Association of mdr1 Gene Expression with Other Prognostic Factors and Clinical Outcome in Human Breast Cancer

BOONNUM PUNYAMMALEE, D.Sc.*,
WICHAI PURISA, B.Sc.*,
SUPHON MANOROMANA, M.D.**,
SUNANTA CHARIYALERTSAK, M.Sc.*,
BUDSABA RERKAMNUAYCHOK, D.M.Sc.***

Abstract

Multidrug resistance of cancer (CA) is one of a major problems in CA chemotherapy that is frequently associated with the expression of P-glycoprotein (P-gp) encoded by mdr1 genes. However, the controversial results exist regarding to the significance of mdr1 gene expression on clinical drug resistance to chemotherapy of breast CA cells. Recent evidence reported a strong correlation between the increased P-gp levels and the prognosis in advanced breast CA. The current study investigated whether mdr1 gene expression has any impact on prognosis and response to chemotherapy in breast CA patients. We determined mdr1 expression in 127 primary and 8 locally relapsed breast CA using a sensitive, specific and quantitative technique based on a RT-PCR and Southern blot hybridization detection by non-radioactive labelled-probe. In patients with primary breast CA, mdr1 expression were negative (mdr1-ve), low (<10 units), high (≥10 units) in 63.8, 8.7 and 27.5 per cent of the patients, respectively. No differences in age, menopause status, tumor size, stage, lymph node involvement, estrogen receptor level and p53 level were observed between mdr1-ve and mdr1+ve expression patients. However, mdr1 gene expression is often associated with number of positive lymph nodes and negative estrogen receptors ($p = 0.008$ and 0.0007, respectively). In locally relapsed cases, mdr1-ve was 62.5 per cent whereas 37.5 per cent were mdr1+ve with high level of mdr1 RNA. No differences in other prognostic factors: lymph nodal involvement, estrogen receptor level and p53 level, were detected in both groups. Response to chemotherapy in primary and recurrent breast CA was not different in mdr1-ve and mdr1+ve patients. Finally, our results show that mdr1 gene expression is frequently present in breast CA both before and after chemotherapy. Association of mdr1 gene overexpression with other two prognostic factors suggests that they may confer a more aggressive nature of the tumor, drug resistance and poor prognosis. Evaluation of these factors may improve the ability to identify and select breast CA patients at high risk for poor prognosis for aggressive treatment. However, in this series response to CMF chemotherapy of primary and locally recurrent breast CA were not affected by the presence or absence of mdr1 gene product.

* Research Division,
** Division of Medical Oncology, National Cancer Institute, Department of Medical Services, Ministry of Public Health, Bangkok 10400,
*** Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand.

Many human CA have been cured by combination chemotherapeutic drugs, however intrinsic and acquired drug resistance remain to be a major hindrance to successful treatment of many other CA[1]. One of the mechanisms responsible for drug resistance is the increased synthesis of P-gp found in cell lines selectively grown in increasing levels of structurally and functionally unrelated hydrophobic compounds such as anthracyclines, vinca alkaloids, dactinomycin, colchicine, epipodophyllotoxins and paclitaxel[2-7]. Cells selected by one of these drugs have cross-resistant phenotypes to all of them. This multidrug resistance is the result of reduced intracellular drug concentration due to pumping drugs out of cells through the action of P-gp[3,8]. The 170 kd transmembrane glycoprotein encoding by a human mdr1 gene is highly expressed in a number of normal tissues such as colon, kidney, liver, adrenal gland and some bone marrow stem cells where it is thought to function as a pump transporting toxins, drugs and hormones out of cells[9-11].

Several human CA both untreated and treated with chemotherapeutic drugs frequently have mdr1 gene expression[10,12-16]. Some CA, including CA of colon, kidney and liver known to be intrinsically resistant to anti-cancer drugs express high mdr1 RNA. Some CA such as breast CA and lymphoma which are initially chemoresponsive but acquire resistance after therapy have increasing P-gp level[17,18]. This information suggests that mdr1 gene expression might play a role in resistance of CA cells to cytotoxic drugs. However, clinical studies reported thus far showed inconsistent correlation between the level of mdr1 gene expression and clinical drug resistance in some CA, such as lung and breast CA[19,20]. Recent studies reported a strong correlation between the presence of increased levels of either P-gp alone or P-gp together with mutant p53, with the shorter survival in locally advanced breast CA patients[21,22]. Despite the small number of patients enrolled in these studies, their findings have stimulated interest in studying prognostic implications of P-gp.

The current study has investigated the association of mdr1 gene expression with other prognostic factors in a large number of breast CA patients. We have also analyzed the impact of mdr1 gene expression on clinical outcome. Tumors of primary CA both untreated and treated and of locally relapse were studied using reverse transcription-polymerase chain reaction (RT-PCR) Southern hybridization technique which enables the measurement of mdr1 mRNA levels in small samples and allows to detect a low level of mdr1 expression in those clinical samples.

MATERIAL AND METHOD

Tissue samples

Tumor samples were obtained from 144 patients who underwent surgery for breast CA at the National Cancer Institute, Thailand. Within 1 h after surgical excision, all samples were stored as small pieces of tissue and kept frozen in liquid nitrogen until use.

