Lack of Correlation of Functional Scintigraphy with ^{99m}Technetium-Methoxyisobutylisonitrile with Histological Necrosis following Induction Chemotherapy or Measures of P-Glycoprotein Expression in High-Grade Osteosarcoma[1]

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ABSTRACT

In osteosarcoma, some studies have suggested P-glycoprotein expression is a prognostic factor. The clearance of ^{99m}technetium hexakis-2-methoxyisobutylisonitrile (^{99m}Tc-MIBI) has been used in some tumor systems as an *in vivo* measure of P-glycoprotein-mediated efflux. In this study we explored the correlation between ^{99m}Tc-MIBI clearance and histological necrosis following induction chemotherapy and P-glycoprotein expression in osteosarcoma. The primary tumors of 20 patients with high-grade osteosarcoma were imaged at diagnosis with ^{99m}Tc-MIBI, and the uptake ratios and biological half-lives were calculated. P-Glycoprotein expression in the tumor tissue was determined immunohistochemically and by measuring mRNA expression of the multidrug resistance-1 gene. The histological necrosis following induction chemotherapy was assessed by the Huvos grading system. The biological half-life of ^{99m}Tc-MIBI ranged from 1.4 to 52.5 h. Seven of the 20 tumor samples had a favorable extent of necrosis following induction chemotherapy. The ^{99m}Tc-MIBI half-life and uptake ratio showed no correlation with histological necrosis following induction chemotherapy. The ^{99m}Tc-MIBI half-life and uptake ratio did not correlate with either measure of P-glycoprotein expression. The results of this pilot study indicate that ^{99m}Tc-MIBI imaging is not an effective predictor of histological necrosis following induction chemotherapy in high-grade osteosarcoma. ^{99m}Tc-MIBI imaging did not correlate with measures of P-glycoprotein expression in the tumor tissue.

INTRODUCTION

OS[3], the most common primary malignant bone tumor, occurs mainly in children and adolescents. In OS, prognostic factors at diagnosis other than clinical stage have not been clearly identified (1). The extent of necrosis following induction chemotherapy (Huvos grade) is a strong predictor of patient outcome, but attempts to improve outcome by intensifying therapy at that time have largely been unsuccessful (2). Because ~60–70% of patients with localized OS are cured with standard approaches, a need exists to be able to identify a "poor risk" subgroup of patients at diagnosis for stratification of therapy (1, 3). Some retrospective studies have suggested that pgp expression at diagnosis may be a prognostic factor for OS (4, 5), whereas other studies have failed to confirm this relationship (6–8). Several potential explanations for these conflicting results exist, but one concern is that immunohistochemistry, which has been relied on in prior studies, may not be an appropriate method to evaluate pgp expression (4–6, 8). It has been reported that immunohistochemistry for pgp does not always correlate with multidrug resistance-1 mRNA levels in OS (9). In other tumor systems, the simultaneous use of multiple methodologies to detect pgp expression and function has been advocated (10).

An imaging procedure that can detect functional pgp expression and thus potentially serve as a prognostic factor provides a number of theoretical advantages, particularly in OS. Frequently the only tumor tissue available from the diagnostic biopsy is paraffin-embedded material, which precludes analysis by any method other than immunohistochemistry. The biopsy represents a small portion of a tumor, which frequently is large and heterogeneous. A series of lipophilic cationic radiopharmaceuticals, including ^{99m}Tc-MIBI (sestamibi), have been identified and validated as transport substrates for pgp, thus enabling functional imaging (11–13). In many tumor systems, including, among others, breast cancer, lung cancer, hepatocellular carcinoma, malignant lymphomas, soft tissue sarcoma, and bone

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[3] The abbreviations used are: OS, osteosarcoma; pgp, P-glycoprotein; ^{99m}Tc-MIBI, ^{99m}technetium hexakis-2-methoxyisobutylisonitrile; RT-PCR, reverse transcription-PCR.

sarcomas, significant correlations have been reported between ^{99m}Tc-MIBI scintigraphy and pgp immunohistochemistry, *in vitro* cytotoxicity assays, chemotherapy response, and/or patient outcome (14–20). In some of the same histologies as well as others, including parathyroid adenomas or head and neck cancer, some published reports have failed to identify a significant correlation between ^{99m}Tc-MIBI scintigraphy and measures of pgp expression or patient outcome (21–24).

