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Expression of Multidrug Resistance-related Transporters in Human Breast Carcinoma

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The expression levels of mRNA for multidrug resistance 1 (*MDR1*) gene, multidrug resistance protein 1 (MRP1), lung resistance-related protein (LRP) and breast cancer resistance protein (BCRP), which confer multidrug resistance *in vitro*, were examined in 43 untreated breast carcinoma patients, of whom 38 subsequently received doxorubicin-based chemotherapy after surgery, in order to elucidate the roles of these genes in drug resistance *in vivo*. The mRNA levels were determined using a semi-quantitative reverse-transcription polymerase chain reaction method in breast carcinoma tissues including at least 80% carcinoma cells. The expression level of *BCRP* gene was low and did not vary markedly in comparison with that of *MDR1*, *MRP1* or *LRP* gene. The expressions of *MDR1* and *MRP1* genes were correlated with each other, but the expression of *BCRP* or *LRP* gene did not correlate with that of other genes. These four gene expressions were independent of age, TNM categories and the status of progesterone or estrogen receptor. The expression levels of these four genes were not related to the relapse or prognosis of the 38 patients treated with doxorubicin-based chemotherapy. P-glycoprotein (P-gp)/MDR1, MRP1 and LRP may play more important roles than BCRP in chemotherapy of human breast carcinoma.

Key words: MDR — BCRP — Breast carcinoma

Intrinsic or acquired drug resistance is a major therapeutic problem for cancer chemotherapy, limiting the efficacy of chemotherapy.[1)] Knowledge of the mechanisms of drug resistance may lead to new treatment strategies for overcoming drug resistance.

MDR is an important mechanism of drug resistance in tumor cell lines, and involvement of *MDR1*, *MRP1* and *LRP* genes has been identified.[2–4)] *MDR1* and *MRP1* are members of the ABC transporter gene family,[5, 6)] expressed in both human solid tumors and hematological malignancies.[7, 8)] The 110-kd LRP, the major vault protein, is frequently overexpressed in multidrug-resistant cells, and has an important role(s) in transportation of drugs from nucleus to cytoplasm.[4)] Further, Akiyama and co-workers demonstrated that ribozyme to *LRP* gene resulted in doxorubicin, VP-16 and paclitaxel resistance in SW620 cells induced to differentiate by treatment with sodium butyrate.[9)] Recently, *BCRP* (*MXR*/*ABCP*) gene, a member of the ABC transporter family, has been described in breast, colon, gastric and fibrosarcoma cell lines.[10–12)] Overexpresssion of BCRP was induced by exposure of the cells to mitoxantrone or doxorubicin/verapamil and resulted in a different resistance profile from the cases of *MDR1* or *MRP1* gene overexpression.[13, 14)] Topoisomerase I inhibitors such as topotecan and camptothecin are substrates for BCRP.[13–15)]

Several reports have appeared on the expression of P-gp/MDR1, MRP1 and LRP in human breast carcinoma using immunohistochemistry or molecular biological methods (Table I). However, their clinical significance in human breast carcinoma is still uncertain. Further, the expression of BCRP in human samples has been analyzed only in hematological malignancy.[16)] In this study, we analyzed the expression levels of *BCRP*, *MDR1*, *MRP1* and *LRP* genes in patients with untreated breast carcinoma.

MATERIALS AND METHODS

Patients and samples Surgical specimens from 43 patients with untreated breast carcinoma were available for this study. The patients underwent surgery in Tokyo Metropolitan Hospital between 1983 through 1999. All the samples were stored at −80°C and embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) immediately before use. The data on clinicopathologic variables such as age, TNM categories and the status of estrogen/

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Abbreviations used: ABCP, human placenta-specific ATP-binding cassette gene; BCRP, breast cancer resistance protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LRP, lung resistance related protein; MDR, multidrug resistance; MRP, multidrug resistance protein; MXR, mitoxantrone resistance protein; P-gp, P-glycoprotein.

