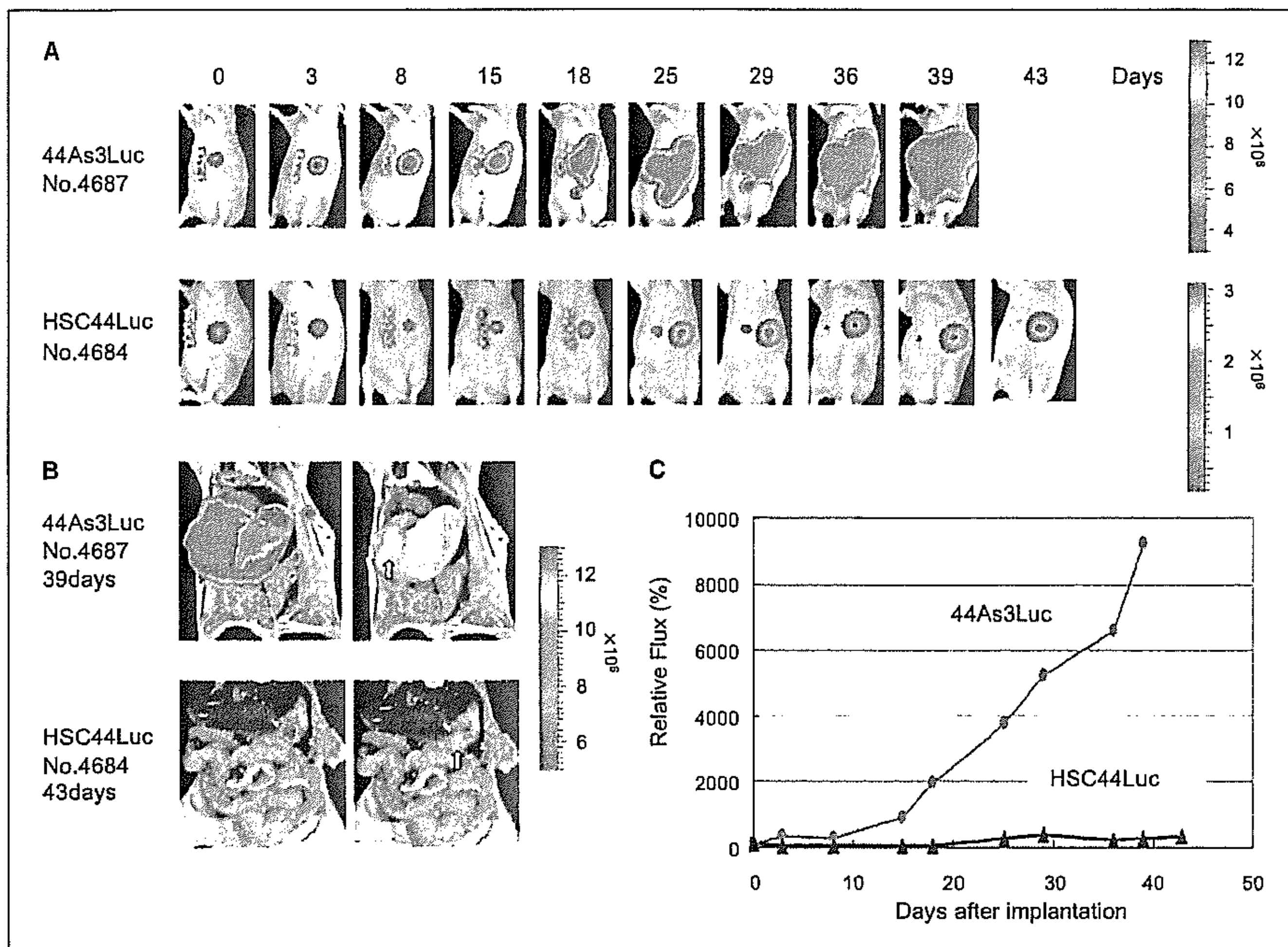

Figure 4. Quantitative photon counting analysis of progression process of peritoneal disseminated metastasis of the highly metastatic and the parent cell lines. *A,* detection of progression process of peritoneal disseminated metastasis. *B,* photon counting analysis of the peritoneal disseminations after orthotopic implantation (*yellow arrow*, site) of the cells. *C,* quantitative analysis of progression process of peritoneal disseminated metastasis of 44As3Luc (●) and HSC44Luc (▲) cell lines ($n = 5$). This experiment was repeated thrice, and similar results were observed each time.

of the survival period (17). A similar evaluation was conducted in the present study using 44As3Luc cells. Figure 5 (*top*) shows a typical example of the photon counting analysis, whereas Figure 5 (*bottom*) shows the time course of the changes in the number of photons. Following three doses of CPT-11 (200 mg/kg/mouse), the tumor gradually decreased in size, reaching a level close to the limit of detection on the 20th day. During the 5th and 6th week, the tumor began to show slow growth in the stomach followed during the 8th/9th week by peritoneal invasion and the onset of cancerous peritonitis accompanied by ascites formation and death of the animals. The survival period was markedly longer in the drug-treated group compared with that in the saline-treated controls. Plotting of the number of photons measured (average of five animals) against time yielded a tumor growth curve, thus allowing quantitative evaluation of drug-induced suppression of the progression of peritoneal dissemination (Fig. 5, *bottom*).

As stated above, the 44As3Luc cells began to proliferate again during the 5th/6th week after implantation in the CPT-11 treatment group. We therefore gave three additional doses beginning on day 28 (after the onset of reproliferation). Figure 5 (*top*) shows a typical example of the bioluminescence signal in such a case. The additional doses of CPT-11 (400 mg/kg/mouse) markedly suppressed the proliferation of the 44As3Luc cells until around day 60; however, proliferation again began to be detected thereafter. By around day 80, the tumor started to grow more rapidly and spread, causing moribund animals to appear by around day 90. The survival period of the animals was markedly prolonged by the additional drug doses. Figure 5 (*bottom*) shows the time course of changes in the number of photons (average of five animals). Quantitative comparison of the proliferation and spread of the tumor cells was possible between the drug treatment group and the control group and between two drug treatment groups, thus allowing objective evaluation of the responses to treatment.

Discussion

Before the present study, very little was known about how scirrhous gastric carcinoma cells invaded and proliferated within the primary lesion, how they exfoliated and thus became free, how they colonized and proliferated within the peritoneal cavity, or how they advanced to the stage of cancerous peritonitis. Herein, we investigated the course of proliferation and spread of gastric cancer

