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Usefulness of Tc-99m MIBI SPECT in predicting multidrug resistance gene expression levels in non-small cell lung cancer—a preliminary report

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In this study we investigated whether Tc-99m hexakis 2-methoxy isobutyl isonitrile (Tc-99m MIBI) single-photon emission computed tomography (SPECT) has a correlation with the multidrug resistance (MDR1) and multidrug resistance-associated protein (MRP1) gene expression levels in non-small cell lung cancer (NSCLC). Fifteen patients with NSCLC were studied. SPECT images were obtained 15 (early) and 120 (delayed) min after injection of Tc-99m MIBI. We chose only one transverse section and set the region of interest over the tumor and out of the body. The mean counts in the tumor on early and delayed images were corrected by using those in the background and represented as Te and Td, respectively. Resected tumor specimens were frozen with liquid nitrogen and each positive control cell line was cultured. After the total ribonucleic acid (RNA) was extracted from specimens and cell lines, the complimentary deoxyribonucleic acid (cDNA) was amplified by the reverse transcription-polymerase chain reaction (RT-PCR) method. Each product was electrophoresed and fluorointensity was measured. The gene expression level was represented as the ratio of that of the positive control cell line. Te and Td indicated a significant correlation with the MDR1 gene expression level ($p = 0.015$ and $p = 0.022$), but not the gene of MRP1 ($p = 0.100$ and $p = 0.145$). In conclusion, Te and Td are useful parameters in predicting the MDR1 gene expression level, but not MRP1 in NSCLC.

Key words: non-small cell lung cancer, Tc-99m MIBI, MDR1, MRP1, RT-PCR

INTRODUCTION

MULTIDRUG RESISTANCE is an important problem in chemotherapy of lung cancer. P-glycoprotein (Pgp) and multidrug resistance associated protein (MRP) have been confirmed to be involved in this phenomenon in *in vitro* studies.[1,2] Pgp is a 170 kDa weight transmembrane protein encoded by a multidrug resistant (MDR1) gene on a region of chromosome 7 and is expressed in normal tissues such as liver and kidney.[3] MRP is 190 kDa glycoprotein encoded by MRP1 gene on a region of chromosome 16, and is overexpressed in cancer cell surfaces.[4] Their physiological functions remain unclear. Although MRP as well as Pgp belongs to the ATP-binding cassette superfamily, MRP excludes a substrate with the cooperation of a glutathione.[5] Since Tc-99m hexakis 2-methoxy isobutyl isonitrile (Tc-99m MIBI) has been confirmed to accumulate on many kinds of tumor *in vivo*[6,7] and to be a substrate for Pgp in *in vitro* studies,[8] it has been reported that Tc-99m MIBI is useful in multidrug resistance through Pgp in *in vivo* studies.[9] Moreover, Hendrikse et al. and Moretti et al. indicated that Tc-99m MIBI is a substrate for not only Pgp but also MRP in *in vitro* studies,[10,11] but few studies show a relationship between MRP and Tc-99m MIBI single-photon emission computed tomography (SPECT) in patients with non-small cell lung cancer (NSCLC). Induction chemotherapy has been recently performed on patients with resectable NSCLC.[12] This protocol of chemotherapy includes some kinds of anticancer drugs such as doxorubicin, vindesine and paclitaxel, which have been confirmed to be substrates for either Pgp

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or MRP in *in vitro* studies.[9,13–15] Therefore, a tumor with overexpression of Pgp or MRP may tend to show resistance to these anticancer drugs. If Tc-99m MIBI reflects these transmembrane proteins or their gene expression levels in NSCLC, it would be possible to select more effective drugs, which are substrates for Pgp or MRP, for complete resection, because the response to chemotherapy may be different in individual cases. The purpose of this study is to investigate whether Tc-99m MIBI SPECT is useful for predicting MDR1 and MRP1 gene expression in patients with NSCLC.

