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Anticancer drug clustering in lung cancer based on gene expression profiles and sensitivity database

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Abstract

background: The effect of current therapies in improving the survival of lung cancer patients remains far from satisfactory. It is consequently desirable to find more appropriate therapeutic opportunities based on informed insights. A molecular pharmacological analysis was undertaken to design an improved chemotherapeutic strategy for advanced lung cancer.

Methods: We related the cytotoxic activity of each of commonly used anti-cancer agents (docetaxel, paclitaxel, gemcitabine, vinorelbine, 5-FU, SN38, cisplatin (CDDP), and carboplatin (CBDCA)) to corresponding expression pattern in each of the cell lines using a modified NCI program.

Results: We performed gene expression analysis in lung cancer cell lines using cDNA filter and high-density oligonucleotide arrays. We also examined the sensitivity of these cell lines to these drugs via MTT assay. To obtain our reproducible gene-drug sensitivity correlation data, we separately analyzed two sets of lung cancer cell lines, namely 10 and 19. In our gene-drug correlation analyses, gemcitabine consistently belonged to an isolated cluster in a reproducible fashion. On the other hand, docetaxel, paclitaxel, 5-FU, SN-38, CBDCA and CDDP were gathered together into one large cluster.

Conclusion: These results suggest that chemotherapy regimens including gemcitabine should be evaluated in second-line chemotherapy in cases where the first-line chemotherapy did not include this drug. Gene expression-drug sensitivity correlations, as provided by the NCI program, may yield improved therapeutic options for treatment of specific tumor types.

Background

While various anti-cancer drugs have been developed, many patients with solid tumors still exhibit poor prognosis. Accordingly, it is now important to determine the appropriate use of such drugs clinically. With respect to treatment of lung cancer, there are many anti-cancer agents in use, such as cisplatin (CDDP), carboplatin (CBDCA), docetaxel, paclitaxel, vinorelbine, gemicitabine, 5-fluorouracil (5-FU), CPT-11, etc. A number of combination therapy regimens employing platinum compounds have proven to be effective[1] and are widely applied to initial treatment for unresected non-small cell lung cancer (NSCLC)[2]. In addition, docetaxel and pemetrexed have been reported to be effective in the context of second-line chemotherapy for NSCLC[3,4]. However, at present, the effect of these therapies on improving patient survival remains far from satisfactory [1-3]. It is consequently desirable to find more appropriate therapeutic opportunities based on informed insights. With the recent near-completion of the human genome sequence, genome-wide gene expression profiling through both cDNA and oligonucleotide arrays has been greatly facilitated [5-7]. There are many reports associated with isolation of molecules involved in drug sensitivity [8-10]. Of particular relevance was the use of DNA array-based methodology by the National Cancer Institute (NCI) to assess the gene expression profiles of 60 human cancer cell lines of diverse tissue origin (NCI60 set), with a view to determining associations with the extensive drug sensitivity data accumulated on this cell line cohort so far[11]. The NCI60 gene expression study was analogous in some respects to assessment of clinical tumors for markers that predict sensitivity to therapy. The essential aim of this study was to utilize similar advanced gene expression profiling technologies and drug sensitivity assays to aid in the selection of appropriate drug combinations for the treatment of lung cancer. We performed gene expression analysis in lung cancer cell lines using cDNA filter and high-density oligonucleotide arrays. We also examined the sensitivity of these cell lines to commonly used anti-cancer agents (docetaxel, paclitaxel, gemcitabine, vinorelbine, 5-FU, SN38, cisplatin (CDDP), and carboplatin (CBDCA)) via MTT assay. We related the cytotoxic activity of each of these agents to the corresponding expression pattern in each of the cell lines using a modified NCI program. To obtain our reproducible gene-drug sensitivity correlation data, we separately analyzed two sets of lung cancer cell lines, namely 10 and 19.

Methods

Clustering on the basis of drug activity and gene expression patterns

Cell lines

We analyzed the expression profiles and sensitivity to anti-cancer drugs of separate two sets of lung cancer cell lines. The first set consisted of PC9, PC7, PC14, A549, Lu65, LK2, H69, N231, Lu135, and SBC3 (Set 1). The second consisted of RERF-LC-KJ, RERF-LC-MS, RERF-LC-AI, PC1, PC3, PC6, PC10, Lu130, Lu139, Lu165, ABC-1, EBC-1, LC2/ad, LC1/sq, LC-1F, SQ5, QG56, MS-1, and SBC5 (Set 2). The PC1, PC3, PC6, PC7, PC9, PC10, PC14, and QG56 cell lines were obtained from IBL (Gumma, Japan). The A549, NCI-H69, and NCI-N231 cell lines were obtained from the American Type Culture Collection (Rockville, MD)[12]. The Lu65 and Lu135 cell lines were provided by Y. Shimosato and T. Terasaki (National Cancer Center Research Institute, Tokyo, Japan)[12]. The LK-2 and SBC-3 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). PC1, PC3, PC6, PC10, Lu130, Lu139, and Lu165 cell lines were provided by S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). RERF-LC-KJ, LC2/ad, SQ5, LC1/sq, LC-1F, RERF-LC-AI, and MS-1 cell lines were obtained from the RIKEN Cell Bank (Ibaraki, Japan). RERF-LC-MS, EBC-1, SBC5, and ABC-1 cell lines were purchased from the Health Science Research Resources Bank (Osaka, Japan). PC7, PC9, PC14, A549, Lu65, RERF-LC-KJ, RERF-LC-MS, PC3, ABC-1, and LC2/ad are adenocarcinoma cell lines. LK-2, RERF-LC-AI, PC1, PC10, EBC-1, LC1/sq, LC-1F, SQ5, and QG56 are squamous cell cancer cell lines. NCI-H69, NCI-N231, Lu135, SBC-3, PC6, Lu130, Lu139, Lu165, MS-1, and SBC5 are small cell lung cancer cell lines.

Assay for drug activity

Estimation of cytotoxicity in the above-mentioned cell types was mediated by a rapid colorimetric assay for mitochondrial dehydrogenase activity, as previously described[13]. Briefly, cells were seeded into 12-well plates (Falcon, Lincoln Park, NJ). Following 24 hr exposure to particular anti-cancer agents, the cells were washed twice and incubated for a further 24 hr in drug-free medium. Subsequently, the cells were incubated with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) for 4 hr. The blue formazan crystals, formed by viable cells, were solubilized by the addition of 10% n-dodecylsulfate sodium salt (SDS) in 0.01N HCl followed by overnight incubation. Samples were then subjected to spectrophotometric analysis at 560 nm (Ultraspec 4050; LKB, Bromma, Sweden).

RNA isolation, cDNA array hybridization and analysis of hybridization signals

Total RNA was isolated from each cell line using standard protocols described previously[14,15]. To avoid variations due to cell culture conditions, we cultured each untreated cell line separately in 6 different flasks. mRNA was then purified from total RNA by incubation with oligo-dT-magnetic beads (Toyobo Co., Osaka, Japan)[16]. The ElectorGene Array System (GeneticLab. Co., Ltd. Sap-

poro, Japan) was used for filter-based cDNA array analysis, as previously reported[16]. Thirteen hundred species of human DNA fragments are spotted in duplicate on a filter. The genes represented on this filter included cancer-related and drug resistance-associated genes, as well as housekeeping genes and non-mammalian genes as negative controls. To prepare the probes, reverse transcription was performed using Reverse Transcriptase, ReverTraAce (Toyobo Co., Osaka, Japan), together with a random 9 mer (Toyobo Co., Osaka, Japan) as the primer and 5 μg of polyA RNA. The probes were labelled with biotin by incorporation of biotin-16-deoxyuracil triphosphate (dUTP) during the synthesis of cDNA. The filters were pre-incubated in 20 ml of PerfectHyb (Toyobo Co., Osaka, Japan) at 68°C for 30 min. The biotin-labeled probes were denatured and added to the pre-hybridization solution. The filters were incubated overnight at 68°C in the hybridization mixture. After washing, specific signals on the filters were detected by the Imaging High – Chemilumi – Detection kit (Toyobo Co., Osaka, Japan). CDP-Star substrate (Tropix, Bedford, MA) was used as the chemiluminescence substrate. A chemiluminescence image of the filter was acquired by Fluor-S (Bio-Rad, Hercules, CA). The gene expression images were quantified by measuring the intensity of the signals using Imagene (BioDiscovery, Los Angeles, CA). The signal intensity among filters was analyzed by ElectorGene Finding System (GeneticLab, Sapporo, Japan). The background threshold was set at a level of 3-fold higher than the negative control. Signal intensities were normalized by comparison with the average values of all probe. We also performed high-density oligonucleotide array analysis using Affymetrix GeneChip technology (Affymetrix, Santa Clara, CA). This oligonucleotide microarray contains 22,282 transcripts (HG-U133A, Affymetrix, Santa Clara, CA). Total RNA was used to synthesize double-strand cDNA with ReverTraAce and a T7-(dT)24 primer (Metabion, Germany). Then, biotinylated cRNA was synthesized from the double-stranded cDNA using the RNA Transcript Labeling kit (Enzo Life Sciences, Farmingdale, NY) and was purified and fragmented. The fragmented cRNA was hybridized to the oligonucleotide microarray, which was washed and stained with streptavidine-phycoerythrin. Scanning was performed with an Agilent Microarray Scanner (Agilent Technologies, Palo Alto, CA). GeneChip analysis was performed based on the Affymetrix GeneChip Mannual (Affymetrix Inc., Santa Clara, CA) with Microarray Analysis Suite (MAS) 5.0, Data Mining Tool (DMT) 2.0, and Microarray Database software. The data we generated by GeneChip was deposited in Gene Expression Omnibus (GEO)(GEO accession: GSE4127)(17).