In this pilot study, the uptake and clearance of ^{99m}Tc-MIBI in patients with newly diagnosed high-grade extremity OS were measured. The scintigraphy results were related to the extent of necrosis (Huvos grade) in the tumor following induction chemotherapy as well as measures of pgp expression, determined both immunohistochemically and by quantitative RT-PCR for multidrug resistance-1 expression in the tumor tissue. This may indicate the potential for ^{99m}Tc-MIBI scintigraphy to predict response to chemotherapy, patient outcome, and pgp expression in high-grade OS.

PATIENTS AND METHODS

Twenty patients with newly diagnosed high-grade extremity OS participated in this study between May 1998 and May 2000. All newly diagnosed extremity OS patients were offered participation in the study. Tumor tissue was obtained from the patients after the patient or guardian provided informed written consent in accordance with a research protocol approved by the Memorial Hospital Institutional Review Board. All patients received standard OS induction chemotherapy comprising four courses of high-dose methotrexate with leucovorin rescue and two cycles of cisplatin and doxorubicin, as has been described previously in the Memorial Sloan-Kettering Cancer Center T10 and T12 protocols (2, 25). Histological necrosis following induction chemotherapy was assessed at the time of definitive surgery based on the Huvos grading system as described previously (25). Thallium scans were performed and analyzed as a routine clinical study as has been described previously (26).

^{99m}Tc-MIBI Scintigraphic Imaging. The interval from initial biopsy to ^{99m}Tc-MIBI scan averaged 8 days (range, 1–15 days). Despite the patient's age, Clark's rule was applied to the radioactivity dosage, which was based on proportional body weights as related to the standard weight mean of 150 pounds, to give 740 MBq (20 mCi) ^{99m}Tc-MIBI (Cardiolite; DuPont Pharma, Wilmington, DE). Serial planar images were acquired for 5 min each at four time points at ~20 min and 1, 2, and 4 h after i.v. administration of radiotracer. These spot images were centered on the lesion and included the opposite limb. A dual-head ADAC Genesys camera with a LEHR collimator, equipped with SUN Spark series computer and Pegasys Image Processing System (ADAC laboratories, Milpitas, CA) was used for image acquisition. Whole-body images were acquired after each planar spot image. All calculations were performed using previously described methods (20). To calculate the biological half-life, we drew a manual region of interest over the entire area of tumor uptake. The count density in this region was determined for each of the time points. These data were adjusted for decay correction and were used to produce a fitted monoexponential curve to derive the tumor effective biological half-life. The uptake ratio was determined by relating the count density in the same region of interest on the 20 min image to the identical area on the contralateral leg, which did not include tumor. For the uptake ratio, the mean of the anterior and posterior images was calculated and presented.

Immunohistochemistry. All paraffin-embedded material was retrieved from the Department of Pathology at Memorial Sloan-Kettering Cancer Center. Immunohistochemistry was performed as has been described previously (4, 8, 27). Briefly, sections from decalcified bone tumor specimens were cut at 4–5 μm, deparaffinized, and rehydrated. Pretreatment comprised digestion with 0.05% trypsin followed by microwave treatment for 10 min. JSB-1 (Signet Laboratories, Dedham, MA) at a dilution of 1:20 and C494 at a dilution of 1:20 (Signet Laboratories, Dedham, MA) were used for pgp. Incubations were at 4°C for 16 h. Color reactions were obtained using a standard avidin and peroxidase-conjugated streptavidin (Dako Corporation, Carpinteria, CA) technique. Slides were counterstained with Harris modified hematoxylin (Fisher, Pittsburgh, PA). Positive controls, constituting normal adrenal gland, as well as negative controls, in which the primary antibody was omitted, were included with each run. A pathologist blinded to patient identity scored each case. The cases were scored as 0 (no staining), 1+ (1–25% of the cells staining positive), 2+ (26–50% positive), 3+ (51–75% positive), and 4+ (76–100% positive). The staining was considered positive only if it localized predominantly to the membrane.