MATERIALS AND METHODS

Patient population

Among the NSCLC patients who attended the department of thoracic surgery of Osaka Medical College Hospital between March 1999 and November 1999, twenty-one had indication of surgery on the basis of chest radiography and computed tomography (CT). Six of these patients were excluded because of previous chemotherapy or histologically proven metastatic lung cancer. Finally, fifteen patients with primary lung cancer who had indication of surgery, including eight with adenocarcinomas and seven with squamous cell carcinomas were involved in this study (mean age: 63.87 ± 8.55 years; age range: 49–75 years), comprising six males and nine females. None had previously received chemotherapy or radiotherapy. The size of the tumor was measured on the basis of chest CT. No tumor revealed histological necrosis. We evaluated only primary lesions in this study. The purpose and procedure of this study were fully explained to these patients, and they gave their informed consent to participation.

Tc-99m MIBI SPECT imaging

Tc-99m MIBI scintigraphy was performed on all patients 2–6 days before surgery. SPECT images were obtained 15 (early) and 120 (delayed) min after injection of 600 MBq of Tc-99m MIBI (Cardiolite, Daiichi Radioisotope, Hyogo), which was labeled in manufactural methods, with a three head gamma camera, GCA9300A (Toshiba Co., Tokyo, Japan) equipped with a parallel hole collimator. The matrix size was 128 × 128. For SPECT images, 90 projections were obtained with a 128 × 128 matrix for 30 sec a view. Image reconstruction was done by filtered backprojection with a Ramp filter. Neither attenuation correction nor scatter correction was done. While compared with chest CT, identical regions of interest (ROIs) were drawn over the tumor uptake on one transverse section which demonstrated the lesion most clearly and which was carefully selected on both the early and the delayed SPECT images. The square ROIs with a size of 20 × 40 pixels were set out of the body on both early and delayed images and mean counts in the ROIs on both images were measured in each case. The tumor uptake ratios were calculated as T/B, where T = mean counts in the tumor; B = mean counts in the background. These ratios at 15 (early) and 120 (delayed) min after injection of Tc-99m MIBI were represented as Te and Td, respectively and the washout rate in the tumor (Twr) was calculated as follows: [Te − Td]/Te × 100 (%).

Tumor tissue and cell lines

The cancer specimens with a diameter of about 10 mm, which were almost the central parts of the tumors and heavier than 500 mg, were carefully resected and washed with 100 m*l* of isotonic sodium chloride solution in a clean environment so that neither the normal or fat tissue nor blood was included in the tumor specimens. They were immediately stored at −140°C with liquid nitrogen. The K562/Adr overexpressing Pgp but not MRP and H69AR cell lines overexpressing MRP but not Pgp, which were kindly provided by Tohoku (Sendai, Japan) and Queens University (Kingston, Ontario, Canada), were cultured with medium containing RPMI1640 (Sigma, Chemical Co.) + 10% bovine serum in a 5% CO_2 and 95% air incubator and used as positive controls for MDR1 and MRP1 gene expression, respectively. The cell line culture was continued until colony grew to an amount as great as the abovementioned NSCLC specimens.

Reverse transcription-polymerase chain reaction (RT-PCR)

First, 0.1 μl of total RNA of each specimen and cell lines were extracted by TRISOL (GIBCO-BRL). OD 260 nm was measured and the quality of gene was confirmed. Ten μl aliquots were made of diluted 0.1 $\mu g/\mu l$ of total RNA with 2 μl of 25 mM $MgCl_2$, 1 μl of RNA PCR buffer, 1 μl of 10 mM dNTPs, 0.25 μl of RNase inhibitor, 0.5 μl of AMV RNase (TaKaRa RNA PCR Kit Ver. 2.1, TaKaRa, Shiga) and 0.5 μl of random 9 mers adjusted with DEPC-H_2O to react at 30°C for 10 min, 45°C for 30 min, 99°C for 5 min and 4°C for 5 min in a thermal cycler (TaKaRa PCR Thermal Cycler MP, TaKaRa, Shiga). Secondly, 0.5 μl of sense and antisense primers of each kind of gene (each concentration of the primer was 20 pmol/μl) were added to the aliquots with 3 μl of $MgCl_2$, 4 μl of RNA buffer, 0.25 μl of TaKaRa Taq. polymerase and 4 μl of RNA PCR buffer. Fifty μl of the diluted liquid was made with DEPC-H_2O to react at 94°C at 30 sec, 72°C at 30 sec, 55°C for 30 sec and 72°C for 30 sec. This process was performed for 30, 35 and 30 cycles to amplify MDR1, MRP1 and β_2 microglobulin, respectively in each case. For MDR1, sense 5′-GTGGAGCATTCAGACTTGTCTTTCAGCA-3′ and antisense 5′-TTCACTCAATCCAAATGCGGCA-TCTTC-3′ were used as primers. For β_2 microgloblin, sense 5′-CGGAAACATCCACGACCCTAATCC-3′ and antisense 5′-ACCTCCTCATTCGCATCCACCTTGG-3′ were used. For MRP1, sense 5′-GCCTGGCAGCTGGAA-GACAAATACACAAAATT-3′ and antisense 5′-CAGACA-GCAGCTGACAGTCCAAGAACAGGACT-3′ were used.