Data analysis

We performed data cleansing for filter arrays as follows. Firstly, the gene expression matrix [T] was scaled by using the average of all probe sets. Each of the filter arrays contained three spots of negative control (pUC), so we figured out their average signal value *M*. We defined 3 *M* as the threshold value, and transformed the numerical signal values < 3 M to "Nan" (not a number). After omitting the rows holding "Nan" more than one, we selected 600 genes for this analysis. Data analysis for the correlation coefficients that related the drug activity patterns to the expression patterns of the genes was principally performed by a modified NCI program[11]. The symbol [A] (GI_{50}) refers to the drug activity matrix in which the rows represent the anti-cancer drugs and the columns represent the human lung cancer cell lines. The symbol [T] (gene expression) refers to the gene expression matrix in which the rows represent individual genes and the columns represent the cell lines. In order to analyze the relationship between gene expression and drug activity, we generated the gene-drug correlation matrix [AT] (correlation coefficient) in which the rows represent the genes and the columns represent the drugs. Firstly, we subtracted its mean value from the matrix [A] in the direction of row and columns for a pre-treatment. Secondly, we normalized each element in the matrix [A] by subtracting its row-wise mean and dividing by its row-wise standard deviation; normalized [T] was generated in a similar way. Finally, we took the inner product of the matrix [A] and the transpose of the matrix [T]. The resulting matrix [AT] implied the Pearson correlation coefficients (※1) that reflected the relationship between drug activity and gene expression.

(※1) The Pearson correlation coefficient r is given by the formula

$$r = \frac{1}{n-1}\sum_{k=1}^{n}\left(\frac{A_k-\bar{A}}{\Phi_A}\right)\left(\frac{T_k-\bar{T}}{\Phi_T}\right)$$

$$= \frac{\frac{1}{n-1}\sum_{k=1}^{n}(A_k-\bar{A})(T_k-\bar{T})}{\sqrt{\frac{1}{n-1}\sum_{k=1}^{n}(A_k-\bar{A})^2}\sqrt{\frac{1}{n-1}\sum_{k=1}^{n}\left(T_k-\bar{T}\right)^2}}$$

Φ_X : the standard deviation of X

$\bar{X}$: the mean of X

Hierarchical clustering helps to comprehend a characteristic of huge volumes of data. With cluster analysis, the elements are divided into groups that show similar patterns by calculating the distances between their respective rows and columns. The AT-clustered image map (CIM), indicating the correlation coefficients between gene expres-

Table 1: Growth inhibitory activities (GI50)(μg/ml) of various anti-cancer agents against 10 human lung cancer cell lines – Set 1

Drug	PC7	PC9	PC14	A549	Lu65	LK-2	H69	N231	Lu135	SBC-3
CDDP	4.55	3.53	2.86	5.46	3.39	4.72	1.64	1.25	0.22	4.84
SN38	5.05	3.45	3.79	6.80	0.48	4.25	0.77	0.66	0.05	0.07
VIN	2.64	1.43	>10	>10	0.06	0.02	0.02	0.01	0.02	0.51
DOC	2.46	21.34	20.38	21.31	3.61	26.31	4.72	6.04	0.53	>50
GEM	<0.05	14.28	>500	>500	79.53	54.61	3.73	3.88	<0.05	<0.05
5FU	2.76	5.84	>10	>10	8.76	>10	>10	>10	5.68	0.51
CBDCA	95	70	>100	46.3	71	0.225	7.54	45	61	3.5
PAC	6.7	39	34	51	34	20	<0.001	7	5.5	0.5

sion and drug sensitivity in the 10 human lung cancer cell lines, was obtained by the linkage-average clustering method, also known as UPGMA (un-weighted pair-group method using arithmetic average). The statistical algorithms and the graphical outputs described here were implemented in MATLAB 6.5 Release 13 (the MathWorks, Inc., US).

Results

Clustering on the basis of drug activity and gene expression patterns

We used filter-based DNA arrays, representing 1,302 cancer-related and drug resistance-associated genes, and Affymetrix GeneChip technology to perform gene expression profile analysis of 10 human cancer cell lines. To avoid the influence of cell culture conditions, we separately cultured each cell line in 6 bottles[16]. The controls including GAPDH, β-actin genes, were located in duplicate at the outer line in the opposite angle. A standard curve was obtained by the calculation of serial diluted spots of GAPDH. The expression level of each gene was calculated by comparison with the internal standard. Drug sensitivity tests, namely by MTT analysis, were performed on the 10 lung cancer cell lines. Eight anti-cancer drugs currently used for lung cancer chemotherapy; docetaxel, paclitaxel, gemcitabine, vinorelbine, 5-FU, SN38, CDDP, and CBDCA, were selected for our analyses. Table 1 shows the growth inhibitory activities (GI50) levels of these anti-cancer agents against the lung cancer cell lines. We then analyzed the gene expression profiling data in relation to the activity profiles of the 8 drugs examined. The drugs were clustered on the basis of Pearson correlation coefficients that related their activity patterns across the 10 cell lines to the expression pattern of the genes in the cell lines[11]. The AT-matrix clustered image map (CIM) summarized the relationship between drug sensitivity and gene expression, as it allows the visualization of patterns of similarity in large sets of high-dimensional data (Fig. 1B)[16]. In this analysis, gemcitabine were located in separate clusters (Fig. 1A). We performed an analogous gene expression profiling screen using Affymetrix GeneChip arrays, receiving the same results with respect to drug clusters (Fig. 1B). The results of the analysis of NSCLC cell lines was similar to that seen with all lung cancer cell lines (Figure 2A,B). However, it is sometimes difficult to consistently reproduce data of the gene-drug sensitivity correlation using cDNA array technique and clinical response data. To obtain reproducible data, we separately performed Affymetrix GeneChip array-based gene expression profile analyses and sensitivity tests on another set of 19 human lung cancer cell lines and examined the sensitivity of these separate sets to 8 commonly used anti-cancer agents. Table 2 shows the GI50 levels of these anti-cancer agents against the lung cancer cell lines (Set 2) [see Additional file 1]. The drugs were clustered using Set 2, 19 cell lines (Set 2)[11]. In this analysis, gemcitabine was again located in separate clusters (Fig. 3A). The results of the analysis of NSCLC cell lines of Set 2 was also similar (Fig. 3B). Several genes, were commonly listed that differentiated gemcitabine from the oth-

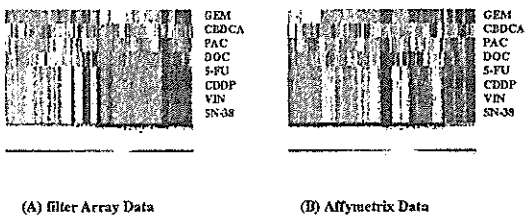

Figure 1
Clustered image map (CIM) relating drug activity to gene expression in lung cancer cell lines – Experiment 1. (A) using filter array, (B) using GeneChip. The cluster trees of drugs (y axis) and gene expression (x axis) were shown in the CIM. Each block of colors represents correlations between clusters of genes and drugs; red (high positive correlation) and blue (negative correlation). In this analysis, gemcitabine belonged to an isolated cluster (Fig. 1A,B). GEM; gemcitabine, PAC; paclitaxel, DOC; docetaxel, VIN; vinorelbine

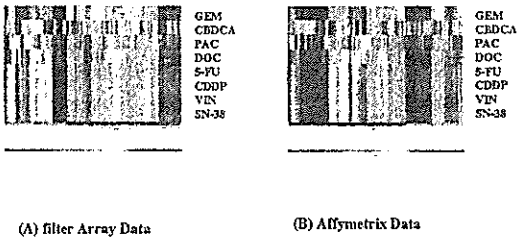

Figure 2
Clustered image map (CIM) relating drug activity to gene expression in NSCLC cell lines – Experiment 1. (A) using filter array, (B) using GeneChip. The cluster trees of drugs (y axis) and gene expression (x axis) were shown in the CIM. Each block of colors represents correlations between clusters of genes and drugs; red (high positive correlation) and blue (negative correlation). In this gemcitabine belonged to an isolated cluster (Fig. 2A,B). GEM; gemcitabine, PAC; paclitaxel, DOC; docetaxel, VIN; vinorelbine

ers in Sets 1 and 2. These were a LEMT1 domain containing gene (Accession No. NM_015416), a dehydrogenase gene (Accession No. AL050217), and a gene of homo sapiens hypothetical protein (Accession No. NM_016402). A LEMT1 domain containing gene was reported to contribute to neoplastic cellular transformation[18]. A dehydrogenase gene constitutes a large protein family of NAD(P)(H)-dependent oxidoreductase[19]. A gene of homo sapiens hypothetical protein is similar to a heat shock 70kDa protein 8 isoform[20]. Presently, their functions involved in drug sensitivity remains unclear.