Cell lines

Drug-sensitive KB-3-1 and multidrug-resistant KB-V-1 epidermoid carcinoma cell lines were kindly provided by Dr Ira Pastan, National Cancer Institute, Bethesda. KB-V-1 contains 100 fold amplification of the mdr1 gene and expresses mdr1 mRNA at a very high level. Cells were grown in Dulbecco's modified Eagle's medium, 15 per cent fetal bovine serum, penicillin-streptomycin and glutamine. KB-V-1 were grown in the presence of 1 μg/ml vinblastine.

RNA isolation

Total cellular RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction[23]. Approximately 0.1 g of breast tissue was chopped into small pieces, placed in 2 ml of denaturing solution (D soln, 4M guanidine isothiocyanate / 25mM sodium citrate *p*H 7.0 / 0.5% sarcosyl / 0.1M 2-mercaptoethanol) and rapidly homogenized with intermittent storing on ice. Sequentially, 0.2 ml 2M sodium acetate *p*H 4.0, 2 ml of water-saturated phenol, and 0.4 ml of a chloroform-isoamylalcohol mixture (49:1 v/v) were added to homogenate with thorough mixing by inversion and vigorously vortexing in the last step. The mixture was stored on ice for 15 min and centrifuged at 3,000 rpm for 40 min at 4°C. The aqueous phase was then transferred to a fresh tube and the RNA was precipitated with ethanol at -20°C overnight. RNA pellet was dissolved in 0.3 ml D soln and was reprecipitated with ethanol at -20°C 1 h, centrifuged 13,000 rpm for 15 min at 4°C, washed once in 70 per cent ethanol, vacuum-dried for 15 min, dissolved in an appropriate amount of diethyl-

pyrocarbonate (DEPC)-water and kept at -80°C until needed. RNA yield was quantitated by UV spectrophotometer at the OD260.

mdr1 RNA levels of tumor specimens and cell lines were determined using RT-PCR-Southern hybridization.

cDNA synthesis

Total RNA was converted to cDNA by reverse transcription reaction (RT) which was carried out as follows. Two hundred and fifty ng of total RNA in 5 µl distilled water was heated to 80°C for 5 min and quickly chilled on ice for 5 min. Afterwards cDNA was synthesized in a mixture containing total RNA, 5 µl of 5xRT buffer (94 mM Tris.HCL *p*H 6.9, 453 mM $MgCl_2$, 750 mM ß-NAD, 50 mM $(NH_4)2SO_4$, BRL), 1 µl of random hexadeoxy-nucleotide primer (100 pmol, Promega), 2 µl of dNTP (1 mM each), 0.5 µl of RNase H^- reverse transcriptase (200 U/, BRL), 1 µl of Dithiothreitol (0.1 M DTT) and 10 µl of distilled water. The mixture was incubated at 25°C for 10 min and followed by 37°C, 30 min. To terminate reaction, the tube was heated at 95°C for 5 min, then chilled immediately on ice and stored until subsequent PCR.

PCR

cDNA aliquots derived from 50 ng of total RNA were used for DNA amplification by PCR using 1 unit of Taq DNA polymerase (Promega) in a final volume of 50 µl. Thirty cycles of PCR were carried out using a DNA thermal cycle model 480 (Perkin-Elmer/Cetus). Each cycle of PCR included 30 sec of denaturation at 94°C, followed by 30 sec of primer annealing at 55°C and 30 sec of extension at 72°C. Primers used for amplification of mdr1-specific sequences and ß2-microglobulin (ß2m)-specific sequences were according to Noonan et al[24]. The sequence of mdr1- and ß2m forward primers were CCCAT CATTGCAATAGCAGG, bases 2596-2615 and ACCCCCACTGAAAAAGATGA, bases 1544-1563 respectively. The mdr1- and ß2m reverse primer were AGTCCTCGTCTTCAAACTTG, bases 2733-2752 and GTAGTACCTC--CAAACTTCTA, bases 2253-2262 and 3508-3517. Each primer was added at 10 pmol per reaction. PCR using mdr1 primers gives 157 base-pair (bp) amplified product and ß2m primers gives 120 bp product. PCR products were separated on 2 per cent Nusieve 3:1 agarose gels (FMC Bioproducts), stained with ethidium bromide and viewed with a transilluminator. Negative and positive control reactions were included in each experiment where 250 ng of total RNA from KB-3-1 drug sensitive and KB-V-1 multidrug resistant cells were used.

