Overexpression of bax Associated with Mutations in the Loop-Sheet-Helix Motif of *p53*

Cheng-long Huang,* Nobuoki Kohno,[†]
Haruhiko Inufusa,[‡] Ken Kodama,[§] Toshihiko Taki,*
and Masayuki Miyake*

From the Department of Thoracic Surgery and Department V of Oncology,* Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka, Japan; the Second Department of Internal Medicine,[†] Ehime University School of Medicine, Ehime, Japan; the First Department of Surgery,[‡] Kinki University School of Medicine, Osaka, Japan; and the Department of Surgery,[§] Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

Recent investigations have revealed that mutations of the loop-sheet-helix motif of *p53* is a significant factor for a poor prognosis in patients with non-small-cell lung cancer (NSCLC). To clarify this mechanism, *bcl-2* and *bax* expression were evaluated in relation to mutations of *p53*. Tumor tissues of 203 patients with NSCLC were analyzed. Immunohistochemistry was performed to evaluate *bcl-2* and *bax* expression, and polymerase chain reaction single-strand conformation polymorphism following direct sequencing was performed to investigate *p53* status. A total of 79 carcinomas were *bcl-2* positive, 146 carcinomas were *bax* positive, and 72 carcinomas had missense mutations of *p53*. There was no difference in *bcl-2* expression in relation to *p53* status. On the other hand, tumors with structural mutations of *p53* had significantly lower expression of *bax* than those with wild-type *p53* ($P = 0.0026$). In contrast, tumors with mutations of the loop-sheet-helix motif of *p53* had significantly higher expression of *bax* than those with wild-type *p53* ($P = 0.0236$). The frequency of a *bcl-2*/*bax* ratio of ≥ 1 was significantly lower in tumors with mutations of the loop-sheet-helix motif than that in tumors with wild-type *p53* ($P = 0.0240$). The *bcl-2*/*bax* ratio status was a significant factor for a prognosis in patients with NSCLC ($P = 0.0083$). Mutations of the loop-sheet-helix motif of *p53* were correlated with overexpression of *bax*, while other mutations of *p53* were correlated with low levels of *bax* expression. This variation in pattern of *bax* expression in relation to mutant *p53* might reflect the biological behavior of tumors in patients with *bcl-2*-positive NSCLC. (*Am J Pathol* 1999, 155:955–965)

p53 is one of the major tumor suppressor genes,^{1–4} and most nonfunctioning *p53* proteins with missense mutations of one *p53* allele coupled to deletion of the second allele seem to cause carcinogenesis.⁵ However, many studies have reported that some mutant *p53* genes could cause not only loss of normal function but also new carcinogenesis.^{6–8} *p53* protein is a DNA-binding protein, with a sequence-specific DNA-binding region in the core domain.^{9,10} Two regions of the core domain of the *p53* protein, the loop-sheet-helix motif (codons 273 to 286) and the L3-loop (codons 237 to 250), are considered to play important roles in contact with target DNA.¹¹ Recently, we have found that the mutation of the loop-sheet-helix motif of *p53* is a significant factor for a poor prognosis in patients with non-small-cell lung cancer (NSCLC).¹² Similarly, mutations in these two regions of *p53* were also reported to have a strong effect on clinical outcome in head and neck cancer.¹³ These results suggested that mutations of these regions of *p53* could not control their target genes and precisely reflect the biological behavior of these types of malignant tumors.