Discussion

Here, we used a DNA array-based gene expression profiling approach, together with assessment of the cytotoxic activity of several widely applied anti-cancer agents, in two collections of human lung cancer cell lines. In particular, we related gene expression and drug sensitivity patterns in these cell lines. According to our separate two combined cytotoxicity and transcriptomic analyses, gemcitabine belonged to an isolated cluster. These results would suggest that combination chemotherapy regimens including gemcitabine could be a candidate for initial treatment, because combinations of drugs belonging to different clusters could expand the spectrum of the chemotherapy. Gemcitabine was deemed from our studies to be a good candidate for the treatment of recurrent or refractory NSCLC. Recently, an *in silico* search was performed to identify genes whose expression was positively or negatively correlated with sensitivity to four platinum compounds (CDDP, CBDCA, oxaliplatin and tetraplatin); the publicly available databases of the National Cancer Institute (NCI)(21) were used for this purpose[22]. CDDP, CBDCA, oxaliplatin and tetraplatin are platinum-based compounds that are classically thought to have a similar spectrum of activities, allowing for one agent to be substituted for the other[23]. Important similarities were noticed between CDDP and CBDCA on one hand, and tetraplatin and oxaliplatin on the other hand[22]. The gene-drug correlations using NCI program in these study may be a valuable tool for the identification of determinants of anticancer drug activity in tumors and for the design of cancer chemotherapy.

Vekris *et al.* described several limitations to the type of study that they have developed. 1. The evaluation of gene expression was performed on a subset of 1416 genes and molecular markers. 2. The level of expression of the 1416 molecular markers was determined with a technique that was still under development and not fully validated. 3. The criterion for drug cytotoxicity that has been retained by the NCI is the 50% growth inhibitory concentration (GI50) rather than the overall number of cells killed. There were several differences between the study of Vekris *et al.* and ours, most notably with respect to the cell lines analyzed in each case. Our study focused on lung cancer cell lines, whereas Vekris *et al.* utilized available information on the NCI60 set, a wide range of cancer cell lines[22]. The evaluation of gene expression by Vekris *et al.* was performed on a subset of 1,416 genes, which represents a relatively small fraction of the total transcriptome. We analyzed gene expression using two different

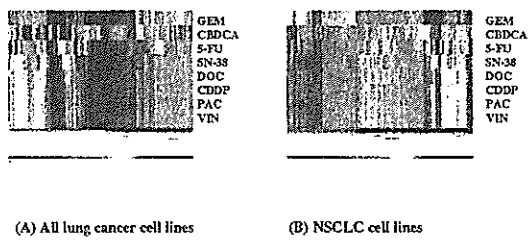

Figure 3
Clustered image map (CIM) relating drug activity to gene expression in lung cancer cell lines using GeneChip – Experiment 2. (A) all lung cancer cell lines, (B) NSCLC. The cluster trees of drugs (y axis) and gene expression (x axis) were shown in the CIM. Each block of colors represents correlations between clusters of genes and drugs; red (high positive correlation) and blue (negative correlation). In this analysis, gemcitabine belonged to an isolated cluster (Fig. 3A,B). GEM; gemcitabine, PAC; paclitaxel, DOC; docetaxel, VIN; vinorelbine

DNA array formats, namely spotted filter (data not shown) and genome-wide GeneGhip arrays, with similar results being obtained. In addition, we separately analyzed two sets of lung cancer cell lines, 10 and 19 lines to obtain our reproducible gene-drug sensitivity correlation data.

Using cDNA array technique and clinical response data, it is sometimes difficult to consistently reproduce gene-drug sensitivity correlation data. These data were often influenced by sampling methods, sample preservation status, tumor size, tumor environment status including tumor vessels and inflammation, etc. In the study of Vekris *et al.* and ours, these influences were small because cancer cell lines were used. However, cell lines differ from tumor cells and should therefore be considered as surrogates that may contain information on the molecular cell biology and molecular pharmacology of cancer.

In the treatment of lung cancer, a number of combination therapy regimens employing platinum compounds have proven to be effective[1] and are widely applied as first-line treatment for unresected NSCLC; for example, CDDP + docetaxel, CBDCA + paclitaxel, CDDP + gemcitabine, CDDP + CPT-11, CDDP + paclitaxel, CDDP + vinorelbine, etc[2]. In addition, docetaxel and pemetrexed have been reported to be effective in the context of second-line chemotherapy for NSCLC[3,4]. However, how were the anti-cancer agents in these reports selected? It is consequently desirable to find more appropriate therapeutic opportunities based on informed insights.

Conclusion

The results of our molecular pharmacological analysis suggest that chemotherapy regimens including gemcitabine should be evaluated in second-line chemotherapy if the initial chemotherapy does not include this drugs. A total design approach to cancer chemotherapy through the gene-drug correlations using NCI program may yield improved therapeutic options.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AG and SK designed this study, analyzed and interpreted the data. CL, YS, and KM carried out sensitivity test, YS, and MS carried out cDNA array analysis. SK, YM, RN, MN, and AY carried out cell culture and RNA extraction. AS, and NO carried out acquisition of cDNA array data, HU carried out statistic analysis. All authors read and approved the final manuscript.

Additional material

Additional File 1

Table 2 Growth inhibitory activities (GI50)(μg/ml) of various anti-cancer agents against 19 human lung cancer cell lines – Set 2

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Alterations in novel candidate tumor suppressor genes, *ING1* and *ING2* in human lung cancer

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Abstract. The *ING1* gene is involved in the regulation of the cell cycle, senescence, and apoptosis and is a novel candidate tumor suppressor gene. *ING2*, another gene in the *ING* family, was identified and cloned. The functions of *ING1* and *ING2* largely depend on the activity of p53. To determine whether an alteration in these genes plays a role in carcinogenesis and tumor progression in lung cancer, we screened 30 human lung cancer cell lines and 31 primary lung cancer tumors for mutations in these genes using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and direct sequencing. Our findings failed to uncover any mutations in these genes. We also examined the expression of *ING1* and *ING2* in lung cancer cell lines that either had or lacked a *p53* mutation, and in a control bronchial epithelium cell line, using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). *ING1* expression was up-regulated in all 7 lung cancer cell lines that had a *p53* mutation, while the expression of *ING2* was down-regulated in 6 of 7 lung cancer cell lines that had a p53 mutation. These results suggest that the *ING1* and *ING2* genes have different roles in lung carcinogenesis and progression, and the *ING2* gene may be an independent tumor suppressor candidate on *p53*.

Introduction

Human cancers develop as a result of the stepwise accumulation of multiple acquired genetic defects that include mutations in tumor suppressor genes. A mutation in the *p53* tumor suppressor gene is the most frequently identifiable defect in human cancers (1). Other candidate tumor suppressor genes have been cloned and are referred to as *ING* (2). *ING1*, which is located on chromosome 13q 33~34 (3) and was found to encode a nuclear protein, consists of exons 1a, 1b, and 2 (4). Four alternatively spliced transcripts of *ING1* encode *ING1a*, *ING1b*, *ING1c* and *ING1d* (4-6). *ING2* was cloned and mapped to human chromosome 4q35 by fluorescence *in situ* hybridization and radiation-hybrid analyses (GenBank accession no. AF053537) (7,8). Over-expression of *ING1* and *ING2* led to growth arrest in the G1 phase of the cell cycle and induced apoptosis in several cell types (2,8,9) that largely depend on the activation of p53 (8,10). ING family proteins contain a region that is homologous to plant homeodomain (PHD) finger domains and have been implicated in chromatin-mediated transcriptional regulation (2,11).

ING1 gene mutations are rare in many human cancers, and no report exists on *ING2* gene mutation analysis in human cancers. Reduced expression of the *ING1* gene has been reported in breast (12) and gastric cancers (13), and in lymphoid malignancies (14).

To determine whether alterations in the *ING1b* and/or *ING2* genes are involved in lung carcinogenesis, lung cancer cell lines and primary tumors were examined for such mutations using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequence analysis. In addition, the expression of *ING1b* and *ING2* was analyzed in lung cancer cell lines using real-time quantitative reverse transcription (RT)-PCR.

Materials and methods

Tissue samples. Primary lung cancers (n=31) and matched control samples were obtained during autopsy of patients previously been admitted to the Fourth Department of Internal Medicine at the Nippon Medical School Main Hospital in Tokyo, Japan, and immediately frozen at -80°C. The samples included 14 adenocarcinomas, 8 squamous cell carcinomas (SCCs), 6 small cell lung cancers (SCLCs), 2 large cell carcinomas, and 1 adenosquamous cell carcinoma.

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Key words: *ING1*, *ING2*, lung cancer, polymorphism, mRNA expression

Table I. Sequence of the polymerase chain reaction (PCR) primer used for amplification of the indicated exons in *ING1b* and *ING2*.