Table I. Expression Level and Clinicopathological Significance of P-gp/MDR1, MRP1, LRP and BCRP in Human Breast Carcinoma

Reporter, Year, Reference#	P-gp/MDR1	MRP1	LRP	BCRP
	Proportion[a)]/Method/CPS[b)]/RC[c)]	Proportion/Method/CPS/RC	Proportion/Method/CPS/RC	Proportion/Method/CPS/RC
Wallner, 1991, 18	27/59 (46)/Slot blot/No/NT[d)]			
Charpin, 1994, 20	2/20 (46.7)/IHS[e)]/Stage, Size/Yes	16/20 (80)/IHS/NT/No	15/20 (75)/IHS/NT/No	
Filipits, 1996, 29	54/134 (40)/RT-PCR/NT/NT 36/63 (57.1)/IHC/NT/NT	134/134 (100)/RT-PCR/NT/NT 63/63 (100)/IHC/Stage/NT		
Linn, 1996, 21	14/30 (46.7)/IHS/Stage/Yes			
Chevillard, 1996, 33	15/53 (28.3)/RT-PCR/NE/Yes			
Linn, 1997, 22	7/43 (16.3)/IHC/NT/Yes			
Nooter, 1997, 30		78/259 (34)/IHS/Stage/Yes		
Lacave, 1998, 25	113/213 (53)/IHS/No/NT			
Mechetner, 1998, 23	28/254 (11)/IHS/NT/Yes			
Pohl, 1999, 28			87/99 (88)/IHC/No/No	
Yang, 1999, 27	29 cases/RT-PCR/No/Paclitaxel			
Present study	43 cases/RT-PCR/No/No	43 cases/RT-PCR/No/No	43 cases/RT-PCR/No/No	43 cases/RT-PCR/No/No

a) Proportion of positive-cases (number of positive cases/examined cases (percentage of positive cases %)).
b) Clinicopathological significance.
c) Effect on response to chemotherapy.
d) Not tested.
e) Immunohistochemistry.

progesterone receptor are shown in Table I. The relapse and prognosis of the patients was investigated on June 30, 2000. Informed consent was obtained from each patient, and Tokyo Metropolitan Komagome Hospital committee approved this project prior to the study. The sections including at least 80% carcinoma cells were used for total RNA preparation.

RT-PCR Total RNA of human breast carcinoma was prepared by using Trizol (Gibco Life Tech, Gatesberg, MD). cDNA was synthesized with 3 μg of total RNA and random hexadeoxynucleotide primer (Gibco Life Tech) in 20 μl of a solution containing reverse transcriptase. cDNA was diluted 1:4 with water and stored at −20°C until use. PCR was performed with cDNA derived from 30 ng of RNA. PCR reactions were carried out in a total volume of 25 μl containing cDNA, dGTP, dATP, dTTP and [α-^{32}P]dCTP at a concentration of 200 μM, 4 μM of each primer and 0.25 unit of ExTaq polymerase (TaKaRa Shuzo, Otsu, Shiga). The PCR condition consisted of 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, followed by 72°C for 10 min. The PCR primer sequences of *MDR1*, *MRP1*, *LRP*, *BCRP* and *GAPDH* which was used as an internal control were as follows: *MDR1* sense primer 5′-CCCATCATTGCAATAGCAGG-3′ and antisense primer 5′-GTTCAAACTTCTGCTCCTGA-3′ corresponding to 167-bp (residues 2733 to 2752); *MRP1* sense primer 5′-ATCAAGACCGCTGTCATTGG-3′ and antisense primer 5′-TCTCGTTCCTACTGAACGTCC-3′ corresponding to 180-bp (residues 1379 to 1559); *LRP* sense primer 5′-CCCCCATACCACTATATCCATGTG-3′ and antisense primer 5′-TCGAAAAGCCACTGATCTCCTG-3′ corresponding to 405-bp (residues 136 to 522); *BCRP* sense primer for: 5′-TGCCCAGGACTCAATGCAACAG-3′ and antisense primer 5′-ACAATTTCAGGTAGGCAATTGTG-3′ corresponding to 172-bp (residues 1979 to 2128); *GAPDH* sense primer 5′-CCCCTGGCCAAGGTCATCCATGACAACTTT-3′ and antisense 5′-GGCCATGAGGTCCACCACCCTGTTGCTGTA-3′ corresponding to 513-bp (residues 515 to 1027).