Southern blotting and hybridization

For a more definite identification of the products, amplified DNA was blotted onto nylon membrane filter (Magnagraph, MSI) by capillary transfer overnight[25]. Next morning the blot was rinsed briefly in 6x saline-sodium citrate buffer (5x SSC) to remove the adhering gel and dried at room temperature (rt) by placing the filter upward. The DNA was fixed to the membrane by baking at 80°C for 2 h. After fixation, filter was prehybridized in 50 per cent formamide, 5x SSC, 1 per cent blocking solution, 0.1 per cent N-lauroylsarcosine, 0.02 per cent sodium dodecyl sulphate (SDS) at 42°C for at least 1 h. Hybridization was performed at 42°C for at least 6 h with two probes, mdr1- and ß2m probe labelled with digoxigenin-dideoxyuridine triphosphate (DIG-ddUTP), simultaneously. After hybridization, the filter was washed twice for 5 min at rt in 2x SSC, 0.1 per cent SDS. Then 0.1x SSC, 0.1 per cent SDS that had been warmed to 42°C was added twice at 15 min intervals. Filter can then be used directly for detection of hybridized DNA or stored air-dried for later detection.

Immunological detection

Hybridized filters were detected by incubating at rt for 30 min with anti-DIG-alkaline phosphatase conjugate. The excess unbound antibody-conjugate was removed by washing twice for 15 min rt in buffer containing 0.1 M maleic acid, 0.15 M NaCl *p*H 7.5. The color reaction was developed at alkaline *p*H by the addition of colorless substrate 5-bromo-4-chloro-3-indoyl phosphate (X-phosphate) and nitroblue-tetrazolium salt (NBT) at rt in dark. When the purple color precipitate was clearly formed (within 1-24 h), the reaction was then terminated by washing the filters for 5 min with 10 mM Tris HCl, 1 mM EDTA *p*H 8.0.

The mdr1 and ß2m bands was quantified by scanning with image analyzer (ID Advanced Programme, AAB, U.S.A.). The values for RT-PCR were determined by substracting the mdr1 value obtained in the KB-3-1 and normalizing against the ß2m results. ß2m expression was used to com-

pensate for the different amounts of RNA loaded and to rule out false negativity.

DNA labelling

Labelling of oligonucleotide primer with DIG-ddUTP : To prepare mdr1 and ß2m probe, mdr1- and ß2m forward primer (100 pmol) were separately labelled at its 3'-end with terminal transferase by incorporation of a DIG-ddUTP using DIG oligonucleotide 3'-end labelling kit (Boehringer).

Statistical analysis

Statistical evaluations were performed with the Chi-square test (χ^2) to compare levels of mdr1 gene expression with menopause status, several prognostic factors (age, tumor size, number of lymph node involvement, estrogen receptor and p53 expression) and clinical outcome. Fisher's exact test was used for a sample size smaller than 15. A p value of less than 0.05 was considered statistically significant.

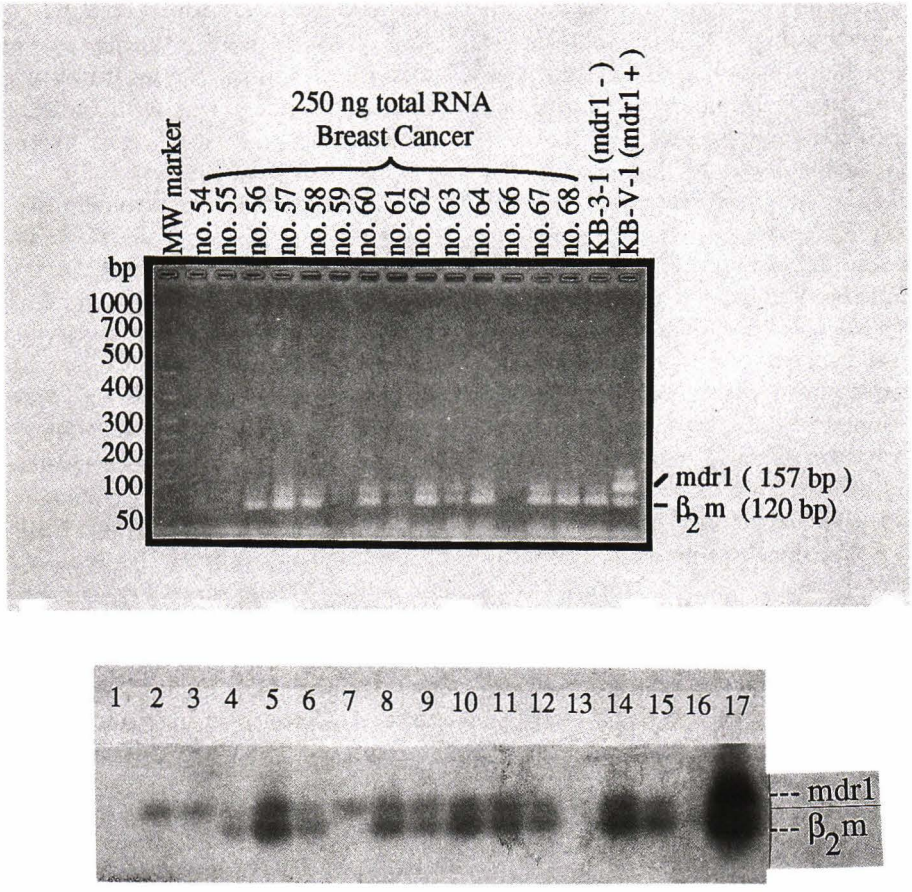

Fig. 1. (A) Amplified mdrl and ß2m-specific PCR products with RNA from breast cancers (lane 2-15), drug sensitive KB-3-1 (lane 16) and multidrug-resistant KB-V-1 (lane 17). After reverse transcription of 250 ng of total RNA, the complementary DNA was amplified by PCR. All aliquots of reaction mixture were subjected to 2% Nusieve agarose gel electrophoresis and stained with ethidium bromide. Arrows indicate the band of ß2m (120 bp) and mdrl (157 bp) sequences. The leftmost lane is 100 bp DNA marker.