To date, genes considered to be target genes of *p53* include *bcl-2*,¹⁴ *bax*,¹⁵ topoisomerase II,¹⁶ insulin-like growth factor binding protein-3 (IGF BP-3),¹⁷ vascular endothelial growth factor (VEGF),¹⁸ and multidrug resistance (MDR).¹⁹ Since many NSCLCs are resistant to radio-chemotherapy in themselves, the poor prognosis of patients with mutations of the loop-sheet-helix motif of *p53* might be associated with cell cycle regulation and apoptosis more than with radio-chemoresistance.⁵ In addition, recent studies have demonstrated that *bcl-2* and *bax*, among members of the *bcl-2* family, regulate not only apoptosis but also the cell cycle.^{20–24} Therefore, we performed the additional study focusing on *bcl-2* and *bax* expression in relation to mutations of *p53* in patients with NSCLC to clarify the mechanism of clinical behavior of these mutations. We also classified the mutations of *p53* into three groups as described previously; mutations from codon 273 to codon 286 were classified as mutations of the loop-sheet-helix motif, mutations from codon 237 to codon 250 as mutations of the L3-loop, and mutations of other regions as structural mutations.^{13,25} In

Supported in part by grants-in-aid from the Ministry of Education, Science, and Culture of Japan (10557115 and 08407040) to M. Miyake.

Accepted for publication May 11, 1999.

Address reprint requests to Dr. Masayuki Miyake, Department of Thoracic Surgery and Department V of Oncology, Kitano Hospital, Tazuke Kofukai Medical Research Institute, 13-3, Kamiyama-cho, Kita-ku, Osaka 530-8480, Japan.

addition, because bax is widely expressed in normal tissues, such as smooth muscle cells and the bronchial wall,²⁶ results by Northern blotting or quantitative reverse transcriptase polymerase chain reaction (RT-PCR) could be confused by bax expression in surrounding normal tissues, and blind RNA analysis was not suitable for this study. Therefore, immunohistochemistry was performed to evaluate bcl-2 and bax expression in this study.

Materials and Methods

Clinical Characteristics of Patients

From January 1991 to April 1995, the patients with NSCLC who underwent surgery at the Department of Surgery of the Osaka Medical Center for Cancer and Cardiovascular Diseases and the Department of Thoracic Surgery of the Kitano Hospital, Medical Research Institute of Osaka, Japan, were studied as described in our previous report.¹² Among these 204 patients, 203 cases were investigated, except one case with wild-type *p53* whose specimen was not available for immunohistochemistry. They included 121 patients with adenocarcinoma, 71 patients with squamous cell carcinoma, and 11 patients with large-cell carcinoma. Tumor-node-metastasis (TNM) staging designations were made according to the postsurgical pathological international staging system.²⁷ Patients' clinical records and histopathological diagnoses were fully documented. This report includes follow-up data as of February 28 in 1998. The median follow-up period for all patients was 41.8 ± 23.6 months.

All patients with N2 or N3 status had mediastinal radiotherapy (50 Gy in 25 fractions for a period of 5 weeks) after surgical resection and then were treated by two cycles of adjuvant chemotherapy including cisplatin (80 mg/m² given intravenously on day 1) and vindesine (4 mg/m² given intravenously on days 1, 8, and 15). Eighteen patients with T3 or T4 status, who were found to have incomplete resection, also received radiotherapy (50 Gy in 25 fractions for a period of 5 weeks) for microscopic-positive margins. The other patients had no radiotherapy before recurrence. Post-operative adjuvant systemic chemotherapy was given according to nodal status. Of 82 node-positive patients, 78 patients underwent treatment by the adjuvant chemotherapy with two cycles of cisplatin and vindesine. Of 121 node-negative patients, 118 patients did not have further adjuvant treatment. We detected distant metastases in 83 patients during the observation period, and among these 83 patients, 14 patients had also local recurrences. Seven patients had locoregional recurrences. After recurrence, locoregional tumors or lymph nodes were principally treated with radiotherapy. Patients with distant metastases were treated with other regimes of chemotherapy, including cisplatin and etoposide.