Exon		Sense primer sequence	Antisense primer sequence
ING1b			
Exon 1		TGCAGTGCTATTTTTTGAGGGG	CGCCCCCGCCCATCCATCA
Exon 2	a	ACGCCTGTCCTTCTTGCCCC	CTTGCCGCTGTTGCCCGCTG
	b	TTCGAGGCGCAGCAGGAGCT	CTTGGCCTTCTTCTCCTTGGG
	c	CAGCAACCACGACCACGACG	TGAGCCCCACGCACGAGAAG
	d	CCTCCCCATCGACCCCAACG	ACATTTTACACTCCTTGCACCTCA
ING2			
Exon 1		TGCATGTGCGGCTGCTGGATG	TGTCACGGGAGAAAGGGAAG
Exon 2	a	CCTTGGAAATGTTGTGTCTGC	TTCTGGTTGGCTGGAATCC
	b	TGCTGAAAGTGAACGAGCCTC	TCCTATCATCTCCCCATAAGACAC
	c	AAAGAAACGCTCCAAGGC	CCCTTTAAAATGTGGATGGCC

Table II. Sequence of primer and probe used in the real-time reverse transcription-polymerase chain reaction (RT-PCR) indicated gene.

Gene	Sense primer sequence	Antisense primer sequence	Probe sequence
ING1b	CGAAATACCAAGAGATCCTGAAGG	TGCGCCCCGTCTGTCT	ACGAGTGCTACGAGCGCTTCAGTCG
ING2	GGACTACCTTGAGTGCGTGGA	TCGCAGCACAGACACGTTC	TCGCTGCCCCACGACATGCA
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTC	CAAGCTTCCCGTTCTCAGCC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Lung cancer cell lines. A total of 15 human non-small cell lung cancers (PC1, PC3, PC7, PC9, PC10, PC13, PC14, Lu65, A427, A549, NCI-H23, NCI-H157, NCI-H358, NCI-H441, and NCI-H520) and 15 human small cell lung cancers (Lu24, Lu130, Lu134, Lu135, Lu138, Lu139, Lu140, Lu141, NCI-H69, NCI-H82, NCI-H526, NCI-N230, NCI-N231, NCI-N417, and SBC5) were used for DNA analysis (15). The Lu24, Lu65, and Lu135 cell lines were provided by Dr Y. Shimosato and Dr T. Terasaki (National Cancer Center Research Institute, Tokyo, Japan). The NCI-N231, A549, A427, NCI-H358, NCI-H157, NCI-H23, NCI-H441, NCI-H520, NCI-H82, NCI-N417, NCI-H526, and NCI-H69 cell lines were obtained from the American Type Culture Collection (Rockville, MD) (16), while the PC1, PC3, PC7, PC9, PC10, and PC14 cell lines were obtained from Immuno-Biological Laboratories (Gunma, Japan). The Lu24, Lu130, Lu134, Lu138, Lu139, Lu140, Lu141, and SBC-5 cell lines were provided by Dr J. Yokota (National Cancer Center Research Institute). We also performed transcriptional studies on 8 lung cancer cell lines (NCI-N231, Lu65, A549, NCI-H69, Lu135, PC7, PC9, and PC14) and on a normal human bronchial epithelial cell line (BET2A; American Type Culture Collection, no. ATCC CRL-9443).

DNA and RNA isolation. Genomic DNA was extracted from tumor and normal cells by proteinase K treatment and phenol chloroform extraction using standard protocols. RNAs were prepared using standard protocols described previously (17).

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. PCR-SSCP analysis was performed as previously described (16,18). Each of the two exons of the *ING1b* and *ING2* genes was amplified separately using the PCR primers shown in Table I. PCR was performed using the Gene AMP XL PCR kit (Perkin-Elmer Corp./Roche, Branchburg, NJ), which was followed by 40 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 30 sec, and extension at 68°C for 90 sec, with a final extension at 68°C for 8 min. The PCR reaction mixture contained XL buffer with 110 μM $Mg(OAc)_2$, 200 μM deoxynucleotide triphosphate, 0.1 mM of each primer labeled with 5-IAF (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.5 units of rTth DNA polymerase, and 25 ng of genomic DNA. The 5-IAF-labeled PCR products were denatured, cooled on ice, and loaded on neutral 6% polyacrylamide gels with or without 5% (vol/vol) glycerol. Following electrophoresis, the gels were analyzed using the FluorImager 595 (Amersham Pharmacia Biotech).

DNA sequence analysis. DNA sequence analysis was performed as previously described (16,18). Aberrant bands were cut from the gel and further amplified by PCR using sequencing primers with the M13 sequence (TGTAAAACG ACGGCCAGT) added to the appropriate PCR primers. PCR was performed as described above, and the products were purified and sequenced using a fluorescent automated sequencer (Perking-Elmer Corp./Applied Biosystem, Inc., Foster City, CA, USA).

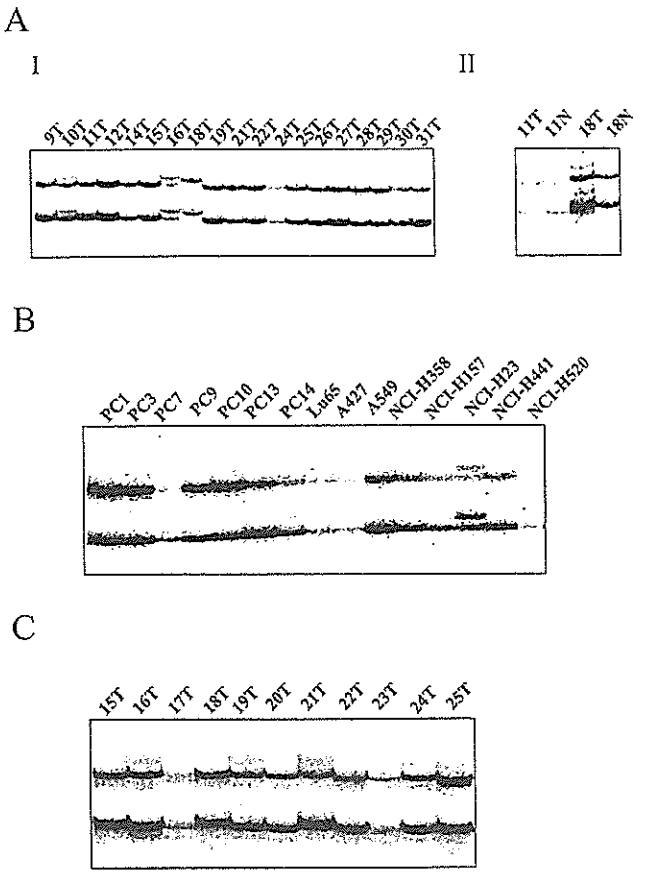

Figure 1. (A) PCR-SSCP analysis of exon 2 of the *ING1b* gene showed mobility shifts in lung cancer tissues [tumors (T) 10, 12, 16, and 18]. Tumor (T) and normal tissue (N) from case 18 showed similar patterns on PCR-SSCP. (B) PCR-SSCP analysis of exon 2 of the *ING1b* gene showed an aberrant band in lung cancer cell line NCI-H23. (C) PCR-SSCP analysis of exon 1 of the *ING2* gene showed mobility shifts in the lung cancer tissues of cases 15, 18, and 21.