Quantitative RT-PCR. RNA was prepared from frozen tumor tissue by use of RNAzol as per manufacturer's instructions (BiotecX Laboratory). Quantitative RT-PCR was performed using methodologies described previously (28–31). Total RNA (5–10 μg) was treated with RNase-free DNase (Boehringer-Mannheim, Indianapolis, IN), reextracted with phenol-chloroform, and reverse transcribed using random hexamers with a cDNA cycle kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Relative gene expression was calculated by determining the ratio between the amount of the radiolabeled PCR product within the linear range of the multidrug resistance-1 gene and the β-actin gene. The PCR conditions and gel electrophoresis have all been described previously (28–31). Radioactivity was quantitated on a Fuji BAS 2500 phosphorimager (Fuji Photo Film, Tokyo, Japan). The primers used were 5′-CCCATCATTGCAATAGCAGG-3′ and 5′-GTTCAAACTTCTGCTCCTGA-3′ for MDR-1, and BA67 and BA68 for β-actin; all primers have been described previously (27–30). The CCRF-CEM cell line served as a negative control, and CEM-VBL, which is known to overexpress multidrug resistance-1 (obtained from Dr. William Beck, University of Illinois at Chicago, Chicago, IL), served as a positive control. Any detectable PCR product of the appropriate size was considered as positive for MDR-1 gene expression.

Statistical Methods. The results of the ^{99m}Tc-MIBI scans were related to the Huvos grade and measures of pgp expression by a *t* test. Measures of pgp expression and the thallium scan results were related to Huvos grade and each other by a Fisher exact test.

RESULTS

The clinical information for the 20 newly diagnosed high-grade extremity OS patients who participated in this study is

Table 1 Clinical characteristics of the high-grade OS patients undergoing ^{99m}Tc-MIBI scans

Patient	Age (years)	Sex	Site	Subtype	Size	Metastases
1	10	M	R[a] distal femur	None	8 × 6.3 × 6.3	None
2	11	F	R proximal tibia	Osteoblastic	5.8 × 3.5 × 3	None
3	19	M	R proximal humerus	Osteoblastic	10 × 4.5 × 2.7	None
4	15	M	R distal femur and rib	Chondroblastic	10.5 × 7 × 7	Lung and bone
5	13	M	R proximal tibia	Osteoblastic	7.5 × 4.5 × 4	None
6	9	F	L distal femur	Osteoblastic and chondroblastic	9.5 × 6.5 × 5.9	None
7	8	M	L proximal tibia	Chondroblastic	4.2 × 3 × 2.5	None
8	16	F	R proximal humerous	Osteoblastic	8.5 × 4 × 3.8	None
9	18	M	R proximal tibia	Giant cell rich	6.6 × 4.3 × 4.1	None
10	27	M	L ilium, proximal and distal femur	None	10 × 4.5 × 4	Bone
11	18	F	R distal femur	MFH type	5.5 × 3.0 × 3.5	None
12	18	F	L distal femur	None	4.9 × 3.8 × 3.8	None
13	15	M	R distal femur	Osteoblastic	5.5 × 2 × 1.4	Lung
14	17	M	L distal femur	Osteoblastic	9.5 × 3.5 × 2.0	None
15	31	F	R proximal tibia	Osteoblastic fibroblastic, and chondroblastic	4.8 × 4.8 × 3.8	None
16	30	M	R proximal humerus	Giant cell rich	15.5 × 3.8 × 3.0	None
17	17	M	R proximal tibia	Osteoblastic and chondroblastic	2 × 2.5 × 2	None
18	11	F	R distal femur	Giant cell rich	7.5 × 5.8 × 4.4	None
19	12	M	L distal femur	Osteoblastic	12.5 × 6.8 × 5.2	None
20	7	F	R proximal tibia	Osteoblastic and giant cell rich	4 × 2.2 × 2	None

[a] R, right; L, left; MFH, malignant fibrous histiocytoma.

