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• LIVER CANCER •

Correlation of expression of multidrug resistance protein and messenger RNA with ^{99m}Tc-methoxyisobutyl isonitrile (MIBI) imaging in patients with hepatocellular carcinoma

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Abstract

AIM: To explore whether P-glycoprotein (Pgp) and other pumps, multidrug resistance-associated protein (MRP) and lung resistance protein (LRP), could affect tumor accumulation and efflux of ^{99m}Tc-MIBI in liver cancer.

METHODS: Surgically treated 78 liver cancer patients were included in this study. Before surgery, ^{99m}Tc-MIBI SPECT was performed 15 min and 120 min after injection of 20 mCi ^{99m}Tc-MIBI, respectively. Early uptake, delayed uptake (L/Nd), and washout rate (L/Nwr) of ^{99m}Tc-MIBI were obtained. Expressions of Pgp, MRP and LRP were investigated with Western blotting and immunohistochemistry. Messenger RNA (mRNA) level of Pgp, MRP and LRP was determined by RT-PCR.

RESULTS: No ^{99m}Tc-MIBI uptakes in tumor lesions of 68 of 78 (87.2%) patients with hepatocellular carcinoma were found on ^{99m}Tc-MIBI SPECT. P-gp expression was observed in tumor tissues of the patients with no uptake of ^{99m}Tc-MIBI ($P<0.017$). No appreciable correlation was found between liver cancer ^{99m}Tc-MIBI images and expression of MRP or LRP on the level of protein or mRNA.

CONCLUSION: ^{99m}Tc-MIBI SPECT is noninvasive, and useful in predicting the presence of MDR1 gene-encoded Pgp in patients with hepatocellular carcinoma.

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INTRODUCTION

Multidrug resistance (MDR) is the main barrier to efficient chemotherapy of human malignancies. MDR has been closely associated with overexpression of multidrug resistance genes (MDRI)[1] and has been observed in hepatocellular carcinomas (HCC)[2]. The MDR phenotype has been defined on the basis of cellular drug targets involved Pgp, MRP, LRP and atypical MDR (mediated through altered expression of topoisomerase type II)[3-6]. Pgp, encoded by MDRI gene, is a 170-ku transmembrane glycoprotein and acts as an adenosine triphosphate (ATP)-driven drug efflux pump to reduce drug accumulation[7]. MRP is a 190-ku membrane-bound glycoprotein and can act as a glutathione S-conjugate efflux pump by transporting drugs that are conjugated or cotransported with glutathione[8,9]. Both Pgp and MRP are integral membrane proteins belonging to the ATP-binding cassette (ABC) superfamily of transporter proteins, which appear to confer resistance by decreasing intracellular drug accumulation[10]. In contrast, LRP is not an ABC transporter protein. LRP has recently been identified as a vault protein, which is a typical multisubunit structure involved in nucleocytoplasmic transporter[11]. Determination of these MDR proteins at the time of diagnosis is imperative to the development of rational therapeutic strategies for preventing drug resistance.

^{99m}Tc-MIBI is a cationic lipophilic agent, widely used for myocardial perfusion imaging to detect various tumors[12-18]. Recent evidence has shown that ^{99m}Tc-MIBI is a suitable transport substrate for Pgp and may provide additional information about the Pgp status of tumor cells[19,20]. It has been reported that MIBI is accumulated within mitochondria and cytoplasm of cells based on transmembrane electrical potentials. Malignant tumors show increased transmembrane potential as a result of increased metabolic requirements that induce increased accumulation of MIBI in tumors[21]. The potential advantage of ^{99m}Tc-MIBI imaging lies in its superiority in detecting the presence of Pgp overexpression *in vivo* noninvasively[22,23]. Recently, ^{99m}Tc-MIBI efflux has been shown to be a substrate for MRP *in vivo*[24].

^{99m}Tc-MIBI imaging or SPECT was performed in various cancers[25,26], but no clinical studies in HCC have been found. The aim of this study was to determine whether Pgp and other pumps, MRP and LRP, could affect tumor accumulation and efflux of ^{99m}Tc-MIBI in hepatocellular carcinoma.