(B) Southern hybridization analysis of mdrl and ß2m-specific PCR products with mdrl and ß2m-specific probe labelled with DIG-ddUTP. After 6 h hybridization, the hybridized probes were detected by anti-DIG-alkaline phosphatase conjugate and then developed color in X-phosphate substrate and NBT solution.

RESULTS

Quantitation of mdr1 RNA

Determination of mdr1 RNA levels was possible in 135 samples while in 9 samples mdr1 RNA levels were excluded from evaluation because of poor quality of the isolated RNA. 127 of the evaluable samples were obtained from patients with primary breast CA at the time of initial diagnosis (124 cases) or at the delayed surgery after chemotherapy (3 cases). Eight were from locally relapsed disease following conventional therapy (surgery and/or chemotherapy). All samples were analyzed by RT-PCR Southern hybridization. As shown in Fig. 1A, some variations in the intensity of mdr1 and ß2m PCR band were evident between samples. Both drug-sensitive cell control KB-3-1 and multidrug-resistant KB-V-1 showed a comparable signal of ß2m product but only KB-V-1 gave a prominent mdr1-specific signal. These PCR products were blotted and probed to confirm the evidence of mdr1 and ß2m expression (Fig. 1B). Variations in the mdr1 signals on ethidium bromide gel occurred in parallel with variations in the intensity of hybridization signals, indicating minor uneveness in blotting. Surprisingly, ß2m signal in KB-3-1 negative control observed on ethidium bromide gel gave very low signal intensity after hybridization step. This could be due to the detection error. An arbitrary value of 100 units (U) was assigned to the mdr1 RNA expression of 250 ng of total RNA from KB-V-1 cells. Mdr1 expression level in each sample was expressed relatively to the signal obtained from KB-V-1 which usually gave a reproducible and distinct signal.

Mdr1 RNA levels in breast CA

Mdr1 RNA levels were negative in 81/127 (63.8%) and positive in 46/127 (36.2%) of the primary breast CA with low expression (<10 U) in 11/127 (8.7%) and high expression (≥10 U) in 35/127 (27.5%) of the samples (Table 1). Among 127 primary breast CA, there were 3 samples from patients previously treated by 4, 6 and 12 courses of CMF (cyclophosphamide + methotrexate + 5-fluorouracil), 1 had no detectable level of mdr1 RNA, 1 had low mdr1 and 1 had high mdr1 expression level. In local relapses, mdr1 RNA levels were negative in 5/8 (62.5%) and positive in 3/8 (37.5%). Only high expression was observed in all 3 mdr1+ve cases. Although this is a limited number of patients, it is of interest because it may reflect the acquired expression of mdr1 after previous therapy and may represent acquired resistance secondary to this overexpression.

Relationship to clinicopathologic characters and prognostic factors

The age, histology, tumor size, lymph node involvement, metastasis, stage, estrogen receptor (ER) status, p53 protein expression and mdr1 RNA expression of the patients with primary breast CA are summarized in Table 2 and 3. The mean age of 127 patients was 50.2 ± 12, ranged 28-81 years. 73 were premenopausal and 54 postmenopausal women. In regard to age or menopause status, there was no significant difference in the incidence of mdr1 gene expression (χ^2 = 2.37 and 2.3, respectively).

Among 127 cases of primary breast CA, 124 were classified as ductal adenocarcinoma: 115 invasive ductal carcinoma (IDC), 9 intraductal carcinoma (ITDC). 3 were classified as lobular carcinoma. Mdr1+ve was observed in 41/115 (35.7%) of IDCs and 4/9 (44.4%) of ITDCs (data not shown). Thus, mdr1+ve was 45/46 (98%) in ductal adenocarcinoma type and 1/46 (2%) in lobular carcinoma type. Analogous results of mdr1-ve were 79/81 (98%) and 2/81 (2%) in ductal and lobular carcinoma, respectively. Size of primary tumors were not different between mdr1 RNA-ve and mdr1 RNA+ve patients (χ^2 = 2.3). The distribution of lymph node-negative versus node-positive was nearly the same in mdr1-ve and mdr1+ve cases (χ^2 = 0.745). Expression of the mdr1 gene was never detected in untreated primary tumor of the 5 patients who had distant metastases but 1

Table 1. MDR1 gene expression in primary and locally relapsed breast cancers.

MDR1 levels	Primary cases (%)	Locally relapsed cases (%)
Negative	81/127 (63.8 %)	5/8 (62.5 %)
Positive	46/127 (36.2 %)	3/8 (37.5 %)
Low (<10 U)	11 (8.7 %)	0
High (≥10 U)	35 (27.5 %)	3 (37.5%)

Table 2. MDR1 expression levels and clinical data of patients with primary breast cancer.