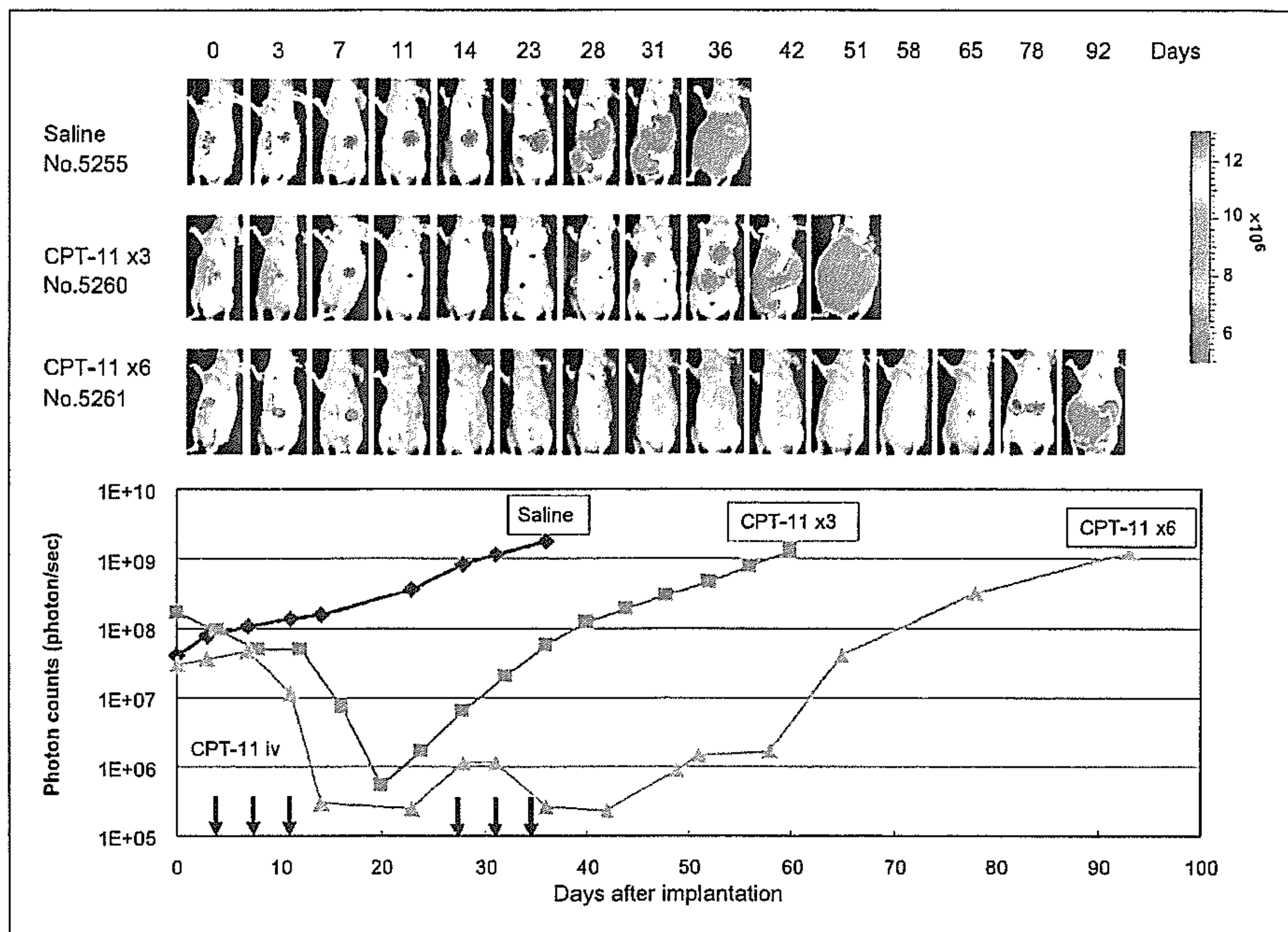

Figure 5. Quantitative photon counting analysis of the effect of CPT-11 on peritoneal disseminated metastasis 44As3Luc mouse model. Effects of CPT-11 in the peritoneal dissemination mouse model established using orthotopically implanted 44As3Luc cells. Mice receiving CPT-11 (*arrow*) or vehicle alone as control ($n = 5$; $P < 0.001$) were monitored twice weekly for the development of peritoneal dissemination. Similar results were obtained in a second experiment conducted independently.

cells by sacrificing the animals at different points of time after orthotopic implantation of the highly metastatic tumor cell line 44As3 (17) and conducted anatomic and histopathologic examinations in the sacrificed animals. In this experiment, the sequence of findings seems to endorse the previous contention that gastric cancer cells invade deeper layers of the gastric wall to reach the serosa and then exfoliate, thereby being released into the peritoneal cavity, resulting in peritoneal dissemination.

The growth of tumors in the gastric wall and the subsequent progression to cancerous peritonitis are difficult to monitor extracorporeally unlike s.c. tumors. For monitoring the progression of tumor dissemination, the only possible method was to implant the tumor cells into groups of mice and sacrifice the animals at different points of time for autopsy and observation; quantitative comparison was still not possible by this method (10–12, 18–25). All of these problems were resolved in the present study by introduction of the luciferase gene into tumor cells with a high metastasizing potential and subsequent *in vivo* photon counting analysis. In the first step, we confirmed that the results of the conventional method of evaluation in relation to proliferation of our gastric carcinoma cells were consistent with the results of our photon counting analysis. We then conducted an experiment on a model of peritoneal dissemination. Using the *in vivo* photon counting technique, it was possible to observe the same animals successively, beginning from the growth of the tumor at the site of implantation to peritoneal dissemination and, finally, the formation of ascites. Furthermore, it was possible to observe the processes of dissemination progression on a real-time basis, allowing quantitative analysis and comparison of the course of proliferation and progression within the living body after implantation of a cell line with high metastasizing potential and its parent cell line based on changes in the photon number.

Needless to say, it is important to develop a screening model for exploring substances effective against tumors and ultimately developing clinically useful anticancer agents. We previously reported that an animal model of peritoneal dissemination established using the highly metastatic cell lines (44As3, 58As1, and 58As9) established by our group satisfied all of the requirements of a model for drug screening (17, 44). However, before this model can be applied as a universally valid drug evaluation system, the following problems must be resolved: (*a*) methods for appropriate observation and objective evaluation are urgently needed,

(*b*) excellent operative skill is indispensable for orthotopic implantation with high reproducibility, and (*c*) large numbers of animals are needed. With the establishment of this experimental system, the conventional problems associated with the evaluation of peritoneal dissemination have been overcome and highly reliable data are now obtainable. Therefore, a stage has been reached where this model of peritoneal dissemination can also be applied as a system for evaluation of the effects of drugs. Furthermore, because photon counting analysis allows noninvasive evaluation of the fate of cancer cells *in vivo* on a real-time basis, the pain experienced by experimental animals may be reduced, such that this technique would also be useful from the viewpoint of animal welfare (45).

We have used the bioluminescence signal from the luciferase reporter gene in our peritoneal metastasis model. Luciferase genes in our tumor cells can function stably over significant periods in tumors and in their metastases. To date, several other peritoneal metastasis models of human stomach cancer in animals have been reported (28, 31). For example, Hasegawa et al. (28) used green fluorescent protein (GFP) retroviral-infected human stomach cancer. In this nude mouse model, tumor cells were peritoneally injected and GFP transduction allowed visualization of the subsequent metastatic process. A major advantage of GFP labeling is that imaging requires no preparative procedures and hence allows for direct visualization in living tissue (26, 27, 29, 32, 34). In contrast, photon counting technique requires exogenous injection of luciferin substrate, which can stress the animals, and in addition, the intensity of the luciferase signal may sometimes be variable and unstable (46). Furthermore, Ray et al. (32) reported that red fluorescent protein imaging is ~1,000 times stronger than that of luciferase *in vivo*. Therefore, for monitoring the tumor metastasis process at the single-cell level, fluorescence imaging may be the more practical method. In fact, fluorescence-based orthotopic metastatic models have been used to study mechanisms and for drug discovery (14, 30, 33, 35).

In conclusion, our photon counting analysis involving a highly metastatic cell line, 44As3Luc, seems to be a useful model for studies, such as those designed to clarify the mechanism of peritoneal dissemination progression in intractable scirrhous gastric carcinoma, and for the development of new agents effective against such tumors.

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Gene expression profiling of ATP-binding cassette (ABC) transporters as a predictor of the pathologic response to neoadjuvant chemotherapy in breast cancer patients

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Key words: ATP-binding-cassette (ABC) transporters, breast cancer, class prediction, neoadjuvant chemotherapy, oligonucleotide microarray

Summary

Drug resistance is a major obstacle to the successful chemotherapy. Several ATP-binding cassette (ABC) transporters including ABCB1, ABCC1 and ABCG2 have been known to be important mediators of chemoresistance. Using oligonucleotide microarrays (HG-U133 Plus 2.0; Affymetrix), we analyzed the ABC transporter gene expression profiles in breast cancer patients who underwent sequential weekly paclitaxel/FEC (5-fluorouracil, epirubicin and cyclophosphamide) neoadjuvant chemotherapy. We compared the ABC transporter expression profile between two classes of pretreatment tumor samples divided by the patients' pathological response to neoadjuvant chemotherapy (residual disease [RD] versus pathologic complete response [pCR]) ABCB3, ABCC7 and ABCF2 showed significantly high expression in the pCR. Several ABC transporters including ABCC5, ABCA12, ABCA1 ABCC13, ABCB6 and ABCC11 showed significantly increased expression in the RD ($p < 0.05$). We evaluated the feasibility of developing a multigene predictor model of pathologic response to neoadjuvant chemotherapy using gene expression profiles of ABC transporters. The prediction error was evaluated by leave-one-out cross-validation (LOOCV). A multigene predictor model with the ABC transporters differentially expressed between the two classes ($p \leq 0.003$) showed an average 92.8% of predictive accuracy (95% CI, 88.0–97.4%) with a 93.2% (95% CI, 85.2–100%) positive predictive value for pCR, a 93.6% (95% CI, 87.8–99.4%) negative predictive value, a sensitivity of 88.1%(95% CI, 76.8–99.4%), and a specificity of 95.9% (91.1% CI, 87.8–100%). Our results suggest that several ABC transporters in human breast cancer cells may affect the clinical response to neoadjuvant chemotherapy, and transcriptional profiling of these genes may be useful to predict the pathologic response to sequential weekly paclitaxel/FEC in breast cancer patients.

Introduction

Resistance to chemotherapy is a significant obstacle to appropriate treatment of cancer patients. Various cellular pathways may play a role in drug resistance and ATP-binding cassette (ABC) transporters are one of the most well known mediators leading to drug resistance and treatment failure. To date 49 ABC transporter genes have been identified and classified into seven groups, ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG (database of ABC transporters available at http://nutrigene.4t.com/humanabc.htm).

