Influence of histological type, smoking history and chemotherapy on survival after first-line therapy in patients with advanced non-small cell lung cancer

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The usual primary endpoint in clinical trials for first-line chemotherapy in advanced non-small cell lung cancer is overall survival. Second-line chemotherapy can also prolong overall survival. Non-smoking history has been associated with a treatment effect for epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) versus placebo for overall survival. We performed a retrospective analysis to identify prognostic factors for progression-free survival and overall survival in patients with advanced non-small cell lung cancer treated with first-line carboplatin/paclitaxel, and to examine the effect of second-line therapy on progression-free survival and overall survival. Ninety-eight patients (median age 61 years, 35 female, 74 adenocarcinoma, 68 smokers, 56 performance status 0) fulfilled our criteria, of which 75 patients (78%) received more than second-line therapy (docetaxel [54%] gefitinib [48%] erlotinib [4%]). For overall survival, smoking history and histology were significant prognostic factors. The 2-year overall survival rates were as follows: smokers, 17%; non-smokers, 52%, $P < 0.0001$; adenocarcinoma, 40%; other 15%, $P = 0.0017$. Multivariate analysis in patients who received second-line therapy showed treatment with EGFR-TKI was an independent predictor of overall survival. Smoking history and adenocarcinoma histology were prognostic factors for an improved outcome with carboplatin/paclitaxel in patients with non-small cell lung cancer. Our study results suggest that the use of EGFR-TKI after first-line treatment may be associated with an improvement in overall survival. (Cancer Sci 2007; 98: 226–230)

L ung cancer is the malignant tumor with the highest mortality rates in the world.[1] Approximately 80% of all lung cancer cases are non-small cell lung cancer (NSCLC) and patients with postoperative recurrence or advanced NSCLC may be treated with systemic chemotherapy. Platinum-based chemotherapy is widely used as first-line treatment. Various combination regimens are available – the Four-Arm Cooperative Study (FACS) conducted in Japan between October 2000 and June 2002 did not demonstrate any superiority of three experimental platinum-based regimens (cisplatin/gemcitabine, cisplatin/vinorelbine and carboplatin/paclitaxel) compared with the reference arm of cisplatin/irinotecan.[2,3] However, due to its good tolerability, ease of use and experience in Western countries, carboplatin/paclitaxel is currently the standard first-line chemotherapy for NSCLC in Japan.

Docetaxel has been widely used as second-line therapy for NSCLC in Japan. However, since its approval in July 2002, the use of gefitinib (IRESSA), an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), has been increasing each year. Erlotinib, another EGFR-TKI, which is approved in a number of Western markets has also been used in clinical registration trials in some Japanese medical institutions. Gefitinib was the first molecular targeted agent to be approved for

the treatment of NSCLC in Japan. Two international cooperative Phase II studies (IRESSA Dose Evaluation in Advanced Lung Cancer Trial: IDEAL1 and 2) demonstrated efficacy (response rates, 12.0–18.9%) and favorable tolerability of gefitinib in the treatment of NSCLC after failure of platinum-based chemotherapy.[4,5] Furthermore, the results of subset analyses of IDEAL1 indicated that the patient characteristics of Japanese nationality, female gender and adenocarcinoma histology were associated with longer overall survival (OS).[4]

In a placebo-controlled Phase III study (BR21) erlotinib significantly prolonged OS compared with placebo in patients with previously treated NSCLC.[6] A similar Phase III study (IRESSA Survival Evaluation in Lung Cancer [ISEL]) of gefitinib in refractory, advanced NSCLC showed an improvement in survival compared with placebo in the overall study population, which did not reach statistical significance.[7] However, in a subset analysis, statistically significantly longer survival was demonstrated in patients of Asian origin and in patients who had never smoked.[7] With the availability of new second-line anti-cancer agents such as gefitinib and erlotinib, it is necessary to consider more fully the influence of second-line treatment on evaluation of OS following standard first-line treatment. Since the opening of our department in October 2002, carboplatin/paclitaxel has been used as the standard first-line therapy for NSCLC, while the use of gefitinib as second-line therapy is increasing each year. In this study we performed retrospective analyses of data from patients who had received carboplatin/paclitaxel, in order to identify prognostic variables affecting OS and progession-free survival (PFS), and also to determine the contribution of second-line and subsequent treatment to prolongation of OS.

Patients and Methods

Patients. This retrospective study recruited patients with NSCLC who had received chemotherapy at the Thoracic Oncology Division, Shizuoka Cancer Center, Japan, between October 2002 and September 2005. Patients met all of the following criteria:[1] clinical stage IIIB or IV;[2] patients were administered carboplatin area under the curve (AUC) 6 + paclitaxel 200 mg/m² as first-line chemotherapy; and[3] performance status (PS) 0 or 1.

Target patients were identified in our electronically controlled clinical database and the following information extracted from their data:[1] patient demographics at the start of first-line chemotherapy (age, gender, smoking history, histology, stage);[2] objective tumor response;[3] time to disease progression;[4] OS;

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Review Article

Genes Regulating the Sensitivity of Solid Tumor Cell Lines to Cytotoxic Agents: A Literature Review

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In order to review gene alterations associated with drug responses *in vitro* to identify candidate genes for predictive chemosensitivity testing, we selected from literature genes fulfilling at least one of the following criteria for the definition of '*in vitro* chemosensitivity associated gene': (i) alterations of the gene can be identified in human solid tumor cell lines exhibiting drug-induced resistance; (ii) transfection of the gene induces drug resistance; (iii) down-regulation of the gene increases the drug sensitivity. We then performed Medline searches for papers on the association between gene alterations of the selected genes and chemosensitivity of cancer cell lines, using the name of the gene as a keyword. A total of 80 genes were identified, which were categorized according to the protein encoded by them as follows: transporters ($n = 15$), drug targets ($n = 8$), target-associated proteins ($n = 7$), intracellular detoxifiers ($n = 7$), DNA repair proteins ($n = 10$), DNA damage recognition proteins ($n = 2$), cell cycle regulators ($n = 6$), mitogenic and survival signal regulators ($n = 7$), transcription factors ($n = 4$), cell adhesion-mediated drug resistance protein ($n = 1$), and apoptosis regulators ($n = 13$). The association between the gene alterations and chemosensitivity of cancer cell lines was evaluated in 50 studies for 35 genes. The genes for which the association above was shown in two or more studies were those encoding the major vault protein, thymidylate synthetase, glutathione S-transferase pi, metallothionein, tumor suppressor p53, and bcl-2. We conclude that a total of 80 *in vitro* chemosensitivity associated genes identified in the literature are potential candidates for clinical predictive chemosensitivity testing.

Key words: chemotherapy — sensitivity — drug resistance — solid tumor

INTRODUCTION

Malignant neoplastic diseases remain one of the leading causes of death around the world despite extensive basic research and clinical trials. Advanced solid tumors, which account for most malignant tumors, still remain essentially incurable. For example, 80% of patients with non-small cell lung cancer have distant metastases either at the time of the initial diagnosis itself or at the time of recurrence after

surgery for the primary tumor. Systemic chemotherapy against malignant tumors remains of limited efficacy in spite of the development in the recent past of several new chemotherapeutic agents; therefore, patients with distant metastases rarely live for long (1).

Tumor response to chemotherapy varies from patient to patient, and clinical objective response rates to standard chemotherapeutic regimens have been reported to be in the range of 20–40% for most common solid tumors. Thus, it would be of great benefit it became possible to predict chemosensitivity of various tumors even prior to therapy. DNA, RNA and protein-based chemosensitivity tests have

been performed in an attempt to predict the clinical drug response, but the precise gene alterations that might be predictive of the chemosensitivity of the tumors are still unknown. Here we aimed to review the gene alterations that may be associated with the drug response *in vitro* (*in vitro* chemosensitivity associated genes) in order to identify candidate genes for predictive chemosensitivity testing in the clinical setting. The association between these gene alterations and clinical chemosensitivity in lung cancer patients has been reported elsewhere (2).

METHODS

In vitro chemosensitivity associated genes were identified from the medical literature as described previously (2). Briefly, we conducted a Medline search for papers on tumor drug resistance published between 2001 and 2003. This search yielded 112 papers, including several review articles. Manual search of these papers led to identification of 134 genes or gene families that were potentially involved in drug resistance based on their function. We conducted a second Medline search for *in vitro* studies of the 134 genes or gene families using the name of the gene as a keyword. Genes

that fulfilled at least one of the following criteria for the definition of *in vitro* chemosensitivity associated gene were selected from the 134 genes: (i) alterations of the gene can be identified in a human solid tumor cell lines exhibiting drug-induced resistance: (ii) transfection of the gene induces drug resistance: (iii) down-regulation of the gene or of the protein encoded by it increases the drug sensitivity. For this last category, we included studies in which the gene expression or function was suppressed by antisense RNA, hammerhead ribozyme, or antibody against the gene product. Finally, a Medline search for papers on the association between gene alterations and chemosensitivity of solid tumor cell lines was performed using the name of the gene as a keyword. Papers in which the association was evaluated in 20 or more cell lines were included in this study. The name of each gene was standardized according to the Human Gene Nomenclature Database of National Center for Biotechnology Information (NCBI).

RESULTS

Of the 134 genes or gene families, gene alterations were found in cells exhibiting drug-induced resistance, transfection of the gene increased or decreased the drug resistance,

Table 1. Transporters and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
ABCA2	U	—	S	Estramustine	—	1
ABCB1	U	R	S	DOX, PTX, VCR, VBL	Yes (lung, DOX)	2–11
					No (lung, DOX)	12
ABCB11	--	R	--	PTX	--	13
ABCC1	U	R	S	CPT, DOX, ETP, MTX, VCR	Yes (lung, CDDP, DOX)	11,14–21
					No (lung, PTX)	22
ABCC2	U	R	S	CDDP, DOX, MTX, VCR	No (lung, DOX)	18, 21, 23–25
ABCC3	NC, U	R	—	ETP, MTX	Yes (lung, DOX)	21, 25–28
ABCC4	NC, U	NC, R	--	MTX	No (lung, DOX)	12, 25, 29–31
ABCC5	NC, U	NC	--	DOX, MIT	Yes (lung, ETP)	12, 25, 31–34
ABCG2	M, U	R	—	DOX, MIT, MTX, SN38, TOP	–	35–43
MVP	U	—	NC	DOX	Yes (brain, CDDP, DOX)	44–47
					Yes (lung, DOX)	10
ATP7A	U	–	--	CDDP	–	48
ATP7B	U	R	--	CDDP	--	48–52
SLC29A1	U	--	--	5-FU	No (NCI-panel)	52, 53
SLC28A1	–	S	—	5'-DFUR	No (NCI-panel)	53, 54
SLC19A1	D	S	--	MTX	Yes (NCI-panel)	55–58

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; M, mutated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive.
Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; ETP, etoposide; MIT, mitoxantrone; MTX, methotrexate; PTX, paclitaxel; SN38, irinotecan metabolite; TOP, topotecan; VBL, vinblastine; 5-FU, 5-fluoruracil; 5'-DFUR, 5'-deoxy-5-fluorouridine, capecitabine metabolite.

