Table 2. Genes that show differential expression levels in 44As3 cells as compared with those in HSC-44PE cells

Ratio	Symbol	Gene name	Function and description
Upregulated gene			
29.63	*MMP1*	Matrix metalloproteinase 1	Proteolysis and peptidolysis, collagenase
11.25	*LOC57402*		Cell signaling
9.68	*I_1109564*	H19	Unknown function
4.92	*LGALS1*	Lectin, galactoside-binding, galectin 1	Apoptosis, cell adhesion
4.84	*I_930461*	Tropomyosin 2 (beta)	Contractile proteins
4.47	*AGR2*	Anterior gradient 2 homolog (Xenepus laevis)	Oncogenesis
4.40	*BOK*	BCL2-related ovarian killer	Induction of apoptosis
4.03	*NAP1L4*	Nucleosome assembly protein 1-like 4	Nucleosome assembly
3.99	*TNC*	Tenascin C (hexabrachion)	Binding, cell adhesion
3.98	*HSPB1*	Heat shock 27 kDa protein 1	Regulation of translational initiation
3.95	*HGD*	Homogentisate 1,2-dioxygenase	Tyrosine catabolism, phenylalanine catabolism
3.83	*PIGPC1*		Plasma membrane protein actvated by p53, cell death
3.52	*PIASY*		Apoptosis
3.48	*SYP*	Synaptophysin	Regulating neurotransmitter release
3.38	*RPS12*		Ribosomal protein S12
3.36	*CTSL*	Cathepsin L	Associated with highly invasive tumors
3.35	*HSPC018*		Unknown function
3.23	*INSIG1*	Insulin induced gene 1	Metabolism, cell proliferation
3.20	*I_962761*	Insulin induced gene 1	Metabolism, cell proliferation
3.17	*I_963048*		Immunity
Downregulated gene			
0.14	*THBS1*	Thrombospondin 1	Endopeptidase inhibitor, signal transducer, cell adhesion
0.34	*PRG1*	Proteoglycan 1, secretory granule	Proteoglycan
0.41	*CUL4B*	Cullin 4B	Cell cycle
0.41	*MCM3*	MCM3	Adenosinetriphosphatase, DNA binding
0.42	*NFE2L3*	Nuclear factor (erythroid-derived 2)-like 3	Transcription coactivator, transcription factor
0.43	*THBS4*	Thrombospondin 4	Heparin binding, calcium ion binding, cell adhesion
0.45	*CD59*	CD59 antigen p18–20	Lymphocyte antigen, defense response, signal transduction
0.46	*VBP1*	von Hippel-Lindau binding protein 1	Protein binding
0.46	*LOC51659*		Unkown function
0.47	*PODXL*		Lymphocyte adhesin and homing
0.47	*H2BFB*		H2B histon family member B
0.47	*ZNF195*	Zinc finger protein 195	
0.47	*MCT-1*		Cyclin, cell cycle
0.48	*CD44*	CD44 antigen	Transmembrane glycoprotein, extracellular matrix attachment
0.48	*NDUFA1*	NADH dehydrogenase (ubiquinone)	Energy pathways

kidney also. Although only rarely, pleural effusion and ovarian micrometastases were also noted (data not shown). Orthotopic injection of the parent cell line (HSC-58), however, resulted in the mice becoming moribund approximately 68 days after the implantation (Table 1, Fig. 2E). When the dead animals were autopsied, mild peritoneal dissemination was noted, but ascites were observed in only a very small number of animals (Table 1).

Comparison of gene expression between the highly metastatic- and the parent cell lines. The parent cell lines with a low potential for peritoneal dissemination were compared with the highly metastatic cell lines, using a cDNA microarray (approximately 30 000 genes; Agilent). The differences in the gene expression levels between the two types of cell lines were assessed by measuring the ratios of their expression. The ratio was rated as significant when it was over 2:1. The first 15 genes ranked in terms of this ratio are shown in Tables 2 and 3. When the highly metastatic cell line 44As3 was compared with its parent cell line HSC-44PE, the expression of 89 genes, such as that of MMP1 and cathepsin L, was more intense and that of 19 genes; for example, thrombospondin 1 was less intense in the 44As3 cells in comparison to the parent cell line (Table 2). Table 2 shows the results of a similar comparison of 58As1 and 58As9 cells (highly metastatic cell lines showing a marked increase of proliferative potential) with the parent cell line, HSC-58. Compared to that in the parent cell line, 58As1 cells showed more intense expression of 40 and less intense expression of 20 of the genes examined, while 58As9 cells showed more intense expression of 36 and less intense expression of 32 of the genes examined. In addition to the MMP1 and cathepsin L genes, genes encoding molecules associated with cell adhesion, motility, proliferation, apoptosis, metabolic enzymes and so on, also showed altered expression.

Then, the expression levels of MMP1 and cathepsin L were confirmed at the protein level and compared with the expression levels of known metastasis-associated genes (Table 4). Weak MMP1 protein expression was seen in 44As3 cells as well as 58As9 cells. The cathepsin L gene was expressed in HSC-44PE cells, but even stronger expression was observed in the 44As3 cells (Fig. 3A,B). Intense expression of this gene was also seen in the metastatic cell line, 58As1 (Fig. 3C). Moderate expression of the cathepsin L gene was observed in 58As9 cells, whereas expression of this gene was totally absent in the parent cell line (Fig. 3D). Molecules whose expression levels differed markedly between the parent cell line and the 58As1 or 58As9 cells were dysadherin, CD44, integrin β4, EGFR (Fig. 3E,F), HGF, and VEGF (Fig. 3G,H). While dysadherin was not expressed in the HSC-58 cells, it was expressed intensely in all the highly metastatic subclones (Fig. 3I,J). Intense expression of nm23

Table 3. Genes that show differential expression levels in 58As1 and 58As9 compared with that in HSC-58 cells

Ratio	Symbol	Gene name	Function and description
Upregulated genes 58As1			
19.41	*ADH1C*	Alcohol dehydrogenase 1C, gamma polypeptide	Zinc ion binding, electron transporter, metabolism
18.59	*ADH1B*	Alcohol dehydrogenase IB, beta polypeptide	Zinc ion binding, electron transporter, metabolism
17.70	*FABP1*	Fatty acid binding protein 1, liver	Lipid transporter, fatty acid metabolism, cell signaling
15.21	*ADH1A*	Alcohol dehydrogenase 1A, alpha polypeptide	Zinc ion binding, electron transporter, metabolism
14.15	*PLAT*	Plasminogen activator, tissue	Proteolysis and peptidolysis, blood coagulation
11.01	*MTP*	Microsomal triglyceride transfer protein subunit precursor	Lipid metabolism, Small molecule-binding protein
10.47	*AKR1C2*	Aldo-keto reductase family 1, member C2	Bile acid electron transporter, metabolism
4.71	*CYP1B1*	Cytochrome P450, family 1, subfamily B, polypeptide 1	Cytochrome P450, electron transporter, morphogenesis
4.67	*AKR1C3*	Aldo-keto reductase family 1, member C3	Electron transporter, metabolism, cell proliferation
4.04	*PON2*	Paraoxonase 2	Arylesterase
4.01	*RDHL*		NADP-dependent retinol dehydrogenase/reductase
3.73	*SERPINE2*	Serine proteinase inhibitor, clade E, member 2	Serpin, development
3.60	*PPP1R14A*	Protein phosphatase 1, regulatory subunit 14A	
3.54	*TGFBI*	Transforming growth factor, beta-induced, 68 kDa	Integrin binding, tumor suppressor, cell adhesion
3.45	*PROCR*	Protein C receptor, endothelial (EPCR)	Receptor, inflammatory response
Upregulated genes 58As9			
7.03	*AKR1C2*	Aldo-keto reductase family 1, member C2	Bile acid transporter, binding, electron transporter
6.08	*I_1109564*	H19, imprinted maternally expressed untranslated mRNA	Unknown function
5.95	*MKNK2*	MAP kinase-interacting serine/threonine kinase 2	Phosphorylation, signal transduction
4.10	*APOC1*	Apolipoprotein C-I	Lipid metabolism
4.07	*FLJ21841*		Unknown function
4.02	*SERPINE2*	Serine proteinase inhibitor, clade E, member 2	Serpin, development
3.77	*CTSL*	Cathepsin L	Cathepsin L, associated with highly invasive tumors
3.28	*AKR1C3*	Aldo-keto reductase family 1, member C3	Electron transporter, metabolism, cell proliferation
3.11	*SIAT8B*	Sialyltransferase 8B (alpha-2, 8-sialyltransferase)	Metabolism, embryogenesis and morphogenesis
3.01	*FKBP1B*	FK506 binding protein 1B	Popeptidylprolyl isomerase
2.82	*KIAA1247*		Member of the sulftase family
2.80	*I_1000731*	GRB2-associated binding protein 2	Protein-protein and protein–lipid interactions
2.77	*STMN3*	Stathmin-like 3	Neurogenesis, SCG10 like-protein, tumor progression
2.75	*ANK3*	Ankyrin 3, node of Ranvier (ankyrin G)	Cytoskeletal anchoring, vesicle transport
2.74	*CEBPE*	CCAAT/enhancer binding protein (C/EBP), epsilon	Transcription activating factor, defense response
Downregulation 58As1			
0.11	*LAMR1*	Laminin receptor 1	Signal transduction, cell adhesion, invasive growth
0.13	*S100A4*	S100 calcium binding protein A4	Calcium ion binding, invasive growth
0.15	*RARRES1*	Retinoic acid receptor responder	Negative regulation of cell proliferation
0.15	*HLA-DQB1*	HLA complex, class II, DQ beta 1 precursor	Immune response
0.15	*KLK6*	Kallikrein 6 (neurosin, zyme)	Serine-type peptidase, pathogenesis
0.16	*TM4SF4*	Transmembrane 4 superfamily member 4	Negative regulation of cell proliferation, glycosylation
0.16	*HLA-DRA*	Major histocompatibility complex, class II, DR alpha	Immune response
0.17	*CTNNB1*	Catenin (cadherin-associated pritein), beta 1, 88 kDa	Tumor suppressor, cell adhesion, transcription
0.19	*HLA-DRB3*	Major histocompatibility complex, class II, DR beta 3	Immune response
0.20	*SAT*	Spermidine/spermine N1-acetyltransferase	Diamine N-acetyltransferase, modulates tumorigenicity
0.20	*HLA-DRB5*	Major histocompatibility complex, class II, DR beta 5	Immune response
0.20	*I_966873*		Strong similarity to human HLA-DRB1
0.21	*FOS*	v-fos FBJ murine osteosarcoma viral oncogene homolog	Transcription, methylation, cell growth, oncogenesis
0.21	*I_965396*		Unknown, high similarity to charcterized human AG2
0.22	*CEACAM6*	Carcinoembryonic antigen-related cell adhesion molecule 6	Signal transduction, cell-cell signaling
Downregulation 58As9			
0.06	*CEACAM8*	Carcinoembryonic antigen-related cell adhesion molecule 8	Tumor antigen, immune response, cell adhesion
0.07	*TM4SF3*	Transmembrane 4 superfamily member 3	Signal transducer, tumor antigen, pathogenesis
0.07	*LAMR1*	Laminin receptor 1 (ribosomal protein SA, 67 kDa)	Signal transduction, cell adhesion, invasive growth
0.09	*CEACAM6*	Carcinoembryonic antigen-related cell adhesion molecule 6	Signal transduction, cell-cell signaling, cell adhesion
0.12	*CTNNB1*	Catenin (cadherin-associated pritein), beta 1, 88 kDa	Tumor suppressor, cell adhesion, oncogenesis
0.12	*KRT19*	Keratin 19	Structural constituent of cytoskeleton, differentiation
0.13	*KRTHA3A*	Keratin, hair, acidic, 3 A	Cell shape and cell size control
0.13	*CEACAM3*	Carcinoembryonic antigen-related cell adhesion molecule 3	Tumor antigen, immune response, cell adhesion
0.14	*I_966690*		Strong similarity to human HLA-DRB4
0.17	*S100A4*	S100 calcium binding protein A4	Calcium ion binding, invasive growth
0.20	*FOS*	v-fos FBJ murine osteosarcoma viral oncogene homolog	Transcription, methylation, cell growth, oncogenesis
0.22	*DAF*	Decay accelerating factor for complement (CD55)	Decay accelerating factor
0.23	*MYC*	v-myc myelocytomatosis viral oncogene homolog (avian)	Transcription factor, cell cycle, pathogenesis
0.24	*CRIP1*	Cysteine-rich protein 1 (intestinal)	Zinc ion binding, cell proliferation
0.24	*KRTHB6*	Keratin, hair, basic, 6	Monilethrix

 doi: 10.1111/j.1349-7006.2005.00054.x

Table 4. Expression of metastasis-related genes in the highly metastatic and the parent gastric cancer cell lines