PCR and quantitative analysis of PCR products In order to evaluate the amplified PCR products semi-quantitatively, the optimal conditions for the detection of *MDR1*, *MRP1*, *LRP*, *BCRP* and *GAPDH* genes were determined using cDNA derived from placenta. At 40 cycles of PCR, the relative yields of PCR products were similar, indicating that this number of cycles corresponded to the plateau. At 25 cycles or less, expression of each gene could not be clearly distinguished (data not shown). Thus we used 35 PCR cycles for the detection of each gene. The amplified cDNA fragment was electrophoresed on 6% polyacryl-

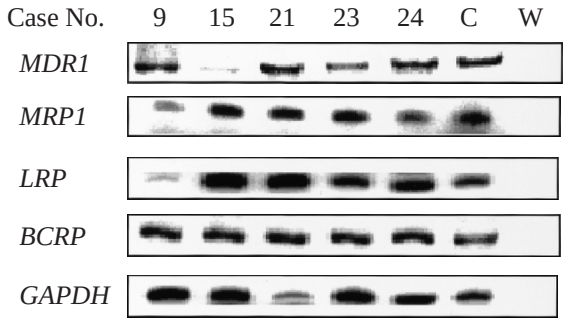

Fig. 1. *MDR*, *MRP1*, *LRP* and *BCRP* gene expression in human breast carcinomas. A. Expression of *MDR*, *MRP1*, *LRP* and *BCRP* genes in 43 patients with breast carcinoma, determined by RT-PCR, as described in "Materials and Methods." Lanes C: RNA of placenta and HCT15 cells was used as positive controls for the detection of *MDR1*, *MRP1*, *BCRP* and *LRP* genes. Lanes W: PCR was performed without each cDNA. Data were expressed relative to the expression of *GAPDH* gene in each breast carcinoma.

amide gel (Fig. 1). The quantitative analysis of the amplified PCR products was performed using a BAS 2000 imaging plate (Fuji, Kanagawa).

Statistical analysis Association of continuous variables was evaluated using the Mann-Whitney *U* test. The relationship between *MDR1*, *MRP1*, *LRP* or *BCRP* gene expressions and potential explanatory variables, including age, pT category, pN category, pM category and hormonal receptor status was determined by use of the χ^2 test. The statistical analyses were performed using Statview J 5.0 software (Abacus, CA). Two-sided *P* values were calculated and were considered significant when less than 0.05.

RESULTS

Expression and clinical significance of *BCRP, MDR1, MRP1* or *LRP* genes in human breast carcinoma Forty-three primary breast carcinoma tissues were used for the detection of *BCRP*, *MDR1*, *MRP1* or *LRP* gene expres-

Table II. Relationship of *MDR1*, *MRP*, *LRP* and *BCRP* Gene Expression Levels and Clinicopathological Variables in Patients with Breast Carcinoma