Table 2 Correlation between the biological half-life and uptake ratio of ^{99m}Tc-MIBI and histological necrosis following preoperative chemotherapy and measures of pgp expression

Patient	^{99m}Tc-MIBI scan		Histological necrosis (Huvos grade)	pgp immunohistochemistry	MDR-1 RT-PCR
	Half-Life	Uptake ratio			
1	1.4	1.8	2	NEG[a]	NEG
2	2.2	1.9	2	NEG	NEG
3	2.3	1.7	2	NEG	POS
4	2.9	2.9	1	NEG	POS
5	3.1	3.5	3	1+	NEG
6	3.8	2.8	3	4+	POS
7	3.8	2.4	2	NEG	POS
8	4.2	2	2	4+	NEG
9	4.3	4.8	4	4+	NEG
10	4.4	1.2	1	NEG	POS
11	4.8	5.5	2	NEG	NEG
12	4.9	2.4	3	1+	NEG
13	4.9	1.7	3	1+	NEG
14	5.7	2.9	1	1+	NEG
15	6.9	2.8	1	NEG	POS
16	8.2	2.3	1	NEG	NEG
17	13.2	3.7	2	NEG	NEG
18	22.7	1.5	4	NEG	NEG
19	45.6	1.6	2	NEG	NEG
20	52.5	1	3	NEG	POS

[a] NEG, negative; POS, positive.

presented in Table 1. Twelve males and 8 females participated (age range, 7–31 years; median, 15 years). Seventeen of the patients had lesions around the knee, with 10 involving the distal femur and 7 affecting the proximal tibia. Three patients had lesions involving the proximal humerus. The three patients with metastases at diagnosis were Enneking stage IIIb, all others were stage IIb. A variety of histological subtypes were observed (Table 1). Overall the clinical features of this population of patients were typical for those diagnosed with this disease.

All patients received induction chemotherapy. Limb-sparing surgical resection was performed in 18 of the 20 patients. One patient (patient 7) underwent a rotationplasty procedure, and one patient (patient 10) underwent an above-knee amputation. The interval between diagnosis and surgical resection of the tumor averaged 86 days (range, 75–101 days), which is typical for the induction chemotherapy administered.

The biological half-life of ^{99m}Tc-MIBI ranged from 1.4 to 52.5 h (Table 2). The uptake ratio of ^{99m}Tc-MIBI is also

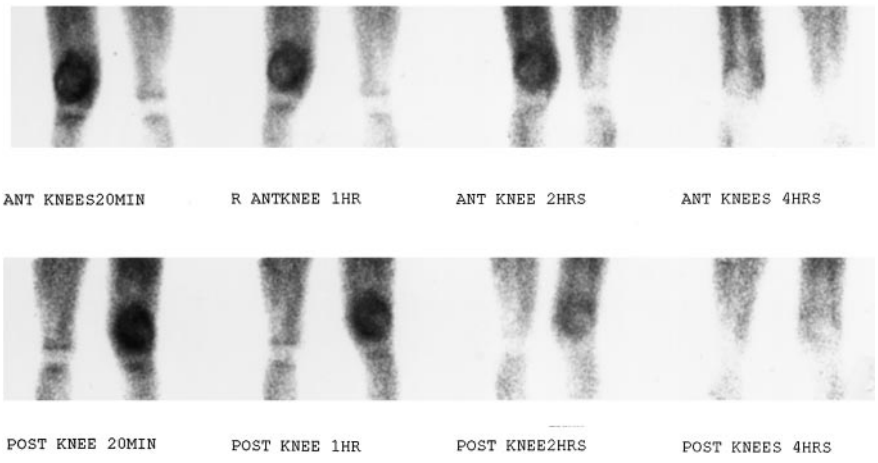

Fig. 1 Representative scintigraphic imaging of ^{99m}Tc-MIBI at 20 min and 1, 2, and 4 h after i.v. administration of radiotracer with anterior and posterior views. This tumor of the right distal femur is easily visualized.