	All patients	MDR1 negative	MDR1 positive			χ^2 test	Correletion p value
			Total	Low	High		
n	127	81	46	11	35		
Age (yr)							
Mean ± SD	50.2 ± 12	50.6 ± 12.4	49.4 ± 11.3	49.4 ± 12.6	49.4 ± 11	2.37	.585
Range	28 - 81	28 - 81	35 - 80	35 - 80	36 - 77		
Menopause status							
Premenopausal	73 (57%)	42 (52%)	31 (67%)	11	20	2.3	.09
Postmenopausal	54 (43%)	39 (48%)	15 (33%)	4	11		
Histology							
Ductal adenocarcinoma	124 (98%)	79 (98%)	45 (98%)	15 (100%)	30 (98%)	.51	.195
Lobular carcinoma	3 (2%)	2 (2%)	1 (2%)	0	1 (2%)		
Tumor size							
T1	25 (20%)	17 (21%)	8 (17%)	1	7	2.3	82
T2	76 (60%)	48 (59%)	28 (61%)	9	19		
T3	8 (6%)	4 (5%)	4 (9%)	1	3		
T4	18 (14%)	12 (15%)	6 (13%)	4	2		
Lymph node							
N0	56 (44%)	35 (43%)	21 (46%)	8	13		
N1,2,3	69 (54%)	46 (57%)	23 (50%)	7	16	.745	.008 *
Nx	2 (2%)	0	2 (4%)	0	2		
Metastasis							
M0	116 (91%)	72 (89%)	44 (96%)	15	29		
M1	5 (4%)	5 (6%)	0	0	0	.45	.873
Mx	6 (5%)	4 (5%)	2 (4%)	0	2		
Tumor staging							
I	15 (12%)	10 (12%)	5 (11%)	1	4		
IIA	46 (36%)	27 (34%)	19 (41%)	6	13	.30	
IIB	37 (29%)	25 (31%)	12 (26%)	3	9	.15	
IIIA	8 (6%)	4 (5%)	4 (9%)	1	3	.11	.547
IIIB	16 (13%)	10 (12%)	6 (13%)	4	2	.017	
IV	5 (4%)	5 (6%)	0	0	0	4.35 *	

All comparisons are not significant ($p > .05$) except *

Table 3. Association between MDR1 expression levels and estrogen receptors and p53 expression in patients with primary breast cancer.

	Total patients	MDR1 negative	MDR1 positive			χ^2 test	Correlation p value
			Total	Low	High		
n	126	80	46	15	31		
ER-negative	67 (53%)	42 (53%)	25 (54%)	7	18	.15 NS	.0007 S
ER-positive	59 (47%)	38 (47%)	21 (46%)	8	13		
n	120	75	45	15	30		
p53-negative	83 (69%)	48 (64%)	35 (78%)	9	26	3.42 NS	.201 NS
p53-positive	37 (31%)	27 (36%)	10 (22%)	6	4		

NS = No Significance ; S= Significance

Table 4. MDR1 expression levels and clinical data of patients with locally relapsed breast cancer.

	All patients	MDR1 negative	MDR1 positive	Correlation p value
n	8	5	3	
Age (yr)				
Mean ± SD	47.5 ± 6.48	50.8 ± 5.26	42 ± 4.36	.104
Range	39 - 57	46 - 57	39 - 47	NS
Menopause status				
Premenopausal	5 (63%)	2 (40%)	3 (100%)	.141
Postmenopausal	3 (37%)	3 (60%)	0	NS
Histology				
Ductal adenocarcinoma	7 (88%)	5 (100%)	2 (67%)	.42
Lobular carcinoma	1 (12%)	0	1 (33%)	NS
Tumor size				
T1	0	0	0	
T2	3 (38%)	1 (20%)	2 (67%)	.662
T3	1 (12%)	1 (20%)	0	NS
T4	4 (50%)	3 (60%)	1 (33%)	
Lymph node				
N0	3 (38%)	2 (40%)	1 (33%)	.798
N1,2,3	5 (62%)	3 (60%)	2 (67%)	NS
Metastasis				
M0	6 (75%)	3 (60%)	3 (100%)	.5
M1	1 (12.5%)	1 (20%)	0	NS
Mx	1 (12.5%)	1 (20%)	0	
Tumor staging				
I	0	0	0	
IIA	1 (12.5%)	1 (20%)	0	
IIB	2 (25%)	0	2 (67%)	.751
IIIA	1 (12.5%)	1 (20%)	0	NS
IIIB	3 (37.5%)	2 (40%)	1 (33%)	
IV	1 (12.5%)	1 (20%)	0	

NS = No Significance

treated primary CA patient without metastasis who had mdr1+ve, developed metastasis 3 months later. No difference in tumor stage was found in the two mdr1 groups except stage IV tumor where all tumor had mdr1 RNA-ve. (χ^2 = 4.35 $p<.05$; significance). Estrogen receptors were detected in 59/126 (47%) of the primary CA but no significant difference in receptor positivity was seen between mdr1-ve and mdr1+ve group (χ^2 = 0.15). P53 overexpression was detected in 31 per cent of the patients, nevertheless, p53 levels were independent of mdr1 RNA levels (χ^2 = 3.42). A correlation between mdr1 RNA level and lymph node involvement and negative ER was found in primary breast CA (p = 0.008 and 0.0007, respectively). There exists no relationship between p53 accumulation and mdr1 expression status (p = 0.201). Approximately 3/127 (2%) of primary CA received chemotherapy before surgical resection. The results described above did not significantly change when the 3 treated patients were omitted from analyses.