MATERIALS AND METHODS

Patients

Seventy-eight patients (30 women, 48 men, aged 24-71 years, mean 54±11.6 years) with HCC were enrolled in the study, 70 of 78 (89.7%) patients were hepatitis B surface antigen positive, 2 (2.6%) were anti-hepatitis C virus positive, and the remaining had no known cause of HCC. No patients previously received chemotherapy. All patients underwent ^{99m}Tc-MIBI SPECT prior to surgery. All tumor samples were analyzed with RT-PCR, Western blotting and immunohistochemistry.

^{99m}Tc-MIBI SPECT

Liver imaging was performed with a double-head gamma camera equipped with a high-resolution parallel-hole collimator (PRISM 2000; Marconi Medical Systerms, Cleveland, OH). Images were obtained 15 and 120 min after injection of 20 mCi ^{99m}Tc-MIBI, respectively. Early and delayed SPECT of the liver was performed

on all patients. For SPECT of the liver, 72 projections were obtained using 64×64 matrix at 45 s per view. Image reconstruction was performed using filtered back projection with Butter-worth and ramp filters. Transverse, coronal, and sagittal sections were reconstructed. Attenuation correction was not applied.

SPECT images were compared with liver CT images, and accumulation in liver tumors was interpreted by nuclear medicine physicians. The findings on ^{99m}Tc-MIBI livers were measured semiquantitatively. Regions of interest (ROIs) were manually defined on the transaxial tomograms showing the lesion's highest uptake in center of the tumor. ROIs placed on the lesions (L) encompassed all pixels that had uptake values of >90% of the maximum uptake in that slice, and the average rate in each ROI was calculated. Another ROI of the same size was then drawn over the normal lung (N) on the same transverse section. The early uptake (L/Ne) and the delayed uptake (L/Nd) were obtained. The washout rate (L/Nwr) was calculated using the following formula: L/Nwr = (L/Ne-L/Nd)×100 (L/Ne).

Immunohistochemical study

After resection of HCC, immunohistochemiscal study of the biopsy or resected tumor tissues and surrounding nontumorous liver parenchyma was performed. Four-micrometer-thick, formalin-fixed, paraffin-embeded tissue sections were cut from the specimens and mounted on poly-L-lysine-coated glass slides (Sigma Chemical Co., St. Louis, MO). The standard avidin-biotin-peroxidase complex (ABC) technique was used for immunostaining using a LSAB kit (Dako Co., Carpinteria, CA).

After deparaffinization and rehydration, the sections were treated with 1 mL/L methanol hydrogen peroxide for 20 min to block endogenous peroxidase activity, incubated with normal horse serum for 30 min at 37 ℃, and with primary antibody, JSB-1 (1:20), MRP1 (1:10), LRP-56 (1:10) overnight in a moist chamber at 4 ℃. The tissue sections were incubated with avidin-biotin-peroxidase complex. The final reaction product was revealed by exposure to 0.3 g/L diaminobenzidine, and the nuclei were counterstained with Mayer's hematoxylin.

A negative control was obtained by staining the sample with secondary antibody and a positive control by inclusive of a normal liver. The results of immunostaining were interpreted independently by two pathologists who were unaware of the imaging studies. Expressions of Pgp, MRP and LRP were scored as follows: -, negative; +, ≤10% positive tumor cells; ++, ≤30% positive tumor cells; +++, >30% positive tumor cells.