Table 2. Drug targets, the associated proteins, and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
TUBB	IEC, M	—	—	PTX	—	59–63
TUBB4	U	—	S	PTX	Yes (NCI-panel, PTX)	59, 60, 63–66
TUBA	IEC, M	R	—	PTX	—	64, 67, 68
TYMS	U	R	S	5-FU	Yes (renal cell, 5-FU)	69–74
					No (NCI-panel, 5-FU)	75
					Yes (lung, DOX)	10
TOP1	M	R*	—	CPT	—	76–84
TOP2A	M, D	—	—	ETP, DOX	No (lung, DOX)	10, 82–91
TOP2B	D	—	—	ETP	—	86, 87
DHFR	M, U	R*	—	MTX	—	92–96
MAP4	—	S	—	PTX	—	97
MAP7	—	S	—	PTX	—	98
STMN1	U	R	—	PTX	—	99, 100
KIF5B	—	R	R	ETP, PTX	—	101, 102
HSPA5	—	R	—	ETP	—	103
PSMD14	—	R	—	CDDP, DOX, VBL	—	104
FPGS	D	—	—	5-FU	—	105

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; IEC, isoform expression change; M, mutated; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; ETP, etoposide; MTX, methotrexate; PTX, paclitaxel; VBL, vinblastine; 5-FU, 5-fluorouracil.
*Over-expression of the mutant gene.

and down-regulation of the gene altered the drug sensitivity for 45, 57 and 32 genes, respectively, and a total of 80 genes fulfilled the criteria for the definition of an 'in vitro chemosensitivity associated gene'. The genes were categorized according to the protein encoded by them as follows: transporters ($n = 15$, Table 1), drug targets ($n = 8$, Table 2), target-associated proteins ($n = 7$, Table 2), intracellular detoxifiers ($n = 7$, Table 3), DNA repair proteins ($n = 10$,

Table 3. Intracellular detoxifiers and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
GSTP1	U	—	S	CDDP, DOX, ETP	Yes (lung, DOX)	10, 106, 107
					Yes (NCI-panel)	108
GPX	—	R, NC	—	DOX	Yes (lung, CDDP)	109–112
GCLC	U	R	S	CDDP, DOX, ETP	Yes (NCI-panel)	106, 108, 113–121
GGT2	U	R	—	CDDP, OXP	—	114, 117, 122, 123
MT	U, NC	R	—	CDDP	Yes (urinary tract, CDDP)	118, 124–130
					Yes (lung, DOX)	10, 131
RRM2	U	R	—	5-FU, GEM, HU	—	71, 132–134
AKR1B1	U	—	—	DNR	—	135

Alterations in drug-induced resistance cells (DIRC): NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive. Drugs: CDDP, cisplatin; DNR, daunorubicin; DOX, doxorubicin; ETP, etoposide; GEM, gemcitabine; HU, hydroxyurea; OXP, oxaliplatin; 5-FU, 5-fluorouracil.

Table 4. DNA damage recognition and repair proteins and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
HMGB1	U	–	–	CDDP	–	136
HMGB2	--	S	–	CDDP	--	137
ERCC1	U	R	S	CDDP	–	138--140
XPA	U	R	–	CDDP	No (NCI-panel)	141--143
XPD	--	R	...	CDDP	Yes (NCI-panel)	142--144
MSH2	D, NC	–	...	CDDP	–	145, 146
MLH1	D, NC	–	--	CDDP	–	145--147
PMS2	D, NC	--	--	CDDP	--	146, 147
APEX1	--	R	...	BLM	--	148
MGMT	–	R	S	CPM, ACNU	Yes (lung, DOX)	10, 149--152
BRCA1	U	S	R	PTX	--	153--155
GLO1	--	R	...	DOX	...	156

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea; BLM, bleomycin; CDDP, cisplatin; DOX, doxorubicin; PTX, paclitaxel.

Table 4), DNA damage recognition proteins ($n = 2$, Table 4), cell cycle regulators ($n = 6$, Table 5), mitogenic and survival signal regulators ($n = 7$, Table 6), transcription factors ($n = 4$, Table 6), cell adhesion-mediated drug resistance protein ($n = 1$, Table 6), and apoptosis regulators ($n = 13$, Table 7).

The association between the gene alterations and *in vitro* chemosensitivity was evaluated in one study for 25 genes, in two studies for seven genes, in three studies for two genes, and in five studies for one gene, and in a total of 50 studies for 35 genes (Table 8). Significant association was found between chemosensitivity and alterations of genes encoding transporters, drug targets and intracellular detoxifiers (Table 8). Genes for which such association was shown in

two or more studies were those encoding the major vault protein/lung resistance-related protein (*MVP*) (Table 1), thymidylate synthetase (*TYMS*) (Table 2), glutathione S-transferase pi (*GSTP1*), metallothioncin (*MT*) (Table 3), tumor suppressor protein p53 (*TP53*), and B-cell CLL/ lymphoma 2 (*BCL2*) (Table 7).

DISCUSSION

We identified a total of 80 *in vitro* chemosensitivity associated genes. These genes have been the subject of considerable research, and of numerous scientific publications. In addition, we may also have to expect the existence of many other genes associated with chemosensitivity

Table 5. Cell cycle regulators and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
RB1	--	R	..	DOX	Yes (lung, DOX)	157--159
					No (lung, CDDP, DOX)	160
CML		S	---	MMC, PTX	Yes (lung, CDDP)	161--163
CDKN1A	U	R, S	S	CDDP, BCNU, PTX	...	164--171
CCND1	–	R, S	S	CDDP, MTX, PTX	No (lung, DOX)	10, 172--176
CDKN2A	---	S, R	---	CDDP, 5-FU, PTX, TOP	Yes (brain, 5-FU)	177--184
CDKN1B	--	R	–	DOX	---	185

Alterations in drug-induced resistance cells (DIRC): U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: BCNU, carmustine; CDDP, cisplatin; DOX, doxorubicin; MMC, mitomycin C; MTX, methotrexate; PTX, paclitaxel; TOP, topotecan; 5-FU, 5-fluorouracil.

Table 6. Mitogenic and survival signal regulators, integrins, transcription factors and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
ERBB2	–	R, NC	S	CDDP, PTX	Yes (lung, DOX)	10, 22, 186–191
EGFR	–	R	–	DOX	No (lung, CDDP, DOX, PTX)	10, 22, 112, 192
KRAS2	–	R*	–	CDDP	–	193
HRAS	–	R*, NC	–	Ara-C, DOX, PTX	No (lung, DOX)	10, 193–197
RAF1	–	R	–	DOX	–	198
AKT1	–	NC, R	S	CDDP, DOX, PTX	–	199–201
AKT2	–	R	S	CDDP	–	200, 202
ITGB1	–	–	S	ETP, PTX	–	203, 204
JUN	–	R	–	CDDP	No (lung, DOX)	10, 205
FOS	U	R	S	CDDP	No (lung, DOX)	10, 206–208
MYC	NC, U	S, R	R, S, NC	CDDP, DOX	No (lung, DOX)	10, 209–216
NFKB1	U	–	S	5-FU, DOX, ETP	–	217–222

Alterations in drug-induced resistance cells (DIRC): NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs)): NC, no change; R, resistant; S, sensitive. Drugs: Ara-C, 1-beta-D-arabinofuranosylcytosine; CDDP, cisplatin; DOX, doxorubicin; ETP, etoposide; PTX, paclitaxel; 5-FU, 5-fluorouracil.
*Up-regulated with mutated K-ras gene.

Table 7. Apoptosis regulators and in vitro evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
TP53	–	S, R*	R, S	CDDP, DOX	Yes (brain)	223–229
					Yes (NCI-panel)	230
					No (breast, DOX)	231
					No (breast, DOX, PTX)	232
					No (lung, PTX)	22
MDM2	–	S, R	S	CDDP, DOX, PTX	–	169, 233–238
TP73	–	–	R	CDDP, ETP	–	239, 240
BCL2	U, D	R	–	CDDP, CPT, DOX	Yes (breast, DOX)	164, 198, 231, 241–244
					Yes (lung, PTX)	22
					No (breast, DOX)	232
BCL2L1	NC	R	S	CDDP, PTX	–	243–251
MCL1	–	–	S	DTIC	–	252
BAX	NC	S	R	CDDP, ETP, 5-FU	No (breast, DOX)	231, 244, 253–260
					No (lung, PTX)	22
BIRC4	–	NC	S	PTX	–	261, 262
BIRC5	–	R	S	CDDP, ETP	–	263–265
TNFRSF6	NC	–	S	CDDP	Yes (lung, DOX)	10, 242
CASP3	–	S	–	CDDP, DOX, ETP	No (lung, DOX)	10, 266–268
CASP8	–	–	R	CDDP	–	261
HSPB1	C	R	S	DOX	–	52, 269–273

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive. Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; DTIC, dacarbazine; ETP, etoposide; PTX, paclitaxel; 5-FU, 5-fluorouracil.
*Resistant in mutant *TP53* over-expressed cells.