As shown in Table 4 and 5, relapsed patients had a mean age less than 50 years (mean = 47.5, range 39-57), 63 per cent were premenopausal and had high-risk characteristics of several parameters: large tumor size >2 cm 5/8 (62 %), negative ER status 3/7 (43%) and overexpression of p53 protein 3/8 (37%). However, no significant changes in mdr1 expression were associated with any established prognostic factor mentioned above.

Table 5. Association between MDR1 expression levels and estrogen receptors and p53 expression in patients with locally relapsed breast cancers.

	Total patients	MDR1 negative	MDR1 positive	Correlation p value
n	7	4	3	
ER-negative	4 (57%)	2 (50%)	2 (67%)	.252 NS
ER-positive	3 (43%)	2 (50%)	1 (33%)	
n	8	5	3	
p53-negative	5 (63%)	4 (80%)	1 (33%)	.516 NS
p53-positive	3 (37%)	1 (20%)	2 (67%)	

NS = No Significance

Table 6. Association between MDR1 expression levels and clinical outcome in chemotherapy- and non-chemotherapy treated breast cancer patients.

	Chemotherapy [a]					Non-chemotherapy [b]		
	Total	CR	PR	NR	NE	Total	CR	NR
Primary breast cancer								
n	91	68	4	12	7	36	34	2
MDR1-negative	54	41 (60%)	3 (75%)	7 (58%)	3 (43%)	27	25 (74%)	2 (100%)
MDR1-positive	37	27 (40%)	1 (25%)	5 (42%)	4 (57%)	9	9 (26%)	0
Low		9	0	0	2		4	
High		18	1	5	2		5	
χ^2		.12 (NS)						
Correlation, p value		.246 (NS)						
Locally relapsed breast cancer								
n	6	3		3				
MDR1-negative	3	1		2				
MDR1-positive	3	2		1				
χ^2		1.0 (NS)						
Correlation, p value		.53 (NS)						

a cyclophosphamide + methotrexate + 5-fluorouracil
b surgery only or surgery + hormonal therapy
CR: Complete Response; PR: Partial Response; NR: No Response; NE: No Evaluation or loss to clinic; NS: No Significance

In this small sample size, distant metastasis was never detected in all 3 positive mdr1. In contrast, 1 of the 3 mdr1-ve patients had metastasis.

Relationship to clinical outcome

Chemotherapy response is shown in Table 6. Of the 91 primary CA treated with adjuvant chemotherapy (CMF regimen), complete remission of the tumor was identified in 68 patients, 4 had partial response, 12 had no response while 7 were not evaluated or lost to clinic. 54 of 91 patients had mdr1 RNA-ve whereas the other 37 had mdr1 +ve. However, there was no direct association between the level of mdr1 RNA and the initial response to chemotherapy (χ^2 = 0.12). Moreover, intrinsic mdr1 RNA levels did not correlate with the incidence of relapse after surgery since mdr1 +ve and mdr1-ve patients developed local relapses at the same rate (13.5% and 13%, respectively) within 9 to 36 months after complete remission (data not shown). When considered the 3 cases treated with chemotherapy before resection, complete response of the tumor was identified in 1 patient having mdr1-ve and 1 having low mdr1 expression (4 U). The latter case developed metastasis after 8 months and died. The last patient (mdr1 = 11U) had partial response and had poor evolution.

Of the 8 relapsed patients, clinical outcome was available on 6 patients and two were lost from the clinic after surgery. High mdr1 RNA expression was detected in 3 cases. All were treated with CMF combination therapy, 1 had no response to chemotherapy and 2 had complete response. For those 3 patients who had mdr1-ve, 2 had no response and 1 had complete remission. Again, no significant association in chemotherapy response was found between mdr1 gene-ve and mdr1 gene+ve tumors (Fisher's exact test = 1.0).

DISCUSSION

The controversy regarding whether or not overexpression of mdr1 gene results in clinical drug resistance and poor response of breast CA to chemotherapy urged us to investigate the association of mdr1 expression with response to chemotherapy. Our results demonstrate the increased mdr1 RNA levels in 36.2 per cent of primary breast CA with high levels (10-66 U) in 27.5 per cent of the tumor samples. Our results are in agreement with several reports in which increased mdr1 expression had been detected in primary breast CA using different approaches. Goldstein et al[13] reported mdr1 RNA levels between 2 and 29 U in 15 per cent of breast CA biopsies while Keith et al[18] demonstrated increased mdr1 gene expression in 51 per cent of breast CA. Likewise, Wallner et al[26] reported elevated mdr1 RNA levels in 46 per cent primary breast CA with high levels in 17 per cent of the specimens. However, two independent studies employing Northern blot analysis showed that increased mdr1 RNA was never detected in 219 and 34 of both untreated and treated primary CA[19,27]. The variations in the incidence of tumors with detectable mdr1 RNA may result from variations in the detection techniques, tumor etiology, tumor grading and genetic background of individuals.