Variables	All patients	*MDR1*		Significance[a)]	*MRP*		Significance	*LRP*		Significance	*BCRP*		Significance
		Low	High		Low	High		Low	High		Low	High	
Total	43												
Age (yr)													
Median		52	50	NS[b)]	52	51	NS	50	52	NS	52	50	NS
Range		(36−80)	(31−80)		(41−65)	(31−80)		(30−65)	(36−80)		(31−80)	(30−65)	
pT category				NS			NS			NS			NS
T1	2	1 (50%)	1 (50%)		2 (100%)	0 (0%)		2 (100%)	0 (0%)		1 (50%)	1 (50%)	
T2	20	9 (45%)	11 (55%)		9 (45%)	11 (55%)		10 (50%)	10 (50%)		9 (45%)	11 (55%)	
T3	11	6 (55%)	5 (45%)		4 (36%)	7 (64%)		5 (45%)	6 (55%)		3 (27%)	8 (73%)	
T4	10	5 (50%)	5 (50%)		6 (60%)	4 (40%)		4 (40%)	6 (60%)		8 (80%)	2 (20%)	
pN category				NS			NS			NS			NS
N0	11	6 (55%)	5 (45%)		5 (45%)	6 (55%)		4 (36%)	7 (64%)		6 (55%)	5 (45%)	
N1	26	13 (50%)	13 (50%)		12 (46%)	14 (54%)		14 (54%)	12 (46%)		9 (35%)	17 (65%)	
N2	2	2 (100%)	0 (0%)		2 (100%)	0 (0%)		1 (50%)	1 (50%)		2 (100%)	0 (0%)	
N3	2	0 (0%)	2 (100%)		1 (50%)	1 (50%)		1 (50%)	1 (50%)		2 (100%)	0 (0%)	
Nx	2	0 (0%)	2 (100%)		1 (50%)	1 (50%)		1 (50%)	1 (50%)		2 (100%)	0 (0%)	
pM category				NS			NS			NS			NS
M0	33	18 (55%)	15 (45%)		17 (52%)	16 (48%)		14 (42%)	19 (58%)		13 (40%)	20 (60%)	
M1	7	3 (43%)	4 (57%)		3 (43%)	4 (57%)		4 (57%)	3 (43%)		6 (86%)	1 (14%)	
Mx	3	0 (0%)	3 (100%)		1 (33%)	2 (67%)		3 (100%)	0 (0%)		2 (67%)	1 (33%)	
ER[c)]				NS			NS			NS			NS
Positive	25	11 (44%)	14 (56%)		9 (36%)	16 (67%)		12 (48%)	13 (52%)		12 (48%)	13 (52%)	
Negative	17	9 (53%)	8 (47%)		11 (65%)	6 (35%)		6 (35%)	11 (65%)		6 (40%)	9 (60%)	
PR[d)]				NS			NS			NS			NS
Positive	26	10 (38%)	16 (62%)		11 (42%)	15 (58%)		15 (58%)	11 (42%)		12 (46%)	14 (54%)	
Negative	15	9 (60%)	6 (40%)		8 (53%)	7 (47%)		6 (40%)	9 (60%)		9 (60%)	6 (40%)	

a) Mann-Whitney *U* test or χ^2 test was used.
b) NS, not significant.
c) Estrogen receptor.
d) Progesteron receptor.

sion. Table II summarizes the patients' characteristics at the time when the samples were obtained at surgery.

MDR1, *MRP1*, *LRP* and *BCRP* gene expression levels are shown in Fig. 2. Although *MDR1*, *MRP1*, *LRP* or *BCRP* gene expression varied more than 1000-fold overall, one very high expression case was found for each of *LRP* and *BCRP*. Excluding this, *BCRP* gene expression level did not differ much in comparison with *MDR1*, *MRP1* or *LRP* gene expression levels.

Based on the median value of the mRNA expression of each gene, we examined the relationship between clinicopathologic variables and each gene expression. The expression levels were independent of age, TNM category and status of estrogen/progesterone receptors (Table II), and every other category examined.

Concerning the effects of the expression levels of these genes on doxorubicin-based chemotherapy in human breast carcinoma, we analyzed the relationship between relapse and the expression levels of *MDR1*, *MRP1*, *LRP* and *BCRP* among 38 cases treated with doxorubicin after surgery. The proportions of the cases with relapse were relatively similar in relation to *MDR1* (first quartile, recurrence/cases, 4/9; second, 4/9; third, 6/9 and fourth, 4/11), *MRP1* (4/9, 5/9, 4/9, 4/11), *LRP* (5/9, 5/9, 4/9, 4/11) and *BCRP* gene expression levels (5/9, 4/9, 6/9, 3/11) (Fig. 2). Box-plot analysis also revealed no difference of expression levels between the cases with relapse and those without (Fig. 3). The expression levels of these genes did not appear to have any marked effect in patients with breast carcinoma (data not shown).

