discordant results might also be explained by the heterogeneous distribution of Pgp in tumors, in which a small biopsy specimen might not always represent whole tumor Pgp expression. However, any patient with heterogeneous tetrofosmin washout from the lesion was not observed in our study. Burak et al.[29] have advised the application of small standard ROIs using single-photon emission tomography in determining regional washout rates because of the possible insufficiency of planar imaging.

The optimum time for delayed imaging regarding ^{99m}Tc-sestamibi is under debate in the literature. Taki et al.[28] preferred a delay of 3 hr for late images. On the other hand, Burak et al.[29] showed that 1-hr late imaging could also be used to define the clearance of sestamibi. Thus, the timing of the late images was planned as 90 min after injection in current study. Our results revealed that 90 min delay after injection could be sufficient to demonstrate the different washout patterns of tetrofosmin between Pgp positive and Pgp negative tumors. However, complete washout in 90 min was observed in only one lesion (1/16) among lesions with Pgp expression. This ratio is very low when compared to the ratio obtained from the study in which 3 hr delayed imaging was used with sestamibi by Taki et al. (7/15).[28] From this point of view, it could be argued that later imaging times at least 120 min after injection may be preferred if the images are to be analyzed visually.

Doxorubicin is a chemotherapeutic agent which is included in therapy protocols in malignant bone and soft-tissue tumors, and is generally effective. It has been shown to be a transport substrate for Pgp.[7] Functional identification of Pgp before initiation of chemotherapy might provide important information and might be valuable in management of patients with malignant bone and soft-tissue tumors. If modulation of Pgp becomes feasible in clinical practice, ^{99m}Tc-tetrofosmin scintigraphy would be useful as a Pgp function monitoring method.

CONCLUSIONS

According to our results, ^{99m}Tc-tetrofosmin uptake in bone and soft-tissue tumors were not related to Pgp overexpression. Pgp overexpression was found to be correlating to the washout rate of the tracer. ^{99m}Tc-tetrofosmin scintigraphy with washout analysis may not only be a useful method for evaluating Pgp overexpression but also its function. Tumor imaging with ^{99m}Tc-tetrofosmin may be a promising clinical tool in monitoring patients with malignant bone and soft-tissue tumors. But further prospective studies are needed to evaluate the prognosis and event-free survival of patients.

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