Cell line	MMP-1	Cathepsin L	Cell adhesion					Oncogenes					Angiogenesis				nm23	Smad4
			CD44	E-cadherin	Dysadherin	β-catenin	Integrin α6β4	EGFR	c-erb-B-2	cript	c-met	HGF	bFGF	VEGF	IL-6	IL-8		
44As3	+	++	+++a	++	++	++	—	++	−	+	+	−	−	+	−	−	++	−
HSC-44PE	−	+	+++a	++	++	++	−++	++	−	+	+	+	−	+	−	−	−	−
58As1	−	++	++	−	++	++	−++	+	−	+	++a	++	+	++	−	−	++	−
58As9	+	+	++	−	++	+	−+	++	−	+	++a	+	+	++	−	−	+	−
HSC-58	−	−	+	−	−	++	—	−	−	+	++a	−	+	−	−	−	−	−

Immunohistochemical staining was carried out as described in a previous study.[8] ++, Moderate or strong staining intensity, or staining of > 75% of the cells; +, weak staining intensity, or staining of < 25% of the cells; −, negative staining, or staining of < 1% of the cells. a, gene amplification.

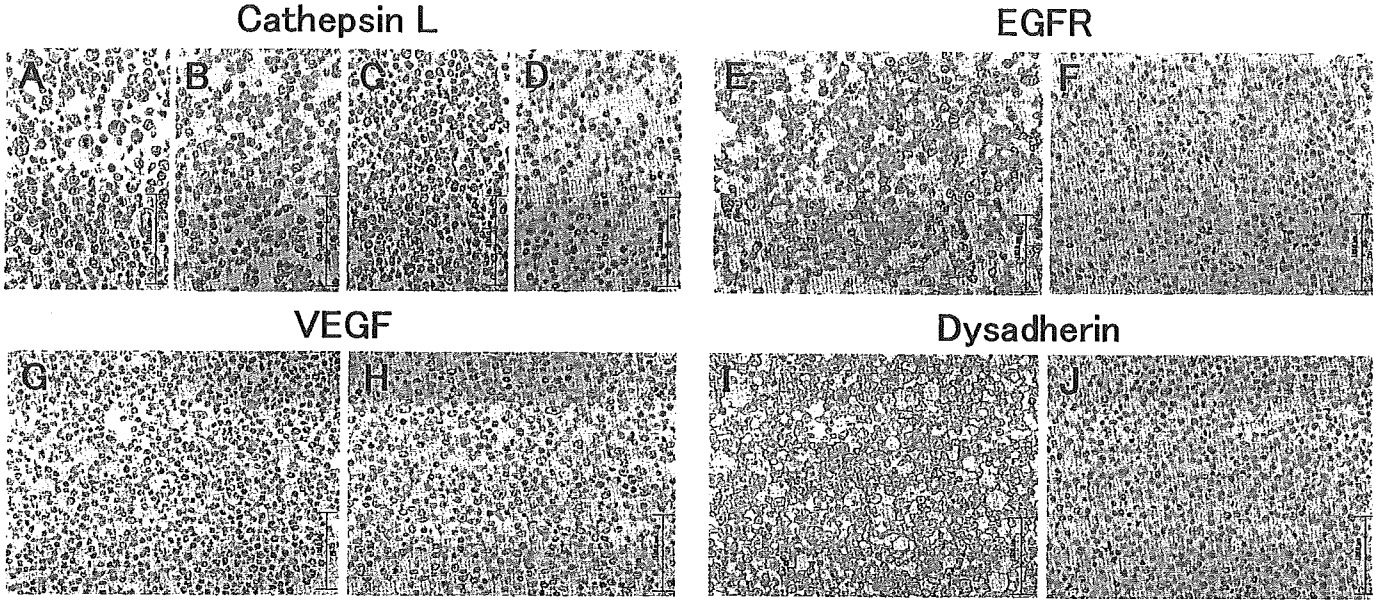

Fig. 3. Immunohistochemical analyses of Cathepsin L, EGFR, VEGF and Dysadherin in the highly metastatic and the parent cell lines. The metastatic 44As3 (A) and 58As1 (C) expressed strongly detectable cathepsin L in the cytoplasm. Immunoreactivity for cathepsin L observed weakly at the cytoplasm in HSC-44PE (B), but immunoreactivity completely absent from HSC-58 (D). (E), Expression of EGER is observed in the membranes of the 58As1 subclone. (F), Immunoreactivity for EGER was completely absent from HSC-58 cells. (G), Expression of VEGF is observed in the cytoplasm of the 58As9 cells, but immunoreactivity absent from HSC-58 (H). (I), Expression of dysadherin is observed at the cell-cell boundaries in 58As9 subclone. (J), Immunoreactivity for dysadherin was completely absent from HSC-58 cells.

was also observed in highly metastatic cell lines, while Smad4 expression was not seen in these cell lines.

Usefulness of the model as a tumor metastasis model for the evaluation of drugs. All of the highly metastatic cell lines served as highly reproducible models of peritoneal dissemination, and a quantitative relationship was observed between the number of inoculated cells and the animal survival rate (incidence of tumor) (data not shown). Next we evaluated antitumor effects of antitumor agents in this model. We selected CPT-11[17] and GEM[18] as representative cytotoxic agents. Figure 4A shows the survival curve of the 58As1 implanted mice treated with CPT-11. Most of the animals belonging to the untreated control group died of extensive peritoneal dissemination approximately 30 days after the implantation. In the CPT-11-treated group (200 mg/kg/head), however, 60 days passed before the first animal death was noted. Thus, treatment with CPT-11 significantly ($P < 0.001$, unpaired t-test) prolonged the survival of the animals injected with the tumor cells, and dose-dependency was evident when the data from multiple groups were compared. Similar results were also obtained for 44As3 cells (Fig. 4B). Figure 4(C) shows the results of the experiment in which GEM was administered intravenously following orthotopic inoculation of 58As1 cells. The survival period was significantly prolonged in the GEM-treated group (100 mg/kg/head). Similar results were also obtained for mice implanted with the 44As3 cells (data not shown).

To identify the stage of tumor metastasis suppressed by these agents, RT-PCR analysis was performed with sets of primers specific for human and mouse β actin, respectively. Cells collected from the intraperitoneal lavage fluid 21 days after orthotopic implantation of 58As1 cells served as the samples. Autopsy examination revealed that there was no macroscopic tumor formation in the gastric wall of the drug-treated animals, while peritoneal dissemination was noted in the untreated control group. Figure 4(D) shows two typical animals used for each experimental group. In the untreated control group, RT-PCR product represents human-derived β actin gene was clearly identified (lanes 3 and 4). In the CPT-11- and GEM-treated groups, however, the gene sequence of human origin was less clear (lanes 5, 6 and 7, 8, respectively). These results suggested that

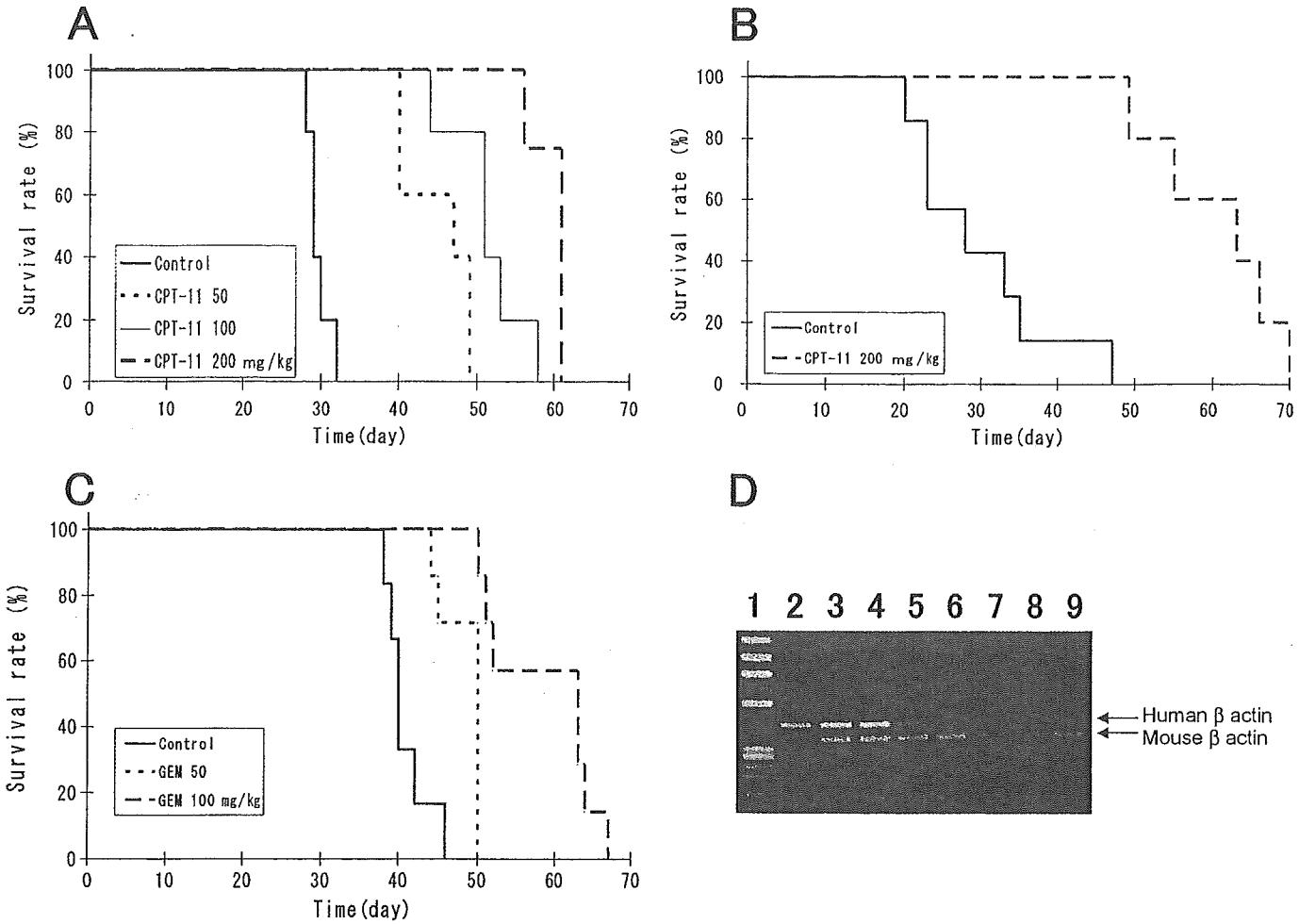

Fig. 4. Effects of CPT-11 and GEM in the peritoneal dissemination mouse model established using orthotopically implanted 44As3 or 58As1 cells. Mice receiving CPT-11 or GEM, or vehicle alone as control, were monitored daily for the development of peritoneal dissemination. (A), Survival of 58As1-tumor-bearing mice after CPT-11 treatment ($n = 5$; $P < 0.001$). This experiment was repeated thrice and similar results were observed each time. (B), Survival of 44As3-tumor-bearing mice after CPT-11 treatment ($n = 6$; $P < 0.001$). (C), Survival of 58As1-tumor-bearing mice after GEM treatment ($n = 7$; $P < 0.001$). Similar results were obtained in two independent experiments. (D), CPT-11 and GEM inhibit dissemination of cancer cells into the peritoneal cavity *in vivo*. RT-PCR was performed on the disseminated cells isolated from the intraperitoneal lavage fluid (2 mL of PBS) using human-specific and mouse-specific primers against β-actin. The total amount of RNA (200 ng) was equalized in all the samples. Lane 1, maker; lane 2, human gastric cell line HSC-39; lanes 3 and 4, untreated control group; lanes 5 and 6, 200 mg/kg CPT-11-treated group; lanes 7 and 8, 100 mg/kg GEM-treated group; lane 9, murine leukemia cell line P388.