These PCR products were electrophoresed on 3% NuSieve agarose gel after staining with ethidium bromide. The fluorointensity of each lane was measured with a fluorescent image analyzer (FMBIO II Multi-View, TaKaRa, Shiga). MDR1 and MRP1 gene expression levels were corrected by dividing with that of the β_2 microglobulin, and represented as the ratio to K562/Adr and H69AR, respectively.

Statistical analysis

Data are shown as the mean ± SD. Spearmann's rank test or Mann-Whitney's U test was used for statistical analysis. A p value less than 0.05 was considered significant in both tests.

Table 1 Patients characteristics

Patient No.	Age	Sex	Size (cm)	Type	Stage	Location	Te	Td	Twr	MDR1	MRP1
1	55	F	5.5×4.0	Ad	T2N2M0	LUL	74.36	30.05	59.59	0.18	0.19
2	73	M	2.8×2.4	Sq	T1N1M0	LUL	25.98	12.87	50.46	0.19	0.12
3	63	M	3.5×3.0	Ad	T2N3M0	RUL	133.33	64.55	51.51	0.06	0.06
4	73	M	1.5×1.0	Sq	T1N1M0	LLL	43.23	18.37	57.51	0.11	0.12
5	74	F	1.5×1.5	Ad	T1N1M0	RLL	172.00	61.20	64.42	0.03	0.03
6	49	F	3.2×2.0	Sq	T2N2M0	RUL	271.93	130.07	52.17	0.03	0.04
7	68	F	2.6×2.2	Sq	T1N1M0	LLL	160.11	109.61	31.54	0.02	0.27
8	58	F	3.2×2.0	Sq	T2N2M0	LLL	138.25	66.83	51.66	0.08	0.02
9	57	M	1.5×1.0	Sq	T3N2M0	LUL	266.00	131.81	50.45	0.06	0.03
10	62	F	1.5×1.2	Ad	T1N0M0	LUL	28.39	15.44	45.26	0.08	0.11
11	65	F	2.5×2.0	Ad	T1N2M0	RUL	26.72	15.45	42.18	0.36	0.50
12	75	M	2.0×1.8	Ad	T3N1M0	LUL	231.16	124.75	46.03	0.14	0.44
13	73	M	2.6×2.2	Sq	T1N2M0	LUL	70.00	18.80	73.14	0.30	0.48
14	60	F	2.2×2.0	Ad	T1N0M0	RUL	30.05	17.72	41.03	0.03	0.07
15	52	F	4.5×4.0	Ad	T2N2M0	RML	29.63	14.93	49.61	0.28	0.37
mean ± SD							113.41±90.08	55.50±46.92	51.10±10.02	0.13±0.11	0.19±0.18

Ad, adenocarcinoma; Sq, squamous cell carcinoma; Type, histological type; Te, tumor/background ratio on early image; Td, tumor/background ratio on delayed image; Twr, washout rate in the tumor calculated as [Te − Td]/Te × 100 (%); LUL, left upper lobe; RUL, right upper lobe; LLL, left lower lobe; RLL, right lower lobe; RML, right middle lobe; MDR1, MDR1 gene expression level relative to that of K562/Adr cell line; MRP1, MRP1 gene expression level relative to that H69AR cell line