Extensive studies have been conducted on the individual proteins or genes of ABC transporter members regarding their role in chemoresistance. ABCB1 (MDR1-P-gp) [1,2], ABCC1 (MRP1) [3], and ABCG2 (MXR) [4] are particularly well known as mediators leading to resistance to several chemotherapeutic agents including paclitaxel [5], topoisomerase inhibitors [6], anthracyclin [7] and tyrosine kinase inhibitors [8]. Although little has been known about most of ABC transporter members, other members of this family sharing sequence and structural homology may play roles in absorption, distribution, and excretion of chemotherapeutic agents and probably influence the response to chemotherapy.

Recently, using ABC transporter gene expression profiling, studies on the relationship of drug resistance and ABC transporter were performed in cancer cell lines [9,10].

The characterization of the comprehensive expression of these genes in relation to the clinical response to chemotherapy may be useful to determine on an individual basis the patient's underlying risk and choose the optimal therapeutic regimen to which the individual cancer patient is most likely to respond. We studied the relationship between ABC transporter gene expression and the responsiveness to chemotherapy in early breast cancer patients who underwent sequential weekly paclitaxel/FEC (5-fluorouracil, epirubicin and cyclophosphamide) neoadjuvant chemotherapy and evaluated the feasibility of developing a multigene predictor model of pathologic response using differentially expressed ABC transporters on the basis of microarray data.

Materials and methods

Patient and sample preparation

This study was performed at the National Cancer Center Hospital, Tokyo, Japan. This study was approved by the institutional review boards of the National Cancer Center. Twenty-one pretreatment samples were obtained from breast cancer patients who underwent neoadjuvant chemotherapy from 2002 to 2004. All patients underwent pretreatment core needle biopsy (CNB) of the primary tumor tissue before starting neoadjuvant chemotherapy. The core needle biopsy was done using 14–16 gauge needles.

The patients received 4 cycles of FEC (5-Fluorouracil 500 mg/m^2, Epirubicin 100 mg/m^2 and Cyclophosphamide 500 mg/m^2) every three weeks followed by 12 cycles of weekly paclitaxel (80 mg/m^2). Additionally, in the case of HER2 positive determined by immunohistochemical staining (IHC), the specific inhibitory antibody of HER2 receptor, Trastuzumab (Herceptin®) was added in the course of the paclitaxel (Herceptin 4 mg/kg on day1 then 2 mg/kg weekly). Samples that showed 3+ IHC staining were considered as HER2 positive.

Every patient underwent surgery on the completion of the neoadjuvant chemotherapy, and histopathologic examination was performed. As described previously [11], pathologic complete response (pCR) was defined as no pathologic evidence of any residual invasive cancer cells in the breast and axillary lymph nodes, and residual disease (RD) was defined as any residual cancer cells on the histopathologic examination. Informed consent was obtained from all patients for voluntary participation in the study.

Tissue preparation and microarray

Samples for the microarray were collected into tubes containing Isogen (Nippon gene, Toyama) and stored at −80 °C. Total RNA was extracted by the single step method of Chomczynski et al. [12] with acid guanidinium thiocyanate phenol chloroform after homogenizing the tissue using a high speed homogenizer. The mean yield of RNA was 23.1 μg (ranged from 12.3 to 31.6 μg) from each collected samples. RNA that had distinct ribosomal RNA band by electrophoresis and had A_{260}/A_{280} absorbance ratio ranging from 1.8 to 2.1 was used for cDNA synthesis. Gene expression profiles were analyzed on a high-density oligonucleotide microarray (GeneChip® HG-U133 Plus 2.0; Affymetrix, Santa Clara, CA) containing 54,675 probe sets. The oligonucleotide microarray procedure for generation of the biotin-labeled cyclic RNA (cRNA) by *in vitro* transcription, hybridization to the array and scanning were performed according to the manufacturer's instructions. The amplification cycle of RNA to cDNA and cDNA to cRNA was performed using the GeneChip® 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit including SuperScript II reverse transcriptase and a T7-$(dT)_{24}$ primer (Affymetrix). The synthesized cRNA was biotinylated using GeneChip 3′-amplification reagents for IVT labeling. The labeled cRNA was then purified and chemically fragmented at 94 °C for 35 min using the GeneChip Sample Cleanup Module (Affymetrix). The labeled fragmented cRNA was next hybridized to the GeneChip® at 45 °C for 16 h according to the manufacturer's instructions. The hybridized probe array was washed and stained with streptavidin-phycoerythrin. The stained probe array was scanned with a GeneChip® Scanner3000 (Affymetrix) at 570 nm. The signal intensity of the gene expression level was calculated by GeneChip Operating Software, Ver.1 (Affymetrix).

Data analysis

Microarray data analyses were performed with BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam. (http://linus.nci.nih.gov/BRB-ArrayTools.html) which provides a variety of tools for the analysis of gene expression profile. Gene expression data were log transformed (base 2) and normalized to the median expression value of all genes on each array. Any genes in which the expression levels did not differ by at least by 1.5 fold from the median in at least 20% of the arrays were filtered out, for the exclusion of the genes showing minimal variation across the set of arrays. In addition, if an expression value was missing or filtered out in more than 50%, these data were excluded. The final data set included 50,508 clones, and contained all 49 ABC transporter genes. The list of transcripts on ABC transporters was obtained using GeneSprings software (http://www.silicongenetics.com/cgi/SiG.cgi/index.smf) from Agilent Technologies (Waldbronn, Germany). (Supplementary data).

Class comparison

To identify informative genes differentially expressed between the two classes of patients grouped by their pathologic response, we used supervised classification methods applying the random variance t-test to data using the BRB Array Tools and was accompanied by multivariate permutation tests in order to minimize false-positives with the maximum allowed number of

false positives set at 10, a false discovery rate of 0.1, and confidence 90%. Genes with a parametric p-value less than 0.05 were considered statistically significant.

Class prediction

To develop a prediction model of pathologic response using the ABC transporter gene expression profiles, we used the class prediction tools of BRB ArrayTools in which six multivariate classification methods were available including a compound covariate predictor [13], a K-nearest neighbor analysis ($K = 1, 3$), a nearest centroid analysis, a support vector machine [14] and a diagonal linear discriminate analysis.

For the evaluation of the feasibility of developing a multigene predictor model of response to neoadjuvant chemotherapy using differentially expressed ABC transporters, six different multivariate classification models were examined. Firstly, we determined the number of genes that were included in the classifier model using a paired t-test applying multiple univariate parametric significance thresholds, and developed a classifier model based on these selected genes at the univariate parametric significance thresholds. With changes in the parametric significance thresholds, the multivariate classification algorithms were performed iteratively evaluating the classification error and the classifier p-value to identify the best classifier, and the processes were iteratively performed for each number of genes included in the classifier (determined by the significance threshold). The prediction error of each model was evaluated by leave-one-out cross-validation (LOO-CV) [15]. This validation procedure was performed in a manner that removed the left-out sample before selecting the discriminate genes [15,16]. The classifier p-value, the probability that similar low error rate happen by chance, was obtained by a random permutation test performed 2000 times.

Results

The patient characteristics

All the patients received 4 courses of FEC (5-fluorouracil, epirubicin and cyclophosphamide) combination chemotherapy followed by 12 courses of weekly paclitaxel. In those patients who were HER-2 positive by IHC, Trastuzumab (Herceptin®) was added in the course of the treatment. We divided the patients into two groups from the results of the histopathologic examination performed after the completion of the neoadjuvant chemotherapy. Pathologic data were available for nineteen patients. Patients with no pathologic evidence of any residual invasive cancer cells in breast were classified as 'pCR', and if any residual cancer cells were found in the histopathologic study, these patients were classified as 'RD' group. Thirty-six point eight percent (7) of the nineteen patients showed no pathologic evidence of any residual invasive cancer cells in the breast and were classified as pCR and 63.2% (12) of patients were classified as RD.

Gene expression profiling of differentially expressed ABC transporters

Using gene expression data of the pretreatment tumor sample, we compared the ABC transporter gene expression profile between the two groups (RD versus pCR). A probe set on all of the 49 human ABC transporters genes known so far was contained in the microarray chip we used (HG-U133 Plus 2.0; Affymetrix). To identify differentially expressed ABC transporter genes potentially associated with the clinical response to neoadjuvant chemotherapy, a supervised class comparison analysis was performed. The random variance model t-test was used to discover differentially expressed genes and was accompanied by a multivariate 1000 permutation tests in order to minimize false–positives with the maximum allowed number of false positives set at 10, a false discovery rate of 0.1 and 90% confidence.

By comparing the average expression level of each transcript on ABC transporters between the two classes of patients, the median expression level in the RD group was 107.8 (range 15.8–6009.1) and 104.4 in the pCR group (range 17.9–5690.6). The median of fold difference (RD: pCR) of transcripts on the ABC transporters was 1.0, ranging from 0.3 to 7.6. Several ABC transporters showed prominently high expression at over 50 fold of the median value although the tumor samples were all from the pretreatment chemotherapy-naïve patients. The highest average expression level in the RD group, 6009.1, was observed in ABCC5 (AF146074, RD: pCR = 6009.1:2427.5, fold ratio 2.48) and the highest expression level in the pCR group, 5690.6, was observed in TAP1 (ABCB2, NM_000593, RD: pCR = 4551.4:5690.6, fold ratio 0.8), the transporter associated with antigen processing (Table 1).