Table 8. Gene categories and association with in vitro chemosensitivity

Category	No. of genes	Total no. of studies	No. of studies showing association (%)
Transporter	15	13	7 (54)
Drug target	8	5	3 (69)
Target associated protein	7	0	0 (0)
Intracellular detoxifier	7	6	6 (100)
DNA repair	10	3	2 (67)
DNA damage recognition protein	2	0	0 (0)
Cell cycle	6	5	3 (60)
Mitogenic signal	5	3	1 (33)
Survival signal	2	0	0 (0)
Transcription factor	4	3	0 (0)
Cell adhesion-mediated drug resistance protein	1	0	0 (0)
Apoptosis	13	12	5 (42)
Total	80	50	22 (44)

but not selected in the current study, because they have never caught the scientific eye for some reasons. Thus, the results of this study may be significantly influenced by publication bias. Nonetheless, we do believe that these genes have been selected reasonably carefully, and that they may be helpful for establishing a clinical predictive chemosensitivity test.

While the association between alterations of the 80 genes and the chemosensitivity of various cell lines was evaluated in 50 studies, significant association was observed in only 22 (44%) (Table 8). The cellular functions of a gene vary among cell types and experimental conditions. The evaluation of the gene functions, however, was conducted under only limited cellular contexts in these studies, as expected. Thus, for example, the conditions of a gene transfection experiment may differ from those of an experiment to evaluate the chemosensitivity for many cell lines. The gene functions may not necessarily be examined under all possible conditions, but the evaluation must be conducted under conditions similar to those in the clinical setting in order to develop clinical chemosensitivity testing using these genes.

The other possibility for the poor correlation to *in vitro* chemosensitivity may be that more than one gene alterations are involved in the chemosensitivity of tumors. This may be discussed from the standpoint of the signal transduction pathway and from the cellular standpoint. From the standpoint of the signal transduction pathway, more than one gene may be involved in the reaction to a cytotoxic agent. One of the best examples is cooperation of *TP53* with another

member of the p53 family, p73 (*TP73*), in the response to both DNA damage and chemosensitivity (3,4). From the cellular standpoint, several pathways may work additively, antagonistically, or complementally in determining the chemosensitivity of the cell. This can be understood well from the context of induction and inhibition of apoptosis being controlled by pro-apoptotic and anti-apoptotic pathways. Thus, it would be important to study several pathways at the same time, or to evaluate the net effect of the involvement of various pathways.

Complex factors influencing the cellular chemosensitivity may be operative on a tumor *in vivo*, in such a way that the tumor may exhibit highly heterogeneous gene alterations; that the tumor cells may interact with various host cells, including immune cells, fibroblasts and vascular endothelial cells; and that the differences in the distance between each tumor cell and blood vessels may affect the exposure level of tumor cells to a drug. No systematic approach has been developed to include this complex interplay of factors in the study of cellular chemosensitivity, although studies on cell adhesion-mediated drug resistance may be partly helpful.

Among the six genes for which the association was shown in two or more *in vitro* studies, four encode classical drug resistance proteins which are known to inhibit the drug–target interaction. These proteins are relatively specific for the drug as well as the cell type; e.g. *TYMS* is critical for 5-fluorouracil sensitivity. Thus, *TYMS* is a good candidate for chemosensitivity testing in patients with colorectal cancer who are treated with 5-fluorouracil (Table 2). *MVP* is involved in the transport of doxorubicin, therefore, it would be of interest to examine the association between the expression of *MVP* and the drug response in patients with breast cancer; the association of *MVP* with chemosensitivity has been evaluated only for brain tumor and lung cancer cell lines, to date (Table 1). However, the remaining two of the six genes, *TP53* and *BCL2*, are associated with apoptosis, and therefore may be relatively cell-type specific. Since all the three *in vitro* studies using breast cancer cell lines failed to show any associations between alterations of these genes and the chemosensitivity, the association should be evaluated in other tumor types in the clinical setting (Table 7).

The recently developed cDNA microarray technique allows analysis of the mRNA expression of more than 20 000 genes at once, and as many as 100–400 genes have been statistically shown as potentail chemosensitivity-related genes in various studies (5–7). The 80 genes in the current study were selected theoretically based on their known functions, and their contribution to *in vitro* chemosensitivity was shown in the experiments. Thus, it would be of interest to evaluate the expression profiles of these genes by cDNA microarray analysis, even if the difference in expression between sensitive and resistant cell lines does not reach statistical significance.

In conclusion, 80 *in vitro* chemosensitivity associated genes were identified from a review of the literature, which

may be considered to be future candidates for clinical predictive chemosensitivity testing.

Acknowledgments

Conflict of interest statement

None declared.

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Genomic profiling of malignant pleural mesothelioma with array-based comparative genomic hybridization shows frequent non-random chromosomal alteration regions including *JUN* amplification on 1p32

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Genome-wide array-based comparative genomic hybridization analysis of malignant pleural mesotheliomas (MPM) was carried out to identify regions that display DNA copy number alterations. Seventeen primary tumors and nine cell lines derived from 22 individuals were studied, some of them originating from the same patients. Regions of genomic aberrations observed in >20% of individuals were 1q, 5p, 7p, 8q24 and 20p with gains, and 1p36.33, 1p36.1, 1p21.3, 3p21.3, 4q22, 4q34-qter, 6q25, 9p21.3, 10p, 13q33.2, 14q32.13, 18q and 22q with losses. Two regions at 1p32.1 and 11q22 showed a high copy gain. The 1p32.1 region contained a protooncogene, *JUN*, and we further demonstrated overexpression of *JUN* with real-time polymerase chain reaction analysis. As MPM cell lines did not overexpress *JUN*, our findings suggested that induction of *JUN* expression was involved in the development of MPM cells *in vivo*, which also might result in gene amplification in a subset of MPM. Meanwhile, the most frequent alteration was the 9p21.3 deletion, which includes the *p16^{INK4a}/p14^{ARF}* locus. With polymerase chain reaction analysis, we determined the extent of the homozygous deletion regions of the *p16^{INK4a}/p14^{ARF}* locus in MPM cell lines, which indicated that the deletion regions varied among cell lines. Our results with array comparative genomic hybridization analysis provide new insights into the genetic background of MPM, and also give some clues to develop a new molecular target therapy for MPM. (*Cancer Sci* 2007; 98: 438–446)

M PM, a highly lethal neoplasm of the serosal lining of the pleural cavity, is thought to develop from superficial mesothelial cells.[1] In up to 80% of patients, MPM occurs within about 30 years of exposure to asbestos.[2–4] The incidence of MPM is expected to increase dramatically over the next few decades. It has been estimated that 250 000 people will die of MPM in Europe in the next three decades, and 2500–3000 new cases are diagnosed each year in the USA.[5,6] In Japan, a recent report has shown that there will be approximately 100 000 deaths due to MPM in the next 40 years using an age-cohort model.[7] Survival of patients with MPM is very poor, with a median survival of 7–11 months after diagnosis, especially in advanced-stage patients, regardless of a recent advancement in chemotherapeutic modalities that combines cisplatin and antifolate.[8–10]

The long latency period between asbestos exposure and tumor development implies that multiple, and likely diverse, genetic changes are required for the malignant transformation of

mesothelial cells. Many studies have been conducted to determine underlying key genetic and epigenetic events responsible for the development of MPM, some of which may be directly caused by asbestos fibers. Traditional karyotype analysis using primary samples or cell lines uncovered multiple non-random chromosomal abnormalities that are frequently detected in most human MPM specimens, which include chromosomes 1p, 3p, 6q, 9p and 22q.[11–18] Subsequent studies of such common regions with allele loss, which indicate the sites of TSG, have identified the target genes of MPM, including *p16^{INK4a}/p14^{ARF}* on chromosome 9p21 and *NF2* at 22q. The *p16^{INK4a}/p14^{ARF}* gene, one of the most frequently mutated TSG of human malignancies, has been shown to be inactivated in ~90% of MPM, with most cases being targeted by homozygous deletion.[19,20] The *NF2* gene at the 22q12 locus, which is responsible for a familiar cancer syndrome of neurofibromatosis type II, has also been shown to be inactivated in 40–50% of MPM, mainly with nonsense mutation or homozygous deletion.[21,22] In contrast, the *p53* gene, another of the most frequently mutated TSG in human malignancies, is only occasionally mutated in MPM, with approximately 25% of MPM specimens being inactivated.[23,24] Meanwhile, MPM does not show frequent mutation of known protooncogenes including *KRAS*, *NRAS* and *EGFR*.[25–28] Thus, it has been suggested that there are other yet unidentified TSG or protooncogenes responsible for the development of MPM. Recently, a CGH technique introduced to search for additional genes that are potentially involved in MPM biology has identified other regions with alterations, including 1q, 4q, 5p, 6p, 7p, 8p, 8q, 10p13-pter, 13q, 14q, 15q, 17p12-pter, 17q and 20.[29–34]

In the present study, we carried out array CGH analysis with 17 resected MPM samples (from 16 patients) and nine MPM cell lines from a total of 22 individuals. We confirmed the same chromosomal alterations as described before in the literature and further identified new regions such as 8q24 and 13q33.2. We also identified high copy gain at 1p32, which includes the *JUN* protooncogene. The present study provides new insights

Table 1. Summary of malignant pleural mesotheliomas analyzed with array comparative genomic hybridization (CGH)

KD number[†]	Sex	Subtype	Asbestos exposure	Cell line	p16^{INK4a}/p14^{ARF}[‡]	NF2[§]	JUN
332	Male	Epithelioid	+		HL	(+)	No Amp
355	Male	Epithelioid	−			(+)	No Amp
471	Male	Epithelioid	Unknown		HL	(+)	No Amp
476	Male	Biphasic	−	Y-MESO-8 A, -8D	HD	+	No Amp
905	Male	Epithelioid	Unknown		HL	del(533–537)	No Amp
977	Male	Epithelioid	Unknown			(+)	No Amp
1032	Male	Biphasic	+			(+)	No Amp
1033	Male	Epithelioid	+			(+)	Amp
1038	Male	Epithelioid	+			(+)	No Amp
1039	Male	Duciduoid	+			(+)	Amp
1041	Male	Duciduoid	+		L	(+)	Amp
1043	Female	Epithelioid	+			del(468–479)	No Amp
1044	Male	Epithelioid	−		L	(+)	No Amp
1045	Male	Epithelioid	−		L	(+)	No Amp
1046	Male	Biphasic	+		L	(+)	No Amp
1048	Male	Epithelioid	+	Y-MESO-9	HD	del(527–528)	No Amp
1049	Male	Epithelioid	+			(+)	No Amp
	Female	Epithelioid	−	Y-MESO-12	HD	+	No Amp
	Female	Epithelioid	−	ACC-MESO-1	HD	Q389X	No Amp
	Male	Epithelioid	+	ACC-MESO-4	HD	+	No Amp
	Male	Unknown	Unknown	NCI-H28	HD	[+]	No Amp
	Male	Unknown	Unknown	NCI-H2052	HD	R341X	No Amp
	Unknown	Unknown	Unknown	MSTO-211H	HD	[+]	No Amp

†KD Number indicates primary tumors available for array CGH analysis. Two primary tumors were obtained from the same patient at surgical resection (KD1039) and autopsy (KD1041). ‡p16^{INK4a}/p14^{ARF} status was indicated as follows: HL, high-level loss; L, loss; HD, homozygous deletion (detected in cell lines). § +, No point mutation was detected with PCR sequencing analysis of exons 1–17 covering the entire open reading frame of NF2, and homozygous deletion was not detected in the corresponding cell line; (+), no point mutation was detected in exons 1–17, but homozygous deletion was not determined due to possible contamination of non-cancerous DNA; [+], undetectable point mutation for exons 2, 5, 7, 8, 9, 10, 11 and 12. Data of p16^{INK4a}/p14^{ARF} and/or NF2 of Y-MESO-8A, Y-MESO-8D, ACC-MESO-1, ACC-MESO-4, NCI-H28, H2052 and MSTO-211H referred to Sekido et al. and Usami et al.[21,28] Amp, amplification.

into the genetic background of MPM, and also gives some clues to developing a new molecular target therapy for MPM.