Quantitative RT-PCR

RT was performed with random primers with a complementary DNA (cDNA) synthesis kit (Promega, Madison, WI). RT-reaction reagents were added as follows: 2 μL of $MgCL_2$ (50 mmol/L), 2 μL of reverse transcription buffer (Tris-HCL [PH8.3], 100 mmol/L, KCL 500 mmol/L and Triton X-10 010 g/L), 2 μL of deoxynucleotide mixture (10 mmol/L), 0.5 μL of RNase inhibitor (20U), 2 (15U) of avian myeliblastoma virus reverse transcriptase, 1 μL of random primers (500 μg/mL) and 5 μg substrate RNA. The final volume of the reaction (20 μL) was completed with RNase free water. First strand cDNA synthesis was carried out at 42 ℃ for 30 min in the DNA thermal cycler (PTC-100, MJ Reserch Inc., Watertown, MA). Afterwards, the tubes were incubated at 99 ℃ for 5 min to stop the reaction. Then each tube was kept at 4 ℃ until PCR was performed. Expression of the target genes (MDR1, MRP and LRP) and endogenous reference β-actin was quantified using the primers and standards. The primers were designed using the software Primer Express (Applied Biosystems) (Table1).

RT -PCR

Expressions of the target genes (MDR1, MRP and LRP) and GAPDH gene were quantified using the primers and standards. The primers were designed using the software Primer Express (Applied Biosystems) (Table2).

RT-PCR was performed according to the TaqMan 2-step

Table 1 MDR1, MRP, LRP primers for RT-PCR amplifications

Gene	Quantification method	Sequence	cDNA
MDR1	Forward primer	5- CATTGGTGTGGTGAGTCAGG-3	1523-1542
	Reverse primer	5- CTCTCTCTCCAACCAGGGTG-3	1679-1698
MRP	Forward primer	5- CTACCGAGAGGACCTGGACT-3	4099-4118
	Reverse primer	5- GTCTAGCTTGTCAGGAAGGG-3	4437-4456
LRP	Forward primer	5- TAAGGGCTTCCAGCACCAAC-3	148-167
	Reverse primer	5- GGAGTTCTCGCTTCTCGTCC-3	520-539
β-actin	Forward primer	5- GTGTTTGGCCGAGTCCTCACC-3	
	Reverse primer	5- CTCCTGCAAGGAAAAGCTCTG-3	

Table 2 Oligonucleotides used for PCR amplifications

Gene	Quantification method	Sequence	cDNA
MDR1	Forward primer	5'- CCCAGGAGCCCATCCTGT-3'	3774-3791
	Revease primer	5'- CCCGGCTGTTGTCTCCATA-3'	3838-3821
	Probe	5'-(FAM)TGACTGCAGCATTGCTGAGAACATTGC(TAMRA)-3'	3793-3819
MRP	Forward primer	5'- AAGCGCCTCGAGTCGGT-3'	3617-3633
	Revease primer	5'- TCGAATGACGCTGACCCC-3'	3694-3677
	Probe	5'-(FAM)AGCCGCTCCCCGGTCTATTCCC-(TAMRA)-3'	3635-3656
LRP	Forward primer	5'- TTTCGATGACTTCCATAAGAACTCA-3'	1881-1905
	Reverse primer	5'- TTCCGAGGTCTCAAAGCCAA-3'	1950-1931
	Probe	5'(FAM)-CCCGCATCATTCGCACTGCTGT- (TAMRA)3'	1907-1928
GAPDH	Forward primer	5'- GAAGGTGAAGGTCGGAGTCA-3'	
	Revease primer	5'- GAAGATGGTGATGGGA-3'	
	Probe	5'-(JOE)CAAGCTTCCCGTTCTCAGCC(TAMRA)-3'	

method using the ABI PRISM 7 700 sequence detection system (Applied Biosystems). The nontemplate controls, standard dilutions, and samples were assayed. A 25-mm volume of PCR reaction mixture was used, containing 200 ng of the sample cDNA, TaqMan buffer, 200 mmol/L deoxy-ATP, deoxycytidine triphosphate, and deoxy-guanosine triphosphate, 400 mmol/L deoxyuridine triphosphate, 5.5 mmol/L magnesium chloride, 0.025 U/mL AmpliTaq Gold DNA polymease (Applied Biosystems), 0.01 U/mL AmpErase uracil N-glycosylase (Applied Biosystems), 200 nmol/L forward and reverse primers, and 100 nmol/L probe. PCR cycling conditions included an initial phase at 50 ℃ for 2 min, followed by at 95 ℃ for 10 min for AmpErase, 40 cycles at 95 ℃ for 15 s, and at 60 ℃ for 1 min. Quantification of the PCR products was based on the TaqMan 5' nuclease assay using the ABI PRISM 7 700 sequence detection system. The starting quantity of a specific mRNA in an unknown sample was determined by preparing a standard cDNA. The standard curve was generated on the basis of the linear relationship between CT value (corresponding to the cycle number at which a significant increase in fluorescence signal was first detected) and logarithm of the starting quantity. The unknown samples were quantified by the software of the ABI PRISM 7 700 sequence detector system, which calculated the CT value for each sample and then determined the initial quantity of the target using the standard curve. The amount of expressed target gene was normalized to that of GAPDH.