treatment with the agents reduced the number of cancer cells in the peritoneal cavity. It was also found that the drugs directly inhibited the growth of s.c. tumors following implantation of 58As1 and 44As3 cells (data not shown). From these results, it is considered highly likely that while the agents suppress metastasis of these tumor cells by suppressing tumor formation at the implanted site, the small number of tumor cells remaining within the peritoneal cavity gradually proliferate, making it difficult to obtain better therapeutic results than some prolongation of the survival period.

Discussion

In the present study, we isolated 44As3 cells from HSC-44PE, and 58As1 and 58As9 cells from HSC-58, and succeeded, in a reliable manner, in establishing a model in which peritoneal dissemination occurred from a primary lesion of gastric carcinoma. The 44As3, 58As1 and 58As9 sublines were generated by the conventional method, that is by selection of highly metastatic clones (found in small numbers among cancer cells showing poor metastatic potential) *in vivo*.[19] On the basis of a study using clinical samples for microarray analysis of primary and metastatic lesions, investigators recently suggested that primary cancers with a high metastatic potential may differ in nature from those having a poor metastatic potential.[20] However, the results of our study support the conventional view that clones with a high metastatic potential contained in small amounts among the cancer tissues are responsible for the formation of metastatic lesions.

We consider that the establishment of this model is significant in the following respects: (i) it allows reproduction of all the steps in the development of cancerous peritonitis, from the stage of infiltrative growth of the tumor within the gastric mucosa to peritoneal dissemination and formation of ascites; (ii) it is an animal model of metastatic gastric cancer that closely resembles that in clinical cases; (iii) quantitative analysis is possible with this model, because it is established from cultured cells. The model is expected to be useful for the study of the continuity or association between infiltrative growth/peritoneal dissemination and gene expression, mechanism of formation of bloody ascites, and analysis of the microenvironmental factors influencing the development of the metastases. Comparison of the expression

 doi: 10.1111/j.1349-7006.2005.00054.x

levels of the relevant genes among different cell lines with markedly varying metastatic potential may be expected to allow isolation of new molecules involved in the peritoneal dissemination of tumors. Furthermore, these sublines are also expected to contribute to advancement of the functional analysis of the involvement of the newly identified molecules in peritoneal dissemination.

Following recent advances in the comprehensive analysis of gene expression, it has been gradually revealed that gene expression patterns undergo complex alterations during the course of metastasis of gastric carcinoma.[21–27] cDNA array analysis carried out using cell lines with varying metastatic potentials in the present study revealed altered expressions of numerous genes in these cells, including those involved in adhesion, proliferation and metabolism. Among others, markedly increased expression of the MMP1 gene[27,28] was observed in 44As3 cells; however, the expression of MMP1 at the protein level was low in these cells, suggesting that MMP1 may not be closely involved in metastasis. Cathepsin L, involved in the degradation of the extracellular matrix,[29] was intensely expressed in not only 44As3, but also 58As1 cells. This finding was confirmed by immunostaining. Intense cathepsin L expression was also seen in 58As9 cells. These results suggest that this molecule may be closely associated with the metastatic potential of these tumor cells. Meanwhile, it is known that invasion and metastasis of gastric cancer occur as a result of accumulation of changes in several genes.[21] These include genes encoding cell adhesion-related molecules (E-cadherin,[30,31] β-catenin,[30] integrin α6β4,[8] dysadherin,[32] CD44,[33,34] etc.), molecules associated with proliferation, loss of intercellular adhesion and matrix degradation (EGF, c-erbB-2,[35] cript,[36] etc.), motility-associated molecules (HGF, c-met,[37] etc.), molecules associated with vascularization (VEGF,[38] IL-6,[39] IL-8,[40] bFGF,[41] etc.), a tumor metastasis suppressor gene (nm23)[42] a gene associated with the malignant course of tumors (Smad),[43] and so on. When the expression of these genes was analyzed, markedly increased expression of MMP1, cathepsin L and nm23 was observed in the highly metastatic 44As3 cell line as compared with that in the poorly metastatic parent cell line. Molecules expressed specifically in the highly metastatic cell lines 58As1 and 58As9 included cathepsin L, dysadherin, CD44, integrin β4, EGFR, HGF and VEGF. Although these molecules seemed to be closely related to peritoneal dissemination of gastric carcinoma, it would be desirable to determine the exact causal relationship between these molecules and tumor metastasis using *in vivo* models. Nonetheless, our results suggest that: (i) there may be multiple pathways involving different molecules for the apparently single process of tumor metastasis, and (ii) the genes contributing to the metastatic potential of tumor cells may differ between the parent cells and the clones selectively isolated from it.

As stated, the presence of peritoneal metastasis represents an advanced stage of cancer associated with a poor prognosis, and no effective therapy for this condition is available as yet. It is therefore important to devise a new therapeutic strategy based on the aforementioned novel viewpoints. One such strategy that has been discussed is the development of anticancer agents based on molecular targeting. To seek such agents, a model allowing appropriate evaluation of drugs is essential, and models to be used for drug evaluation *in vivo* need to satisfy the following six requirements: (i) the tumor should undergo proliferation, spread, dissemination and metastasis akin to those seen in clinical cases; (ii) the tumor cell survival rate in the gastric wall following orthotopic implantation should be 100%; (iii) the frequency of metastasis should be 90–100%; (iv) the model should be highly reproducible; (v) the interindividual variance should be small, to allow easy comparison among different test groups; (vi) application to experiments using many animals should be relatively easy. When the animal model of peritoneal dissemination established in this study was evaluated according to these criteria, all the three highly metastatic cell lines established satisfied all of these six requirements. We evaluated the antitumor activities of two antitumor agents (CPT-11[17] and GEM[18]) using the animal models implanted with 58As1 and 44As3 cells. Treatment with these agents suppressed the proliferation and spread of the tumor and significantly prolonged the survival of the animals. For each of the cases studied, a dose-response relationship was observed, and the experiments were highly reproducible. Another advantage of this animal model is that the length of time from implantation to tumor formation is short (causing death within 40 days); this feature may be expected to contribute to shortening of the evaluation period. The advantages of this model may prove to be useful for the development of drugs based on molecular targeting.

In the past, no approach was known for isolation of host factors involved in the cascade of tumor proliferation in the primary lesion to formation of ascites, or for the functional analysis of this cascade (e.g. analysis of interactions). The model established in the present study is expected to contribute greatly to the advancement of studies in these fields and in other applied research.

Acknowledgments

We are grateful to Dr A. Ochiai (Pathology Division, National Cancer Center Research Institute East) for fruitful discussions. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan.

References

1 Japanese Gastric Cancer Association. Japanese Classification of Gastric Carcinoma – 2nd English Edition. *Gastric Cancer* 1998; **1**: 10–24.
2 Tahara E. Endocrine tumors of the gastrointestinal tract: classification, function and biological behavior. In: Watanabe S, Wolff M, Sommers SC, eds. *Digestive Disease Pathology*. Philadelphia: Field andWood, 1988; 121–47.
3 Fenoglio-preier C, Carneiro F, Correa P *et al.* Gastric carcinoma. In: Hamilton SR, Aaltonen LA, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Digestive System*. Lyon: IARC Press, 2000; 37–52.
4 Maruyama K. Results of surgery correlated with staging. In: Preece PE, Cuschieri A, Wellwood JM, eds. *Cancer of the Stomach*. Orlando: Grune and Stratton, 1986; 145–63.
5 Moriguchi S, Maehara Y, Korenaga D, Sugimachi K, Nose Y. Risk factors which predict pattern of recurrence after curative surgery for patients with advanced gastric cancer. *Surg Oncol* 1992; **1**: 341–6.
6 Yashiro M, Chung YS, Nishimura S, Inoue T, Sowa M. Peritoneal metastatic model for human scirrhous gastric carcinoma in nude mice. *Clin Exp Metastasis* 1996; **14**: 43–54.
7 Fujita S, Suzuki H, Kinoshita M, Hirohashi S. Inhibition of cell attachment, invasion and metastasis of human carcinoma cells by anti-integrin β1 subunit antibody. *Jpn J Cancer Res* 1992; **83**: 1317–26.
8 Ishii Y, Ochiai A, Yamada T *et al.* Integrin α6β4 as a suppressor and a predictive marker for peritoneal dissemination in human gastric cancer. *Gastroenterology* 2000; **118**: 497–506.
9 Kotanagi H, Saito Y, Shinozawa N, Koyama K. Establishment of a human cancer cell line with high potential for peritoneal dissemination. *J Gastroenterol* 1995; **30**: 437–8.
10 Kaneko K, Yano M, Tsujinaka T *et al.* Establishment of a visible peritoneal micrometastatic model from a gastric adenocarcinoma cell line by green fluorescent protein. *Int J Oncol* 2000; **16**: 893–8.
11 Yanagihara K, Seyama T, Tsumuraya M, Kamada N, Yokoro K. Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the c-myc oncogene. *Cancer Res* 1991; **51**: 381–6.
12 Yanagihara K, Kamada N, Tsumuraya M, Amano F. Establishment and characterization of a human gastric scirrhous carcinoma cell line in serum-free chemically defined medium. *Int J Cancer* 1993; **54**: 200–7.
13 Yanagihara K, Ito A, Toge T, Numoto M. Antiproliferative effects of

isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Res* 1993; **53**: 5815–21.
14 Yanagihara K, Tanaka H, Takigahira M *et al.* Establishment of two cell lines from human gastric scirrhous carcinoma that possess the potential to metastasize spontaneously in nude mice. *Cancer Sci* 2004; **95**: 575–82.
15 Luo L, Salunga RC, Guo H *et al.* Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat Med* 1999; **5**: 117–22.
16 Hughes TR, Mao M, Jones AR *et al.* Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. *Nat Biotechnol* 2001; **19**: 342–7.
17 Kanzawa F, Saijo N. *In vitro* interaction between gemcitabine and other anticancer drugs using a novel three-dimensional model. *Semin Oncol* 1997; **24**: S7-8–S7-16.
18 Saijo N. Preclinical and clinical trials of topoisomerase inhibitors. *Ann N Y Acad Sci* 2000; **922**: 92–9.
19 Fidler IJ. Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. *Cancer Metast Rev* 1986; **5**: 29–49.
20 Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. *Nat Genet* 2003; **33**: 49–54.
21 Yokozaki H, Yasui W, Tahara E. Genetic and epigenetic changes in stomach cancer. *Int Rev Cytol* 2001; **204**: 49–95.
22 Yasui W, Oue N, Ito R, Kuraoka K, Nakayama H. Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications. *Cancer Sci* 2004; **95**: 385–92.
23 Hippo Y, Yashiro M, Ishii M *et al.* Differential gene expression profiles of scirrhous gastric cancer cells with high metastatic potential to peritoneum or lymph nodes. *Cancer Res* 2001; **61**: 889–95.
24 Hippo Y, Taniguchi H, Tsutsumi S *et al.* Global gene expression analysis of gastric cancer by oligonucleotide microarrays. *Cancer Res* 2002; **62**: 233–40.
25 Hasegawa S, Furukawa Y, Li M *et al.* Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23 040 genes. *Cancer Res* 2002; **62**: 7012–7.
26 Weiss MM, Kuipers EJ, Postma C *et al.* Genomic profiling of gastric cancer predicts lymph node status and survival. *Oncogene* 2003; **22**: 1872–9.
27 Inoue T, Yashiro M, Nishimura S *et al.* Matrix metalloproteinase-1 expression is a prognostic factor for patients with advanced gastric cancer. *Int J Mol Med* 1999; **4**: 73–7.
28 Sakurai Y, Otani Y, Kameyama K *et al.* Therole of stromal cells in the expression of interstitial collagenase (matrix metalloproteinase-1) in the invasion of gastric cancer. *J Surg Oncol* 1997; **66**: 168–72.
29 Dohchin A, Suzuki JI, Seki H, Masutani M, Shiroto H, Kawakami Y. Immunostained cathepsins B and L correlate with depth of invasion and different metastatic pathways in early stage gastric carcinoma. *Cancer* 2000; **89**: 482–7.
30 Hirohashi S. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* 1998; **153**: 333–9.
31 Behrens J, Mareel MM, Van Roy FM, Birchmeier W. Dissecting tumor cell invasion: epithelial cells acquire invasive; properties after the loss of uvomorulin-mediated cell-cell adhesion. *J Cell Biol* 1989; **108**: 2435–47.
32 Ino Y, Gotoh M, Sakamoto M, Tsukagoshi K, Hirohashi S. Dysadherin, a cancer-associated cell membrane glycoprotein, down-regulated E-cadherin and promotes metastasis. *Proc Natl Acad Sci USA* 2002; **99**: 365–70.
33 Yokozaki H, Ito R, Nakayama H, Kuniyasu H, Taniyama K, Tahara E. Expression of CD44 abnormal transcripts in human gastric carcinomas. *Cancer Lett* 1994; **83**: 229–34.
34 Nishimura S, Chung YS, Yashiro M, Inoue T, Sowa M. CD44H plays an important role in peritoneal dissemination of scirrhous gastric cancer cells. *Jpn J Cancer Res* 1996; **87**: 1235–44.
35 Tsugawa K, Yonemura Y, Hirono Y *et al.* Amplification of the c-met, c-erbB-2 and epidermal growth factor receptor gene in human gastric cancers: correlation to clinical features. *Oncology* 1998; **55**: 475–81.
36 Kunuyasu H, Yoshida K, Yokozaki H *et al.* Expression of cript, a novel gene of the epidermal growth factor family, in human gastrointestinal carcinomas. *Jpn J Cancer Res* 1991; **82**: 969–73.
37 Kunuyasu H, Yasui W, Yokozaki H, Kitadai Y, Tahara E. Aberrant expression of c-met mRNA in human gastric carcinomas. *Int J Cancer* 1993; **55**: 72–5.
38 Takahashi Y, Cleary KR, Mai M, Kitadai Y, Bucana CD, Ellis LM. Significance of vessel count and vascular endothelial growth factor and its receptor (KDR) in intestinal-type gastric cancer. *Clin Cancer Res* 1986; **2**: 1679–84.
39 Huang SP, Wu MS, Wang HP, Yang PS, Kuo ML, Lin JT. Correlation between serum levels of interleukin-6 and vascular endothelial growth factor in gastric carcinoma. *J Gastroenterol Hepatol* 2002; **17**: 1165–9.
40 Kitadai Y, Haruma K, Sumii K *et al.* Regulation of angiogenesis in human gastric carcinomas by interleukin-8. *Am J Pathol* 1998; **152**: 93–100.
41 Ueki T, Koji T, Tamiya S, Nakane PK, Tsuneyoshi M. Expression of basic fibroblast growth factor and fibroblast growth factor in advanced gastric carcinoma. *J Pathol* 1995; **177**: 353–61.
42 Nakayama H, Yasui W, Yokozaki H, Tahara E. Reduced expression of nm23 is associated with metastasis of human gastric carcinomas. *Jpn J Cancer Res* 1993; **84**: 184–90.
43 Ijichi H, Ikenoue T, Kato N *et al.* Systemic analysis of the TGF-beta-smad signaling pathway in gastrointestinal cancer cells. *Biochem Biophys Res Commun* 2001; **289**: 350–7.

 doi: 10.1111/j.1349-7006.2005.00054.x

British Journal of Cancer (2005) 92, 1877–1880

www.bjcancer.com

Gefitinib treatment affects androgen levels in non-small-cell lung cancer patients

M Nishio*[,1], F Ohyanagi[1], A Horiike[1], Y Ishikawa[3], Y Satoh[2], S Okumura[2], K Nakagawa[2], K Nishio[4] and T Horai[1]

[1]Division of Internal Medicine, Cancer Institute Hospital, Japanese Foundation For Cancer Research, Ariake 3-10-6, Koto-ku, Tokyo 135-8550, Japan; [2]Department of Chest Surgery, Cancer Institute Hospital, Japanese Foundation For Cancer Research, Tokyo, Japan; [3]Department of Pathology, Cancer Institute, Japanese Foundation For Cancer Research, Tokyo, Japan; [4]Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan

Gefitinib, an inhibitor of the epidermal growth factor receptor (EGFR, HER1/ErbB1) tyrosine kinase, has been shown to have clinical activity against non-small-cell lung cancers (NSCLCs), especially in women nonsmokers with adenocarcinomas. The aim of the present study was to clarify the relationship between androgen levels and gefitinib treatment in patients with advanced NSCLCs. Sera from 67 cases (36 men and 31 women) were obtained pretreatment and during treatment with gefitinib monotherapy (days 14–18) for examination of testosterone, dehydroepiandrosterone sulphate (DHEA), and dehydroepiandrosterone sulphate (DHEAS) levels. Testosterone and DHEA during treatment were significantly lower than the pretreatment values in both women and men, and the DHEAS levels during treatment were also significantly lowered in women. Gefitinib treatment significantly suppressed androgen levels, especially in women who had no smoking history. In addition, hormone levels in women responding to gefitinib were significantly lower during the treatment than in women who did not respond. Gefitinib-associated decrease in serum androgen levels may play a role in its clinical efficacy.
British Journal of Cancer (2005) **92,** 1877–1880. doi:10.1038/sj.bjc.6602585 www.bjcancer.com
Published online 3 May 2005

Keywords: sex hormone; epidermal growth factor receptor; tyrosine kinase inhibitor

Translational Therapeutics

Non-small-cell lung cancer (NSCLC) is a major health problem worldwide for both men and women (Ferlay *et al*, 2001). Usually at the time of diagnosis more than 50% of the patients have advanced or metastatic disease. While cytotoxic chemotherapy slightly prolongs survival among advanced NSCLC patients, it exerts clinically significant adverse effects (Non-Small-Cell Lung Cancer Collaborative Group, 1995; Schiller *et al*, 2002). An effective, palliative, low-toxicity treatment for patients with advanced NSCLC is therefore needed and for this purpose the epidermal growth factor receptor (EGFR/HER1) is a promising target. Gefitinib (ZD 1839, Iressa; AstraZeneca, London, UK) is an orally active, selective HER1-tyrosine kinase inhibitor (Wakeling *et al*, 2002), which has been shown to elicit objective responses in NSCLC cases, particularly in women nonsmokers with adenocarcinomas (Fukuoka *et al*, 2003; Kris *et al*, 2003). Recently, active mutations of EGFR have been identified in such cases (Paez *et al*, 2004; Pao *et al*, 2004) and may be linked with the sensitivity to gefitinib (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004). However, the reason why mutations frequently occur in these particular individuals is poorly understood.

Androgens are important hormones that play definitive roles in the differentiation of males and females. They can modify the activity of the epidermal growth factor network and EGFR signaling is essential for androgen-induced proliferation (Klein and Nielsen, 1993; Dammann *et al*, 2000; Torring *et al*, 2003). A receptor for androgens has been reported to occur in NSCLCs (Beattie *et al*, 1985; Kaiser *et al*, 1996) and there may be cooperative interaction between the hormones and active mutations of EGFR during the development of lung cancer. Previous reports have suggested that smoking increases the levels of androgens in men and women (Law *et al*, 1997; Trummer *et al*, 2002) and carcinogens from cigarette smoke may disrupt androgen function by reducing androgen receptor (AR) levels in androgen-responsive organs (Lin *et al*, 2004).

On the basis of these reports, we hypothesised that androgens may play an important role in the efficacy of gefitinib in NSCLC cases. In the present study, we therefore evaluated androgen levels in patients treated with gefitinib and the relationship with clinical efficacy.

PATIENTS AND METHODS

Between September 2002 and May 2004, 67 advanced or recurrent NSCLC patients were analysed in this study. All 67 were treated at our institution with gefitinib monotherapy (250 mg oral doses of gefitinib once daily) until disease progression occurred. Response evaluation and confirmation were performed in accordance with the WHO criteria (WHO, 1979). In brief, complete response (CR) was defined as complete disappearance of all lesions in imaging studies for at least 4 weeks without the appearance of any new lesions. Partial response (PR) was defined as a >50% decrease under the baseline in the sum of the products of the

*Correspondence: Dr M Nishio; E-mail: mnishio@jfcr.or.jp
Received 13 December 2004; revised 21 March 2005; accepted 21 March 2005; published online 3 May 2005

perpendicular diameters of all measurable lesions and at least stabilisation of all nonmeasurable lesions over a minimum period of 4 weeks. Progressive disease (PD) was defined as a >25% increase in the sum of the products of all measurable lesions, an unequivocal increase of nonmeasurable disease, or the appearance of new lesions. Cases were classified as having stable disease (SD) if none of the criteria for classifying responses as a CR, PR, or PD were met.

Blood was drawn before and during gefitinib administration. A previous report indicated the median time to symptom improvement with gefitinib to be only 8 days (Fukuoka *et al*, 2003), and we therefore checked the hormone levels at days 14–18, when serum was sampled between 10:00 and 14:00 and stored at 80°C for subsequent analyses. Serum levels of testosterone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulphate (DHEAS) were all measured at the SRL Laboratory (Tokyo, Japan). For testosterone, an electrochemiluminescence immunoassay was applied (ECLusys testosterone; Roche Diagnostics KK, Tokyo, Japan) and radioimmunoassays were used for DHEA and DHEAS (DPC DHEA and DPC DHEAS kits; Diagnostic Products Corporation, Los Angeles, CA, USA). The detection limits for testosterone, DHEA, and DHEAS were 5, 0.2, and 20 ng ml^{-1}, respectively. Inter- and intra-assay coefficients of variation were 6 and 8% for testosterone, 8 and 9% for DHEA, and 4 and 4% for DHEAS, respectively.

Appropriate ethical review boards approved the study, which followed the recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

Statistical analysis

A paired *t*-test was used to compare the androgen levels between the two time periods. Patients were grouped into responders (CR and PR) and nonresponders (SD and PD) and the variables in each group were compared with an unpaired *t*-test. All statistical analyses were performed using SPSS version 8 statistical software (SPSS Inc., IL, USA).