Materials and Methods

Cell lines and tumor specimens. Twelve MPM cell lines and one non-malignant mesothelial cell line (MeT-5A) were used. ACC-MESO-1, ACC-MESO-4, Y-MESO-8A, Y-MESO-8D, Y-MESO-9 and Y-MESO-12 were established in our laboratory,[28] whereas NCI-H28 (CRL-5820), NCI-H2052 (CRL-5915), NCI-H2373 (CRL-5943), MSTO-211H (CRL-2081) and MeT-5 A (CRL-9444) were purchased from the American Type Culture Collection (Rockville, MD, USA). NCI-H290 and NCI-H513 were gifts from Dr Adi F. Gazdar. All MPM cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1× antibiotic–antimycotic (Invitrogen) at 37°C in a humidified incubator with 5% CO_2. MeT-5 A was cultured according to the instructions of the American Type Culture Collection. Nineteen MPM samples from 18 Japanese patients were obtained at Aichi Cancer Center Hospital, Nagoya University Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Second Red Cross Hospital and Kasugai City Hospital (KD332, KD355, KD471, KD476, KD905, KD977, KD1032, KD1033, KD1038, KD1039, KD1041, KD1042, KD1043, KD1044, KD1045, KD1046, KD1048, KD1049 and KD1050; of these, KD1039 and KD1041 originated from the same patient at surgery and autopsy, respectively). MPM samples and clinical data were collected after obtaining appropriate institutional review board approval and written informed consent from all patients. To confirm that there was no cross-contamination of clinical samples and cell lines, the uniqueness or identity of MPM tissues and established

cell lines were evaluated by analysis of STR polymorphisms using the AmpFLSTR Identifier Kit (Applied Biosystems, Foster City, CA, USA), including the 16 STR loci D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, Amelogenin, D5S818, and FGA. Primary tumors and cell lines used in the present study are summarized in Table 1.

Preparation of DNA and RNA. Genomic DNA was extracted using a standard phenol–chloroform method.[35] Normal DNA was prepared from peripheral blood of healthy male donors and non-cancerous lung tissue of the patients. Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. DNase treatment was carried out on columns during RNA purification using an RNase-Free DNase Set (Qiagen, Germantown, MD, USA). Random-primed, first-strand cDNA was synthesized from 2 μg total RNA using Superscript II according to the manufacturer's instructions (Invitrogen).

Genome-wide array-based CGH. A genome-wide scanning array with 2304 BAC and PAC clones covering the whole human genome at a resolution of roughly 1.3 Mb was used as described previously.[36] In brief, clones were isolated from bacterial cultures containing the requisite antibiotics and extracted using a Plasmid Mini-kit (Qiagen). The location of all clones used for the array CGH was confirmed by standard fluorescence in situ hybridization analysis. BAC and PAC clones were amplified using degenerate oligonucleotide-primed PCR and spotted on glass slides. DNA preparation from cells, labeling, hybridization and scanning analysis were carried out as described previously[37] with minor modifications.[36,38,39] The data obtained were processed to detect chromosomal imbalances as described.[40]

Southern blot analysis. Genomic DNA from patient samples (7 μg) was digested with EcoRI restriction enzyme, electrophoresed,
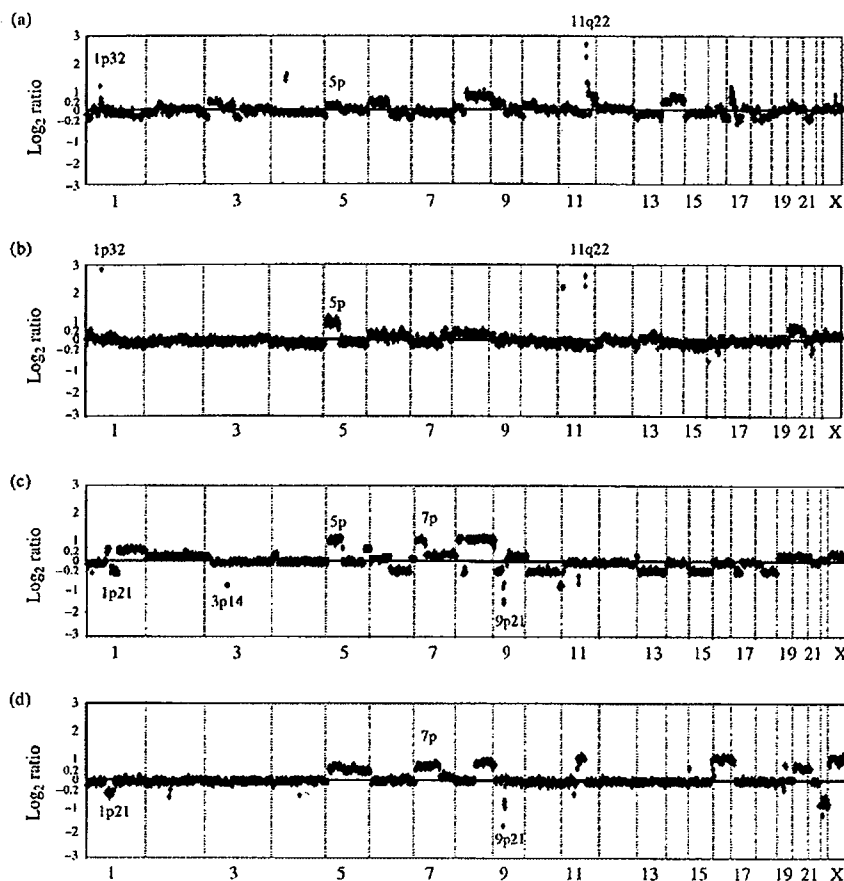
Fig. 1. Array comparative genomic hybridization profile of malignant pleural mesothelioma from three primary tumors and one cell line. Log₂ ratios are plotted for all clones based on chromosome position, with vertical dotted lines showing separation of the chromosome. Clones are ordered from chromosomes 1–22 and X within each chromosome on the basis of the Sanger Center Mapping Position, July 2004 version. (a) KD1033 sample shows chromosomal gain of 1p32.1-p32.3, 2p16, 3p22.2-pter, 3p12, 4q12, 5p, 6pter-q14.1, 8q, 9p, 10p, 11q22.1-q22.3, 11q23.3-qter, 14, 17p12-pter and 20p11.21-p12, and loss of 1p36.13-pter, 1q32-q42, 2q37.1-qter, 3q11-q13.31, 4q34.3-qter, 6q14.3-q21, 6q25-qter, 7q35-qter, 9q34.12-qter, 13q12.11-q13.3, 13q34, 16q23-qter, 17q11.2-qter, 18p, 18q12.2-qter and 21qcen-q22.2. (b) KD1041 primary sample shows chromosomal gain of 1p36.13-p36.32, 1p32.1, 5p, 6p22-pter, 6p12-p21.1, 8, 11p15.2-p15.3, 11q22.1, 20, 22q12-q13.2 and X, and loss of 3p21.31, 4q, 5q35.1-qter, 9p21.3, 11q23-qter, 13q12, 13q33.2, 15q22.3-qter, 16p13.2, 16q11-q12.2 and 21q22. (c) KD471 primary sample shows chromosomal gain of 1p22.2-p31.1, 1q, 2, 4p15-pter, 5p, 5q33.1-qter, 7, 8p21.1-pter, 8q, 9q, 12q24, 19 and 20, and loss of 1p36.31-p36.33, 1p36.13, 1p12-p22.1, 3p14.3-p21.31, 6q14-q25.1, 8p12-p21.1, 9p21.2-pter, 10, 11q12.1, 13, 15, 17p and 18q. (d) Y-MESO-12 cell line shows chromosomal gain of 5, 7pter-q21.3, 8q21-qter, 11qcen-q14.3, 15q11, 16, 19q13.2 and 20, and loss of 1p21-p31.1, 2p11, 4q22.1, 9p21.3, 11p12, 19p13.11 and 22.

and transferred to Hybond N+ (Amersham Biosciences, Piscataway, NJ, USA). Hybridization and washing were carried out using standard techniques.[35] The DNA probes were made by RT-PCR using normal lung cDNA. RT-PCR of *JUN* and *β-actin* were carried out using the primer sets: C-jun-S1, 5′-GACCTTATGGCT-ACAGTAACCC-3′ (sense) and C-jun-AS1, 5′-CTGCTCATCTG-TCACGTTCT-3′ (antisense); and B-Actin-S, 5′-CTGTGGCAT-CCACGAAACTA-3′ (sense) and B-Actin-AS, 5′-AGGAAAGACA-CCCACCTTGA-3′ (antisense).