The ABC transporters, which were significantly differentially expressed with a parametric p-value of less than 0.05, are listed in Table 2. Several transcripts (ABCC5, TAP2/ABCB3) selected overlapped for the microarray chip (HG-U133 Plus 2.0) containing 54,675 probe sets, more than 30,000 human transcripts were detected, derived from more than 20,000 loci within the human genome and some transcripts represented the same human gene.

ABC transporters, the expression of which in the RD group was significantly increased, included ABCC5 (fold ratio 2.48, $p = 0.000368$), ABCA12 (fold ratio 7.64, $p = 0.000795$), ABCA1 (fold ratio 3.30, $p = 0.000859$), ABCC13 (fold ratio 7.54, $p = 0.0194$), ABCB6 (fold ratio 2.17, $p = 0.0271$), and ABCC11 (fold ratio 2.71, $p = 0.0486$) (Table 2). These genes all showed over 2 fold increases in RD compared with pCR tumors. ABCC5 was recently reported to confer resistance to

Table 1. Clinical characteristics of the patients

	No. of patients
Age, years	
Median	51
Range	30–61
Menstruation status	
Pre menopause	12
Post menopause	7
TNM stage	
IIA	8
IIB	7
IIIA	2
IIIB	2
Histology	
Invasive ductal	17
Mixed ductal/lobular	
Invasive lobular	1
Invasive mucinous	1
Nuclear grade	
1	1
2	9
3	9
HER2 status	
HER2-positive	4
HER2-negative	15
ER status	
ER-positive*	5
ER-negative	14
Pathologic response	
Pathologic complete response	7
Residual disease	12
Treatment arm	
A[a]	15
B[b]	4

*Cases in which more than 10% of tumor cells stained positive for ER by IHC classified as ER positive.
[a]Treatment arm A; 4 courses of FEC* followed by 12 courses of weekly paclitaxel.
[b]Treatment arm B; 4 courses of FEC* followed by 12 courses of weekly paclitaxel with Trastuzumab.
*FEC combination chemotherapy (5-fluorouracil, epirubicin and cyclophosphamide).

5-fluorouracil [17] selected with the lowest p-value and it showed the highest gene expression level in tumors with decreased response. (AF146074, expression level RD: pCR = 6009.1: 2427.5, fold ratio 2.48).

CFTR (NM_000492, ABCC7, fold ratio 0.27, $p =$ 0.007030), ABCF2 (NM_005692, fold ratio 0.32, $p =$ 0.015901) and ABCB3 (M74447, TAP2, fold ratio 0.54, $p =$ 0.019345), the transporter associated with antigen processing, showed increased expression in the pCR group but the biological significance concerning responsiveness to chemotherapy remains to be elucidated. The differentially expressed ABC transporter genes are shown in Figure 1 in hierarchical clustering view.

Development of multigene predictor model using the ABC transporter gene expression profile

To evaluate the feasibility of developing a multigene predictor model of response to neoadjuvant chemotherapy using the ABC transporter expression profile, six different multivariate classification models were examined.

Firstly, we determined the number of discriminate genes that were included in the classifier model by applying multiple univariate parametric significance thresholds, and developed a classifier model based on these selected genes at the significance thresholds. With changes in the parametric significance thresholds, the classification error and classifier p-value for each multivariate classification algorithms were evaluated iteratively by LOOCV (leave one out cross validation) [15] and the random permutation test to identify the best classifier model. The classifier p-value, the probability that a similar low error rate could happen by chance, was calculated by 2000 random permutation tests. We calculated the average of the classification error and the classifier p-value of six classifier models at each significance threshold. Figure 2 shows the change in the average classifier p-value for six multivariate classification models from the permutation test and the average of the classification error rate relative to multiple univariate parametric significance thresholds.

During this iterative process, the average estimated misclassification error and classifier p-value also dropped as the significance threshold decreased to 0.003, but applying further stringent significance thresholds caused a steep increase in the classification error. When the ABC transporters differentially expressed between the two classes at a significance threshold level of 0.003 were used for class prediction, the average of the classification error was minimal, 0.072 (92.8% of predictive accuracy, 95% CI, 88.0–97.4%), with the classifier p = 0.012, 93.2% (95% CI, 85.2–100%) positive predictive value for the pCR group, 93.6% (95% CI, 87.8–99.4%) negative predictive value, sensitivity for the pCR group 88.1% (95% CI, 76.8–99.4%), and a specificity of 95.9% (91.1% CI, 87.8–100%). The respective values for each model are represented in Table 3. On applying the compound covariate predictor classifier model, the predictive accuracy reached 100% with a classifier p-value of 0.0005. The ABC transporters selected as the best classifiers are presented in Table 4. The list included ABCA1, ABCA12 and ABCC5, recently reported to confer resistance to cyclic nucleotides including 5-fluorouracil [17].

Our results suggest that the ABC transporter genes expression pattern may be useful in predicting the pathologic response to sequential weekly paclitaxel/FEC in breast cancer patients.

Discussion

To determine the optimal therapeutic regimen to which the individual cancer patient is most likely to respond on

Table 2. Differentially expressed ABC transporters ordered by significance

Gene symbol	Genbank	Parametric *p*-value*	% CV support	RD[a]	pCR[b]	Fold difference[c]	Description
ABCC5	AF146074	0.000368	100	6009.1	2427.5	2.48	ABC, sub-family C (CFTR/MRP), member 5
ABCC5	BE550362	0.000463	100	3571.5	1234.4	2.89	ABC, sub-family C (CFTR/MRP), member 5
ABCA12	AL080207	0.000795	100	711.7	93.1	7.64	ABC, sub-family A (ABC1), member 12
ABCA1	AL833227	0.000859	100	166.8	50.5	3.3	ABC, sub-family A (ABC1), member 1
CFTR	NM_000492	0.007030	100	27.7	104.4	0.27	cystic fibrosis transmembrane conductance regulator, ABC (sub-family C, member 7)
ABCF2	NM_005692	0.015901	100	49.4	154.1	0.32	ABC, sub-family F (GCN20), member 2
TAP2	M74447	0.019345	89	543.4	1008.5	0.54	Transporter 2, ABC, sub-family B (MDR/TAP)
ABCC13	NM_172025	0.019377	100	157.5	20.9	7.54	ABC, sub-family C (CFTR/MRP), member 13
ABCB6	NM_005689	0.027077	89	1471.9	677.5	2.17	ABC, sub-family B (MDR/TAP), member 6
TAP2	AA573502	0.042069	58	1740.5	2802	0.62	Transporter 2, ABC, sub-family B (MDR/TAP)
ABCC11	AF352582	0.048626	42	160.9	59.4	2.71	ABC, sub-family C (CFTR/MRP), member 11

Table sorted by *p*-value. * *p* by random variance *t*-test.
[a]Geometric mean of intensities in the RD group.
[b]Geometric mean of intensities in the pCR group.
[c]Fold difference of geometric means RD: pCR.

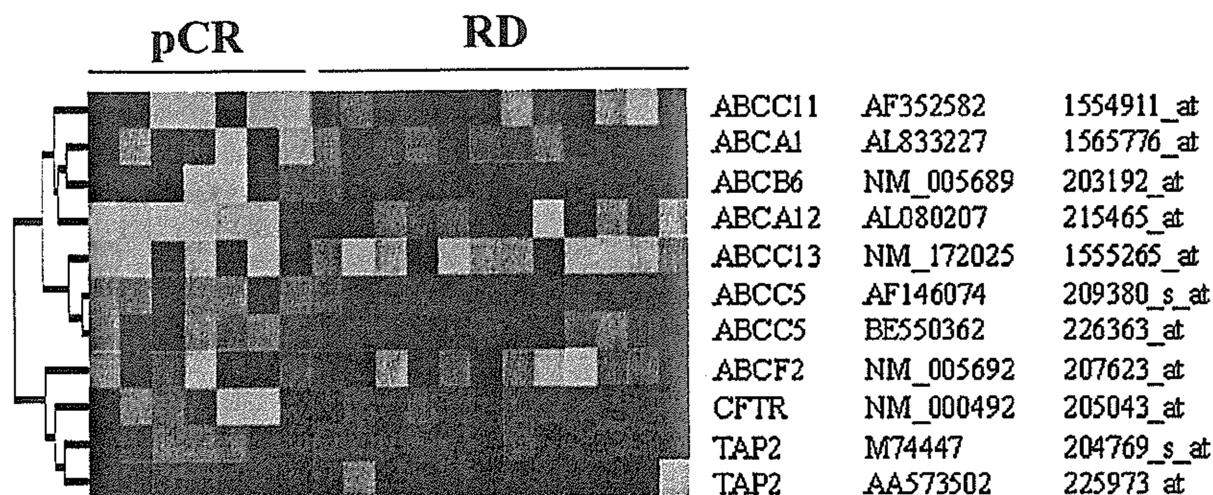

Figure 1. Hierarchical clustering of differentially expressed ABC transporters associated with the response to neoadjuvant chemotherapy in breast cancer patients. The cluster image map shows patterns of differential ABC transporter gene expression in breast cancer patients in respect to the response to neoadjuvant chemotherapy. The hierarchical clustering on each axis was performed using the complete linkage algorithm. Relatively highly expressed genes are shown in red, low expressed genes are shown in green.