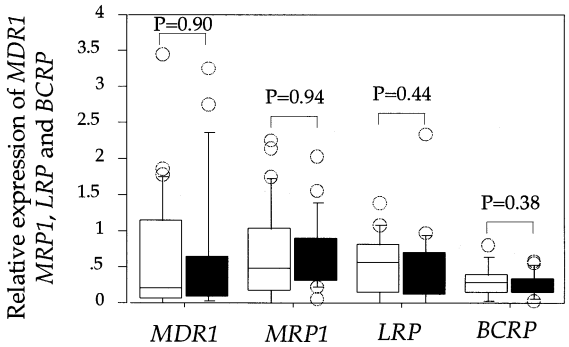

Fig. 3. Association of *MDR1*, *MRP1*, *LRP* or *BCRP* gene expression with relapse in 38 patients with breast carcinoma. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. *P* values were assessed by Student's *t* test. □ recurrence, negative; ■ recurrence, positive.

BCRP* gene expression is independent of *MDR1*, *MRP1* or *LRP To observe the expression pattern of *MDR1*, *MRP1*, *LRP* and *BCRP* genes in human breast carcinoma, the expression levels were plotted in a graph (Fig. 4). A moderate correlation was observed between *MDR1* gene expression and *MRP1* (R=0.515). *BCRP* or *LRP* gene

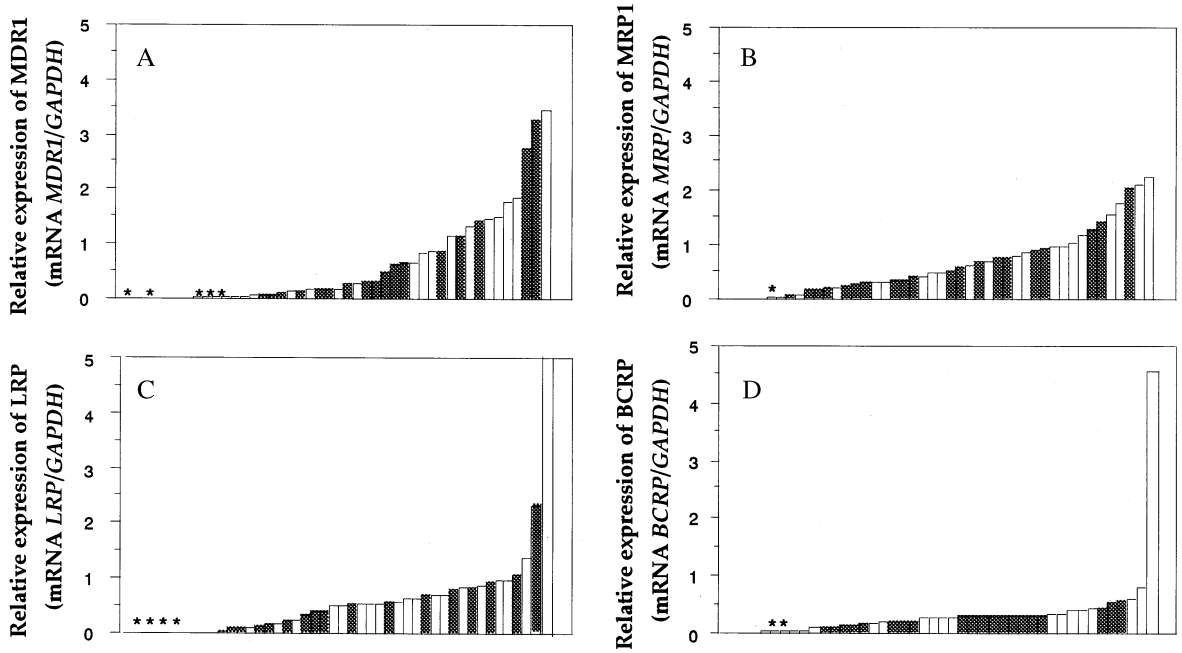

Fig. 2. Expression levels are arranged in order of magnitude for *MDR1* (A), *MRP1* (B), *LRP* (C) and *BCRP* gene (D). The hatched bar and ∗ indicate patients with relapse after surgery.

expression showed no association with any other gene (*BCRP* vs. *MDR1*, R=0.012; vs. *MRP1*, R=0.023; vs. *LRP*, R=0.0256).