Quantitative real-time PCR. The reaction mixture for real-time PCR using first-strand cDNA contained TaqMan universal PCR Master Mix (Applied Biosystems) and 200 nM of each primer, *JUN* (Hs 00277190_s1; Applied Biosystems) and *FOS* (Hs 00170630_m1). All real-time PCR assays were done in MicroAmp optical 96-well reaction plates on an ABI PRISM 7900 Sequence Detector System (Applied Biosystems) according to the manufacturer's instructions. For normalization between samples, PCR amplification of *GAPDH* (Hs 00266705_g1; Applied Biosystems) was included for each sample at each run. Fluorescence measurements and melting curve analyses were carried out using SDS 2.1 software (Applied Biosystems). The relative quantification of gene expression was computed using the comparative threshold cycle method with a mathematical formula described previously, and results are shown as a fold induction of mRNA.[41] We classified them into high-level expresser of *JUN* or *FOS* (defined as >0.15 of *JUN* or *FOS* mRNA expression relative to *GAPDH* mRNA expression), middle-level expresser (defined as >0.025 but <0.15), and low-level expresser (defined as <0.025).

Deletion mapping of 9p21. Information on 16 microsatellite markers and one sequence-tagged site marker at 9p21 was searched, and their sequences were obtained from the Human Genome Database (GDB) and the Ensembl Genome Browser. Three primer sets for exons 1, 2 and 3 of *p16^{INK4a}* were as described previously.[28]

and the primer set of exon 1β of *p14^{ARF}* was p14ARF-F, 5′-CACCTCTGGTGCAAAGGGC-3′ (sense) and p14ARF-R, 5′-CCTAGCCTGGGCTAGAGACG-3′ (antisense).

Mutation analysis of *NF2*. Mutation analysis of *NF2* was carried out by direct sequencing after PCR amplification of genomic DNA. Seventeen primer sets covering the entire coding region of *NF2* were described previously.[28]

Results

Genomic profiles and data analysis of MPM. Array CGH analysis was carried out using genomic DNA samples extracted from 19 MPM primary tumors and nine MPM cell lines (ACC-MESO-1, ACC-MESO-4, Y-MESO-8A, Y-MESO-8D, Y-MESO-9, Y-MESO-12, NCI-H28, NCI-H290 and MSTO-211H). Among 19 primary tumors, we did not detect any significant genomic alterations in two tumors, which was probably due to much contamination of genomic DNA from non-malignant cells, and we excluded these tumors for further analysis. Of the 26 MPM analyzed successfully, there were paired samples from the same individuals: the Y-MESO-8 A and Y-MESO-8D cell lines were established from the KD476 primary tumor, Y-MESO-9 was established from KD1048, and the other two primary tumors (KD1039 and KD1041) were obtained from the same patient at surgical resection and autopsy, respectively. Thus a total of 22 individual MPM were studied (Table 1). All of the clones on chromosome X were analyzed separately because of sex mismatching. Copy number changes were detected at high-resolution for genomes as a whole for primary tumor samples as well as cell lines. We defined regions of gain or amplification as log₂ ratio > +0.2, and regions suggestive of heterozygous loss or deletion as log₂ ratio < −0.2. Figure 1 shows representative data of the entire genomic profiles of three MPM primary tumors and one cell
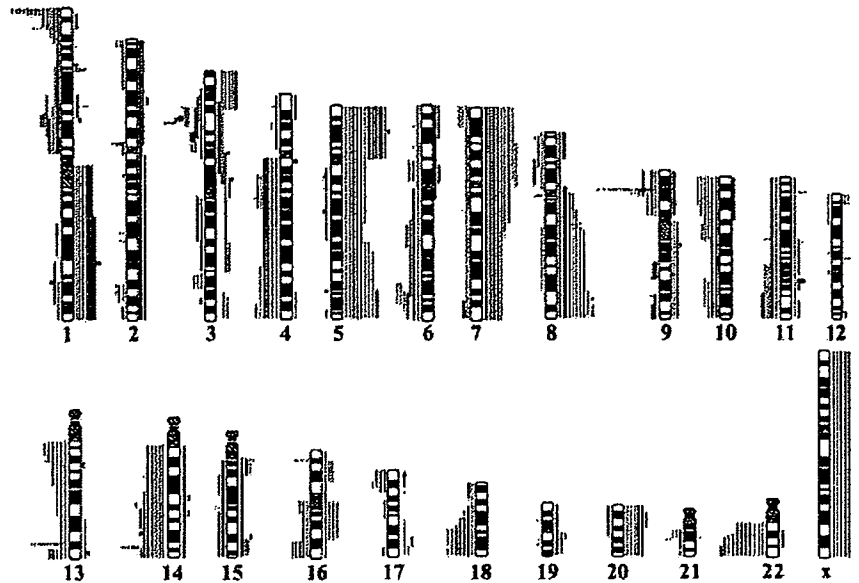
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Fig. 2. Summary of chromosome imbalance detected in 17 malignant pleural mesothelioma patients (black lines) and nine cell lines (red lines). Regions of loss and gain are shown by vertical lines on the left (loss) and right (gain) sides of each ideogram. Regions of high-level amplification are presented by thick lines.

line from different individuals, with some shared altered regions being detected. For example, KD1033 (Fig. 1a) and KD1041 (Fig. 1b) showed shared regions including gain of 1p32.1, 5p, 8q, 11q22.1 and 20p and loss of 13q12 and 21q22. Figure 2 is a summary of chromosome imbalance detected in 17 MPM samples (black lines) and nine cell lines (red lines). Regions of high-level gain or amplification (defined as $\log_2$ ratio > +1.0) and those of homozygous loss or deletion (defined as $\log_2$ ratio < −1.0) are presented by thick lines. A summary of frequent chromosomal regions of gain and loss, and those of high-level copy gain or amplification, or homozygous loss or deletion detected in 17 MPM samples and nine cell lines is presented in Table 2. We also found that paired samples shared many chromosomal imbalances, although there were several different regions of gains and losses, or regions with relatively weak signals especially in the primary samples. The weak signals were thought to be due to contamination of non-malignant cell DNA (data not shown). Recurrent chromosomal imbalances found in at least six samples consisted of gain on chromosomes 1q (eight tumors/ seven individuals), 5p (12/11), 7p (9/8), 8q24 (9/9), 20p (6/6) and loss on chromosomes 1p36.33 (13/13), 1p36.1 (7/7), 1p21.3 (7/6), 3p21.3 (10/8), 4q22 (7/6), 4q34-qter (6/6), 6q25 (7/6), 9p21.3 (16/16), 10p (6/5), 13q33.2 (11/9), 14q32.13 (13/11), 18q (7/6) and 22q (10/8).

High-level gain at 1p32.1 includes *JUN* protooncogene amplification. The array CGH analysis of 26 MPM revealed that 1p32.1 and 11q22.1 were two distinct regions with high-level gains, which were detected in at least two individual samples (Table 2). Interestingly, these high-level gains were observed simultaneously in the two individuals of KD1033 (Fig. 1a) and KD1041 (Fig. 1b). Another sample, KD1039, was also detected for 1p32.1 amplification (data not shown), and KD1039 and KD1041 were derived from the same patient, with the former at the initial surgical resection and the latter at autopsy. Whereas the KD1033 primary tumor showed a larger gain of five consecutive clones at 1p32.1 including the RP11-63G10 clone, KD1039 showed only a gain of the RP11-63G10 clone but not of the neighboring clones, and KD1041 showed only a gain of the two clones RP11-63G10 and RP11-363E22, with RP11-363E22 located toward the centromeric direction from RP11-63G10 1.9 MB apart (data not shown). Thus, the gain of RP11-63G10 seemed to be a very specific, common genetic event for these MPM, and this BAC clone was found to contain the protooncogene *JUN* (Table 2).

Because previous studies have suggested that asbestos fibers induce *JUN* expression in rat pleural mesothelial cells,[42] we studied the *JUN* status of MPM cells in further detail. We carried out Southern blot analysis with nine primary tumors and nine cell lines, and confirmed *JUN* high-level amplification in the three samples but not in the remaining 15 samples (Fig. 3a). To determine whether these MPM overexpress the transcripts of *JUN*, we carried out quantitative real-time PCR with 11 MPM samples available for RNA analysis together with seven MPM cell lines and one non-malignant mesothelial cell line, MeT-5 A. We found that KD1041, with high-level amplification of *JUN*, overexpressed mRNA of *JUN* (Fig. 3b). Interestingly, we noticed that there seemed to be three groups with distinct levels of *JUN* expression. We classified MPM into three groups according to the levels of *JUN* expression: high-level expresser (defined as >0.15) for three tumors (KD977, KD1041 and KD1044), middle-level expresser (defined as 0.025 < *JUN* < 0.15) for eight tumors (KD1032, KD1033, KD1045, KD1046, KD1048, KD1049, ACC-MESO-4 and H290), and low-level expresser (defined as <0.025) for seven tumors (KD471, KD476, ACC-MESO-1, Y-MESO-8A, Y-MESO-8D, H28 and MSTO-211H) and MeT-5 A. Among the seven MPM cell lines, ACC-MESO-4 and H290 were classified into middle-level expresser and the remaining five into low-level expressers. We also studied the *FOS* expression to determine whether *JUN* coexpresses with *FOS* in MPM cells (Fig. 3c). Most of the MPM cells classified into either high- or middle-level expresser of *JUN* simultaneously expressed *FOS* equal or greater than 0.025, and most expressers of both genes were primary tumors.

Alterations of *p16^{INK4a}/p14^{ARF}* at 9p21.3 and *NF2* at 22q12.2. We found frequent deletions of RP11-149I2 located at 9p21.3 in seven MPM samples and nine MPM cell lines, with five samples (two primary tumors and three cell lines) showing high-level loss. This BAC clone included *p16^{INK4a}/p14^{ARF}*, which is one of the most frequently mutated TSG in human malignancies, and we showed previously that *p16^{INK4a}/p14^{ARF}* was deleted in all MPM cell lines studied.[28] To determine whether the 9p21 deletion region in MPM extends further beyond the *p16^{INK4a}/p14^{ARF}* gene locus, which may indicate another target TSG of MPM in this region, we further carried out PCR analysis using multiple primer sets for comparison with locations of BAC and PAC clones on 9p21. Besides the nine MPM cell lines, another three MPM cell lines (NCI-H290, NCI-H513 and NCI-H2373) were also studied.