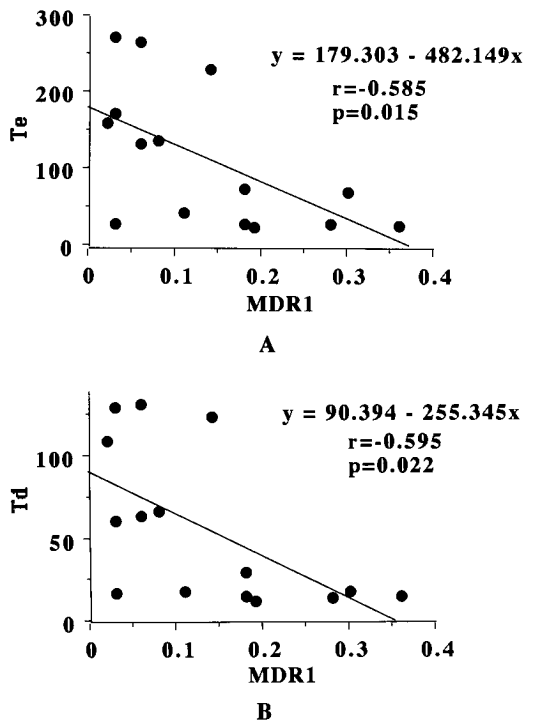

Fig. 1 Significant inverse correlation between MDR1 gene expression level and either Te (A) or Td (B).

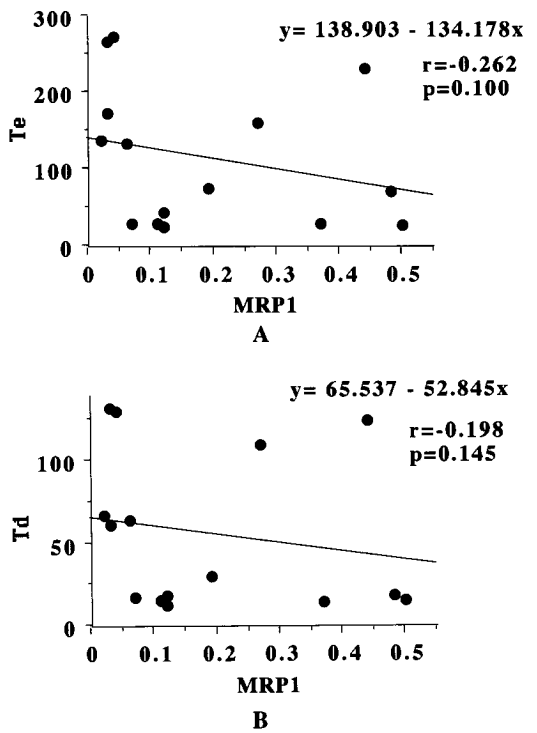

Fig. 2 No significant inverse correlation between MRP1 gene expression level and Te (A) or Td (B).

RESULTS

Relationship between parameters of Tc-99m MIBI SPECT and multidrug resistance gene expression levels

The results are summarized in Table 1. The median values for MDR1 and the MRP1 gene expression level were 0.08 and 0.12, respectively, which were regarded as the cut off values for MDR1 and MRP1 overexpression, because the number of patients was so small that deviation of samples might influence the mean value in this study. Te was significantly correlated with Td ($p < 0.001$). Te as well as Td indicated a significant inverse correlation with the MDR1 gene expression level ($p = 0.015$ and $p = 0.022$, respectively) (Fig. 1), but not MRP1 ($p = 0.100$ and $p = 0.145$, respectively) (Fig. 2). Neither of these gene expression levels was significantly related to Twr ($p = 0.689$ for MDR1 and $p = 0.385$ for MRP1).

Difference between patients with adenocarcinoma and squamous cell carcinoma in parameters and gene expression levels

Neither parameters nor gene expression levels were significantly different in patients with adenocarcinoma ($n = 8$) and squamous cell carcinoma ($n = 7$) ($p = 0.817$, 0.817, 0.729, 0.685 and 0.203 for Te, Td, Twr, MDR1 and MRP1, respectively).

Representative case

Tc-99m MIBI SPECT transverse section indicated accumulation in the lesions on both the early and the delayed images in this case (Patient No. 3). Te and Td were 133.23 and 64.55, respectively. MDR1 and MRP1 gene expression levels were 0.06 and 0.06, respectively, in this tumor specimen (Fig. 3).