an individual basis, there is a real need to develop an appropriate predictor to identify those cancer patients most likely to require or benefit from particular therapies. Resistance to chemotherapy is significant obstacle to appropriate treatment of cancer patients and affects the treatment outcome. Numerous cellular mechanisms exist which are responsible for the treatment failure due to chemoresistance. ABC transporters are the one of the major factors leading to drug resistance. Extensive study has been conducted on the ABC transporters, and ABCB1 (MDR1-P-gp) [1,2], ABCC1-MRP1 [3], and ABCG2-MXR [4] are particularly well known for their role in resistance to several chemotherapeutic agents. Because the members of the ABC transporters are grouped by sequence homology, the remained members may play roles in absorption, distribution, and excretion of chemotherapeutic agent and probably be related to drug resistance although little has been known about most of the functions of these genes. Characterization of the expression of the genes related to chemoresistance is an interesting subject and may lead to clinically useful predictors of response to chemotherapy. The profiling of ABC transporter genes in relation to the clinical response to chemotherapy may also be useful to determine the patient's underlying risk and choose the optimal therapeutic regimen to which the individual cancer patient is most likely to respond.

Focusing on the ABC transporters, we analyzed the gene expression profile in breast cancer patients using microarray data that contain the transcripts of all the

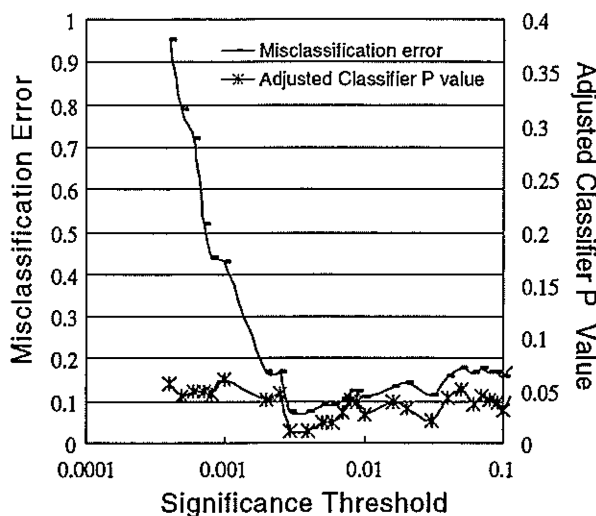

Figure 2. Multivariate predictive classification models in leave-one-out cross-validation and permutation test with an increasing significance threshold at which genes were selected as a classifier. The x-axis represents the significance threshold p value used to select the discriminate genes as classifiers. The y-axis shows the average of the misclassification error rate determined by leave-one-out cross-validation and the average classifier p-value, the probability that a similar low error rate could happen by chance calculated after 2000 permutations. Classifier genes selected as differentials between the 2 classes at a significance threshold $p = 0.003$ level showed the highest discriminate value.

members of ABC transporter family. We compared the expression pattern of the ABC transporters between two classes of pretreatment tumor samples divided by the pathologic response to neoadjuvant chemotherapy (RD versus pCR).

On microarray analysis, several ABC transporters showed differential expression between the two groups of tumors. Of interest, several ABC transporters showed increased expression in the pCR group, including CFTR (NM_000492, ABCC7, fold ratio 0.27, $p = 0.007030$), ABCF2 (NM_005692, fold ratio 0.32, $p = 0.015901$) and ABCB3 (M74447, TAP2, fold ratio 0.54, $p = 0.019345$). ABCB3 is known to be involved in antigen presenting by transporting peptides necessary for the assembly of major histocompatibility complex (MHC) class I molecules from the cytoplasm to the endoplasmic reticulum [18]. It is also known that its reduced expression is associated with HLA class I deficient human tumor cell lines [19] and it has been suggested that it is related to the aggressive features of some kinds of tumors [20–22]. Its increased expression has been found to be associated with pathological complete response in our clinical samples, but any clinical significance in the treatment of in breast cancer remains to be elucidated.

Five ABC transporters ABCC5 (AF146074, fold ratio 2.48, $p = 0.000368$), ABCA12 (AL080207, fold ratio 7.64,

Table 3. Performance of the multivariate classifier; the sensitivity, specificity, PPV and NPV for the pCR group of each predictor model at a significance threshold of $p = 0.003$

	CCV[a]	1NNC[b]	3NNC[c]	NCC[d]	SVM[e]	LDD[f]	Average[g]
Sensitivity	100	85.7	85.7	85.7	71.4	100	88.1
Specificity	100	91.7	91.7	100	100	91.7	95.9
PPV	100	85.7	85.7	100	100	87.5	93.2
NPV	100	91.7	91.7	92.3	85.7	100	93.6
Misclassification error	0	0.05	0.11	0.11	0.05	0.11	0.072
Percent correctly classified	100	95	89	89	95	89	92.8
Classifier P	5.00E-04	0.014	0.025	0.006	0.023	0.005	0.01225

[a]Compound covariate predictor classifier.
[b]1-Nearest neighbor classifier.
[c]3-Nearest neighbor classifier.
[d]Nearest centroid classifier.
[e]Support vector machine classifier.
[f]Linear diagonal discriminant analysis classifier.
[g]Average value of six multivariate classifier models.

Table 4. ABC transporters selected as best classifiers at a significance threshold of 0.003

Gene symbol	Genbank	t-Value	Parametric p-value*	% CV support	RD[a]	pCR[b]	§Fold difference	Description
ABCC5	AF146074	4.43	0.000368	100	6009.1	2427.5	2.48	ABC, sub-family C (CFTR/MRP), member 5
ABCC5	BE550362	4.32	0.000463	100	3571.5	1234.4	2.89	ABC, sub-family C (CFTR/MRP), member 5
ABCA12	AL080207	4.07	0.000795	100	711.7	93.1	7.64	ABC, sub-family A (ABC1), member 12
ABCA1	AL833227	4.04	0.000859	100	166.8	50.5	3.30	ABC, sub-family A (ABC1), member 1

Table sorted by p value.
*Parametric p-value by random variance t-test.
[a]Geometric mean of intensities in the RD group.
[b]Geometric mean of intensities in the pCR group. §Fold difference of geometric means; RD: pCR.

$p =$ 0.000795), ABCA1 (AL833227, fold ratio 3.30, $p =$ 0.000859), ABCC13 (NM_172025, fold ratio 7.54, $p =$ 0.0194), ABCB6 (NM_005689, fold ratio 2.17, $p =$ 0.0271) and ABCC11 (AF352582, fold ratio 2.71, $p =$ 0.0486) showed significantly increased expression in the RD group associated with a decreased responsiveness to sequential weekly paclitaxel/FEC (5-fluorouracil, epirubicin and cyclophosphamide) neoadjuvant chemotherapy. Of these, ABCC5 was selected with the highest significance ($p =$ 0.000368) and the highest expression level (RD: pCR 6009.1: 2427.5) although correlation between the gene expression level and the functional protein level remains to be seen. The ABCC5 (MRP5) transporter on human chromosome 3q27 has been known to be involved in the transport of nucleoside analogs [23] and has been reported to confer resistance to several drugs including methotrexate, GW1843 and ZD1694 (raltitrexed) [24]. Recently, Pratt et al. demonstrated that ABCC5 confers resistance against 5-fluorouracil [17] that was used in our neoadjuvant chemotherapy regimen. These results suggest that ABCC5 mediates transport of several chemotherapeutic agents and may contribute to resistance against 5-fluorouracil which is presently used in neoadjuvant chemotherapy.

In our clinical trial setting, ABCB1, known to confer resistance to several chemotherapeutic agents including paclitaxel, did not significantly increase in tumors with decreased response to neoadjuvant chemotherapy. Samples used in this study were all from chemotherapy-naïve patients and the time of exposure to the drug may not have been sufficient to induce the gene expression of this transporter. Although several ABC transporters showed high expression levels in the pretreatment samples, ABCB1 did not show significantly high expression. ABCB1 may thus play a greater role in resistance to chemotherapy in a secondary chemotherapy clinical setting than in first line chemotherapy when the exposure time is sufficiently long to induce the gene expression of the transporters known to be inducible by exposure to that chemotherapeutic agent [25,26].