Table 2. Chromosomal regions with frequent imbalances or high copy gain or loss detected in malignant pleural mesothelioma

Alteration	Chromosomal region	No. patients (n = 17)	No. cell lines (n = 9)	No. individuals (n = 22)	Gene[†]	BAC/PAC[‡]
Gain						
	1p32.1[§]	3	0	2	JUN	RP11-63G10
	1q	4	4	7		
	5p	8	4	11	CDH10	RP11-116O11
	7p	5	4	8		
	8q24	4	5	9	MYC	RP1-80K22
	11q22.1[§]	2	0	2	IAP	RP11-864G5
	20p	3	3	6		
Loss						
	1p36.33	12	1	13	KIT	RP11-181G12
	1p36.1	4	3	7	NM_018125	RP11-473A10
	1p21.3	2	5	6	RPL5	RP4-716F6
	3p21.3	7	3	8	PFKFB4	RP5-1034C16
	4q22	2	5	6	TMSL3	RP11-309H6
	4q34-qter	3	3	6	Q9P2F5	RP11-739P1
	6q25	3	4	6	PLEKHG1	RP11-291C6
	9p21.3[§]	7	9	16	$p16^{INK4a}/p14^{ARF}$	RP11-14912
	10p	2	4	5		
	13q33.2	7	4	9	DAOA	RP11-166E2
	14q32.13	8	5	11	CHGA/ITPK1	RP11-862G15
	18q	4	3	6	MALT1	RP11-4G8
	22q	7	3	8	NF2	RP1-76B20

[†]Representative genes are listed at each region when bacterial artificial chromosome (BAC) and P-1-derived artificial chromosome (PAC) clones of continuously ordered gain or loss of maximum overlapped clones were less than 10, when known protooncogenes or tumor suppressor genes shown to be involved in human malignancies were located, or when only a few genes were located in this region. [‡]A representative BAC/PAC clone was listed when continuously ordered gain or loss of maximum overlapped region was less than 10 clones, and the clone at the mid-point of the overlapped region was chosen. [§]High copy gain or loss was observed.

After we confirmed homozygous deletions of exons 1, 2 and 3 of the $p16^{INK4a}$ gene and exon 1β of the $p14^{ARF}$ gene in all 12 (100%) MPM cell lines except MSTO-211H, which showed a partial retention of the gene, we used 16 microsatellite markers and one sequence site-tagged marker for the analysis (Fig. 4). For the telomeric direction, the INF-α cluster of genes was homozygously deleted in two cell lines but not in the remaining 10. For the centromeric direction, two cell lines (NCI-H290 and H2052) showed a larger deletion with consecutive losses at markers including D9S259, suggesting that these two cell lines had at least 4 Mb homozygous deletion. Meanwhile, four cell lines (Y-MESO-8A, -8D, NCI-H28 and H513) had a smaller homozygous deletion that was limited within D9S1749 and D9S790, suggesting that the maximum deletion size was less than 482 kb.

Finally, we studied any point mutations of the NF2 gene in 17 primary tumors. After sequencing 17 exons covering the entire coding region of NF2, we found that three tumors had small deletions, all of which resulted in a frameshift mutation (Table 1). Because genomic DNA extracted from snap-frozen primary tumor tissues was used for the analysis, the existence of homozygous deletion was not determined due to possible contamination of non-cancerous DNA.

Discussion

In the present study, we analyzed 17 MPM primary tumors and nine MPM cell lines using array CGH and identified regions of genomic gain and loss. Regions of genomic aberrations observed in >20% of individuals were 1q, 5p, 7p, 8q24 and 20p with gains, and 1p36.33, 1p36.1, 1p21.3, 3p21.3, 4q22, 4q34-qter, 6q25, 9p21.3, 10p, 13q33.2, 14q32.13, 18q and 22q with losses. We confirmed the same chromosomal alterations as reported earlier by other groups and further identified high gain or amplification regions including 1p32, which harbors the JUN

protooncogene. To our knowledge, our present study provides the first detailed array CGH data on chromosomal imbalances in MPM patient tumors and cell lines.

Traditional allelotyping and karyotype analyses revealed non-random chromosomal abnormalities including 1p, 3p, 4p15.1-p15.3, 4q25-q26, 4q33-q34, 6q, 9p, 14q11.1-q12, 14q23-q24 and 22q.[11-18,43,44] Subsequently, chromosomal CGH (also known as conventional CGH) has been carried out to detect more detailed abnormalities in MPM (Table 3). For example, Krismann et al. showed a total of 77 cases of MPM in the main histological subtypes (epithelioid type, sarcomatoid type and biphasic type) using chromosomal CGH.[34] They reviewed common gains at the chromosomal regions of 1q23/1q32, 7p14-p15, 8q22-q23 and 15q22-q25, and common losses at the chromosomal regions of 1p21, 3p21, 4p12-p13, 4q31-q32, 6q22, 9p21, 10p13-pter, 13q13-q14, 14q12-q24, 17p12-pter and 22q in all subtypes. In the present study with array CGH analysis, we also detected similar aberrations of multiple loci that have been found in previous studies.[29-35] These regions include gains of 1p32, 1q and 7p, and losses of 1p21, 9p21 and 22q. In addition to these regions, we have identified new regions such as 8q24 and 13q33.2, which had not been detected with chromosomal CGH analysis. The gain of 8q24 locus was detected by array CGH in nine cases (nine individuals) of these 26 samples. A single BAC, RP1-80K22, which includes the known protooncogene MYC, was located at the overlapped regions of 8q24 amplification. As a previous study showed a significant increase in signal strength of MYC in the mesothelioma tissues from an experimental animal model, compared with basal expression in non-neoplastic mesothelial cells, our findings also support the importance of MYC alteration in the development of MPM.[45]

Previous reports of chromosomal CGH analysis of MPM samples identified the region of gain at 1p32, although a specific candidate target gene was not referred to in detail.[34,46] Using array CGH, we found that a single BAC clone, RP11-63G10,
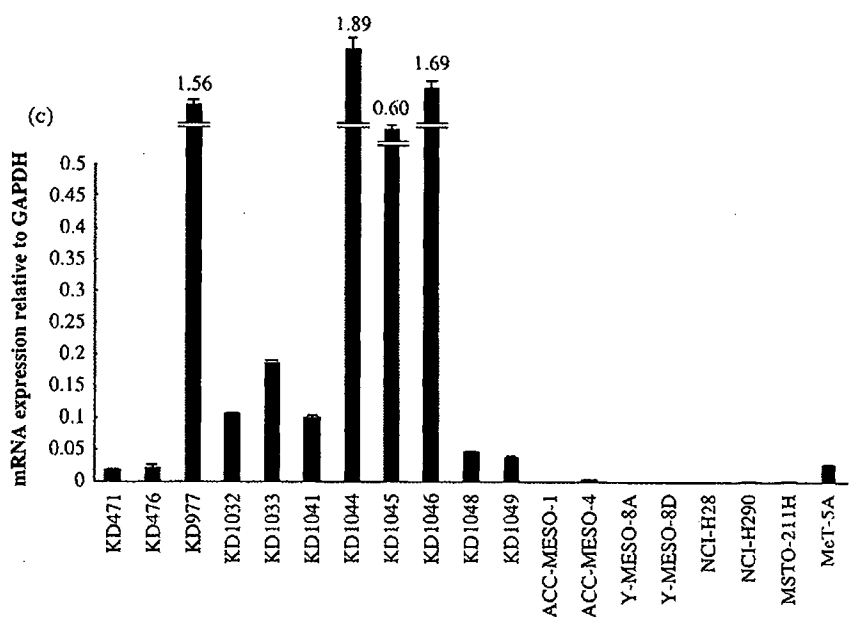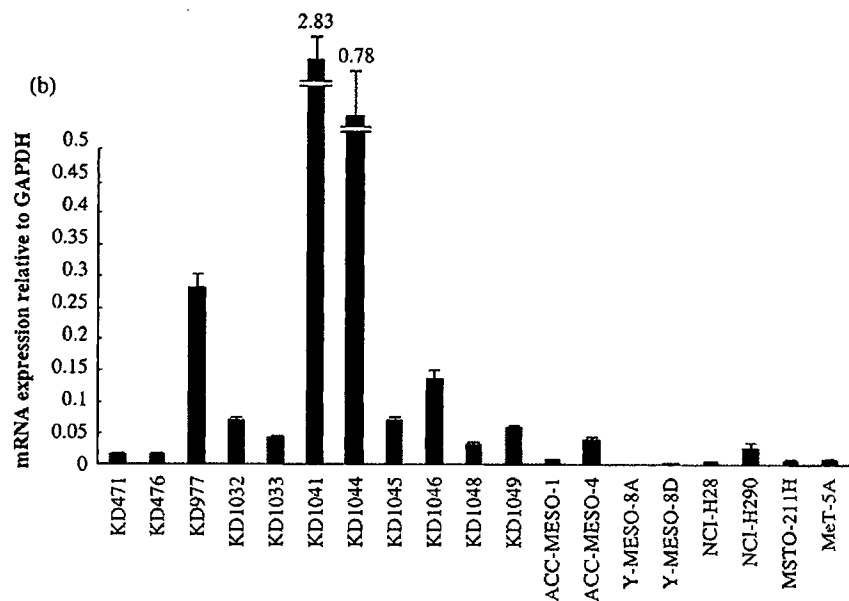
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Fig. 3. *JUN* amplification at 1p32.1 and expression of *JUN* and *FOS* messages in malignant pleural mesothelioma. (a) Southern blot analysis of *JUN*. Each lane was loaded with 7 μg genomic DNA from MPM samples. Southern blot shows high-level amplification of *JUN* in KD1039 and KD1041 and low-level amplification in KD1033. (b,c) Diagrammatic presentation of quantitative real-time polymerase chain reaction data for (b) JUN and (c) FOS mRNA from 11 primary samples, seven MPM cell lines and MeT-5 A. The results were averages of at least three independent experiments with error bars showing standard deviations. MPM were classified into three groups of JUN status expression: high-level expresser (defined as >0.15) for three tumors (KD977, KD1041 and KD1044), middle-level expresser (defined as 0.025 < *JUN* < 0.15) for eight tumors (KD1032, KD1033, KD1045, KD1046, KD1048, KD1049, ACC-MESO-4 and H290), and low-level expresser (defined as <0.025) for the remaining seven tumors and MeT-5A. MPM were also classified into three groups according to *FOS* expression status: high-level expresser (defined as >0.15) for five tumors (KD977, KD1033, KD1044, KD1045 and KD1046), middle-level expresser (defined as 0.025 < *FOS* < 0.15) for four tumors (KD1032, KD1041, KD1048 and KD1049) and MeT-5 A, and low-level expresser (defined as <0.025) for the remaining nine tumors.

detected the region of gain at 1p32.1 in three tumors from two individuals. The RP11-63G10 clone was the only clone that showed overlapping at this region, and harbored only one known gene, the *JUN* protooncogene. Whereas KD1033 showed relatively wide-range amplification including five consecutive clones, KD1039 and KD1041 showed only RP11-63G10 amplification or with another neighbor clone for the latter (data not shown). It is noteworthy that KD1039 and KD1041 were from the same patient at surgical resection and autopsy, respectively, but the ranges of amplification of the *JUN* locus were slightly different. Furthermore, except for 1p32, these two samples also

showed distinct regions of chromosomal alteration for each locus, including a gain at 13q34 for KD1039, and gains at 11p15.2 and 11q22.1 and a loss at 13q33.2 for KD1041 (data not shown). Although we confirmed the identity of these two samples with 16 STR repeats, it remains unclear whether the KD1041 cells originated from a subclonal cancer cell population that existed in the KD1039 tumor and acquired another chromosomal alteration during propagation.