Our findings of a broad range of mdr1 gene expression in untreated and treated primary breast tumors and high level expression in local relapses support the hypothesis that drug-resistant cancer cells exist already in untreated tumors rather than induced by chemotherapy. These cells are then subsequently selected by chemotherapy, clonally expand and probably acquire increased expression of the mdr1 gene.

We did not find an association of mdr1 gene expression with response to chemotherapy in both primary and relapsing tumors. Two possible explanations are that the anti-cancer drugs we used are not the substrates which can bind to P-gp, thus it is likely that expression of the mdr1 gene in breast CA cells did not affect tumor response of breast CA patients. Another likely explanation is that other mechanisms of drug resistance may play a role. Our study on clinical follow-up in patients receiving chemotherapy did not directly answer whether mdr1 expression has a role in chemoresistance of breast tumor. However, the evidence from *in vitro* study[18] where human breast CA cells with high mdr1 RNA levels required higher drug concentrations for growth inhibition comparing to cells with low mdr1 levels implies that mdr1 gene expression may have the impact on tumor response in breast CA patients if drugs bind to P-gp (eg anthracyclines). Therefore, evaluation of mdr1 gene expression in CA cells should be considered prior to chemotherapy in breast CA patients.

The prognosis of breast CA and the response to therapy of individual tumors are contingent on biological behavior of the disease. The most well-characterized indicators of poor prognosis include poor nuclear grade, large tumor size and increasing numbers of lymph nodes involved. Other potential markers of poor prognosis are a high DNA content, a high proliferative index, overexpression of c-erbB2 oncoprotein and / or overexpression of mutated p53 tumor suppressor gene. The presence of the hormonal receptors: ER and progesterone receptor (PR), is of particular interest in breast cancer prognosis[29,30]. Overexpression of mdr1 gene product, P-gp, has been reported to have association with a poor prognosis for some groups of patients. Indeed, association of P-gp overexpression with other prognostic factors such as c-erbB2 (HER-2/neu) oncoprotein and mutant p53 tumor suppressor protein has been found in locally advanced breast CA[22,28]. Expression of P-gp alone or co-expression of P-gp with p53 is strongly associated with shorter survival in these patients[21,22]. In our experience, although no association of mdr1 expression with mutant p53 was detected, we found association of mdr1 expression with other two established prognostic factors, positive lymph node and negative ER status. Combination of these factors may result in a more aggressive nature of the tumor, drug resistance and poor prognosis. Our finding suggests that mdr1 status might be used to identify breast CA patients at high risk for poor prognosis and thus serve as a prognostic marker to select patients for aggressive treatment. Clinical follow-up in this study population will help to establish the significance of mdr1 expression on disease-free survival and overall survival of breast CA patients.

Local recurrent breast CA are relatively common in younger premenopausal Thai women who had high-risk phenotypes of several biological parameters. Although we have seen the similar pattern of the distribution of mdr1 expression in both primary and local recurrent breast tumors, we found no correlation of mdr1 expression with lymph node, ER or other parameters in the latter group. This might be due to the limited number of patients in this group.

In conclusion, our study demonstrates that mdr1 is expressed in both primary and locally recurring breast CA at the same rate. Its expression is often associated with the number of metastatic lymph nodes and low expression of hormonal receptors in the group of primary tumors only and suggests that mdr1 expression status of the primary breast tissue might be used in combination with other prognostic markers to predict patients with poor prognosis. Nevertheless, in our series response to CMF chemotherapy of both primary and local recurrent breast tumor was not affected by the presence or absence of mdr1 gene product.

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ความสัมพันธ์ของยีนดื้อยากับปัจจัยช่วยการพยากรณ์โรคและการตอบสนองต่อเคมีบำบัดในมะเร็งเต้านม

บุญนำ พันธุ์แย้มมาลี, D.Sc., สุพล มโนรมณ์, พ.บ.**,*
วิชัย ปุริสา, วท.บ., สุนันทา จริยาเลิศศักดิ์, วท.ม.*, บุษบา ฤกษ์อำนวยโชค, D.M.Sc.****