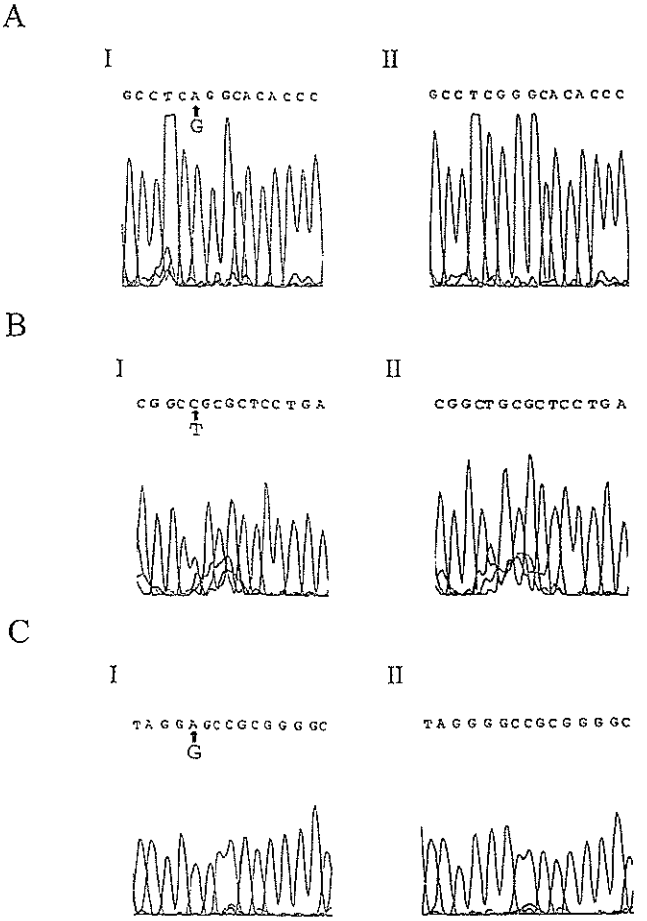

Figure 2. (A), Sequence analysis revealed a nucleotide substitution (G→A, serine to serine) at codon 173 of exon 2 of the *ING1b* gene in case 16. I, DNA sequence of case 16; II, control. (B) DNA sequence of exon 1 of the *ING2* gene revealed a nucleotide substitution (T→C, alanine to alanine) at codon 13. I, case 15; II, control. (C) DNA sequence of exon 1 of the *ING2* gene revealed a nucleotide substitution (G→A) 6 bp downstream of exon 1. I, case 15; II, control.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). RT quantitative-PCR was performed using the ABI Prism 7700 sequence detector (Perkin-Elmer Corp./ Applied Biosystem, Inc.). The PCR primers and the Taq Man fluorescence probes (Table II) were designed using the Primer Express software program (Perkin-Elmer Corp./Applied Biosystem, Inc.). The total RNA sample (1 μg) was reverse transcribed using a random hexamer and a pre-amplification system (Life Technologies). A portion of each cDNA was used for quantitative PCR in a volume of 50 μl, and the reaction mixture contained the designed primers, Taqman probes, and Master Mix that included the PCR buffer, $MgCl_2$, dATP, dCTP, dGTP, dUTP, AmpErase UNG, and AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp./Applied Biosystem, Inc.). PCR was carried out at 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The degree of gene expression was reported as the ratio of a given gene's mRNA in a particular sample to the level of *GAPDH* mRNA in that sample.

Results

Mutation analysis of the ING1b and ING2 genes in human lung cancers and lung cancer cell lines. PCR-SSCP analysis of exon 2 of the *ING1b* gene revealed aberrant bands in 6 of 31 lung cancer tissues (cases 5, 6, 10, 12, 16 and 18) with or without normal bands (Fig. 1A), and 1 of the 30 lung cancer cell lines i.e. NCI-H23, which is a non-small cell lung cancer (Fig. 1B). DNA sequence analysis of these bands revealed a G to A substitution at codon 173 (Fig. 2A), which does not result in an amino acid substitution. Matched normal tissues had these same aberrant bands, which suggested that they were due to polymorphisms. In exon 1 of the *ING2* gene, we detected aberrant bands in 6 of 31 lung cancer tissues (cases 5, 8, 12, 15, 18 and 21) with or without normal bands (Fig. 1C). Sequencing of these bands showed a T to C substitution at codon 13 (Fig. 2B) that did not alter the encoded amino acid, and a G to A substitution in the non-coding region of intron 1 of the *ING2* gene 6 bp downstream of exon 1 (Fig. 2C), which also appeared to be due to polymorphisms.

Expression of mRNA for ING1b and ING2 in human lung cancers and lung cancer cell lines. The expression of *ING1b* mRNA was up-regulated in 7 of 8 lung cancer cell lines compared to the human bronchial epithelium cell line,

Table III. *ING1b* mRNA levels in lung cancer cell lines.

cDNA	*ING1b/GAPDH*	Fold difference[a]
NCI-N231	6.743	6.643
Lu65	5.298	5.220
A549	1.490	1.468
NCI-H69	10.952	10.790
Lu135	5.710	5.626
PC7	14.297	14.086
PC9	2.241	2.208
PC14	2.281	2.247

[a]Fold difference, intensity of *ING1b* expression of sample/intensity of its expression in BET2A.

Table IV. *ING2* mRNA levels in lung cancer cell lines.

cDNA	*ING2/GAPDH*	Fold difference[a]
NCI-N231	0.382	0.288
Lu65	0.737	0.555
A549	0.340	0.256
NCI-H69	0.375	0.282
Lu135	0.381	0.287
PC7	0.280	0.211
PC9	0.086	0.065
PC14	0.491	0.370

[a]Fold difference, intensity of *ING2* expression of sample/intensity of its expression in BET2A.

Table V. *p53* status in lung cancer cell line.

Cell line	Type of mutation	Codon	Exon	Change
NCI-N231	Missense	298	8	GAG→TAG
Lu65	Missense	11	2	GAG→CAG
A549	Wild-type			
NCI-H69	Missense	171	5	GAG→TAG
Lu135	Missense	244	7	GGC→TGC
PC7	Missense	214	6	CAT→CGT
PC9	Missense	248	7	CGG→CAG
PC14	Missense	248	7	CGG→TGG

BET2A (Table III). All 7 cell lines with up-regulated *ING1b* mRNA had a *p53* mutation, and the remaining cell line expressed wild-type *p53*. The expression of *ING2* mRNA was down-regulated in 7 of 8 lung cancer cells (Table IV). Six of the 7 cell lines that had a *p53* mutation also showed reduced *ING2* mRNA expression. The mean relative intensity of *ING1b* and *ING2* expression (intensity of *ING* expression of sample/intensity of its expression in BET2A) was 6.036 (1.468-14.086) and 0.289 (0.065-0.555), respectively ($p<0.01$ using a paired t-test).

Discussion

Mutation of the *ING1* gene has previously been reported in neuroblastoma (2) and gastrointestinal cancer cell lines (13), breast tumors (12), esophageal squamous cell tumors (19), and head and neck squamous cell carcinomas (4). Although *ING1* gene mutations are rare in many human cancers, *ING1* expression is down-regulated in several types of human cancers including breast (12), gastric (13), esophageal (19), and lymphoid cancers (14). The *ING1* gene may serve as a type II tumor suppressor since it inactivates cellular function at transcriptional and post-transcriptional levels (20).

We studied the degree of mutation and expression of the *ING1b* and *ING2* genes in human lung cancer cell lines and tumors. No point mutations in the *ING1b* and *ING2* genes were found in human lung cancers. However, we did observe a single polymorphism at codon 173 (G-to-A) in exon 2 of the *ING1b* gene. This polymorphism has previously been reported in Indian patients with oral squamous cell carcinoma (21) and Japanese breast cancer patients, but not in Canadian cancer patients (12). We also detected a single polymorphism in exon 1 and intron 1 of the *ING2* gene. There were differences in the frequency of polymorphisms between cancer tissues and cell lines in this study. The cancerous tissues were obtained from Japanese patients, while the cell lines were nearly all established in the U.S. Thus, this polymorphism may be more frequent in Japanese patients.

ING proteins have a PHD finger motif that plays a role in chromatin remodeling (11,22), and nuclear localization sequences (NLS) and nucleolar targeting sequences (NTS) (23) that can target ING proteins to nucleoli when ING proteins are overexpressed. One such protein, *ING1b*, has been shown to bind to proliferating cell nuclear antigen (PCNA) through the PCNA-interacting protein (PIP) domain after DNA damage, and to regulate the induction of apoptosis (24) and enhance the repair of UV-damaged DNA (25). This protein contains histone acetyltransferase (HAT) (26-29) and histone deacetyltransferase (HDAC) (30,31). On the other hand, *ING1a* was shown to inhibit histone acetylation by binding to HDAC1 complexes (29). These findings suggest that *ING1b* may regulate the switch from DNA replication to DNA repair.

Previous studies reported that *ING2* mRNA expression was up-regulated in human colon cancers (7). By Western blot analysis, the expression of ING2 protein was found to be diminished in colorectal and hepatocellular carcinomas, and prostate and pancreatic cancers (8). *ING2* was shown to negatively regulate cell proliferation through the induction of *p53* acetylation at lysine 382 (8), and activation of *p53* by acetylation reportedly resulted in the induction of growth inhibitor genes and proteins that activated the mitochondrial apoptotic pathway through the release of cytochrome C (32). In this study, *ING2* mRNA expression was found to be primarily down-regulated in lung cancer cell lines. Previous studies have reported that the overexpression of *ING1b* and *ING2* negatively regulated cell growth through the induction of apoptosis and G1-phase cell cycle arrest in a *p53*-dependent manner (2,8,9). It is interesting that lung cancer cells with inactivated p53 had reduced expression of the *ING2* gene in our study (Table V). It has been reported that

the expression of *ING1* and *ING2* was independent of *p53* status (8,33).

Our results suggest that mutations in the *ING1b* gene are rare, and its mRNA expression is primarily up-regulated in lung cancers. Inactivation of the *p53* gene in lung cancers may play a role in the overexpression of the *ING1b* gene. Our results suggest that the inactivation of *ING2* may play an important role in the development and/or progression of lung cancer, even in cancers that exhibit a *p53* mutation. Since the *ING2* gene was not mutated in these cancer cells, the *ING2* gene may have been negatively transcriptionally regulated, possibly as a result of hypermethylation of the gene promoter or transcriptional factor.