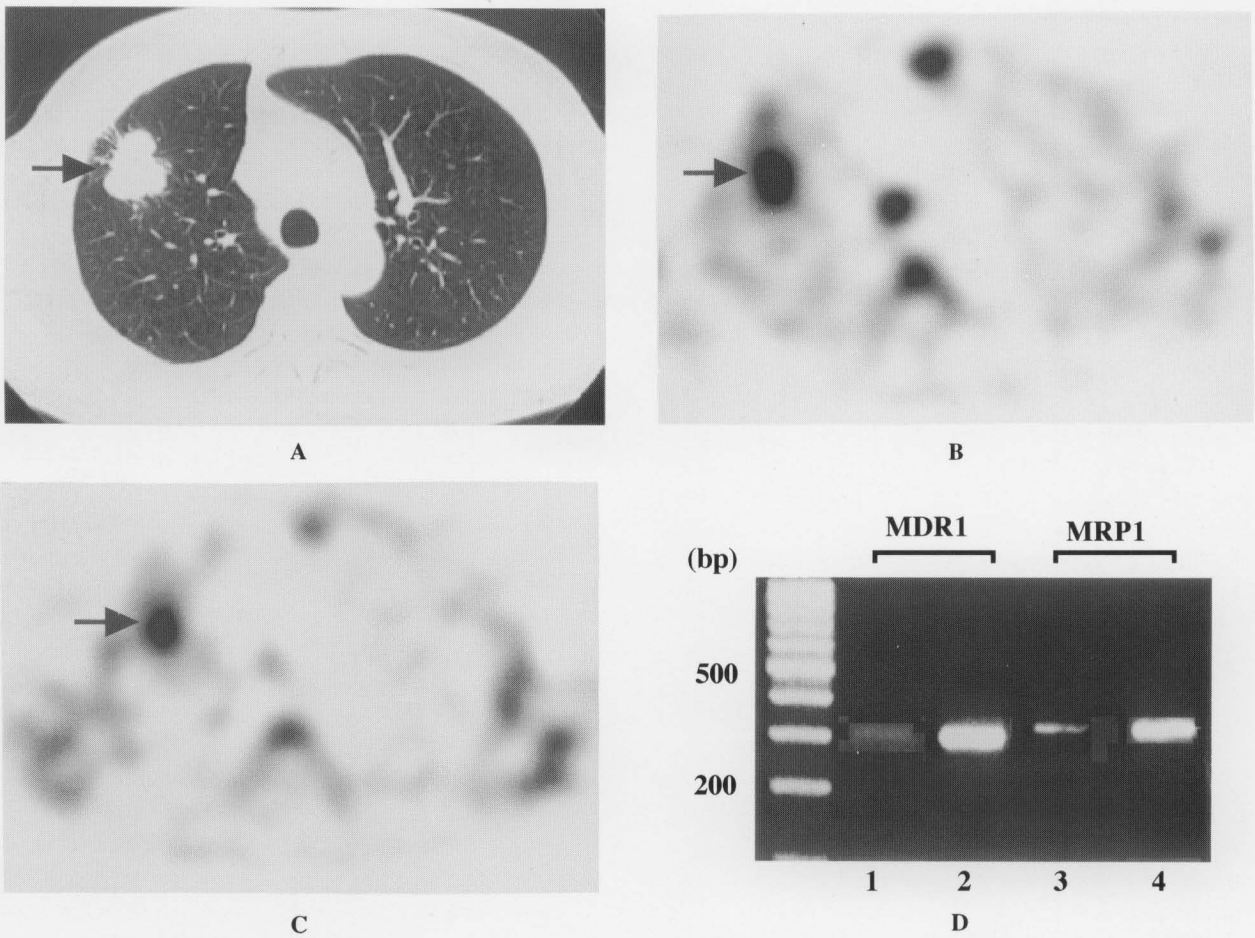

Fig. 3 Representative case: 73-year-old male with squamous cell carcinoma. The chest CT demonstrates tumor shadow with diameter of 3.5 cm in the right upper lobe (A). Tc-99m MIBI SPECT transverse section shows accumulation in the lesions on both the early (B) and the delayed images (C), respectively. In this patient, Te and Td was 133.23 and 64.55, respectively. Lane Nos. 2 and 4 represents the PCR product of each positive control cell line (K562/Adr and H69AR, respectively) by electrophoresis, whereas Nos. 1 and 3 refers to that of MDR1 and MRP1 in this case, respectively (D). The gene expression level was represented as a ratio to that of each positive control cell line. MDR1 and MRP1 gene expression level was 0.06 and 0.06 in this tumor specimen.