RESULTS

Patient characterisation

Data for patient characteristics are listed in Table 1. Of the 67, 31 (46.3%) were women. The median age was 61 years (range, 42–80 years). There were 26 patients (38.8%) who had never smoked and adenocarcinoma was the primary histological finding in 56 cases (83.6%). There was no prior chemotherapy in 16 (23.9%) of the patients, and the remainder had received platinum-based chemotherapy.

Response to treatment could only be evaluated in 64 of the 67 cases. We observed 20 PR (29.8%), and of these, 13 (65%) were women and seven (35%) were men ($P=0.074$). The median and range of treatment duration with gefitinib were 2.1 and 0.2–21 months. In all, 10 (50%) of 20 responders and 29 (66%) of 44 nonresponders had a smoking history ($P=0.226$).

Effects of gefitinib treatment on androgens levels in NSCLC patients

Testosterone, DHEA, and DHEAS were detected in the serum of all 67 patients (see Table 2). There was a significant difference observed between men and women for serum testosterone levels ($P<0.0001$), but not for serum DHEA or DHEAS (DHEA; $P=0.267$, DHEAS; $P=0.0565$).

In women, testosterone, DHEA, and DHEAS levels at pretreatment were significantly higher than during treatment (testosterone; $P=0.025$, DHEA; $P=0.0065$, DHEAS; $P=0.0326$). In men, pretreatment testosterone and DHEA levels were significantly higher than during treatment, but there was no significant difference for DHEAS (testosterone, $P=0.0009$; DHEA, $P=0.0085$; DHEAS, $P=0.33$). In addition, we compared hormone levels between smokers and nonsmokers. Pretreatment, there were no significant differences between women with and without a smoking history. On the other hand, hormone levels were significantly suppressed by gefitinib treatment in the 21 women who had no smoking history (testosterone, $P=0.0016$; DHEA,

Table 1 Patient characteristics

Variable	No. of patients	%
Total	67	
Sex		
Male	36	53.7
Female	31	46.3
Age (years)		
Median	61	
Range	42–80	
Smoking history		
Never	26	38.8
Former/current	41	61.2
Performance status		
0, 1	48	71.6
>2	19	28.4
Histology		
Ad	56	83.6
Non-Ad	11	16.4
Stage		
II–III	17	25.4
IV	27	40.3
Recurrence after surgery	23	34.3
Response		
PR	20	29.8
SD/PD	44	64.7
NE	3	4.5
Prior chemotherapy		
No	16	23.9
Yes	51	76.1

Ad = adenocarcinoma; non-Ad = nonadenocarcinoma; PR = partial response; SD = stable disease; PD = progressive disease; NE = not evaluable.

Table 2 Androgen levels in patients treated with gefitinib

	Pretreatment		During treatment		
Variable	***n***	**Mean±s.d.**	***n***	**Mean±s.d.**	**Paired t-test**
Testosterone (ng ml^{-1})					
Female		21.5±12.0	31	13.8.0±11.0	$P=0.025$
Male	37	409.7±129.8	37	350.8±135.7	$P=0.0009$
DHEA (ng ml^{-1})					
Female	31	2.21±2.03	31	1.33±0.83	$P=0.0065$
Male	37	1.78±1.06	37	1.49±0.92	$P=0.0085$
DHEAS (ng ml^{-1})					
Female	31	854.4±579.5	31	645.8±365.6	$P=0.0326$
Male	37	1137.4±607.7	37	1103.0±601.5	$P=0.33$

s.d. = standard deviation; DHEA = dehydroepiandrosterone; DHEAS = dehydroepiandrosterone sulphate.

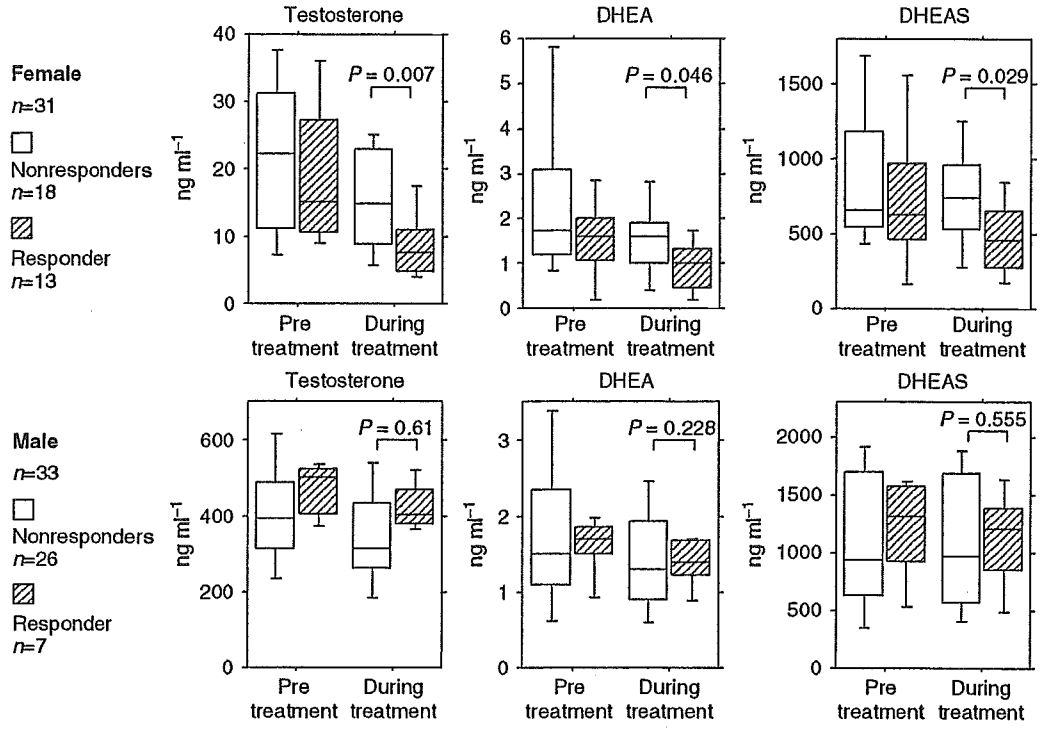

Figure 1 Serum testosterone, DHEA, and DHEAS levels, pretreatment and during the gefitinib administration. Each androgen levels are depicted in accordance to clinical response of gefitinib treatment (responders, PR; nonresponders, SD or PD). Error bars showed standard deviation.

$P=0.0157$; DHEAS, $P=0.0441$), but not in the 10 who had a smoking history (testosterone, $P=0.6159$; DHEA, $P=0.2487$; DHEAS, $P=0.4740$). Figure 1 depicts the androgen levels for women after dividing the group into responders *vs* nonresponders. Testosterone, DHEA, and DHEAS levels in women responders during treatment were significantly lower than those observed in women nonresponders (testosterone, $P=0.007$; DHEA, $P=0.046$; DHEAS, $P=0.029$). When men were included in the analysis, DHEA and DHEAS levels during treatment in the responders ($n=20$) were still significantly lower than in the nonresponders ($n=44$) (DHEA, $P=0.0324$; DHEAS, $P=0.0447$).

DISCUSSION

The present study of androgen levels (testosterone, DHEA, and DHEAS) in advanced NSCLC patients treated with gefitinib monotherapy revealed treatment-related decrease, especially in women who had no smoking history. The clinical response of gefitinib treatment appeared to be correlated with the suppression of the hormone levels.

To our knowledge, there have been no previous reports of effects of gefitinib treatment on levels of androgens in patients, although a number of authors have examined relationships between androgens and activity of the epidermal growth factor network (Klein and Nielsen, 1993; Dammann *et al*, 2000; Torring *et al*, 2003). There is evidence that EGFR expression is involved in prostate cancer development and in progression to androgen independence (Di Lorenzo *et al*, 2002), and an *in vitro* study has provided evidence that androgens increase the EGFR levels in androgen-sensitive prostate cancer cells and that EGFR signaling is essential for androgen-induced proliferation and survival (Torring *et al*, 2003). Although there has been no indication of any relationship between androgens and EGFR in NSCLCs, expression of ARs has been detected in NSCLC cell lines and biopsy samples of primary lung cancers (Kaiser *et al*, 1996). Additionally, expression has been detected more frequently in women with adenocarcinoma, and thus this may be a prognostic factor for use of gefitinib in NSCLCs (Fukuoka *et al*, 2003; Kris *et al*, 2003; Miller *et al*, 2004). The data suggest that there is a correlation between the AR and EGFR functions in lung cancer. In agreement with this hypothesis, our results demonstrated clinical responses to gefitinib treatment to correlate with suppression of androgen levels.

One reason for lower androgen levels in responders than nonresponders might be that smokers are resistant and have higher androgen levels. However, there were no significant difference in smoking history between responders and non-responder in our study and there was no significant difference of the pretreatment levels of androgens between smokers and nonsmokers. On the other hand, gefitinib treatment significantly suppressed androgen levels in women who had no smoking history, but not in smokers. Smoking may disrupt the correlation between EGFR and androgen.

Both gefitinib and androgens are metabolised by CYP3A4/5; therefore, it can be speculated that gefitinib may affect the metabolisms of androgens. On the other hand, there are no direct evidences demonstrating PK interaction between gefitinib and androgens. PK interaction between gefitinib and other drugs metabolised by CY3A4/5 such as docetaxel or irinotecan were reported (Fandi *et al*, 2003; Furman *et al*, 2004). These reports suggested that gefitinib may decrease the clearance of these drugs and it may be due to CYP3A4/5 substrate competition. If there are any PK interactions between gefitinib and androgens, androgens clearance may decrease and androgen levels may increase by gefitinib treatment. However, we showed that gefitinib treatment decreased the levels of androgens and it suggested that the effect may not be due to change of CYP3A4/5 activity.

With single estimations of testosterone and DHEA, it is necessary to take into account the circadian rhythms. In this study, all blood was therefore taken at approximately the same time, that is, between 10:00 and 14:00, although this does not preclude any influence of cycles. On the other hand, several reports have suggested that there is no circadian rhythm for serum DHEAS levels (Molta and Schwartz, 1986; Hall *et al*, 1993; Kos-Kudla *et al*, 2001). Therefore, the differences seen in the DHEAS levels in this study presumably reflect actual effects of gefitinib treatment. This would suggest that the data for the other hormones might also have clinical significance.

In conclusion, the results of the present small, retrospective study indicate that androgen levels in NSCLC patients are affected by gefitinib treatment and that they may be factors determining sensitivity to this chemotherapeutic agent. Further large-scale prospective trials are needed in the future to confirm these results and to examine inter-relationships among androgens, smoking, gefitinib sensitivity, and EGFR mutations.

ACKNOWLEDGEMENTS

A part of this study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Grants-in-Aid for Cancer Research from the Ministry of Health, Labour, and Welfare of Japan.