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Weekly Administration of Irinotecan (CPT-11) plus Cisplatin for Non-small Cell Lung Cancer

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Abstract. *Background: Weekly administration of irinotecan plus cisplatin was evaluated for untreated patients with non-small cell lung cancer (NSCLC). Patients and Methods: Sixty mg/m^2 of irinotecan plus 30 mg/m^2 of cisplatin were administered on days 1, 8 and 15 every 4 weeks. Patients with no evidence of disease progression were treated with at least two cycles (8 weeks). Of the 39 patients, 29 were provided an antidiarrheal program consisting of sodium bicarbonate and magnesium oxide. Results: There were 13 partial responses and an overall response rate of 33.3% [95% CI: 20%-50%]. The median time to progression and survival were 64 days and 12.8 months, respectively. Grade 4 neutropenia occurred in 15.4% of the patients, and Grade 3 and 4 diarrhea was observed in 12.8% and 2.6%, respectively. The incidence of leukopenia of grade 3-4 was significantly lower in patients provided with the antidiarrheal program due to lack of decrease in the lymphocyte count. Conclusion: This phase II study indicated that weekly irinotecan plus cisplatin administration was a promising treatment for untreated NSCLC.*

Irinotecan (CPT-11), a topoisomerase I inhibitor, in combination with cisplatin (CDDP) administration for previously untreated patients with extensive small cell lung cancer (SCLC) resulted in improvement in overall survival, compared with CDDP/etoposide in a randomized phase III study (1). CPT-11 plus CDDP was also active for refractory or relapsed SCLC, after treatment with etoposide in combination with a platinum compound of either CDDP or carboplatin (2). In terms of published results on the use of CPT-11 for non-small cell lung cancer (NSCLC), CPT-11 alone at 100 mg/m^2 on days 1, 8 and 15 within 4-week periods was shown to be active, with a response rate of 34.3% in a phase II study (3). In another phase II study, when 80 mg/m^2 of CDDP on day 1 was combined with 60 mg/m^2 of CPT-11 on days 1, 8 and 15 within 4-week periods, the response rate increased (4) and a randomized phase III study, the Four-Arm Cooperative Study (FACS), compared CPT-11 plus CDDP by this regimen to gemcitabine plus CDDP, paclitaxel plus carboplatin or vinorelbine plus CDDP (5). Regarding to survival time, the FACS showed the same efficacy for CPT-11 plus CDDP [median survival time (MST): 14.2 months] as gemcitabine plus CDDP (MST: 14.8 months), paclitaxel plus carboplatin (MST: 12.3 months), or vinorelbine plus CDDP (MST: 11.4 months) (5).

The maximum synergistic effect on tumor cells was observed by the simultaneous combination of CDDP and CPT-11 *in vitro* (6). We confirmed that the cytotoxicity of CDDP is dependent on the area under the concentration-time curve (AUC) (7), indicating that if the AUC per month was maintained, anti-tumor activity of CDDP was not weakened by divided administration of the maximal dose. In 1998, two phase I studies of weekly administration of irinotecan plus CDDP were performed, one by Saltz *et al.* (8) and another one by our group (9). Saltz *et al.* increased

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the dose of irinotecan, while fixing the dose of cisplatin at 30 mg/m^2 and investigated the pharmacokinetics of irinotecan. In our study the cisplatin dose was increased and the dose of irinotecan was fixed at 60 mg/m^2 and the theoretical validity of dividing the maximum dose of cisplatin was addressed and the pharmacokinetics of cisplatin were clarified. Regardless of the differences in the study design, the treatments in the two studies were similar, *i.e.* the former group concluded that the recommended dose of cisplatin and irinotecan was 30 mg/m^2 and 65 mg/m^2, respectively, on a weekly schedule for 4 out of 6 weeks. Similarly, we reported that the MTD was 33 mg/m^2 of cisplatin and 60 mg/m^2 of irinotecan on a weekly schedule for 3 out of 4 weeks (8, 9).

Recently, a phase II study of weekly administration of CPT-11 plus CDDP for refractory or relapsed SCLC was conducted by our group (2). The treatment schedule was 60 mg/m^2 of CPT-11 plus 30 mg/m^2 of CDDP on days 1, 8 and 15 within 4 weeks. The results indicated that this regimen was both safe and promising. The current study, employing the same treatment schedule, was conducted in chemotherapy-naïve patients with NSCLC.

Patients and Methods

Patients. This study was performed in accordance with the Helsinki Declaration (1964, amended in 1975 and 1983) of the World Medical Association. Prior to their participation in the study, patients admitted to the six institutions of the East Japan Chesters Group (EJCG), were examined to ensure that they met the following criteria: (a) histological or cytological diagnosis of NSCLC, (b) no previous therapy, (c) measurable disease, (d) performance status (PS) of 2 or above on the Eastern Cooperative Oncology Group scale; (e) adequate bone marrow function (white blood cell (WBC) count≥4,000/mm^3, plt≥100,000/mm^3, Hb≥9.5 g/dl); (f) adequate hepatic function (T-Bil≤1.5 mg/dl, transaminases less than twice the upper limit of normal); (g) adequate renal function (S-Cr≤1.5 mg/dl); (h) age 15-75 years; (i) no brain metastasis and (j) no medical problems severe enough to prevent compliance with the study requirements. All patients provided informed consent before enrollment in the study, according to institutional guidelines.

Treatment schedule. Based on the experience with our phase I study of weekly CPT-11 plus CDDP (9), the doses of CPT-11 and CDDP were set at 60 mg/m^2 and 30 mg/m^2, respectively, on days 1, 8, and 15 every 4 weeks. Initially, CPT-11 was administered in 500 ml normal saline as a 90-min *i.v.* infusion. CDDP was then given over a 60-min period. Patients with no evidence of disease progression were treated with at least 2 cycles.

For prevention of emesis, 5-hydroxytryptamine-3-receptor antagonist and dexamethasone were given *i.v.*, prior to the administration of CPT-11 and CDDP. To avoid CDDP-induced renal damage, *d.i.v.* hydration with a total of 1,500 ml was performed.

During performance of this study, it was shown that an antidiarrheal program would prevent CPT-11-induced side-effects (10). Therefore, from the eleventh patient, the antidiarrheal program of oral alkalization (OA) and control of defecation (CD) were employed (10). Coinciding with day one of CPT-11 infusion and for four days thereafter, the following were administered: sodium bicarbonate powder, 2.0 g/day t.i.d. (between meals) and magnesium oxide powder, 1.5 g/day, t.i.d. (after meals). Each dose was to be accompanied by a glass of basic water (pH>7). The dose of magnesium oxide was increased or decreased at the time of constipation or diarrhea, respectively.

During the course of treatment, the doses of CPT-11 and CDDP were withheld on the day it was due in the presence of leukopenia (<3,000/mm^3) and/or diarrhea in excess of grade 1. In the previous phase I study (9), the nadir WBC count was found to be significantly correlated to the ratio of WBC on day 8, to that on day 1 ($r=0.603$, $p=0.0081$). Therefore, the doses of CPT-11 and CDDP were also withheld on day 8, when the WBC count had dropped by more than 30%, compared to day 1.

Granulocyte colony-stimulating factor (G-CSF) was administered when grade 4 leukopenia (<1,000/mm^3) and/or neutropenia (<500/mm^3) were observed. Erythropoietin was not used. Delayed diarrhea, which typically presented 6 days after and beyond the initial CPT-11 administration, was treated with a high dose of loperamide, according to previous reports (11). Persistent, grade 3 or greater diarrhea, despite loperamide therapy, warranted the use of *i.v.* hyperalimentation for fluid management.

Evaluation. Patients underwent staging evaluation by physical examination, chest X-ray, bone scintiscan, computed tomography of the head, chest and abdomen, and fiberoptic bronchoscopy. Staging procedures followed those of the tumor-node-metastasis system.

Prior to the first course, each patient was subjected to chest X-rays and chest CT. The former was assessed at least once every two weeks after the initial evaluation and the latter was planned to be checked once every two months. Tumor response was extramurally reviewed and classified in accordance with World Health Organization criteria. A complete response (CR) was defined as the disappearance of all clinical and radiological evidence of tumor, for at least 4 weeks; a partial response (PR) was defined as a decrease of 50% or more in the sum of products of the longest perpendicular diameters of all measurable lesions, for at least 4 weeks; and progressive disease (PD) was defined as an increase of more than 25% in the sum of products of the longest perpendicular diameters of all measurable lesions or the appearance of new lesions. All other circumstances were considered to indicate stable disease (SD). The time-to-progression (TTP) and survival curves were drawn using the Kaplan-Meier method, and the median survival time was calculated from the day of the first treatment until the death or the last follow-up.

Also, before the first course, a complete blood cell count (CBC), serum chemistry for renal and hepatic function, electrolyte analysis, and urinalysis were performed. These studies were repeated at least once a week after the initial evaluation. The NCI Common Toxicity Criteria was used to grade organ system damage.

Statistical analysis. Sample size, calculated by the response rate, was determined to be 38 patients, to evaluate this phase II study. A 50% response rate was chosen as the desirable target level and a 20% response rate was considered uninteresting. Our design had a power in excess of 90% and a less than 10% type I error. Bayesian data monitoring of the response rate for an early interruption of the study was performed throughout the study (12).