Western blotting

Liver cancer samples were analyzed for the presence of Pgp, MRP and LRP protein. Samples were washed in PBS and homogenized in a lysis buffer[27], Protein supernatants were quantitated using the Lowry assay, and equal amounts of protein from each sample were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were probed with mAb recognizing Pgp, MRP and LRP (Sigma, Co), respectively. Enhanced chemiluminescence was used for protein detection.

Statistical analysis

The results of L/Ne, L/Nd, and L/Nwr were expressed as mean±SD. The differences in L/Ne, L/Nd, and L/Nwr between patients with (-), (+), and (++) Pgp, MRP, and LRP expressions were determined using Student *t* test. The differences in L/Ne, L/Nd, and L/Nwr between patients with high and low Pgp mRNA, MRP mRNA, and LRP mRNA expressions were determined using Student *t* test. If *P* was <0.05, the difference was considered statistically significant.

RESULTS

All the 78 surgically obtained tissue samples were assessed to estimate the levels of Pgp, MRP, and LRP expression on protein and mRNA. Table 3 summarizes the immunohistochemical results and RT PCR data.

Correlation of ^{99m}Tc-MIBI results with immunohistochemical results

Significant MIBI uptake on ^{99m}Tc-MIBI SPECT was noted in tumor lesions of 10 (12.8%) patients with HCC, but not in tumor lesions of 68 (87.2%) patients with HCC. In patients with MIBI uptake, immunohistochemical analysis of tumor tissues showed no detectable P-glycoprotein-positive cells. But immunohistochemical analysis of tumor lesions in patients without MIBI uptake revealed uniformly distributed P-gp-positive cells. We noted a significant correlation between ^{99m}Tc-MIBI SPECT findings and P-gp expression in tumors of patients with HCC.

MRP and LRP protein expression was found in tumor lesions of 7 and 4 patients, respectively, and no correlation was found with ^{99m}Tc-MIBI.