REFERENCES

Beattie CW, Hansen NW, Thomas PA (1985) Steroid receptors in human lung cancer. *Cancer Res* **45:** 4206–4214

Dammann CE, Ramadurai SM, McCants DD, Pham LD, Nielsen HC (2000) Androgen regulation of signaling pathways in late fetal mouse lung development. *Endocrinology* **141:** 2923–2929

Di Lorenzo G, Tortora G, D'Armiento FP, De Rosa G, Staibano S, Autorino R, D'Armiento M, De Laurentiis M, De Placido S, Catalano G, Bianco AR, Ciardiello F (2002) Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clin Cancer Res* **8:** 3438–3444

Fandi A, Gatzemeier U, Smith R, Averbuch S, Manegold C (2003) Final data from a pilot trial of gefitinib (ZD1839) in combination with docetaxel in patients with advanced or metastatic non-small-cell lung cancer (NSCLC): Safety and pharmacokinetics. *Proc Am Soc Clin Oncol* **22:** 655

Ferlay J, Bray F, Pisani P, Parkin DM (2001) *GLOBOCAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Version 1.0 (IARC, CancerBase No. 5).* Lyon, France: IARC Press

Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard J-Y, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong R-P, Baselga J (2003) Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* **21:** 2237–2246

Furman WL, Daw NC, Crews KR, Stewart CF, McCarville B, Santana VM, Hawkins D, Rodriguez-Galindo C, Navid F, Houghton PJ (2004) A phase I study of gefitinib and irinotecan (IRN) in pediatric patients with refractory solid tumors. *Proc Am Soc Clin Oncol* **22:** 8521

Hall GM, Perry LA, Spector TD (1993) Depressed levels of dehydroepiandrosterone sulphate in postmenopausal women with rheumatoid arthritis but no relation with axial bone density. *Ann Rheum Dis* **52:** 211–214

Kaiser U, Hofmann J, Schilli M, Wegmann B, Klotz U, Wedel S, Virmani AK, Wollmer E, Branscheid D, Gazdar AF, Havemann K (1996) Steroid-hormone receptors in cell lines and tumor biopsies of human lung cancer. *Int J Cancer* **67:** 357–364

Klein JM, Nielsen HC (1993) Androgen regulation of epidermal growth factor receptor binding activity during fetal rabbit lung development. *J Clin Invest* **91:** 425–431

Kos-Kudla B, Ostrowska Z, Marek B, Ciesielska-Kopacz N, Kudla M, Kajdaniuk D, Siemidska L, Strzelczyk J (2001) Circadian serum levels of dehydroepiandrosterone sulphate in postmenopausal asthmatic women before and after long-term hormone replacement. *Endocr Regul* **35:** 217–222

Kris MG, Natale RB, Herbst RS, Lynch Jr TJ, Prager D, Belani CP, Schiller JH, Kelly K, Spiridonidis H, Sandler A, Albain KS, Cella D, Wolf MK, Averbuch SD, Ochs JJ, Kay AC (2003) Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* **290:** 2149–2158

Law MR, Cheng R, Hackshaw AK, Allaway S, Hale AK (1997) Cigarette smoking, sex hormones and bone density in women. *Eur J Epidemiol* **13:** 553–558

Lin P, Chang JT, Ko JL, Liao SH, Lo WS (2004) Reduction of androgen receptor expression by benzo[alpha]pyrene and 7, 8-dihydro-9, 10-epoxy-7, 8, 9, 10-tetrahydrobenzo [alpha] pyrene in human lung cells. *Biochem Pharmacol* **67:** 1523–1530

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350:** 2129–2139

Miller VA, Kris MG, Shah N, Patel J, Azzoli C, Gomez J, Krug LM, Pao W, Rizvi N, Pizzo B, Tyson L, Venkatraman E, Ben-Porat L, Memoli N, Zakowski M, Rusch V, Heelan RT (2004) Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. *J Clin Oncol* **22:** 1103–1109

Molta L, Schwartz U (1986) Gonadal and adrenal androgen secretion in hirsute females. *Clin Endocrinol Metab* **15:** 229–245

Non-Small Cell-Lung Cancer Collaborative Group (1995) Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Non-small Cell Lung Cancer Collaborative Group. *BMJ* **311:** 899–909

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304:** 1497–1500

Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M, Varmus H (2004) EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* **101:** 13306–13311

Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, Zhu J, Johnson DH (2002) Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* **346:** 92–98

Torring N, Dagnaes-Hansen F, Sorensen BS, Nexo E, Hynes NE (2003) ErbB1 and prostate cancer: ErbB1 activity is essential for androgen-induced proliferation and protection from the apoptotic effects of LY294002. *Prostate* **56:** 142–149

Trummer H, Habermann H, Haas J, Pummer K (2002) The impact of cigarette smoking on human semen parameters and hormones. *Hum Reprod* **17:** 1554–1559

Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, Gibson KH (2002) ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res* **62:** 5749–5754

WHO (1979) *World Health Organization: WHO Handbook for Reporting Results of Cancer Treatment*, Vol 48, Geneva, Switzerland: WHO Offset Publication

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

Ikuo Sekine, MD, John D. Minna, MD,† Kazuto Nishio, MD,‡ Tomohide Tamura, MD,* and Nagahiro Saijo, MD**

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

(*J Thorac Oncol.* 2006;1: 31–37)

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.

*Divisions of Thoracic Oncology and Internal Medicine, National Cancer Center Hospital, Tokyo, Japan; †Hamon Center for Therapeutic Oncology Research, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX; ‡Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan.

ISSN: 1556-0864/06/0101-0031

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-

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

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, Vinorelbine	Real-time PCR	Low	13	46	0.39
				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, gemcitabine	Real-time PCR	Low	23	52	0.38
				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 Hybridization	C/C	54	54	0.61
				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 Hybridization	G/G	100	48	1.08
				G/A or A/A	8	50	(0.26-4.57)
At codon 312							
Camps et al.[39]	Non-small cell	Cisplatin, gemcitabine	PCR Sequencing	G/G	18	17	3.33
				G/A or A/A	15	40	(0.66-16.7)
At codon 751							
Camps et al.[39]	Non-small cell	Cisplatin, gemcitabine	PCR Sequencing	A/A	22	23	2.04
				A/C or C/C	16	38	(0.49-8.45)
Ryu et al.[38]	Non-small cell	Cisplatin-based	PCR Hybridization	A/A	96	49	0.74
				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.

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%

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.

ACKNOWLEDGMENTS

This study was supported in part by Lung Cancer SPORE Grant P50CA70907, ATP010019, and Grants-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan. We thank Yuko Yabe and Mika Nagai for their collection and arrangement of numerous papers.

REFERENCES

1. Sekine I, Saijo N. Novel combination chemotherapy in the treatment of non-small cell lung cancer. *Expert Opin Pharmacother* 2000;1:1131–1161.
2. Gazdar AF, Steinberg SM, Russell EK, et al. Correlation of in vitro drug-sensitivity testing results with response to chemotherapy and survival in extensive-stage small cell lung cancer: a prospective clinical trial. *J Natl Cancer Inst* 1990;82:117–124.
3. Cortazar P, Johnson BE. Review of the efficacy of individualized chemotherapy selected by in vitro drug sensitivity testing for patients with cancer. *J Clin Oncol.* 1999;17:1625–1631.
4. Cortazar P, Gazdar AF, Woods E, et al. Survival of patients with limited-stage small cell lung cancer treated with individualized chemotherapy selected by in vitro drug sensitivity testing. *Clin Cancer Res* 1997;3:741–747.
5. Shaw GL, Gazdar AF, Phelps R, et al. Individualized chemotherapy for patients with non-small cell lung cancer determined by prospective identification of neuroendocrine markers and in vitro drug sensitivity testing. *Cancer Res* 1993;53:5181–5187.
6. Mariadason JM, Arango D, Shi Q, et al. Gene expression profiling-based prediction of response of colon carcinoma cells to 5-fluorouracil and camptothecin. *Cancer Res* 2003;63:8791–8812.
7. Kikuchi T, Daigo Y, Katagiri T, et al. Expression profiles of non-small cell lung cancers on cDNA microarrays: identification of genes for prediction of lymph-node metastasis and sensitivity to anti-cancer drugs. *Oncogene* 2003;22:2192–2205.
8. Chang JC, Wooten EC, Tsimelzon A, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet* 2003;362:362–369.
9. Dan S, Tsunoda T, Kitahara O, et al. An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines. *Cancer Res* 2002;62:1139–1147.
10. Girard L, Sekine I, Shah J, et al. Correlation between in vitro drug sensitivity and microarray-based gene expression signatures in lung and breast cancer. In Proceedings of the 95th Annual Meeting of American Association for Cancer Research, Orlando, FL, March 27–31, 2004.Pp. 1098.
11. Ein-Dor L, Kela I, Getz G, et al. Outcome signature genes in breast cancer: is there a unique set? *Bioinformatics* 2006;21:171–178.
12. Michiels S, Koscielny S, Hill C. Prediction of cancer outcome with microarrays: a multiple random validation strategy. *Lancet* 2006;365: 488–492.
13. Armitage P, Berry G, Matthews JNS. Statistical Methods in Medical Research, 4th ed. Oxford: Blackwell Science Ltd, 2002.
14. Sekine I, Saijo N. Polymorphisms of metabolizing enzymes and transporter proteins involved in the clearance of anticancer agents. *Ann Oncol* 2001;12:1515–1525.
15. Ellis M, Hayes DF, Lippman ME. Treatment of metastatic breast cancer. In Harris J, Lippman ME, Morrow M, Osborne CK (Eds.), Diseases of

the Breast, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2003.Pp. 1101-1159.
16. Gambacorti-Passerini CB, Gunby RH, Piazza R, et al. Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias. *Lancet Oncol* 2003;4:75–85.
17. Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol.* 2003;21:4342–4349.
18. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2003;21:2237–2246.
19. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* 2003;290:2149–2158.
20. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139.
21. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304: 1497–1500.
22. Minna JD, Gazdar AF, Sprang SR, et al. Cancer: a bull's eye for targeted lung cancer therapy. *Science* 2004;304:1458–1461.
23. Dingemans AM, Witlox MA, Stallaert RA, et al. Expression of DNA topoisomerase IIalpha and topoisomerase IIbeta genes predicts survival and response to chemotherapy in patients with small cell lung cancer. *Clin Cancer Res* 1999;5:2048–2058.
24. Di Leo A, Isola J. Topoisomerase II alpha as a marker predicting the efficacy of anthracyclines in breast cancer: are we at the end of the beginning? *Clin Breast Cancer* 2003;4:179–186.
25. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002;108:153–164.
26. Dabholkar M, Vionnet J, Bostick-Bruton F, et al. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *J Clin Invest* 1994;94:703–708.
27. Metzger R, Leichman CG, Danenberg KD, et al. ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. *J Clin Oncol* 1998;16:309–316.
28. Yu JJ, Mu C, Lee KB, et al. A nucleotide polymorphism in ERCC1 in human ovarian cancer cell lines and tumor tissues. *Mutat Res* 1997;382: 13–20.
29. Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res* 1999;59:1391–1399.
30. Yeh JJ, Hsu WH, Wang JJ, et al. Predicting chemotherapy response to paclitaxel-based therapy in advanced non-small-cell lung cancer with P-glycoprotein expression. *Respiration* 2003;70:32–35.
31. Kawasaki M, Nakanishi Y, Kuwano K, et al. Immunohistochemically detected p53 and P-glycoprotein predict the response to chemotherapy in lung cancer. *Eur J Cancer* 1998;34:1352–1357.
32. Hsia TC, Lin CC, Wang JJ, et al. Relationship between chemotherapy response of small cell lung cancer and P-glycoprotein or multidrug resistance-related protein expression. *Lung* 2002;180:173–179.
33. Savaraj N, Wu CJ, Xu R, et al. Multidrug-resistant gene expression in small-cell lung cancer. *Am J Clin Oncol* 1997;20:398–403.
34. Rosell R, Scagliotti G, Danenberg KD, et al. Transcripts in pretreatment biopsies from a three-arm randomized trial in metastatic non-small-cell lung cancer. *Oncogene* 2003;22:3548–3553.
35. Dingemans AC, van Ark-Otte J, Span S, et al. Topoisomerase IIalpha and other drug resistance markers in advanced non-small cell lung cancer. *Lung Cancer* 2001;32:117–128.
36. Nakanishi Y, Kawasaki M, Bai F, et al. Expression of p53 and glutathione S-transferase-pi relates to clinical drug resistance in non-small cell lung cancer. *Oncology* 1999;57:318–323.
37. Lord RV, Brabender J, Gandara D, et al. Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. *Clin Cancer Res* 2002;8:2286–2291.
38. Ryu JS, Hong YC, Han HS, et al. Association between polymorphisms of ERCC1 and XPD and survival in non-small-cell lung cancer patients treated with cisplatin combination chemotherapy. *Lung Cancer* 2004; 44:311–316.
39. Camps C, Sarries C, Roig B, et al. Assessment of nucleotide excision repair XPD polymorphisms in the peripheral blood of gemcitabine/cisplatin-treated advanced non-small-cell lung cancer patients. *Clin Lung Cancer* 2003;4:237–241.
40. Gregorc V, Ludovini V, Pistola L, et al. Relevance of p53, bcl-2 and Rb expression on resistance to cisplatin-based chemotherapy in advanced non-small cell lung cancer. *Lung Cancer* 2003;39:41–48.
41. Rodenhuis S, Boerrigter L, Top B, et al. Mutational activation of the K-ras oncogene and the effect of chemotherapy in advanced adenocarcinoma of the lung: a prospective study. *J Clin Oncol* 1997;15:285–291.
42. Krug LM, Miller VA, Filippa DA, et al. Bcl-2 and bax expression in advanced non-small cell lung cancer: lack of correlation with chemotherapy response or survival in patients treated with docetaxel plus vinorelbine. *Lung Cancer* 2003;39:139–143.
43. Takayama K, Ogata K, Nakanishi Y, et al. Bcl-2 expression as a predictor of chemosensitivities and survival in small cell lung cancer. *Cancer J Sci Am* 1996;2:212.