Table I. *Patient characteristics (n=39).*

Male/female	31/8 pts
Median age	64 years old
(Range)	(43-75)
Performance status	
0-1	37 pts
2	2 pts
Stage	
IIIB	11 pts
IV	28 pts
Histology	
Adenocarcinoma	26 pts
Squamous cell carcinoma	9 pts
Adenosquamous	2 pts
Large cell carcinoma	1 pt
Unclassified	1 pt
Antidiarrheal program of OA and CD	
Without OA and CD	10 pts
With OA and CD	29 pts
Courses given	
1	8 pts
2	24 pts
3	6 pts
4	1 pt

OA: oral alkalization; CD: control of defecation.

Table II. *Response rate (n=39).*

Response		CR	PR	SD	PD	NE	Response rate
Histology							
Adenocarcinoma	(n=26)	0	9	12	5	0	34.6%
Squamous	(n=9)	0	3	4	1	1	33.3%
Adenosquamous	(n=2)	0	1	1	0	0	50.0%
Large	(n=1)	0	0	0	1	0	0.0%
Unclassified	(n=1)	0	0	1	0	0	0.0%
Antidiarrheal program +/–							
Without OA & CD	(n=10)	0	2	6	2	0	20.0%
With OA & CD	(n=29)	0	11	12	5	1	37.9%
Overall	(n=39)	0	13	18	7	1	33.3%

Of the 39 patients, there were 13 partial responses and an overall response rate of 33.3% (95% confidence interval, 20%-50%) was obtained.
CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; NE: not evaluable (see Patients and Methods).

Results

Between January 1997 and June 2001, 39 patients participated in the trial. The characteristics of the patients are shown in Table I. Eight of the patients were women and 31 were men and the mean age was 64 years. Eleven and 28 patients exhibited stage IIIB and stage IV disease, respectively. Most of the patients had a good performance status (PS), but two of them were PS 2.

Twenty-six out of the 39 patients (67%) received the full schedule of therapy (CDDP+CPT-11 three times/course) in the first course and the others received two times administration, either on days 1 and 8 or days 1 and 15, because of myelosuppression or diarrhea. The average administration frequency of 60 mg/m^2 CPT-11 and 30 mg/m^2 CDDP was 5.2 times in the average of 2.0 courses, calculating that the average administration was 2.6 times/course. Dose intensities of CPT-11 and CDDP were 156 mg/m^2/course and 78 mg/m^2/course, respectively. The first 10 patients, who did not receive the antidiarrheal regimen, were given 1.8 courses of CPT-11 plus CDDP at dose intensities of 150 mg/m^2/course and 75 mg/m^2/course, respectively. The remaining 29 patients, who received the antidiarrheal regimen, were given 2.1 courses of combined therapy of CPT-11 plus CDDP at dose intensities of 158 mg/m^2/course and 79 mg/m^2/course, respectively. Specifically, patients without the antidiarrheal program were given total doses of CPT-11 and CDDP of 270 mg/m^2 and 135 mg/m^2, respectively, and those with the antidiarrheal program were administered with CPT-11 and CDDP at doses of 332 mg/m^2 and 166 mg/m^2, respectively.

All 39 patients were assessed for response (Table 2). Thirteen patients had PR and 18, and seven had SD or PD, respectively. The overall response rate was 33.3% [95% CI: 20%-50%]. When data were analyzed according to whether or not subjects received the antidiarrheal program, there was no statistical difference in the overall response rate. However, among the ten patients without the antidiarrheal program, two (20%) PRs were observed, while among the 29 patients on this program, 11 (37.9%) PRs were observed. The median TTP of all patients was 64 days (Figure 1A), for the first ten patients it was 58 days and for the remaining 29 patients it was 73 days (Figure 1B). There was a significant difference in TTP between those who did and did not receive the antidiarrheal program (log-rank test: $p<0.05$).

The median survival time (MST) for all patients was 12.8 months, and the 1-year survival rate in patients was 55% [95% CI: 38%-70%] (Figure 2).

All 39 eligible patients were assessable for toxicity (Table III). Leukopenia was the major toxicity. The leukocyte count nadir usually occurred around day 21. Grade 4 leukopenia and neutropenia occurred in two patients (5.1%) and six patients (15.4%), respectively. No treatment-related death was observed. Thrombocytopenia occurred less frequently than leukopenia and was less severe. Thrombocytopenia of grade 3 or more was

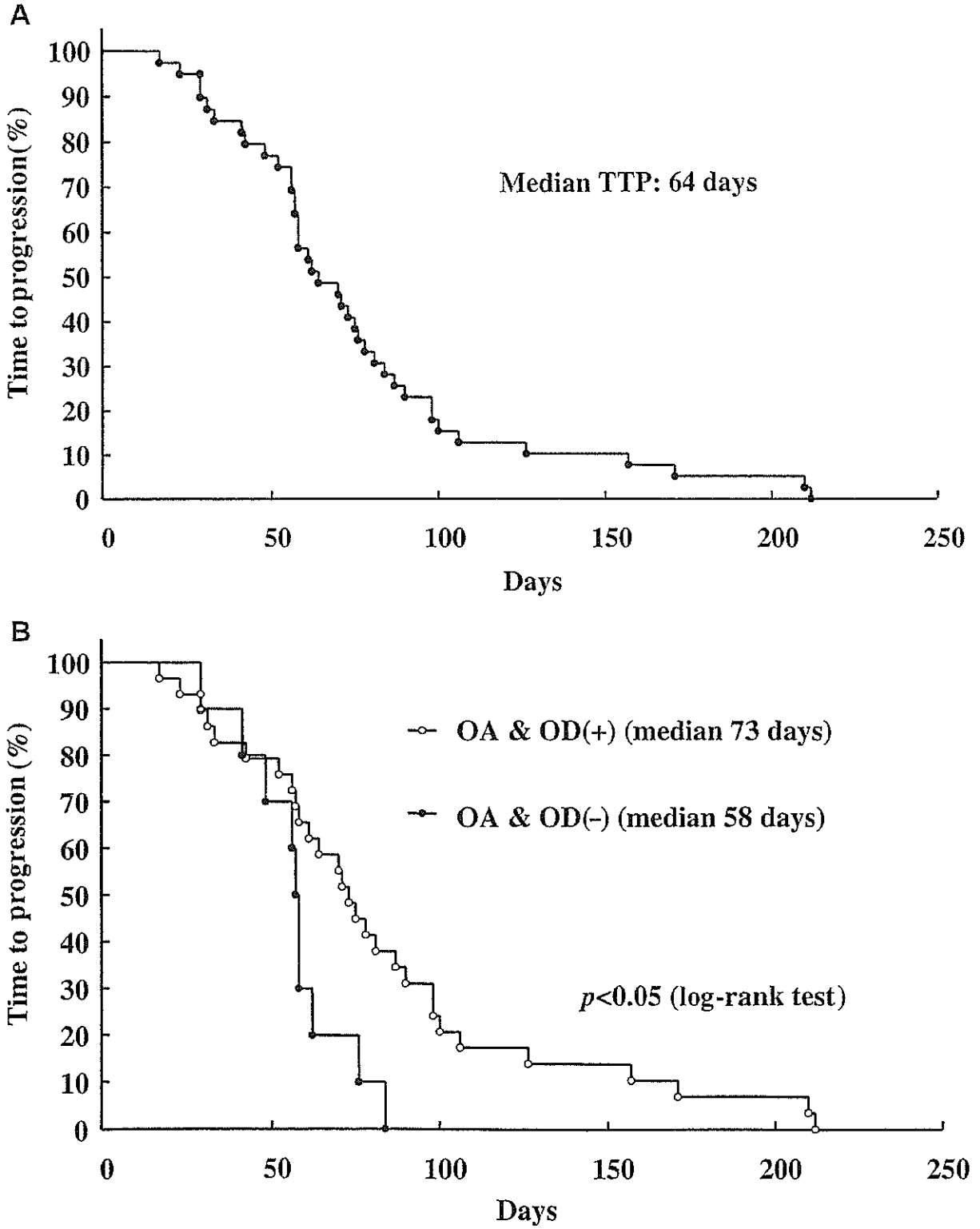

Figure 1. *a) Time to progression (TTP) of all 39 patients. b) TTP of the 29 patients treated by the antidiarrheal program of OA and CD and the 10 patients without the program.*