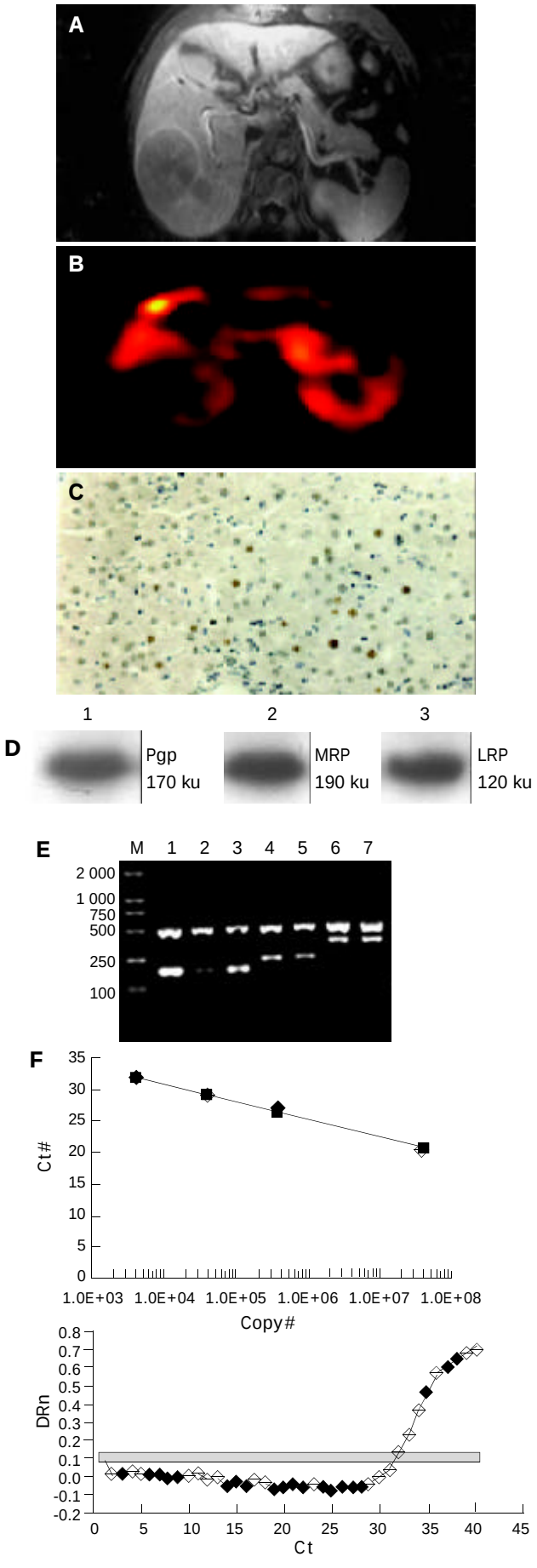

Figure 1 A: CT image of one patient with liver carcinoma. B: ^{99m}Tc-MIBI SPECT Liver image of the patient. C: Immunohistochemical expression of Pgp. D: Western blotting images of Pgp, MRP and LRP. E: MDR1 176 bp, MRP 358 bp, LRP 492 bp shown by RT-PCR image. F: Real time RT-PCR image of Pgp.

Table 3 Patient characteristics and radionuclide imaging

Group	Cases	Tumor size (cm)	^{99m}Tc-MIBI SPECT			Immunohistochemistry			RT-PCR		
			L/Ne	L/Nd	L/Nwr(%)	Pgp	MRP	LRP	mdr1	MRP	LRP
I	68	2.5-15	2.09±0.67	1.25±0.42	54.94±10.23	68 (+)	4 (+)	3 (+)	0.38±0.16	0.04±0.02	0.02±0.01
II	10	1.5-7	1.98±0.56	2.78±0.53	3.07±0.57	10 (-)	3 (+)	1 (+)	0	0.06±0.03	0.03

Correlation of ^{99m}Tc-MIBI with RT-PCR results

No correlation was found between L/Ne and the level of Pgp mRNA, MRP mRNA, and LRP mRNA. The mean L/Nd (2.78±0.64) of the Pgp mRNA low expression group was significantly higher than that (1.25±0.43) of the Pgp high-expression group (P=0.0115, Figure 1). L/Nd was not related to the level of MRP mRNA or LRP mRNA. Statistical support was found toward a significant difference in L/Nwr between the Pgp mRNA high-expression group and the Pgp mRNA low-expression group. L/Nwr was not related to the level of MRP mRNA or LRP mRNA.

The grouping was according to the positive mmunohistochemistry of Pgp, there were 4 cases positive of MRP and 3 cases positive of LRP in group I, there were 3 cases positive of MRP and 1case positive of LRP in group II.