But, some ABC transporters may also play significant role in chemoresistance in early breast cancer. Recently, it was reported that ABCC1 expression predict shorter relapse free survival and overall survival and play important role in resistance to chemotherapy in early breast cancer who underwent CMF (cyclophosphamide, methotrexate, and fluorouracil) adjuvant chemotherapy [27].

A variety of compounds are transported by ABC transporters through the lipid bilayer and still little has been known about the function of individual transporters in transport of chemotherapeutic agents. ABCA1 has been implicated in the control of the extrusion of membrane phospholipids and cholesterol toward specific extracellular acceptors [28] and macrophage interleukin-1 beta secretion and apoptosis [29]. ABCC13, highly expressed in the RD group mapped to chromosome 21q11.2 has been suggested that it might be associated with hematopoiesis. It has also been reported that ABCC13 shows decreased expression during cell differentiates [30]. ABCC11, called MRP8 is known to be a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2′,3′-dideoxycytidine and 9′-(2′-phosphonylmethoxyethyl) adenine [31]. Szakacs et al. [10] suggested ABCC11 mediated resistance may not be confined to nucleoside analog, demonstrating that the ABCC11 transfected cell confers resistance to NSC 671136 by 2–3 fold. ABCB6 is a mitochondrial half transporter that is known to be involved in the transport of a precursor of the Fe/S cluster from mitochondria to the cytosol [32]. A recent report showed that several ABC transporters including ABCB6 amplified drug resistance in a non small cell lung cancer cell line (A549/CPT) in comparison with its parental cell [33].

Although the role in chemoresistant of individual transporters selected in our study to discriminate between the pCR and RD groups remains to be revealed, the transporters may also play roles in response to chemotherapy by influencing absorption, distribution, and excretion of chemotherapeutic agents.

To evaluate the predictive signature of ABC transporters, we examined multigene predictor model of response to neoadjuvant chemotherapy using differentially expressed ABC transporters. Six different multivariate classification models were examined. When the ABC transporters differentially expressed between the two classes at a significance threshold level of 0.003 were used for class prediction, an average 92.8% of predictive accuracy was observed, with a 93.2% positive predictive value for the pCR group, 93.6% negative predictive value, sensitivity for the pCR group of 88.1%, and 95.9% specificity. The classifier p-value, the probability that a similar low error rate could happen by chance, was also low ($p =$ 0.012). The optimum classifier model included ABCC5, ABCA1, and ABCA12. These genes all showed high expression in tumors in the RD group.

Of interest, although we developed the class prediction model from a small subset of genes, i.e., genes belonging only to the ABC transporter family, the predictive accuracy reached above 90% with quite a low classifier p-value although these prediction models based on ABC transporter genes need to be validated in future studies by comparing the classification model with all subsets of genes and with larger numbers of samples.

Our result suggest that several ABC transporters in human breast cancer cells may contribute to the clinical response to neoadjuvant chemotherapy and gene expression profiling of these ABC transporters may be useful in prediction of the pathologic response to sequential weekly paclitaxel/FEC in breast cancer patients.

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A Literature Review of Molecular Markers Predictive of Clinical Response to Cytotoxic Chemotherapy in Patients with Lung Cancer

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Background: To find candidate genes for a predictive chemosensitivity test in patients with lung cancer by using a literature review.
Methods: Using MEDLINE searches, "in vitro chemosensitivity associated genes" and articles on association of the gene alteration with clinical chemosensitivity in lung cancer patients were selected. We calculated odds ratios (ORs) and their 95% confidence intervals (95% CIs) of response rates for patients who had tumors with or without gene alteration. Combined ORs and 95% CIs were estimated using the DerSimonian-Laird method.
Results: Of the 80 in vitro chemosensitivity-associated genes identified, 13 genes were evaluated for association with clinical chemosensitivity in 27 studies. The median (range) number of patients in each study was 50 (range, 28-108). The response rates of lung cancer with high and low P-glycoprotein expression were 0% and 73% to 85%, respectively ($p < 0.001$). Glutathione S-transferase pi expression (OR 0.22, 95% CI 0.06-0.79), excision repair cross-complementing 1 alterations (combined OR 0.53, 95% CI 0.28-1.01; $p = 0.055$), and tumor suppressor p53 mutation (combined OR 0.25, 95% CI 0.12-0.52) were associated with clinical chemosensitivity.
Conclusion: In total, 80 in vitro chemosensitivity-associated genes were identified in the literature, and high and low P-glycoprotein, glutathione S-transferase pi expression, excision repair cross-complementing 1 alterations, and tumor suppressor p53 mutation were candidates for future clinical trials of chemosensitivity tests in lung cancer patients.

Key Words: chemotherapy, drug response, molecular markers, prediction, lung cancer

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Lung cancer is the leading cause of death in many countries despite extensive basic research and clinical trials. Approximately 80% of patients with lung cancer have developed distant metastases either by the time of initial diagnosis or during recurrence after surgery for local disease. Systemic chemotherapy against lung cancer, however, has limitations in efficacy such that patients with distant metastases rarely live long.[1]

Tumor response to chemotherapy varies among patients, and objective tumor response rates to standard chemotherapy regimens are approximately 20 to 40% in patients with non—small-cell lung cancer and 60 to 90% in patients with small-cell lung cancer. Thus, it would be extremely useful to know in advance whether patients have tumors that respond to chemotherapy agents and whether the tumors would be resistant to such therapy. For this purpose, cell culture-based chemosensitivity tests have been investigated for more than 20 years, but they are not widely accepted because of technical problems such as the large amount of material required, a low success rate for the primary culture, length of time required, and poor correlation with the clinical response.[2–5]

To overcome these obstacles, DNA-, RNA-, and protein-based chemosensitivity tests have been created, but gene alterations that are predictive of the clinical drug response are not established. Recently, as many as 400 genes whose expression was associated with drug response were identified by cDNA microarray studies, but their functions do not seem to be related to drug sensitivity or resistance.[6–10] In addition, the genes identified by microarray studies were highly unstable and depended on the selection of patients used for gene identification.[11,12] The purpose of this study was to provide an overview of gene alterations in lung cancer that are associated with chemotherapy drug response to identify candidate genes for predictive chemosensitivity tests in patients with lung cancer.

MATERIALS AND METHODS

Because one set of genes associated with chemosensitivity is those directly involved in drug resistance mechanisms, we conducted a MEDLINE search for articles on tumor drug resistance published in the years 2001–2003. This search yielded 112 studies, including several review articles. By searching manually through these articles, we identified 134 genes or gene families that may be involved in drug resistance based on their function. We conducted the second MEDLINE searches for papers of in vitro studies on the 134 genes or gene families by using their names as a keyword.

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From the 134 genes, we selected genes that met the following definition of "in vitro chemosensitivity associated genes": 1) alteration of the gene was identified in a human drug-induced resistant, solid tumor cell line; 2) transfection of the gene induced drug resistance; or 3) down-regulation of the gene or its encode protein increased drug sensitivity. In this latter category, we included studies in which the gene expression or function was suppressed by antisense RNA, hammerhead ribozyme, or an antibody against the gene product. We excluded studies in which drugs were used to inhibit function because the specificity of the drug against the target may not have been complete. We performed a third MEDLINE search for articles on the association between the gene alteration and chemosensitivity of lung cancer cell lines by using the name of the gene as a keyword. Articles in which the association was evaluated in 20 or more cell lines were included in this study. Finally, we searched MEDLINE for studies on the association between the gene alteration and clinical drug response in patients with lung cancer by using the name of the gene as a keyword. Articles in which the association was evaluated in 25 or more patients with advanced lung cancer were included in this study. Studies in which gene expression was evaluated with microarray were excluded because result analysis and interpretation of this technique have not been established, as indicated by the fact that the list of genes identified by microarray studies was highly variable without overlap between these gene sets.[11,12] Clinical studies on concurrent chemoradiotherapy were excluded. We constructed 2 × 2 tables from the response data and calculated odds ratios (ORs), their variances, and 95% confidence intervals (95% CIs) for the patients who had tumors with gene alteration relative to those who had tumors without gene alteration. Combined ORs and 95% CIs were estimated using the DerSimonian-Laird method.[13] When a response rate was 0, association with gene alteration was evaluated using the χ^2 test because 95% CIs for ORs cannot be calculated. The name of each gene was standardized according to Human Gene Nomenclature Database of National Center for Biotechnology Information.

RESULTS

Of the 134 genes or gene families found, a gene alteration in drug-induced resistant cells, an increased or decreased resistance in transfected cells, and an altered sensitivity in gene down-regulated cells were reported for 45, 57, and 32 genes, respectively. In total, 80 genes met the definition of "in vitro chemosensitivity associated gene" (Table 1).