การดื้อยาของเซลล์มะเร็ง นับเป็นอุปสรรคสำคัญอันหนึ่งต่อการรักษาโรคมะเร็งด้วยยาเคมีเนื่องจากยีนดื้อยา mdr1 ในเซลล์มะเร็งนั้นมีการแสดงออกผลิตสารไกลโคโปรตีน P-gp ออกมาในปริมาณมากเพื่อทำหน้าที่ขับยาออกนอกเซลล์ อย่างไรก็ดีหลักฐานที่แสดงว่ายีนนี้มีความสำคัญต่อการดื้อยาของผู้ป่วยมะเร็งที่ได้รับเคมีบำบัดยังมีความขัดแย้งกันอยู่ และมีหลักฐานที่แสดงว่าการปรากฏของสาร P-gp มีสัมพันธ์กับ prognosis ของมะเร็งเต้านมชนิด advanced การศึกษานี้มีจุดประสงค์เพื่อพิสูจน์ว่าระดับการแสดงออกของยีนดื้อยาสามารถนำมาใช้เพื่อช่วยการพยากรณ์โรคและการตอบสนองต่อการรักษาด้วยยาเคมีของผู้ป่วยมะเร็งเต้านมได้หรือไม่ โดยใช้เทคนิค RT-PCR-Southern Hybridization ซึ่งมีความไว และความจำเพาะในการตรวจวัดดี พบว่าในผู้ป่วยมะเร็งเต้านมชนิด primary 127 ราย มีผู้ที่ไม่มีการแสดงออกของยีนดื้อยา (mdr1 ลบ) ร้อยละ 63.8 ผู้ที่มีการแสดงออกของยีนนี้ (mdr1 บวก) ในระดับต่ำ (<10 ยูนิต) ร้อยละ 8.7 และระดับสูง (≥10 ยูนิต) ร้อยละ 27.5 เมื่อพิจารณาจากปัจจัยต่างๆ เช่น อายุ ภาวะการหมดประจำเดือน ขนาดของก้อนมะเร็ง stage จำนวนของต่อมน้ำเหลืองที่มะเร็งกระจายไปถึง ระดับของ estrogen receptor และระดับของ p53 พบว่ามีจำนวนของผู้ป่วยที่เป็น mdr1 ลบ และ mdr1 บวก กระจายอยู่ในปริมาณใกล้เคียงกัน อย่างไรก็ดีเราพบว่าการแสดงออกของยีนดื้อยามีความสัมพันธ์อย่างมีนัยสำคัญกับจำนวนของต่อมน้ำเหลืองที่มะเร็งกระจายไปถึง และ จำนวนของ estrogen receptor บนผิวของเซลล์มะเร็งน้อยหรือศูนย์ (p = .008 และ .0007 ตามลำดับ) ในกลุ่มผู้ป่วยที่เคยได้รับการรักษาและมีการกลับเป็นของโรคอีก พบที่เป็น mdr1 ลบ ร้อยละ 62.5 และ mdr1 บวก ในระดับสูงอย่างเดียว ร้อยละ 37.5 การกระจายของผู้ป่วย mdr1 ลบ และผู้ป่วย mdr1 บวกไม่มีความแตกต่างกันเช่นเดียวกันในการพิจารณาในทางสถิติเมื่อเปรียบเทียบโดยใช้ปัจจัยต่างๆแต่ละตัว เช่น อายุ ภาวะการหมด ประจำเดือน ขนาดของก้อนมะเร็ง stage จำนวนของต่อมน้ำเหลืองที่มีมะเร็ง ระดับของ estrogen receptor และระดับของ p53 เราพบว่าการตอบสนองต่อการรักษาด้วยยาเคมีในกลุ่มผู้ป่วย primary และกลุ่มที่กลับเป็นอีกมีอัตราใกล้เคียงกัน สรุป ผลของการศึกษานี้แสดงให้เห็นว่าสามารถตรวจพบการแสดงออกของยีนดื้อยาได้ในเนื้อเยื่อมะเร็งเต้านมของผู้ป่วยที่ไม่เคยได้รับ หรือ เคยได้รับการบำบัดด้วยยาเคมีมาก่อน การพบความสัมพันธ์ของการแสดงออกของยีนดื้อยา กับปัจจัยที่ช่วยบอกการพยากรณ์โรค 2 ชนิด คือ จำนวนของต่อมน้ำเหลืองที่มีมะเร็ง และ estrogen receptor ระดับต่ำหรือศูนย์แสดงว่าการปรากฏของปัจจัยต่างๆนี้ร่วมกันคงมีผลทำให้เซลล์มะเร็งมีพัฒนาการไปในทางที่รุนแรงขึ้น เช่น ดื้อต่อยาและมีการพยากรณ์ของโรคไม่ดี การตรวจหาปัจจัยเหล่านี้น่าจะช่วยให้สามารถจำแนกผู้ป่วยที่มีการพยากรณ์โรคที่ไม่ดีได้แน่นอนยิ่งขึ้นเพื่อทำการรักษาอย่าง aggressive ต่อไป อย่างไรก็ดีในกลุ่มผู้ป่วยมะเร็งเต้านมที่เราทำการศึกษานี้ การปรากฏหรือไม่ปรากฏ ของยีนดื้อยาไม่มีผลต่อการรักษาด้วยยาเคมี CMF

* กลุ่มงานวิจัยและค้นคว้า,
** กลุ่มงานเคมีบำบัด, สถาบันมะเร็งแห่งชาติ, กรุงเทพฯ 10400
*** ภาควิชาพยาธิวิทยา, คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี, มหาวิทยาลัยมหิดล, กรุงเทพฯ 10400