DISCUSSION

Several mechanisms are thought to be involved in breast cancer resistance to chemotherapy, including overexpression of the membrane-associated ATP-dependent efflux pump, P-gp encoded by *MDR1*, *MRP1* and *BCRP*, increased level of thymidylate synthetase, altered expression of topoisomerase II, and enhanced detoxification by the gluthathione-linked enzyme system.[1, 17, 18] *MDR1*/P-gp-associated MDR is the best characterized and best understood at the molecular level.[17, 18] However, the contribution of these genes to chemotherapeutic failure in breast carcinoma remains to be proven.[18] Recently, a novel ATP-dependent transporter, BCRP has been identified.[10–12] This transporter was also involved in resistance to mitoxantrone, doxorubicin, topotecan and campthotecin.[13, 14] Therefore, we investigated *BCRP* gene expression level in human breast carcinoma tissues, together with that of *MDR1*, *MRP1* and *LRP* gene, in relation to the clinical data of the patients to clarify whether these genes are involved in clinical drug resistance.

The present study revealed low expression of *BCRP* and small differences of *BCRP* gene expression from tumor to tumor, compared with *MDR1*, *MRP1* or *LRP*. Recently, Scheper and co-workers demonstrated BCRP expression on cytoplasm membrane in several cell lines by immunocytochemical analysis with the antibody BXP-34 against BCRP. In addition, no expression of BCRP was observed in 16 hematological malignancies and 41 human solid tumors, including 17 breast carcinomas.[19] BCRP may not play an important role in progression of breast carcinoma, because its gene expression levels were not also associated with clinicopathologic variables in our study. Similar results have been reported for P-gp/*MDR1*, MRP1 and LRP.[18, 20–23] By contrast, P-gp and MRP1 expression detected by immunohistochemistry was reported to be associated with tumor progression, histopathologic subtype and age in human breast carcinoma.[24–26]

Fojo and co-workers found relationships between *MDR1*/*MRP1* gene expression level and drug sensitivity using 60 NCI drug-screening cell lines.[27, 28] The association of doxorubicin sensitivity and *MDR1*/*MRP1* gene expression was weak in their reports. Furthermore, *BCRP*, *MDR1*, *MRP1* or *LRP* gene expression did not influence the relapse and prognosis of the patients with breast carcinoma treated with doxorubicin in our present study.