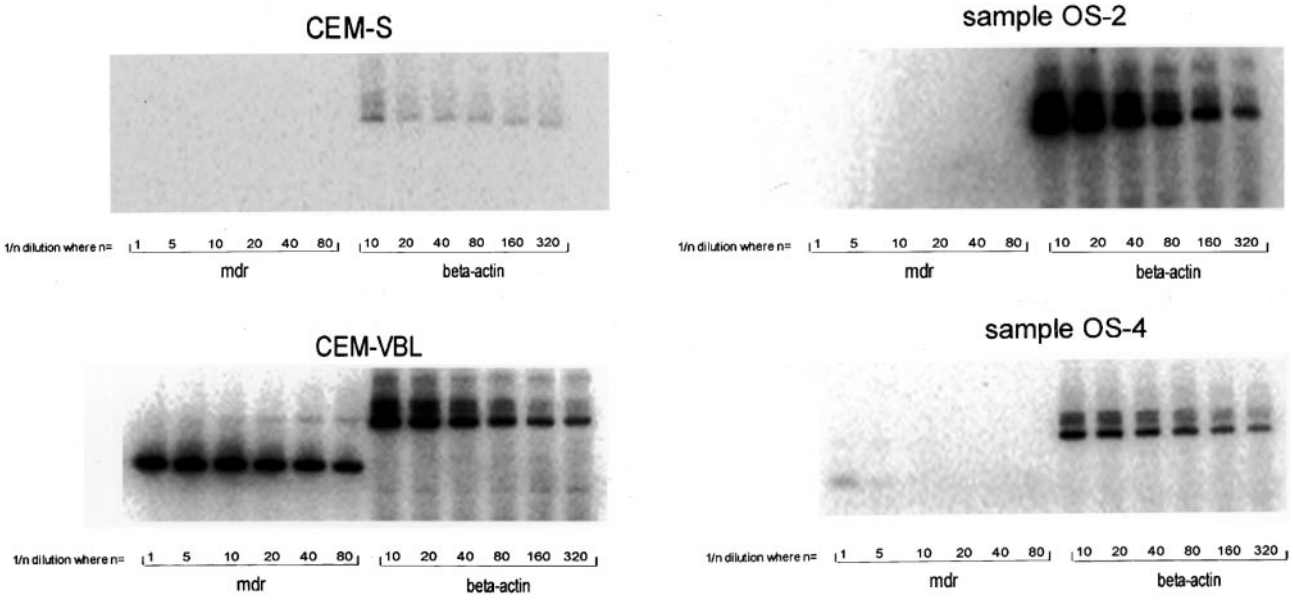

Fig. 2 Representative quantitative RT-PCR for MDR-1 gene expression in two OS tumor samples. *CEM-S* is a negative control, and *CEM-VBL* is a positive control. Sample *OS-4* demonstrates positive MDR-1 gene expression, whereas sample *OS-2* is negative.

presented in Table 2. A representative ^{99m}Tc-MIBI scan is shown in Fig. 1. Seven of the 20 patients had a favorable extent of necrosis (Huvos grade 3 or 4) following induction chemotherapy. Seven of the 20 patients had detectable pgp expression by immunohistochemistry using both the JSB-1 and C494 antibodies. The results obtained with the two antibodies were concordant; therefore, only the C494 results are presented in Table 2. Seven of the 20 patients had detectable multidrug resistance-1 gene expression by quantitative RT-PCR (Fig. 2).

No significant correlation was observed between the ^{99m}Tc-MIBI biological half-life and Huvos grade ($P = 0.41$), with the tumors with a favorable extent of necrosis following induction chemotherapy having a mean biological half-life of 13.7 h and the tumors with less necrosis having a mean biological half-life of 8.1 h. No significant correlation was observed between Huvos grade and the ^{99m}Tc-MIBI uptake ratio ($P = 0.78$). Tumors that did not express pgp immunohistochemically had a higher mean ^{99m}Tc-MIBI biological half-life (13.2 h) compared with those that did not (4.4 h), but this relationship was not statistically significant ($P = 0.19$). No significant correlation was observed between the ^{99m}Tc-MIBI uptake ratio and pgp expression measured by immunohistochemistry or by quantitative RT-PCR for multidrug resistance-1 mRNA. Overexpression of pgp was significantly associated with a favorable extent of necrosis following

Table 3 Correlation between the thallium scan results and histological necrosis following preoperative chemotherapy