Gene alteration was associated with in vitro chemosensitivity in 15 (50%) of 30 studies on 15 (56%) of 27 gene alterations (Table 2). Clinical drug response was evaluated in 27 studies on 13 gene alterations. The methods used to identify gene alteration included immunohistochemical protein expression analysis (n = 18), polymerase chain reaction (PCR)-based mRNA expression analysis (n = 3), and PCR-based mutation analysis (n = 6). All but one clinical study was retrospective, and the median (range) number of patients in each study was 50 (28-108). Gene alteration was associated with clinical response in 8 of the 27 (30%) studies (Table 2).

TABLE 1. In Vitro Chemosensitivity-Associated Genes

Transporters: ABCA2, ABCB1, ABCB11, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCG2, MVP, ATP7A, ATP7B, SLC29A1, SLC28A1, SLC19A1

Drug targets: TUBB, TUBB4, TUBA, TYMS, TOP1, TOP2A, TOP2B, DHFR,

Target-associated proteins : MAP4, MAP7, STMN1, KIF5B, HSPA5, PSMD14, FPGS

Intracellular detoxifiers: GSTP1, GPX, GCLC, GGT2, MT, RRM2, AKR1B1

DNA damage recognition and repair proteins: HMGB1, HMGB2, ERCC1, XPA, XPD, MSH2, MLH1, PMS2, APEX1, MGMT, BRCA1, GLO1

Cell cycle regulators: RB1, GML, CDKN1A, CCND1, CDKN2A, CDKN1B

Mitogenic signal regulators: ERBB2, EGFR, KRAS2, HRAS, RAF1

Survival signal regulators: AKT1, AKT2

Integrin: ITGB1

Transcription factors: JUN, FOS, MYC, NFKB1

Apoptosis regulators: TP53, MDM2, TP73, BCL2, BCL2L1, MCL1, BAX, BIRC4, BIRC5, TNFRSF6, CASP3, CASP8, HSPB1

We evaluated the association between transporter P-glycoprotein/multidrug resistance 1 (ABCB1) expression and clinical chemosensitivity in four studies. The response rate of lung cancer with high ABCB1 expression was consistently 0%, whereas that for lung cancer with low ABCB1 expression was 73 to 85% (Table 3). Among drug targets, only topoisomerase II-beta (TOP2B) expression was associated with clinical drug response in patients with small-cell lung cancer (OR 0.29, 95% CI 0.09-0.95). The intracellular detoxifier glutathione s-transferase pi (GSTP1) was associated with both in vitro and clinical drug response (OR 0.22, 95% CI 0.06-0.79) (Table 4). DNA repair gene excision repair cross-complementing 1 (ERCC1) alterations were associated with drug response among patients with non—small-cell lung cancer with marginal statistical significance; the combined OR (95% CI) for ERCC1 alteration was 0.53 (0.28-1.01; p = 0.055) (Table 5). Tumor suppressor p53 (TP53) mutation was the only alteration associated with drug response among patients with non—small-cell lung cancer among genes involved in cell cycle and apoptosis. A combined OR (95% CI) for TP53 among patients with non—small-cell lung cancer was 0.25 (0.12-0.52) (Table 6). B-cell CLL/lymphoma 2 (BCL2) and its family protein expression was not associated with clinical drug response (Table 7).

DISCUSSION

We identified 80 in vitro chemosensitivity-associated genes in our literature search. Of these, 13 were evaluated clinically in 27 studies; ABCB1, TOP2B, GSTP1, and ERCC1 expression and TP53 mutation were associated with changes to drug responses among patients with lung cancer.

Classical drug resistance is believed to be the result of molecular changes inhibiting the drug-target interaction. ABCB1, an ATP-binding cassette protein, acts as an energy-dependent transmembrane efflux pump and decreases the intracellular accumulation of anticancer drugs, including anthracyclines, vinca alkaloids, taxanes, and epipodophyllotox-

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TABLE 2. Chemosensitivity-Associated Genes and Association with Chemosensitivity

		Association with chemosensitivity					
		In vitro studies (*n*)			Clinical studies (*n*)		
Category	No of Genes	Total	Yes	%	Total	Yes	%
Transporter	15	9	5	55	4	4	100
Drug target	8	2	1	50	5	1	20
Target-associated protein	7	0	0		0	0	
Intracellular detoxifier	7	3	3	100	1	1	100
DNA repair	10	1	1	100	6	0	0
Damage recognition protein	2	0	0		0	0	
Cell cycle	6	4	2	50	2	0	0
Mitogenic signal	5	3	1	33	1	0	0
Survival signal	2	0	0		0	0	
Transcription factor	4	3	0	0	0	0	
Cell adhesion-mediated drug-resistance protein	1	0	0		0	0	
Apoptosis	13	5	2	40	8	2	25
Total	80	30	15	50	27	8	30

TABLE 3. ABCB1 (P-Glycoprotein) and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Expression	Patients (*n*)	RR (%)	Odds ratio
Yeh et al.[30]	Non-small cell	Paclitaxel	IHC	Low	35	80	0
				High	15	0	$p < 0.001$*
Kawasaki et al.[31]	Small cell	CAV or EP	IHC	Low	26	85	0
				High	4	0	$p < 0.001$*
Hsia et al.[32]	Small cell	EP	IHC	Low	37	73	0
				High	13	0	$p < 0.001$*
Savaraj et al.[33]	Small cell	CAV, CEV, or EP	RT-PCR	Low	24	75	0
				High	7	0	$p < 0.001$*
Combined odds ratio for ABCB expression in patients with SCLC: 0							

IHC, Immunohistochemical analysis; RR, response rate; RT-PCR, reverse transcriptase-polymerase chain reaction.
*Calculated using the χ^2 test because the confidence interval cannot be calculated.

ins. Overexpression of this protein gives tumor cells a multidrug resistance phenotype in vitro, which is thought to be associated with clinical chemoresistance.[14] Our review showed that the response rate of tumors with ABCB1 overexpression was 0 in all studies of lung cancer, whereas that for lung cancer tumors with low ABCB1 expression was 73 to 85% (Table 3).

There is a close relationship between drug sensitivity and quantitative and qualitative alterations of the drug's target, including tamoxifen sensitivity and estrogen receptor expression and trastuzumab response and Her-2/neu overexpression in breast cancer,[15] imatinib resistance and BCR-ABL gene amplification and mutations in Philadelphia chromosome-positive leukemias,[16] and imatinib response and KIT gene mutations in gastrointestinal stromal tumors.[17] In all of these cases, the target molecule is a receptor or a mutated tyrosine kinase located at the entry of growth-stimulating signal transduction pathways. Recently, gefitinib, a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR), has been developed, and two large phase II trials showed a response rate of 18% and 12% in patients with non—small-cell lung cancer who were previously treated with conventional chemotherapy.[18,19] Responses to the drug have been unpredictable, but mutations of the EGFR gene were identified in patients with gefitinib-responsive lung cancer.[20,21] Furthermore, all mutations in these tumors were restricted to the activation loop of the kinase domain of EGFR, which are in distinct contrast to mutations in extracelluar and regulatory domains of EGFR in glioblastoma, which are unresponsive to gefitinib.[22] Thus, molecular developments of structure and function of the targets hold the promise of targeted cancer therapy. The target molecules of many anticancer cytotoxic agents have not been clearly defined; therefore, the relationship between the target molecule status and sensitivity to the agent has not been established. TOP2B expression was associated with drug response in patients with small-cell lung cancer, with a response rate of 71% for high TOP2B expression tumors versus 90% for low TOP2B expression tumors (OR 0.29, 95% CI 0.09-0.95).[23] This result, however, is in contrast with the idea that a higher

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TABLE 4. Drug Targets, Intracellular Detoxifier, and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Expression	Patients (n)	RR (%)	Odds ratio (95% CI)
Beta-tubulin class III							
Rosell et al.[34]	Non-small cell	Paclitaxel,	Real-time	Low	13	46	0.39
		Vinorelbine	PCR	High	24	25	(0.09-1.62)
Topoisomerase II-alpha							
Dingemans et al.[23]	Small cell	CEV or EP	IHC	Low	65	85	0.65
				High	23	80	(0.20-2.17)
Dingemans et al.[35]	Non-small cell	Platinum-based	IHC	Low	30	47	0.67
				High	8	38	(0.14-3.40)
Topoisomerase II-beta							
Dingemans et al.[23]	Small cell	CEV or EP	IHC	Low	48	90	0.29
				High	35	71	(0.09-0.95)
Dingemans et al.[35]	Non-small cell	Platinum-based	IHC	Low	18	50	0.86
				High	13	46	(0.21-3.58)
Glutathione s-transferase pi							
Nakanishi et al.[36]	Non-small cell	Cisplatin-based	IHC	Low	17	47	0.22
				High	37	16	(0.06-0.79)

CI, confidence interval; IHC, immunohistochemical analysis; PCR, polymerase chain reaction; RR, response rate; CEV, cyclophosphamide, etoposide, and vincristine; EP, etoposide and cisplatin.