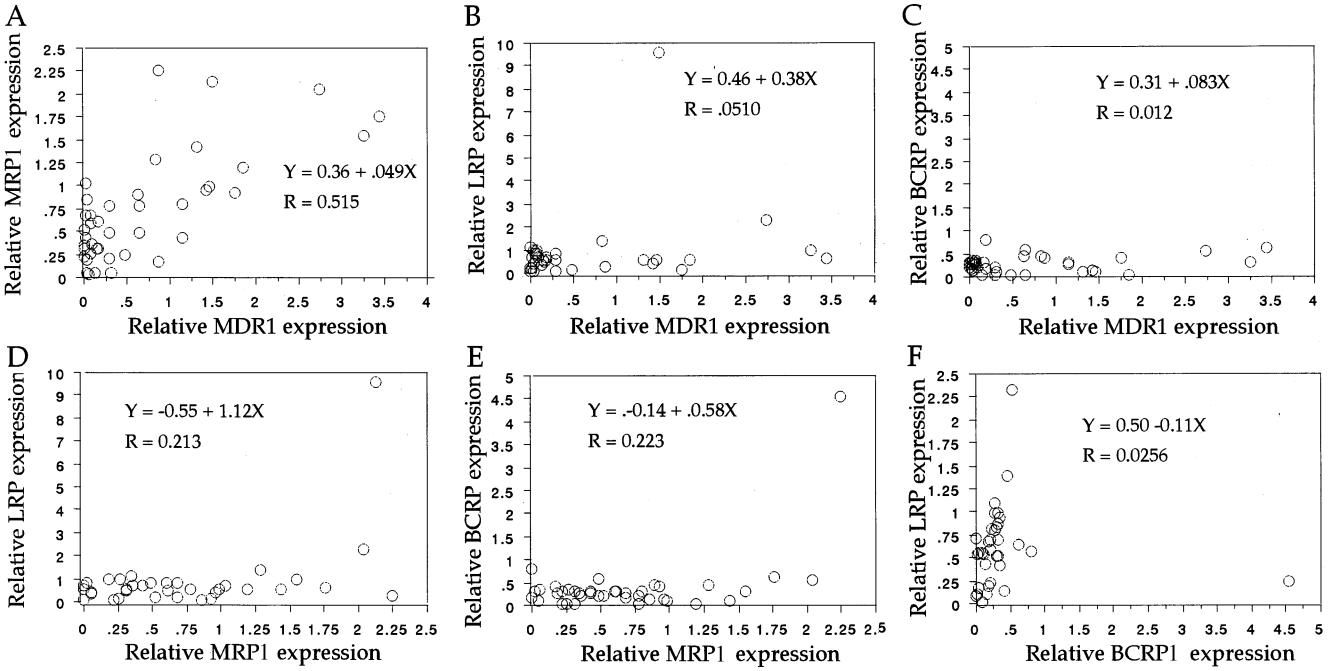

Fig. 4. Correlation among *MDR*, *MRP1*, *LRP* or *BCRP* gene expression in breast carcinomas. Expression of *MDR*, *MRP1*, *LRP* or *BCRP* gene in 43 patients with breast carcinoma, determined by RT-PCR, as described in "Materials and Methods." Each gene expression level is reported relative to *GAPDH* gene. A. *MDR1* vs. *MRP1* gene expression. B. *MDR1* vs. *LRP* gene expression. C. *MDR1* vs. *BCRP* gene expression. D. *MRP1* vs. *LRP* gene expression. E. *MRP1* vs. *BCRP* gene expression. F. *BCRP* vs. *LRP* gene expression.

In previous reports, P-gp expression detected by immunohistochemistry could predict doxorubicin response in human breast carcinoma, but MRP1 or LRP expression could not.[24–26, 29–31] In contrast, the opposite results were reported from other laboratories.[18, 20–23, 25, 26] We used the RT-PCR technique for the quantitative detection of *BCRP*, *MDR1*, *MRP1* or *LRP* gene. Nonmalignant stromal cells as well as peripheral blood lymphocytes, are thought to express detectable levels of *MDR1*, *MRP1* and *LRP* gene.[8, 32, 33] Therefore, immunohistochemical analysis appears to be a better tool for the detection of these proteins than RT-PCR.[24, 29, 31, 34] However, the condition of each section may not always be the same, so in this respect, RT-PCR could be a better tool for analysis of *MDR1*, *MRP1*, *LRP* or *BCRP* gene. First we examined the ratio of carcinoma cells on each section on the H.E. staining image and we used the sections including at least 80% carcinoma cells. We used GAPDH as an internal control to choose good quality sections for RT-PCR. Five cases were excluded because of the low ratio of RNA (<1.4) or failure to detect *GAPDH* by RT-PCR. However, there still remain problems of methodology in the detection of P-gp/*MDR1*, *MRP1*, *LRP* and *BCRP* in human samples.

The co-expression of *MDR1* and *MRP1* was observed in breast carcinoma tissues in this study. In contrast, no association between the expression of *BCRP* or *LRP* and any other gene was observed. Ross and co-workers reported that high expression of *BCRP* did not strongly correlate with high *MDR1* expression.[16] Thus, BCRP may not play an important role in human breast carcinoma. However, the significance of BCRP as well as P-gp/MDR1, MRP1 and LRP in patients with carcinoma expressing these proteins is not still clear. To establish the real significance of these transporters, it may be necessary to observe the clinical effects of specific inhibitors of each transporter.

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