Int. J. Cancer: **118,** 483–489 (2006)

ZD6474 inhibits tumor growth and intraperitoneal dissemination in a highly metastatic orthotopic gastric cancer model

Tokuzo Arao[1,2,4], Kazuyoshi Yanagihara[3], Misato Takigahira[3], Masayuki Takeda[1,2], Fumiaki Koizumi[1,2], Yasushi Shiratori[4] and Kazuto Nishio[1,2]*

[1]*Shien Lab, Medical Oncology, National Cancer Center Hospital, Tokyo, Japan*
[2]*Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan*
[3]*Central Animal Laboratory, National Cancer Center Research Institute, Tokyo, Japan*
[4]*Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*

Angiogenesis inhibitors have been used to treat some cancers, but the therapeutic potential of these agents for gastric cancer has remained unclear. To investigate their therapeutic potential, we examined the effect of ZD6474, an agent that selectively targets vascular endothelial growth factor receptor-2 (VEGFR-2; KDR) tyrosine kinase and epidermal growth factor receptor (EGFR) tyrosine kinase, in a highly metastatic orthotopic model using an undifferentiated gastric cancer cell line, 58As1. ZD6474 (100 mg/kg/day, p.o., 2 weeks) significantly inhibited tumor growth ($p < 0.05$ *vs.* control) and reduced tumor dissemination into the peritoneal cavity ($p < 0.05$ *vs.* control). In addition, to identify putative tumor biomarkers that would reflect the effects of ZD6474 treatment in clinical settings, we examined the gene expression profiles of implanted gastric tumors treated with ZD6474 *in vivo*. Twenty-eight candidate genes were identified, including *IGFBP-3*, *ADM*, *ANGPTL4*, *PLOD2*, *DSIPI*, *NDRG1*, *ENO2*, *HIG2* and *BNIP3L*, which are known to be hypoxia-inducible genes. These genes and gene products may be useful biomarkers for monitoring the effects of ZD6474 treatment. ZD6474 also improved the survival of mice with implanted another undifferentiated gastric cancer cell line, 44As3. In conclusion, our results suggest that ZD6474 may have clinical activity against gastric cancer, particularly undifferentiated gastric cancer with peritoneal dissemination. We also identified putative biomarkers for monitoring the pharmacodynamic effects of ZD6474 by gene expression profiling.

Key words: ZD6474; gastric cancer; intraperitoneal dissemination; VEGF; oligonucleotide microarray

Various anti-cancer agents have been examined for efficacy against gastric cancer over the past two decades, but the median survival time of patients remains around 7 months,[1,2] and the prognosis of gastric cancer patients remains poor. Peritoneal dissemination is common in patients with unresectable gastrointestinal cancer, and many suffer from peritoneal carcinomatosis in the terminal stage. Because undifferentiated gastric cancer is particularly invasive and often accompanied by peritoneal dissemination,[3] a new treatment strategy is needed.

Vascular endothelial growth factor (VEGF) is a key mediator of tumor growth and is known to have multiple functions in angiogenesis, vascular permeability, and the regulation of endothelial cell proliferation and migration.[4–6] VEGF receptors (VEGFR) are activated by ligand stimulation with VEGF and commonly expressed in vascular endothelial cells. VEGFR-2 (KDR/Flk-1) is thought to be important for angiogenesis.[7] Because the VEGF-VEGFR system plays a key role in angiogenesis and tumor growth *in vivo*, the therapeutic potential of many agents targeting this system is being investigated.[8] A recent study has shown that a combination of monoclonal antibody against VEGF and chemotherapy produces a clinically meaningful survival benefit for patients with metastatic colorectal cancer,[5] and these results may lead to changes in the standard treatment for colorectal cancer.[9]

ZD6474 is a novel orally available VEGFR-2 (KDR) tyrosine kinase inhibitor that is also known to selectively target epidermal growth factor receptor (EGFR) tyrosine kinase, both of which are parts of key pathways in tumor growth.[10–13] We demonstrated previously the evidence suggesting that ZD6474 inhibits angiogenesis and tumor growth by targeting EGFR.[14,15]

In our present study, we tested ZD6474 for an inhibitory effect on tumor growth and intraperitoneal dissemination, and for improvement of survival in a newly established, highly metastatic orthotopic gastric tumor model *in vivo*. In addition, we also identified putative biomarkers to monitor the effects of ZD6474 treatment using gene expression profiling.

Material and methods

Reagents

ZD6474 and gefitinib (Iressa®) were provided by AstraZeneca (Cheshire, UK).

Cell cultures

The newly established highly metastatic human signet-ring cell gastric cancer cell lines 58As1 and 44As3 produce large volumes of ascitic fluid and spontaneously metastasize to the peritoneal cavity after orthotopic (gastric wall) implantation.[16,17] 58As1 and 44As3 and human non-small cell lung cancer cell line PC-9 were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Gibco BRL, Grand Island, NY). The PC-9 cells were a gift of Tokyo Medical University. Human embryonic kidney cell line 293 (HEK293) was obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVEC) were maintained in EBM-2 medium (Clonetics, Walkersville, MD) supplemented with EGM-2 kit (Clonetics), according to the manufacturer's instructions.

In vitro *growth-inhibition assay*

The cell-growth inhibitory effects of ZD6474 and gefitinib were assessed by the thiazole blue tetrazolium bromide (MTT) assay (Sigma). Briefly, 180 μl/well of cell suspension was seeded on to Sumilon® 96-well microculture plates (Sumitomo Bakelite, Akita, Japan) and incubated in 10% FBS-containing medium for 24 hr. The cells were then treated with ZD6474 at various concentrations (4 nM–80 μM) and cultured at 37°C in a humidified atmosphere for 72 hr. After the culture period, 20 μl volume of MTT reagent was added, and the plates were further incubated for 4 hr. After centrifuging the plates, the culture medium was discarded and the wells were filled with dimethyl-sulfoxide. The optical density of the cultures was measured at 562 nm with Delta-soft software on a

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant number: 12217165.
***Correspondence to:** Shien Lab, Medical Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81-3-3547-5185. E-mail: knishio@gan2.res.ncc.go.jp
Received 14 March 2005; Accepted after revision 18 May 2005
DOI 10.1002/ijc.21340
Published online 28 July 2005 in Wiley InterScience (www.interscience.wiley.com).

Macintosh computer interfaced to a Bio-Tek Microplate Reader EL-340 (BioMetallics, Princeton, NJ). The experiment was conducted in triplicate.

Immunoblotting

EGFR, phospho-EGFR (specific for Tyr 1068), and anti-rabbit horseradish peroxidase (HRP)-conjugated antibody were purchased from Cell Signalling (Beverly, MA). Cell pellets were lysed in RIPA buffer (Tris-HCl, 50 mM; pH 7.4; NP-40, 1%; Na-deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM; phenylmethyl-sulfonyl fluoride, 1 mM; aprotinin, leupeptin, pepstatin, 1 mg/ml each; Na_3VO_4, 1 mM; NaF, 1 mM). Cell extracts were electrophoresed on 7.5% (w/v) polyacrylamide gels and transferred to a polyvinylidene di-fluoride membrane (Nihon Millipore, Tokyo, Japan). The membrane was incubated in Tris-buffered saline containing 0.5% Tween 20 with 3% BSA and then reacted with the primary antibodies and the HRP-conjugated secondary antibody for 90 min each. Visualization was achieved with an enhanced chemiluminescent detection reagent (Amersham Bioscience, Buckinghamshire, UK).

RT-PCR

A 5 μg of total RNA from each cultured cell line was converted to cDNA with a GeneAmp® RNA-PCR kit (Applied Biosystems, Foster City, CA). The primers used for the PCR were: human-specific beta-actin, forward: 5′-GGAAATCGTGCGTGACATT-3′ and reverse: 5′-CATCTGCTGGAAGGTGGACAG-3′; mouse-specific beta-actin, forward: 5′-GAAATCGTGCGTGACATCAAA-3′ and reverse: 5′-TCATGGTGCTAGGAGCCA-3′; VEGF-A, forward: 5′-GCCTTG-CCTTGCTGCTCTAC-3′ and reverse: 5′-CA-GGGATTTTCTTGTC-TTGC-3′; VEGF-C, forward: 5′-AAACAAGGAGCTGGATGAA-GAG-3′ and reverse: 5′-CAATATGAAGGGACACAACGAC-3′; VEGFR-1, forward: 5′-TAGCGTCACCAGCAGCGAAAGC-3′ and reverse: 5′-CCTTTCTTTTGGGTCTCTGTGC-3′; VEGFR-2, forward: 5′-CAGACGGAC-AGTGGTATGGTTC-3′ and reverse: 5′-ACCTGCTGGTGGAAAGAACAAC-3′; VEGFR-3, forward: 5′-AGCCATTCATCAACAAGCCT-3′ and reverse: 5′-GGCAACAG-CTGGATGTCATA-3′; IGFBP3, forward: 5′- AATGCTAGTGA-GTCGGAGGAAGAC-3′ and reverse: 5′- GGCGACACTGCTTTT-TCTTATAAA-3′; ADM, forward: 5′- CCTGGGTTCGCTCGCCTT-CCTA-3′ and reverse: 5′- GGCTGGAGCCCCGTGTG- CTTGT-3′.

PCR amplification was carried out for 35 cycles (95°C for 45 sec, 56–62°C for 45 sec, and 72°C for 60 sec) with a final extension step at 72°C for 7 min. The bands were visualized by ethidium bromide staining.

Sequencing

Exons 18–21 of the EGFR cDNA from the tumor cell lines were sequenced, and the cDNAs were amplified using the following primers: forward, 5′-TCCAAACTGCACCTACGGATGC-3′, and reverse, 5′-CATCAACTCCCAAACGGTCACC-3′. The PCR amplification consisted of 25 cycles (95°C for 45 sec, 55°C for 30 sec and 72°C for 60 sec). The sequences of the PCR products were determined using ABI prism 310 (Applied Biosystems). Amplification and sequencing were carried out in duplicate for each tumor cell line. The sequences were compared to the GenBank-archived human sequence of EGFR (accession number: NM_005228.3).