JUN is a transcription factor and functions through homo-dimerization or heterodimerization with FOS to form the transcription factor AP-1, which can bind to the promoter region of
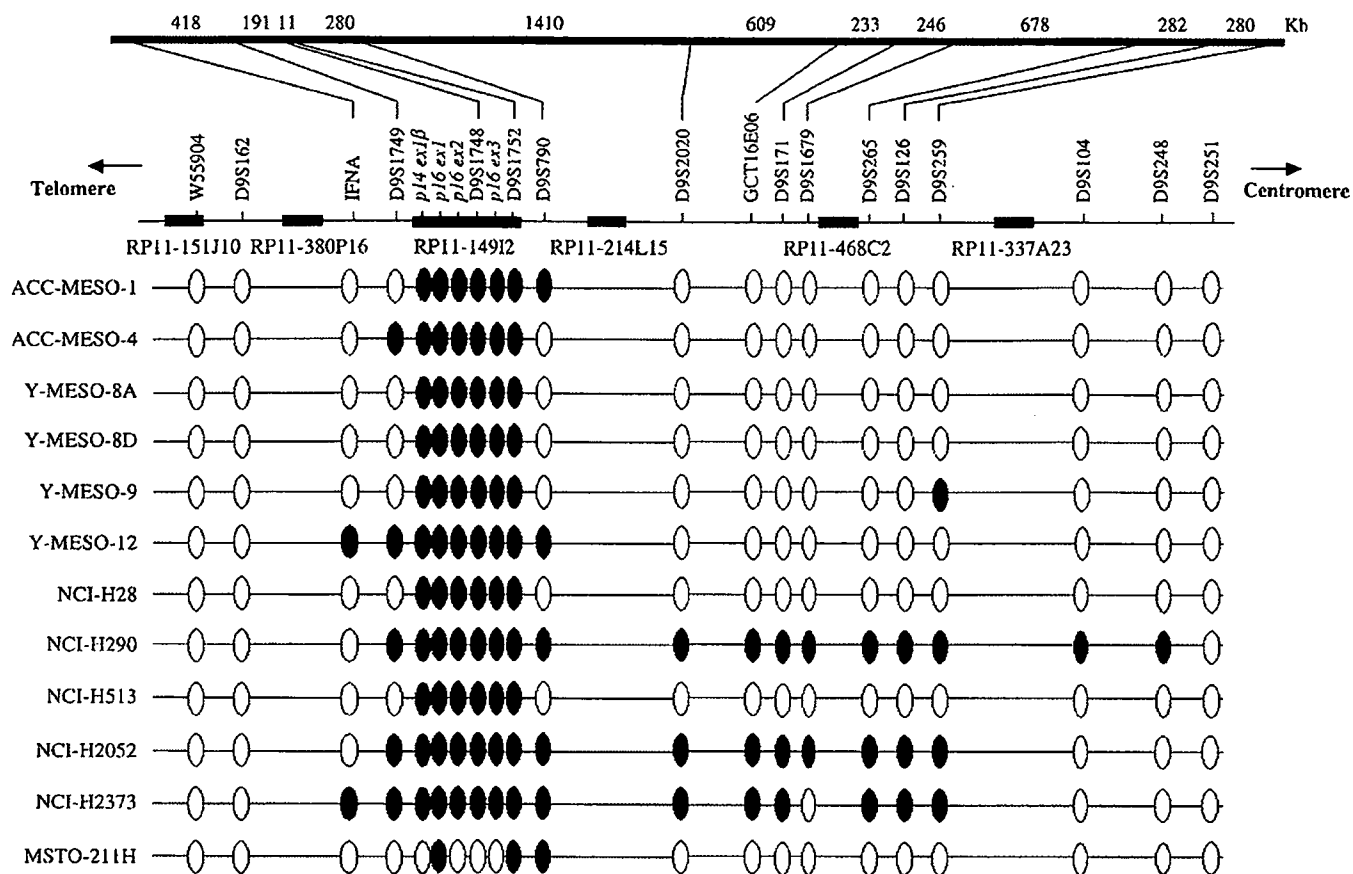
Fig. 4. Homozygous deletion map of the 9p21 region in 12 malignant pleural mesothelioma cell lines. Results of polymerase chain reaction analysis for each locus are shown by open ovals (retention) and closed ovals (homozygous deletion). Locations of genes and markers are according to those of the GDB Human Genome Database and Ensembl Genome Browser. Top bar shows the sizes between the selected markers proportionally: W55904 – (570 kb) – D9S162–(1.71 Mb) – IFNA – (418 kb) – D9S1749 – (191 kb) – D9S1748–(11 kb) – D9S1752 – (280 kb) – D9S790 – (1.41Mb) – D9S2020 – (609 kb) – GCT16E06 – (233 kb) – D9S171 – (246 kb) – D9S1679–(678 kb) – D9S265–(282 kb) – D9S126 – (280 kb) – D9S259 – (2.75 Mb) – D9S104 – (1.15 Mb) – D9S248 – (898 kb) – D9S251.

Table 3. Chromosomal regions with frequent imbalances shown in malignant pleural mesotheliomas from previous reports using chromosomal comparative genomic hybridization (CGH), and the current study using genome-wide array-based CGH

Authors	Year	Samples	Frequent gains	Frequent losses
Kivipensas et al.	1996	11	5p, 6p, 8q, 15q, 17q, 20	1p, 8p, 14q, 22q
Bjorkqvist et al.	1997	27	1cen-qter	4q31.1-qter, 6q22-q24, 9p21-pter, 13, 14q24-qter, 22q13
Bjorkqvist et al.	1998	34	7p, 15q	4q, 6q, 14q
Balsara et al.	1999	24	5p	1p12-p22, 6q25-qter, 9p21, 13q12-q14, 14q24-qter, 15q11.1-q15, 22q
Krismann et al.	2002	77	1q23/1q32, 7p14-p15, 8q22-q23, 15q22-q25	1p21, 3p21, 4p12-p13, 4q31-q32, 6q22, 9p21, 10p13-pter, 13q13-q14, 14q12-q24, 17p12-pter, 22q
Current study		26	1q, 5p, 7p, 8q24, 20p	1p36.33, 1p36.1, 1p21.3, 3p21.3, 4q22, 4q34-qter, 6q25, 9p21.3, 10p, 13q33.2, 14q32.13, 18q, 22q

intermediate genes involved in cell division and other cell functions.[47] Heintz et al. reported that both crocidolite and chrysotile asbestos caused increases in the expression of JUN and FOS in rat pleural mesothelial cells.[42] They demonstrated that, in contrast to phorbol 12-myristate 13-ester, which induced rapid and transient increases in JUN and FOS mRNA, asbestos caused 2–5-fold increases in JUN and FOS mRNA dose-dependently, which persisted for at least 24 h in mesothelial cells. They concluded that by activating the early response gene pathway, asbestos

may induce chronic cell proliferation that subsequently contributes to carcinogenesis in lung and pleura. Thus, our findings of JUN amplification and overexpression detected in MPM tumors is very intriguing, and we also found that three tumors with JUN amplification were from patients with high-grade asbestos exposure. Interestingly, five of seven MPM cell lines were classified into low-level expressers of JUN, compared with three high-level and six middle-level expressers of the 11 primary tumors. This finding suggests that primary MPM tumor cells are

continuously exposed to some stress to induce *JUN* transcription, and that *JUN* transcription is not necessarily induced in the established MPM cell line and MeT-5 A cells under usual tissue culture conditions, which may also indicate that the levels detected in MPM cell culture are of baseline *JUN* expression. Meanwhile, the analysis of *FOS* expression revealed that it was expressed simultaneously with *JUN* in most MPM cases, with high levels of expression of both genes detected mainly in the primary tumors, but not in cell cultures. These findings suggest the possibility that some surgical manipulations cause artificial induction of some genes, including early response genes,[48] which leads to the observation of predominant expression of these genes in the primary tumors. Nevertheless, because gene amplification of *JUN* was indeed identified in three MPM tumors, we think that there were some strong and persistent factors for *JUN* activation during the development of the MPM tumor cells.

JUN has been shown to be induced by other factors such as hypoxia. A recent immunohistochemical analysis detected expression of hypoxia-inducible factor 1α at focal regions in most MPM tumors but not in mesothelial cells, suggesting that hypoxic stress exists in primary MPM tumors.[49] Although the mechanisms and causes of amplification of genes such as *MYC* family members remain poorly understood, amplification of several other genes has been implicated as being induced by carcinogens and other stresses, such as amplification of the dihydrofolate reductase gene via methotrexate treatment.[50] Thus, we speculate that the chronic induction of *JUN* expression might have been induced by multiple stimuli, most importantly by asbestos fibers at the initial stage and possibly by hypoxia and other unidentified factors continuously, and that this might result in gene amplification of *JUN* in a subset of MPM cells during long latency.