DISCUSSION

Since Tc-99m MIBI has been confirmed as substrates for Pgp in *in vitro* studies, several reports have discussed a relationship between the Pgp expression level and the kinetics of Tc-99m MIBI in clinical cancer.[16–18] The MRP expression level, however, should be taken into account in this case, because the Tc-99m MIBI has been confirmed as substrate for MRP as well as Pgp *in vitro* studies.[8,10,11] Recently, induction chemotherapy has been performed on patients with resectable NSCLC. In most of these cases, the standard protocol is a combination of cisplatin with other anticancer drugs such as vindesine and paclitaxel,[12] which are confirmed to be substrates for Pgp or MRP in *in vitro* studies.[9,13–15] Therefore, the prediction of chemosensitivity to these drugs by Tc-99m MIBI SPECT would provide useful information on which is more effective in combination with cisplatin in the induction chemotherapy in patients with NSCLC, because response to these anticancer drugs may be different in individual cases.

In this study, Te indicated an inverse significant correlation with MDR1 gene expression levels, which was in agreement with the findings of Kostakoglu et al. in spite of differences in methods such as immunohistochemistry and scanning time of Tc-99m MIBI SPECT.[17] They obtained early SPECT images 30 min after injection of Tc-99m MIBI. Hassan et al. indicated that accumulation in the lung tumor of Tc-99m MIBI could be seen in the first minute after injection of Tc-99m MIBI and that no significant difference in accumulation was observed between 5–10 and 25–30 min after injection.[19] Koukourakis et al. indicated that Tc-99m MIBI extrusion rate showed a tendency to be higher value in NSCLC with resistance to chemotherapy with etoposide, adriamycin or paclitaxel, which were substrates for Pgp.[20] Kao et al. reported that SPECT, which was obtained at 10 min after injection of Tc-99m MIBI, showed more weaker accumulation in NSCLC with resistance to paclitaxel than in the tumor without it.[21] Pgp expression may be responsible for their findings, because paclitaxel is a substrate for Pgp but not MRP.[15,22] From these findings, our results may suggest that the degree of Tc-99m MIBI accumulation in NSCLC is determined by the Pgp expression level on early phase.

The present study revealed that Td as well as Te was inversely related to MDR1 gene expression in NSCLC but not Twr, which was reported by few authors. In breast cancer, Del Vecchio et al. reported that the fractional retention at 60 as well as 240 min after injection of Tc-99m MIBI was inversely correlated with Pgp expression levels.[23] Moreover, some authors indicated that Tc-99m MIBI accumulation in the cell was based on some factors such as blood flow, capillary permeability and the transmembrane potential of the tumor cells, and its retention depended on mitochondrial activity.[24,25] It is supposed that Te and Td represents accumulation and retention of Tc-99m MIBI, respectively, because as mentioned above, accumulation of Tc-99m MIBI in NSCLC can be seen immediately after injection of this agent. After all, our results suggest that Pgp expression is more dominant than the abovementioned factors in retention as well as accumulation of Tc-99m MIBI in NSCLC. As for Twr, our result suggest that it represents Tc-99m MIBI kinetics, which is dependent on the abovementioned mechanism rather than Pgp or MRP expression levels. Maybe, once Tc-99m MIBI is extruded from the tumor on early image by Pgp-overexpressing cancer cells, Tc-99m MIBI can hardly accumulate in this tumor on the delayed image.

As for the ROI, although some authors set it on normal lung sites for measurement of tumor to normal lung ratio,[26,27] we think this ratio may not be appropriate in assessing the MDR1 or MRP1 gene expression level, because the parameter that was calculated by using Tc-99m MIBI uptake in normal lung may be influenced by MRP or Pgp expression in normal lung sites. Some authors have indicated that abundant MRP or MRP1 gene was detected in normal lung tissue and that overexpression of MRP could be frequently seen in NSCLC.[28–31] Nooter et al. indicated that Pgp expression was relatively low but could be observed in normal lung tissue.[29] In the present study, Te, Td and Twr, which could be calculated without using the ROIs on the normal lung and which were not affected by MRP in normal lung tissues, were therefore used for analysis of Tc-99m MIBI kinetics, but not a tumor to normal lung ratio.