TABLE 5. DNA Repair Genes and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Alteration	Patients (n)	RR (%)	Odd ratio (95% CI)
Excision repair cross-complementing 1 expression							
Lord et al. [37]	Non-small cell	Cisplatin,	Real-time	Low	23	52	0.38
		gemcitabine	PCR	High	24	36	(0.11-1.26)
Excision repair cross-complementing 1 (ERCC1) polymorphism at codon 118							
Ryu et al.[38]	Non-small cell	Cisplatin-based	PCR	C/C	54	54	0.61
			Hybridization	C/T or T/T	53	42	(0.28-1.31)
Combined odds ratio (95% C.I.) for ERCC1 alteration in patients with NSCLC0.53 (0.28-1.01, $p = 0.055$)							
Xeroderma pigmentosum group D polymorphism							
At codon 231							
Ryu et al.[38]	Non-small cell	Cisplatin-based	PCR	G/G	100	48	1.08
			Hybridization	G/A or A/A	8	50	(0.26-4.57)
At codon 312							
Camps et al.[39]	Non-small cell	Cisplatin,	PCR	G/G	18	17	3.33
		gemcitabine	Sequencing	G/A or A/A	15	40	(0.66-16.7)
At codon 751							
Camps et al.[39]	Non-small cell	Cisplatin,	PCR	A/A	22	23	2.04
		gemcitabine	Sequencing	A/C or C/C	16	38	(0.49-8.45)
Ryu et al.[38]	Non-small cell	Cisplatin-based	PCR	A/A	96	49	0.74
			Hybridization	A/C	12	42	(0.22-2.51)
Combined odds ratio (95% CI) for XPD polymorphism in patients with NSCLC: 1.38 (0.68-2.78).							

CI, confidence interval; PCR, polymerase chain reaction; RR, response rate; NSCLC, non—small-cell lung cancer; XPD, xeroderma pigmentosum group D.

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TABLE 6. Cell Cycle Regulators, Mitogenic Signals, Tumor Protein p53, and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Alteration	Patients (n)	RR (%)	Odds ratio (95% CI)
Retinoblastoma 1 expression							
Gregorc et al.[40]	Non-small cell	Cisplatin-based	IHC	Low	61	51	0.45
				High	41	32	(0.20-1.03)
Cyclin-dependent kinase inhibitor 1A, p21 expression							
Dingemans et al.[23]	Small cell	CEV, EP	IHC	Low	63	90	0.57
				High	22	71	(0.17-1.92)
Kirsten rat sarcoma 2 viral oncogene homolog mutation							
Rodenhuis et al. [41, a]	Aenocarcinoma	Ifosfamide, carboplatin	PCR-MSH	Normal	46	26	0.65
				Mutated	16	19	(0.16-2.70)
Tumor protein p53 (P53) mutation							
Nakanishi et al.[36]	Non-small cell	Cisplatin-based	IHC	Normal	11	45	0.19
				Mutated	29	15	(0.04-0.94)
Gregorc et al.[40]	Non-small cell	Cisplatin-based	IHC	Normal	56	57	0.26
				Mutated	46	26	(0.11-0.62)
Combined odds ratio (95% CI) for P53 mutation in patients with NSCLC: 0.25 (0.12-0.52)							
Kawasaki et al.[31]	Small cell	CAV or EP	IHC	Normal	10	70	1.3
				Mutated	20	75	(0.24-6.96)
Dingemans et al.[23]	Small cell	CEV or EP	IHC	Normal	47	85	0.81
				Mutated	45	82	(0.27-2.45)
Combined odds ratio (95% C.I.) for P53 mutation in patients with SCLC: 0.93 (0.37-2.35).							

CI, confidence interval; IHC, immunohistochemical analysis; PCR-MSH, polymerase chain reaction-mutation specific hybridization; RR, response rate; CEV, cyclophosphamide, etoposide, and vincristine; EP, etoposide and cisplatin.
[a]Prospective study.

TABLE 7. B-Cell CLL/Lymphoma 2 (BCL2) Family Expression and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Expression	Patients (n)	RR (%)	Odds ratio (95% CI)
BCL2							
Krug et al.[42]	Non-small cell	Docetaxel, vinorelbine	IHC	Low	26	46	1.75
				High	5	60	(0.25-12.3)
Dingemans et al.[23]	Small cell	CEV or EP	IHC	Low	20	79	1.36
				High	71	85	(0.38-4.86)
Takayama et al.[43]	Small cell	CAV or EP	IHC	Low	17	76	0.50
				High	21	62	(0.12-2.08)
Combined odds ratio (95% CI) for BCL2 expression in patients with SCLC: 0.87 (0.33-2.32)							
BAX (BCL2-associated X protein)							
Krug et al.[42]	Non-small cell	Docetaxel, vinorelbine	IHC	Low	9	56	0.72
				High	19	47	(0.15-3.54)

CI, confidence interval; IHC, immunohistochemical analysis; RR, response rate; CEV, cyclophosphamide, etoposide, and vincristine; EP, etoposide and cisplatin.

expression of topoisomerase II enzymes correlates with greater chemosensitivity in patients with breast cancer.[24]

In addition to genes involved in classical drug resistance, genes that act downstream of the initial damage induced by a drug-target complex are thought to play an important role in chemosensitivity.[25] ERCC1 is a key enzyme in nucleotide excision repair, one of the key pathways by which cells repair platinum-induced DNA damage. High levels of ERCC1 mRNA have been associated with platinum resistance in the treatment of ovarian and gastric cancer.[26,27] The codon 118 in exon 4 of ERCC1 gene is polymorphic with the nucleotide alteration AAC to AAT. Although this base change results in coding for the same amino acid, it may affect gene expression based on the usage frequency of synonymous codons.[28] The associations between drug response and both ERCC1 gene expression and polymorphism at codon 118 in patients with non—small-cell lung cancer have been reported in the literature. A combined OR (95%

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CI) for these ERCC1 alterations was 0.53 (0.28-1.01, p = 0.055), although each study failed to show statistical significant association. Thus, ERCC1 may be a candidate for evaluation of the predictability of drug response in future clinical trials.

TP53, which is mutated or deleted in more than half of lung cancer cells, has a remarkable number of biological activities, including cell-cycle checkpoints, DNA repair, apoptosis, senescence, and maintenance of genomic integrity. Because most anticancer cytotoxic agents induce apoptosis through either DNA damage or microtubule disruption, mutated TP53 may decrease chemosensitivity by inhibiting apoptosis or, in contrast, may increase chemosensitivity by impairing DNA repair after drug-induced DNA damage.[29] This review showed that mutated TP53 was associated with poor drug response in patients with non—small-cell lung cancer (Table 6).

No other genes located downstream (including xeroderma pigmentosum group D, retinoblastoma 1, cyclin-dependent kinase inhibitor 1A, Kirsten rat sarcoma 2 viral oncogene homolog, B-cell CLL/lymphoma 2, and B-cell CLL/lymphoma 2-associated X protein) were associated with clinical drug response (Tables 5-7). The association was evaluated for only 8 of 43 *in vitro* chemosensitivity-associated downstream genes; therefore, key genes may be among the remaining 35 genes. Most clinical studies included a limited number of patients with various background characteristics such as tumor stage and chemotherapy regimen administered, which resulted in low statistical power to identify the association. Finally, because all but one study was retrospective, the quality of tumor samples may vary, and it is therefore unclear whether the gene alteration was detected in all samples. Thus, in future prospective clinical studies, the method of tumor sample collection and preservation, as well as immunohistochemistry and polymerase chain reaction-based methods, should be standardized, and the sample size of patients should be determined with statistical consideration.

The recently developed microarray technique enables investigators analyze mRNA expression of more than 20,000 genes at once, and as many as 100 to 400 genes were selected statistically as chemosensitivity-related genes.[6–8,10] Among them, however, only a limited number of genes were functionally related to chemosensitivity, and only ABCB1 and BAX corresponded with the 80 chemosensitivity-associated genes identified in this literature review, which were picked because of their known function and contribution to in vitro chemosensitivity. Thus, it will be interesting to evaluate the role of expression profile of these genes using microarray analysis.

The association between the expression and alterations of genes and clinical drug responses should be studied further in prospective trials. ABCB1, GSTP1, ERCC1, and TP53, and other genes identified by exploratory microarray analyses should be evaluated in those trials. Simple methods to identify gene alterations, such as immunohistochemistry and polymerase chain reaction-based techniques, will be feasible in future clinical trials because of their simplicity, cost, and time. The median number of patients in retrospective studies analyzed in this review was 50 (range, 28-108). In future prospective trials, sample size consideration for statistical power will also be important.

In conclusion, we identified 80 in vitro chemosensitivity-associated genes in a review of the literature; ABCB1, GSTP1, and ERCC1 expression and TP53 mutation were associated with drug responses among patients with lung cancer.

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