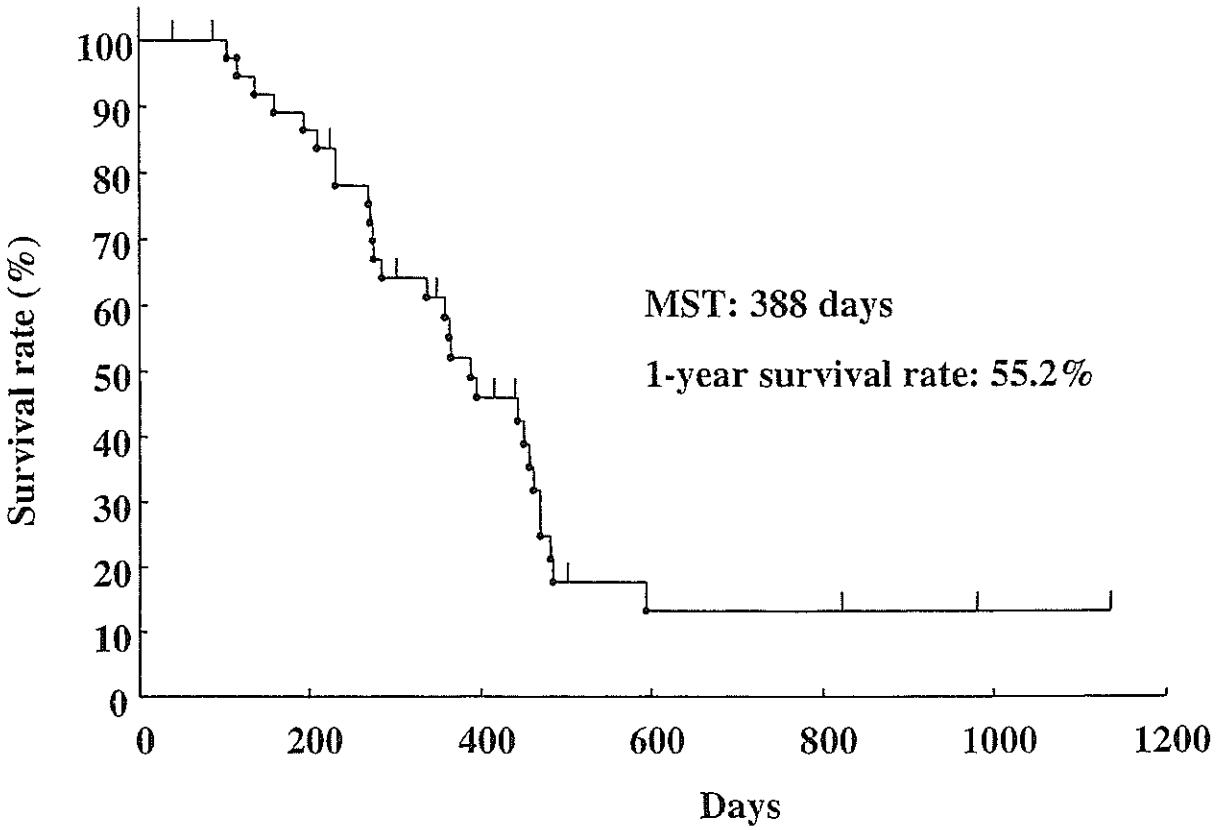

Figure 2. *Median survival time (MST) of all 39 patients after starting weekly CPT-11 plus CDDP.*

observed in one patient (2.6%). No patient required platelet transfusion.

Grade 3 diarrhea was observed in only five patients (12.8%) and grade 4 diarrhea occurred in one patient (2.6%). Although these patients required *i.v.* hydration, they recovered within four to seven days. Of the six patients experiencing diarrhea of grade 3 or higher, three had grades 3 and 4 leukopenia, coincidentally. Although six patients experienced grade 3 nausea or vomiting (15.4%), the other 33 patients experienced nausea of grade 2 or less or no nausea at all, indicating that nausea and vomiting were mild with this regimen. No hepatic, renal and pulmonary toxicities, related to drug administration, were observed in this trial.

Comparing the presence or absence of the antidiarrheal program, the incidence of leukopenia at more than grade 3 was significantly lower in patients receiving the antidiarrheal program (Table IV). This was due to no decrease in lymphocyte count. Anemia, emesis and diarrhea had tendency to be less severe in patients on the antidiarrheal program.

Table III. *Toxicities (n=39).*

	No. of patients with					
CTC grade	1	2	3	4	≥3 (%)	4 (%)
Hematological toxicity						
Leukopenia	9	14	8	2	25.6	5.1
Neutropenia	3	12	12	6	46.2	15.4
Thrombocytopenia	6	5	1	0	2.6	0.0
Anemia	9	9	6	1	17.9	2.6
Other toxicities						
Diarrhea	9	9	5	1	15.4	2.6
Nausea and vomiting	13	8	6	-	15.4	-

Discussion

From the response rate (Table II), survival time and 1-year survival rate (Figure 2), the results of this study indicate that treatment by weekly administrations of CPT-11 plus cisplatin was active in chemotherapy-naïve patients with NSCLC. The

Table IV. *Toxicities with or without antidiarrheal program of OA and CD.*

CTC grade		No. of patients with 1	2	3	4	≥3 (%)	4 (%)
Leukopenia							
Without OA & CD	(n=10)	2	1	4	2	60.0*	20.0
With OA & CD	(n=29)	7	13	4	0	13.8*	0.0
Neutropenia							
Without OA & CD	(n=10)	1	3	3	2	50.0	20.0
With OA & CD	(n=29)	2	9	9	4	44.8	13.8
Thrombocytopenia							
Without OA & CD	(n=10)	1	3	0	0	0.0	0.0
With OA & CD	(n=29)	5	2	1	0	3.4	0.0
Anemia							
Without OA & CD	(n=10)	2	1	3	0	30.0	0.0
With OA & CD	(n=29)	7	8	3	1	13.8	3.4
Diarrhea							
Without OA & CD	(n=10)	2	3	2	1	30.0	10.0
With OA & CD	(n=29)	7	6	3	0	10.3	0.0
Nausea and vomiting							
Without OA & CD	(n=10)	3	3	2	-	20.0	-
With OA & CD	(n=29)	10	5	4	-	13.8	-

OA: oral alkalization; CD: control of defecation.
*p=0.004

randomized phase III study designated, as FACS showed that quality of life (QOL) profiles differed depending on the regimen (5). With the single administration of CDDP combined with CPT-11 in the FACS, half of the patients experienced diarrhea at grade 2 or more and half also experienced vomiting at grade 2 or more, which might have reduced the patients' QOL. Our weekly administrations of CPT-11 plus cisplatin together with the antidiarrheal program provided a practical and well-tolerated regimen (grade 2-3 vomiting: 31%, grade 2-4 diarrhea: 31%) (Table IV).

CPT-11 is a water-soluble semi-synthetic derivative of camptothecin and is metabolized to 7-ethyl-10-hydroxy-camptothecin (SN-38) by carboxyl esterase, mainly in the liver (13). The non-ionic lactone form of SN-38 is the active compound, and the molecular target of SN-38 has been identified as DNA topoisomerase I, a nuclear enzyme implicated in DNA replication and transcription (14). The mechanism of topoisomerase I inhibition involves the formation of a reversible enzyme-drug-DNA ternary complex. Active SN-38 is deactivated to SN38-Glu by conjugation in the liver and is secreted into bile by hepatocytes, with subsequent excretion into the intestine (15, 16). We found that the intestinal absorption of CPT-11 and SN-38 was characteristic of that, with weakly basic drugs (17). At acidic pH, non-ionic lactone forms of CPT-11 and SN-38 were passively transported, and their uptake rates were rapid. On the other hand, the respective anionic carboxylate forms were actively and slowly absorbed at basic pH (17). The uptake rate of SN-38 also correlated with its cytotoxicity (17), indicating that acidic pH in the intestinal lumen might result in a more significant cytotoxic effect of SN-38, on the intestinal epithelium.

In our case-controlled clinical study, we designed an anti-diarrheal program for CPT-11, designated as oral alkalization and control of defecation (OA and CD) (10). The rationale in designing OA and CD was to prevent absorption of active SN-38 lactone by intestinal cells, which should in turn reduce epithelial damage and its impact on subsequent delayed diarrhea. The OA and CD consisted of oral administration of sodium bicarbonate, magnesium oxide and basic water. The three agents have a basic pH and are known to directly mediate alkalization of the intestinal lumen. Sodium bicarbonate also mediates alkalization of bile in the gallbladder. Magnesium oxide demonstrates a laxative action, which should shorten the dwelling time of CPT-11 and SN-38 within the intestine. As a result of the case-control study, the OA and CD induced dose-intensity of CPT-11 and reduced the incidence of delayed diarrhea, nausea, vomiting, myelotoxicity and consumption of loperamide (10).

Sargent *et al.* recommended caution with the use of CPT-11 with fluorouracil and leucovorin for colorectal cancer (18). Deaths occurred within 60 days after initiation of treatment had several characteristics in common: dehydration (resulting from diarrhea, nausea, and vomiting), neutropenia, and sepsis, leading to death. Interpatient variability in CPT-11-induced side-effects is considered to be a major deterrent to clinical use. Therefore, we employed the OA and CD, beginning with the eleventh patient in this study. The weekly administration of CPT-11 plus CDDP with OA and CD was found to be safe for both untreated patients with NSCLC in this phase II study (Table IV) and patients with refractory or relapsed SCLC in the previously reported phase II study (2). The weekly administration of CPT-11 plus CDDP with OA and CD did not reduce activity in either of the phase II studies (2). TTP was significantly longer in NSCLC patients receiving OA and CD than in those not treated (Figure 1). With the use of OA and CD, the lower toxicity might enable administration of the total dose of CPT-11 plus CDDP, resulting in longer TTP.

In conclusion, it is indicated that weekly CPT-11 plus CDDP is a promising treatment for untreated patients with NSCLC, especially when combined with the antidiarrheal regimen of OA and CD. The mildness of side-effects permits administration in an outpatient setting.

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