Using array CGH, we found a region of loss at 9p21 in 16 tumors (16 individuals) that was covered by a single BAC clone, RP11-149I2, which included the $p16^{INK4a}/p14^{ARF}$ gene. It is well known that $p16^{INK4a}/p14^{ARF}$ is one of the most frequently deleted genes in many types of human cancers. Previous studies by other groups identified frequent alteration of $p16^{INK4a}/p14^{ARF}$ in most MPM, and we have also shown that $p16^{INK4a}/p14^{ARF}$ was deleted in all 10 MPM cell lines studied.[28] Although studies with simple PCR techniques reported homozygous deletion of $p16^{INK4a}/p14^{ARF}$ at a relatively lower frequency in MPM tissues than in cell lines, which may be due to contamination of a significant amount of normal stromal cells, we detected frequent deletion at

9p21.3 in seven MPM samples with array CGH. Furthermore, we determined the approximate lengths of deletion regions in 12 MPM cell lines, compared with the locations of DNA markers and BAC or PAC clones. We found that several cell lines showed a relatively small deletion with a maximum deletion size of 482 kb, whereas others showed at least a 4-Mb deletion size. Our findings of the $p16^{INK4a}/p14^{ARF}$ deletion in MPM seem consistent with other reports that the sizes of homozygous deletions vary individually in any given tissue type of malignancy.[51-53] Although it is very clear that $p16^{INK4a}/p14^{ARF}$ is the most important target TSG at the 9p21.3 region, other genes in this homozygous deletion region should also be studied to determine whether any of them play a role in the development of MPM.

Finally, the loss of 3p21.3 locus was detected by array CGH in 10 cases (eight individuals) of the 26 samples. One of the well-known TSG located at this region is *RASSF1A*, which is frequently inactivated by promoter hypermethylation in various types of human malignancies. The frequent hypermethylation of *RASSF1A* was also reported in MPM, which suggests that *RASSF1A* is a strong target TSG at 3p21 during the development of MPM.[54] Meanwhile, we also identified a homozygous deletion including *CTNNB1* (β-catenin) at 3p22.1 in the NCI-H28 cell line, and further demonstrated that the exogenously transfected *CTNNB1* gene inhibited the growth of NCI-H28 cells.[55,56] Thus, because several genes have been suggested as candidate TSG at the 3p21-22 region for various malignancies including MPM, further detailed analysis may be warranted to clarify the most important target TSG in this region for MPM.

To summarize, we subjected MPM samples to array CGH analysis and found genomic regions altered recurrently in MPM, including 1p32 *JUN* protooncogene amplification. Array CGH analysis can thus be expected to provide new insights into the genetic background of MPM and to offer some clues to developing a new molecular target therapy for this highly aggressive fatal tumor.

Acknowledgments

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Genotype-Based Methods for Anticipating Gemcitabine-Related Severe Toxicities May Lead to False-Negative Results

To the Editor: In their recently published clinical study, Sugiyama et al[1] investigated the effects of cytidine deaminase (CDA) genetic polymorphisms on gemcitabine toxicities and altered pharmacokinetics. They conclude, from the observation of a single Japanese patient with the nonsynonimous mutation 208G > A (Ala70Thr) and displaying an abnormal gemcitabine pharmacokinetic profile resulting in subsequent neutropenia, that haplotype *3 harboring the 208 G more than A single nucleotide polymorphism (SNP) could be associated with the occurrence of severe toxicities after gemcitabine administration, and possibly, in combination with other chemotherapy regimens. Such a patient with severe toxicities was actually, repeatedly selected out of a group of five,[2] and then 256[1] carcinoma patients for whom linkage disequilibrium and haplotype analyses were performed in relation to CDA activities, gemcitabine pharmacokinetics analyses, and toxicity monitoring. Little correlation was evidenced among the various diplotype groups, the pharmacokinetic parameters of gemcitabine, and the occurrence of severe toxicities, other than the *3/*3 diplotype recorded in the single patient. Surprisingly, little impact was also reported between CDA activities and gemcitabine exposure levels, an observation contradictory to the pharmacokinetics of this drug,[3] and no data on a possible relationship between CDA phenotypic status and gemcitabine-related toxicities was reported. Finally, although Sugiyama et al claimed that plasma CDA activities correlated well with the CDA genotypes, it was not clear by their data whether the difference was statistically significant, apart from the homozygous *3 carrier.

At our institute, we have phenotyped CDA activity and performed genetic screening, including of the 208G > A mutation reported by Sugiyama et al, in 80 cancer patients (70 white, nine African, and one Asian patient) treated with gemcitabine alone or as part of combinational therapies with platinum derivatives or capecitabine. Four (5%) of 80 patients displayed severe, hematologic toxicities (eg, higher than grade 3 by the National Cancer Institute Common Toxicity Criteria), including a lethal one.[4] We found that all four of these patients with severe toxicities had markedly lower CDA activities (mean deficiency, −75%) than those recorded in the 76 patients showing good gemcitabine tolerance. This observation strongly suggests that CDA downregulation was a culprit for increased toxicities with gemcitabine, including, for the first time, in the toxic-death case we reported. Conversely to what was reported by Sugiyama et al,

genotypic screening at our institute failed to identify genetic polymorphisms associated with the occurrence of toxicities, since for instance, none of our four toxic patients exhibited the 208G > A (Ala70Thr) mutation. This observation is fully consistent with other studies describing controversies regarding genotype-to-phenotype associations with CDA,[5,6] much likely due to the genetic and epigenetic regulations of the CDA gene that remain to be elucidated, and to the possible influence of ethnical origin in the relevance of particular single nucleotide polymorphisms.[7] Taken together, in total contradiction with the Sugiyama study, our own experience strongly suggests that genotypic approaches are probably insufficient to identify patients at risk of gemcitabine toxicity, with an elevated risk of precluding the right diagnostic. Conversely, phenotype-based methods seem to be a safer strategy for ensuring a better outcome in the handling of gemcitabine, a major drug used extensively in clinical oncology.

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In Reply: We appreciate the comments raised by Mercier et al and the opportunity to respond to them. We agree that the reduced intracellular CDA level is one of the major factors increasing gemcitabine-mediated toxicities. We also recognize that the genotyping based on CDA 208G>A (Ala70Thr) itself gives false-negative results with respect to the prediction of hematological toxicities (Table 7 in our article[1]), as is often the case with geno-

typing. Thus, phenotype-based methods are useful for identification of patients at a higher risk toward gemcitabine-mediated toxicities. However, as far as Japanese patients are concerned, the genetic method is fairly useful for predicting severe toxicities of gemcitabine because CDA 208G>A, a tagging SNP of haplotype CDA*3, is one of the factors that reduce CDA activity as clearly demonstrated by us.[1]

According to the letter by Mercier et al, four patients displayed severe hematologic toxicities (> grade 3) without any associations with CDA genotypes in their study. Their observations are quite reasonable from the following points: CDA 208G>A has not been detected in white people, and its allele frequency is relatively low in other populations (probably variable within African populations[2,3]; only nine Africans and one Asian were included in their study); all other genetic polymorphisms that we detected, including CDA 79A>C (*2, Lys27Gln),[4,5] failed to show any significant associations with altered pharmacokinetics and toxicities of gemcitabine and plasma CDA activity.[1] Therefore, we consider that, in white people, no validated genotype is currently available for predicting gemcitabine toxicities.

Mercier et al pointed out that little correlation was evident among the various diplotype groups, the pharmacokinetic parameters of gemcitabine, and the occurrence of severe toxicities, other than the *3/*3 diplotype recorded in the single patient. However, as presented in our article,[1] significant differences were observed between *3/*1 and *1/*1 for pharmacokinetic parameters (our Fig 2), and the incidences of grade ≥ 3 or grade 4 neutropenia in the combined chemotherapies with fluorouracil or platinum-containing drugs were mostly higher in the non-*3/*3 patients than in the non-*3/non-*3 patients (Table 7). Our Figures 3A (gemcitabine as a substrate) and 3B (cytidine as a substrate) show that when plasma CDA activities of the *3/*1 and *3/*2 patients were compared with those of the *1/*1 patients by Dunn's multiple comparison test, statistically significant differences were obtained ($P < .001$ and <0.05 for *3/*1 and *3/*2 groups, respectively, in Fig 3A; $P < .001$ for *3/*1 group in Fig 3B; P values were not provided in our report).[1]

In order to reply to the comments by Mercier et al, we reevaluated the association between grade 4 neutropenia and gemcitabine area under the curve (AUC) or CDA activity (one patient with an extremely high level was excluded) either for the monotherapy or the combined therapy (fluorouracil, carboplatin, or cisplatin) group by the Mann-Whitney test. The median values of AUC were higher in the grade 4 group than in the grade ≤ 3 group (Δ, +9% for the monotherapy; Δ, +30% for the combined therapy), and the median values of plasma CDA levels were lower in the grade 4 group than in the grade ≤ 3 group (Δ, −29% for the monotherapy; Δ, −40% for the combined therapy). Both the increase in AUC and decrease in plasma CDA activity observed in the grade 4 group who received the combined therapies were mainly attributable to the *3-bearing patients. Appropriate cutoff values could not be set for both AUC and plasma CDA activity to effectively screen grade 4 neutropenia since the median values of the two patient groups were not sufficiently different in our hands. Notably, these biomarkers successfully identified the patient who encountered life-threatening toxicities, because he had *3/*3 and showed extremely high AUC and low plasma CDA activity. As for the relationship between plasma CDA activities and AUC values (gemcitabine exposure levels), a moderate but statistically significant correlation was obtained ($r = -0.30$; $P = .0009$). It was reported that CDA released from damaged neutrophils diffuses into blood, and thus CDA activity in the blood is considered to be one of the markers of inflammatory diseases.[5] It must be noted that pretreatment neutro-

phil counts also showed a moderate correlation with CDA activity ($r = 0.37$; $P < .0001$; gemcitabine used as a substrate). Moreover, aging and sex influence on the pharmacokinetic parameters of gemcitabine.[1] Therefore, it is not surprising that very strong correlations were not obtained between plasma CDA activity and the pharmacokinetic parameters of gemcitabine.

Taken together, both predictive genotype (*3) and phenotype markers, gemcitabine AUC and plasma CDA activity, could predict grade 4 neutropenia, but with some false-negative cases and with increased false-positive cases for AUC and plasma CDA. At least, CDA 208G>A is a useful marker to predict gemcitabine toxicities in Japanese and probably East Asians.

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