DISCUSSION

The resistance of tumors to multiple drugs is a major problem in cancer chemotherapy. Pgp, a transmembrane ATP-dependent efflux pump encoded by MDR1 gene, has a central role in multidrug resistance. Increased amounts of Pgp may confer multidrug resistance to cells by preventing intracellular accumulation of a variety of cytotoxic drugs. A unique feature of multidrug resistance is the apparent capacity of Pgp for recognizing and transporting a large group of cytotoxic compounds sharing little structural or functional similarity other than being relatively small hydrophobic and cationic agents, including anthracyclines, Vinca alkaloids, and actinomycin D. Evidence has shown that Pgp as a drug efflux pump extrudes ^{99m}Tc-MIBI and other drugs from cells and that Pgp expression and enhanced efflux of ^{99m}Tc-MIBI from these cells are closely connected[28,29]. In animal models, faster clearance of ^{99m}Tc-MIBI was observed in tumors with or without Pgp expression[30,31]. Our study revealed that the mean L/Nd in Pgp (-) patients was significantly higher than that in Pgp (++) patients (P=0.035). Pgp (++) patients had a higher L/Nwr than Pgp (-)patients (P=0.027). No correlation was found between L/Ne and Pgp expression. The same results were obtained from mRNA level. Moreover, no correlation was found between MRP and ^{99m}Tc-MIBI SPECT. The same result was obtained from LRP protein and mRNA level. ^{99m}Tc-MIBI uptake by tumor is associated with many factors, including direct mechanisms such as negative transmembrane potential and drug efflux pump and indirect mechanisms such as blood flow and capillary permeability. We considered L/Ne to be more affected by blood flow. In contrast, L/Nd and L/Nwr clearly reflected Pgp expression of intrinsic properties of the tumor.

Until now, there have been some reports about the expression of Pgp in tumor tissues of patients with HCC[32,33]. Resistance to cancer chemotherapy in HCC resulted from Pgp expression. The specific localization of Pgp and the incidence of Pgp expression in each histological type of HCC were observed. The analysis indicated that the incidence was the lowest in the compact type of HCC, and it was significantly lower than that in the pseudo glandular and trabecular types[34]. But in our study, mRNA and protein level of Pgp revealed no significant difference in the incidence of Pgp expression in each histological type.

It has been established that MRP belongs to the superfamily of ABC transmembrane transporter proteins and can act as a glutathione S-conjugate efflux pump[35]. ^{99m}Tc-MIBI was shown to be a substrate for MRP *in vitro*[36]. The abilities of Pgp and MRP transporters to wash out ^{99m}Tc-MIBI have been reported to be similar in cell lines, in spite of different possible mechanisms of transport[37]. However, cardiac muscle showed a low L/Nwr of ^{99m}Tc-MIBI and a low level of Pgp expression but a high level of MRP expression[38]. In our study, we did not observe any correlation between tumor accumulation or efflux of ^{99m}Tc-MIBI and expression of MRP on protein level or mRNA level in liver cancer. The mechanisms are also unclear.

LRP has been identified as the vault protein involved in nucleocytoplasmic transport. Recently, subcellular accumulation of drugs was found to be localized in cytoplasm and minimally in nuclei in LRP overexpression cells[39]. As increased cytoplasm concentration of the drug could intensify its contact with the membrane, we proposed that efflux of the drug might be enhanced in LRP overexpression cells. However, we did not find a correlation between tumor accumulation or efflux of ^{99m}Tc-MIBI and expression of LRP. Subcellular accumulation of ^{99m}Tc-MIBI within mitochondria and cytoplasm of cells has been reported to be based on transmembrane electric potentials[40]. Therefore, efflux of ^{99m}Tc-MIBI was rarely affected by expression of LRP.

Until now, there have been few clinical studies on the relation between Pgp expression and ^{99m}Tc-MIBI uptake in HCC. As ^{99m}Tc-MIBI is cleared through the liver, and it is not easy to detect liver tumors. To the best of our knowledge, our study was the first to show an inverse correlation in MDR1/Pgp expression and ^{99m}Tc-MIBI uptake in HCC. But ^{99m}Tc-MIBI SPECT has some limitations, because it depends on the optimal perfusion of tumor tissues. Poor MIBI penetration could be attributable to poor tumor perfusion in tumors larger than 2.5 cm, where tumor necrosis would be expected. Therefore, perfusion studies such as a Tl-201 scan could be used to eliminate the possibility of poor penetration.

In conclusion, our results suggest that L/Nd and L/Nwr of ^{99m}Tc-MIBI are noninvasive and useful in detecting the expression of Pgp in patients with HCC

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