Patient	Histological necrosis (Huvos grade)	Thallium scan results
2	2	No residual activity
3	2	Persistent uptake, unchanged
4	1	Interval increase
5	3	Persistent uptake, less intense
7	2	Persistent uptake, less intense
8	2	Persistent uptake, unchanged
9	4	Small area of residual uptake
10	1	Persistent uptake, less intense
12	3	Small area of residual uptake
13	3	No residual activity
14	1	Persistent uptake, less intense
15	1	Persistent uptake, unchanged
16	1	Persistent uptake, unchanged
18	4	Small area of residual uptake
19	2	Persistent uptake, less intense
20	3	Small area of residual uptake

induction chemotherapy ($P = 0.022$), the opposite of the anticipated finding. No significant correlation was observed between pgp expression as measured by quantitative RT-PCR and the extent of necrosis. No significant correlation was observed between the two measures of pgp expression, immunohistochemistry and quantitative RT-PCR ($P = 0.33$).

Sixteen of the patients who participated in this study had thallium scans performed at diagnosis and before their definitive surgery as a routine clinical study. The results of the thallium scans are presented in Table 3. An interval increase or persistent uptake, even if less intense than the diagnostic scan, after induction chemotherapy was significantly predictive of an inferior extent of necrosis ($P = 0.006$) at the time of definitive surgery. No residual or a small area of residual uptake on thallium scan was predictive of a favorable extent of necrosis.

DISCUSSION

In this pilot study the tumors of a representative cohort of newly diagnosed patients with high-grade OS were evaluated with ^{99m}Tc-MIBI scans. A large variability was observed in the scans, with the biological half-life of the tracer ranging from 1.4 to 52.5 h. Although a higher mean ^{99m}Tc-MIBI biological half-life was observed in tumors that had a favorable extent of necrosis (Huvos grade 3 and 4) at the time of definitive surgery and in tumors that did not express pgp immunohistochemically, the correlation of these factors was not statistically significant. The scan results have not been related to patient outcome because of the short median duration of follow-up. Despite this fact, the extent of necrosis following induction chemotherapy has been a reliable predictor of patient outcome in virtually all prior high-grade OS studies (1–3, 25, 27). Lack of correlation with the extent of necrosis therefore suggests that the ^{99m}Tc-MIBI scans will not correlate with patient outcome. In this same cohort of patients, the results of thallium scans correlated significantly ($P = 0.006$) with the extent of necrosis at the time of definitive surgery, as has been reported previously (26). Unfortunately, thallium scans need to be performed at diagnosis and at definitive surgery to be predictive of the extent of necrosis, limiting their use as a prognostic indicator.

On the basis of this pilot study, pgp expression as measured by ^{99m}Tc-MIBI imaging at diagnosis could not serve as a prognostic factor in OS. An additional concern raised by this study is the lack of correlation between the various measures of pgp expression. This suggests that some of the conflicting results observed in prior studies assessing pgp expression as a prognostic factor in high-grade OS may be secondary to the methodology used (4–9, 27). Some investigators have suggested that pgp expression measured at diagnosis in high-grade OS be used as a basis for stratification of therapy (32). This study does not support that viewpoint.

Several possibilities exist for the lack of correlation. ^{99m}Tc-MIBI is a substrate for other proteins, which can efflux various natural compounds, such as the multidrug resistance-associated protein (33). The status of the multidrug resistance-associated protein in high-grade OS is unknown. An additional possibility is that the demonstration of pgp protein and gene expression does not demonstrate that it is functional. Imaging with ^{99m}Tc-MIBI has a number of theoretical advantages for the assessment of pgp expression in high-grade OS. It can be performed noninvasively *in vivo*, allowing for repeated assessments. Although the scans at diagnosis do not appear to be prognostic indicators, it is possible that evaluations at different time points or changes in the scans obtained for sequential evaluation may have predictive value. An alternative approach would be to administer an multidrug resistance-1 reversal agent, such as PSC833, which may demonstrate whether the efflux of ^{99m}Tc-MIBI can be modulated in the tumor (34, 35). This might have diagnostic utility as well as potential therapeutic implications. Further studies with ^{99m}Tc-MIBI as a measure of functional pgp expression and as a prognostic factor in high-grade OS may be warranted.

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