In this study, neither Te nor Td indicated a significant correlation with MRP1 gene expression levels, which was different from MDR1. Moreover, the tumor specimens, which were almost the central parts of the tumors, were surgically resected in this study. This method was undertaken in order to avoid the influence of Pgp or MRP expression in normal lung tissue around the tumor, but it may be impossible to avoid the existence of normal lung cells in the tumor, because the lung cancer tissue contains not only cancer cells but also normal lung cells. As mentioned above, MRP is confirmed to be expressed more frequently than Pgp in normal lung tissue. Therefore, MRP expression in normal lung cells in the tumor specimen may affect these parameters of Tc-99m MIBI SPECT. Moreover, MRP is confirmed to be expressed occasionally in the cytoplasm as well as the plasma membrane, whereas Pgp is expressed only on the plasma membrane, but not in the cytoplasm.[5,30] Therefore, it is supposed that Tc-99m MIBI is extruded through MRP after accumulation in the cytoplasm of the cancer or normal lung cell, which may result in Tc-99m MIBI accumulation in the tumor specimen. Furthermore, there is a difference between Pgp and MRP in the mechanism of extrusion of Tc-99m MIBI.[5,10] In short, MRP functions as a transporter of a glutathione-S-conjugates, whereas Pgp extrudes a substrate directly from the plasma membrane. In this way, the existence of MRP-overexpressing normal lung cells in the cancer tissue, location of MRP

expression in the cancer or normal lung cells and a difference between Pgp and MRP in mechanism of extrusion of Tc-99m MIBI may account for our findings.

No significant difference in MDR1 or MRP1 gene expression was observed between patients with adenocarcinoma and squamous cell lung cancer in the present study. As for MRP, Wright et al. and Sugawara et al. reported MRP expression was observed more frequently in adenocarcinomas than in squamous cell carcinomas,[31,32] whereas Nooter et al. indicated that MRP was overexpressed in squamous cell lung cancer.[29] Although our RT-PCR method was different from their immunohistochemical analyses, the degree of MRP expression may be independent of histological types in NSCLC.

In the present study, Tc-99m MIBI accumulation was observed in all tumors in spite of MDR1 or MRP1 gene overexpression, whereas many *in vitro* studies demonstrated that no accumulation of Tc-99m MIBI could be observed in Pgp or MRP-overexpressing cell lines.[3,7,9] Furthermore, although no tumors revealed obvious histological necrosis in this study, Ceriani et al. showed that faint or deficient Tc-99m MIBI accumulation might be seen in tumor with necrosis.[26] We think the distribution of these transmembrane proteins in tumor tissue is heterogeneous, whereas that in cell lines selected by anticancer drugs such as doxorubicin and vindesine is homogeneous. Actually, even the maximum level of MDR1 and MRP1 gene expression was 0.36 and 0.50, respectively. Most of tumors without necrosis may therefore be detected by Tc-99m MIBI SPECT even if these gene expression levels are high in NSCLC. Shih et al. reported that the coronal sections of SPECT, which were obtained 60–90 min after injection of Tc-99m MIBI, depicted tumors in twenty-one of the twenty-four patients with NSCLC.[33] Nishiyama et al. demonstrated that Tc-99m MIBI SPECT showed positive in thirty-two and thirty of the thirty-four patients with NSCLC in the early and delayed image, respectively.[34] In their studies, deficient accumulation of Tc-99m MIBI was due to tumor necrosis. On the other hand, Te and Td were likely to be high when tumor location was very close to normal organs such as heart and liver, in which Tc-99m MIBI accumulates physically, in the present study (patient Nos. 6, 9 and 12). We think that scatter radiation from these normal organs tends to enhance the value of these parameters in these cases. The tumor location and necrosis may thus restrict our analysis, which is limitation of this study. Our results suggest, however, that both Te and Td calculated on Tc-99m MIBI SPECT are useful parameters in prediction of MDR1 gene expression level, but not MRP1 in NSCLC. Further study is needed to investigate what degree of Te and Td is valid as the cut off value in assessment of MDR1 gene overexpression in NSCLC.

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