Orthotopic model in vivo

ZD6474 was dissolved in sterile water containing 1% TWEEN 80 (Sigma). Six-week old female BALB/c nude mice were purchased from CLEA Japan Inc. (Tokyo) and maintained under specific pathogen-free conditions. A total of 1×10^6 58As1 cells was inoculated into the gastric wall of each mouse after laparotomy. Three days after the inoculation, the mice were given ZD6474 50 mg/kg/day ($n = 6$) or 100 mg/kg/day ($n = 6$) or a vehicle control ($n = 6$) p.o. for 14 days. After euthanizing the mice on Day 19, tumor volume was measured and tumor samples and intraperitoneal lavage fluid were collected. The tumor samples were formalin fixed ($n = 3$) or stored in Isogen ($n = 3$) (Nippon Gene, Tokyo, Japan). The intraperitoneally disseminated cells were collected from 2 ml of PBS that had been used to wash the peritoneal cavity.

In the survival study, mice were inoculated with 1×10^6 58As1 or 44As3 cells into the gastric wall after laparotomy. Three days after inoculation, the mice were given ZD6474 50 mg/kg/day of ZD6474 p.o. ($n = 7$) or i.p. ($n = 7$) or the vehicle control p.o. ($n = 7$) for 14 days. The "visible ascites," which was evident a few days before death in this model, was used as a surrogate for survival time to consider for animal welfare. Mice were euthanized when ascites became visible, implantation of the gastric cancer cells was confirmed in all of the euthanized mice. No cancer cell was found in one mouse (ZD6474 100 mg/kg/day, 44As3 implanted), and it was excluded from the analysis. The experimental animal protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the Guidelines for Animal Experiments of the National Cancer Center.

Oligonucleotide microarray study

A DNA microarray procedure was used to prepare the *in vitro* transcription products, and oligonucleotide array hybridization and scanning were carried out according to the Affymetrix protocols (Santa Clara, CA). In brief, total RNA extracted from the tumor samples was analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and cRNA was synthesized with the GeneChip® 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). The cRNA were then labeled and purified for use as probes. Hybridization was carried out to the Affymetrix GeneChip HG-U133 Plus2.0 array for 16 hr at 45°C. After washing the glass slides, spot intensity was measured by scanning with a GeneChip® Scanner3000 (Affymetrix) and converted to numerical data with GeneChip Operating Software, Ver.1 (Affymetrix).

Six GeneChips were used to primary implanted 58as1 tumor samples from the vehicle control group ($n = 2$), and the ZD6474-treated group ($n = 2$, 50 mg/kg group; $n = 2$, the 100 mg/kg group).

Statistical analysis

All statistical calculations, except the analysis of the microarray data, were carried out using StatView version 5 software (SAS Institute Inc., Cary, NC). A p-value of <0.05 was considered significant. The microarray data were analyzed with GeneSpring software (Silicon Genetics, Redwood City, CA). The expression data were normalized across the sample set by the 50th percentile of each chip's intensity range. Relative expression data for each probe set were generated by median normalization. The fold change (mean value of the ZD6474-treatment group/mean value of the vehicle control group) was calculated, and genes with >2-fold change or <0.5-fold change were selected.

Results

Cell sensitivity to ZD6474 in vitro *and expression of VEGFR and EGFR*

Cell sensitivity to ZD6474 and the expression levels of EGFR, VEGFR and VEGF were examined in the 58As1 cells. The growth-inhibitory effect of ZD6474 and gefitinib was assessed by an MTT assay. The IC_{50} values of ZD6474 and gefitinib for 58As1 cells were 5.8 ± 1.8 and 11.0 ± 3.0 μM, respectively, suggesting that 58As1 cells are not sensitive to ZD6474 or gefitinib *in vitro*, compared to the "hypersensitive" PC-9 cells (IC_{50} values = 0.09 and 0.03 μM, respectively).[15] The 58As1 cells expressed a relatively high level of EGFR compared to the cells expressing high (PC-9) and low (HEK293) levels of EGFR, but the phosphorylation status was low (Fig. 1*a*). The expression levels of VEGFR and VEGF-A,C were measured by RT-PCR. A low

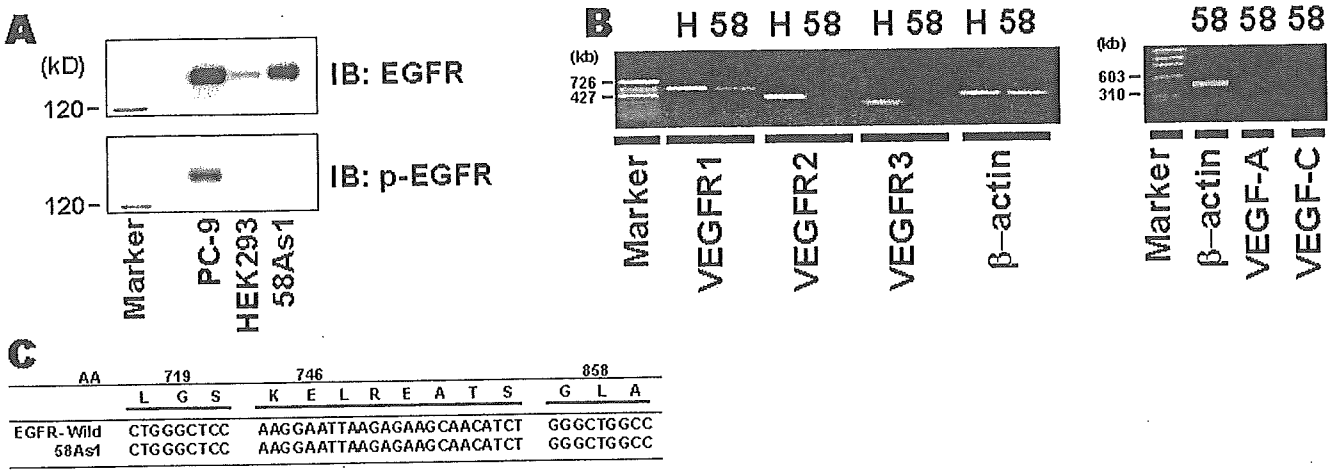

FIGURE 1 – Cellular characteristics of 58As1 cells. (*a*) EGFR expression and phosphorylation levels determined by Western blotting. A moderately high level of EGFR expression was observed in 58As1 cells, compared to cells expressing high (PC-9) and low (HEK293) levels of EGFR. The phosphorylation of EGFR status in 58As1 cells was low under normal culture conditions. IB, immunoblotting. Molecular marker: 120 kD. (*b*) Expression levels of VEGFR and VEGF-A and VEGF-C were measured by RT-PCR. A low level of VEGFR1 expression was detected in 58As1 cells, but no expression of VEGFR-2 or 3 was detected. 58As1 cells expressed VEGF-A but not VEGF-C. H, human umbilical vein endothelial cells. 58: 58As1. (*c*) EGFR sequence in 58As1 cells. No mutations were detected near the ATP-binding domains in 58As1 cells. AA, amino acid.

level of VEGFR1 expression was found in the 58As1 cells, but no VEGFR2 or VEGFR3 expression was detected. The 58As1 cells expressed VEGF-A, but not VEGF-C (Fig. 1*b*). Our results suggest that the lymphatic-metastasis-related VEGF-C and VEGFR3 are not involved in the inhibitory effect of ZD6474 on tumor dissemination observed in our present study *in vivo*.

Because EGFR mutations may be a determinant of tumor cell sensitivity to ZD6474,[15] exons 18–21 of EGFR mRNA from 58As1 cells were sequenced. No mutations near the ATP-binding domains[18,19] were detected, the 58As1 cells were concluded to express the wild-type EGFR.

Growth-inhibitory effect of ZD6474 in the orthotopic model in vivo

To examine the antitumor effect of ZD6474 on gastric cancer, we assessed the growth-inhibitory effect of ZD6474 by measuring implanted tumor volume after 14 days of p.o. treatment *in vivo*. A significant growth-inhibitory effect was observed in the ZD6474 (100 mg/kg/day) group in comparison with the vehicle control group ($p = 0.027$) in athymic mice implanted with 58As1 cells (Fig. 2*a*). Average tumor volume in the vehicle control group, 50 mg/kg/day ZD6474 group and 100 mg/kg/day ZD6474 groups was 106.3 ± 81.8 mm^3, 79.9 ± 70.0 mm^3, and 42.3 ± 24.8 mm^3, respectively.

Histological examination of H&E stained specimens showed a marked reduction in the number of cancer cells in the sub-mucosal lesions and the presence of necrotic tissue in the ZD6474 groups (Fig. 2*b*), suggesting that ZD6474 inhibits the growth of primary gastric tumor *in vivo*.

Inhibitory-effect of ZD6474 on peritoneal dissemination

To monitor the inhibitory effect of ZD6474 on peritoneally disseminated human cancer cells, the mRNA expression ratio of human β-actin/murine β-actin was measured with appropriate specific primers in cells collected from intraperitoneal lavage fluid. A significantly lower level of human-derived β-actin was observed in the 100 mg/kg/day ZD6474 group than in the vehicle control group ($p = 0.049$) (Fig. 2*c*,*d*), indicating that ZD6474 inhibits the intraperitoneal dissemination of gastric cancer in a dose-dependent manner.

Effect of ZD6474 on survival

In the survival experiment, we examined the effect of ZD6474 (p.o. or i.p.) on the survival of mice implanted with 58As1 or 44As3 cells. Both p.o. and i.p. administration of ZD6474 50 mg/kg/day significantly improved the survival of 44As3-implanted mice ($p = 0.0009$, $p = 0004$ *vs.* control, Fig. 3*b*), but did not significantly improve the survival of 58As1-implanted mice ($p = 0.09$, $p = 0.4$ *vs.* control, Fig. 3*a*). The median survival time of the 58As1-implanted mice was 33 days in the control group, 40 days in the i.p. group, and 46 days in the *p.o.* group, whereas in the 44As3-implanted mice, it was 34 days, 43 days and 53 days, respectively. Oral administration of ZD6474 was more effective than i.p. injection ($p = 0.049$) in the 44As3-implanted mice (Fig. 3*b*). These results suggest that ZD6474 is an active against gastric cancer.

Regulation of the gene expression by ZD6474 in vivo

To identify putative tumor biomarkers that reflect the efficacy of ZD6474 in vivo, we analyzed the gene expression profiles of implanted-tumor samples with oligonucleotide microarray. Expression of 26 genes was upregulated by 2-fold or more in the ZD6474 treatment group compared to the control group, whereas 2 genes were downregulated (Fig. 4*a*). Interestingly, of 26 upregulated genes, 9 of these genes were reported previously to be hypoxia-inducible: *IGFBP3* (*insulin-like growth factor binding protein 3*), *ADM* (*adrenomedullin*), *ANGPTL4* (*angiopoietin-like 4*), *PLOD2* (*procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2*), *DSIPI* (*delta sleep inducing peptide, immunoreactor*), *ENO2* (*enolase 2*), *NDRG1* (*N-myc downstream regulated gene 1*) *HIG2* (*hypoxia-inducible protein 2*) and *BNIP3L* (*BCL2/adenovirus E1B 19 kDa interacting protein 3-like*). To confirm upregulation of the genes, we measured the expression levels of representative genes, *IGFBP3* and *ADM*, by RT-PCR in murine tumor samples (Fig. 4*b*).

Discussion

A correlation between somatic EGFR mutations in non-small cell lung cancer cells and sensitivity to EGFR-specific tyrosine kinase inhibitors, including gefitinib and erlotinib, has been demonstrated recently,[18–20] and a similar observation was made in regard to ZD6474 *in vitro*.[15] We demonstrated previously that cells transfected with mutated EGFR were ~60-fold more sensitive to ZD6474 *in vitro*. EGFR tyrosine kinase inhibitors may pro-