Immunohistochemistry

For immunohistochemistry of bcl-2 and bax, a mouse monoclonal antibody for bcl-2 (clone 124, Dako, Glostrup, Denmark) diluted at 1:50 and a rabbit poly-

clonal antibody for bax (N-20, Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:100 were used. Formalin-fixed paraffin-embedded tissue was cut in 4- μ m sections and mounted on poly-L-lysine-coated slides. Sections were deparaffinized and rehydrated. The slides were then heated in a microwave for 10 minutes in a 10- μ mol/L citrate buffer solution at pH 6.0 and cooled to room temperature for 20 minutes. After quenching the endogenous peroxidase activity with 0.3% H₂O₂ (in absolute methanol) for 30 minutes, the sections were blocked for 2 hours at room temperature with 5% bovine serum albumin. Subsequently, duplicate sections were incubated overnight with the primary specific antibodies detecting bcl-2 and bax, respectively. Slides were then incubated for 1 hour with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) for bcl-2 or biotinylated anti-rabbit IgG (Vector Laboratories) for bax. The sections were incubated with the avidin-biotin-peroxidase complex (Vector) for 1 hour, and the antibody binding was visualized with 3,3'-diaminobenzidine tetrahydrochloride. Finally, the sections were lightly counterstained with Mayer's hematoxylin (Figure 1).

Positive staining of lymphocytes infiltrating into the tumor provided an internal control for bcl-2 staining. For bax staining, the internal control was provided by smooth muscle cells in the media of arterioles and in the bronchial wall.²⁶ SP2 supernatants were used as negative controls for both antibodies.

All of the immunostained sections were reviewed by two pathologists who had no knowledge of the patients' clinical status. In cases of multiple areas of low intensity, five areas selected at random were scored, and in sections where all of the staining appeared intense, one random field was selected. The proportion of high- and low-staining tumor cells in each selected field was determined by counting individual tumor cells at high magnification. At least 200 tumor cells were scored per 40 $\times$ field. All sections were scored in a semiquantitative fashion according to the method described previously, which considers both the intensity and percentage of cells staining at each intensity.^{28,29} Intensities were classified as 0 (no staining), +1 (weak staining), +2 (distinct staining), or +3 (very strong staining) in relation to the internal control described above.²⁶ For each slide, a value designated as the HSCORE was obtained by application of the following algorithm: $HSCORE = \Sigma (I \times PC)$, where *I* and *PC* represent staining intensity and percentage of cells that stain at each intensity, respectively, and corresponding HSCOREs were calculated separately. When the HSCORE in a given specimen was ≥ 50 , the sample was classified as bcl-2 positive or bax positive, and when the HSCORE was < 50 , the sample was classified as negative. In addition, the bcl-2 HSCORE was divided by the bax HSCORE in each tumor to obtain a bcl-2/bax ratio as described previously.²⁹

PCR-SSCP and Sequencing

To investigate the mutations of *p53*, we performed PCR single-strand conformation polymorphism (PCR-SSCP)

Figure 1. Immunohistochemical staining of human non-small-cell lung cancer tissues using the avidin-biotin-peroxidase complex procedure. Original magnification, $\times 200$. **A:** Adenocarcinoma with positive bcl-2 expression. **B:** Squamous cell carcinoma with positive bcl-2 expression. **C:** Adenocarcinoma with positive bax expression. **D:** Squamous cell carcinoma with positive bax expression.

and direct sequencing as described in our previous report.¹² Briefly, genomic DNA was extracted from frozen specimens by using proteinase K digestion and phenol/chloroform extraction. After 40 cycles of PCR amplification of exons 5 to 8 of *p53* were performed, electrophoresis for SSCP was done to detect mutant bands that were stained with ethidium bromide and visualized under ultraviolet light. Then, to ascertain base changes and exclude the non-missense mutations detected by PCR-SSCP, direct sequencing was performed.

Statistical Analysis

Overall cancer-specific survival was defined from the date of operation to the date of cancer-related death. The statistical differences in bcl-2 and bax expression and mutations of *p53* in relation to several clinical and pathological parameters were assessed by the χ^2 test and the *t*-test. Moreover, the statistical significance of bcl-2 and bax expression among each mutant region of *p53* was also analyzed by the *t*-test and the χ^2 test. The Kaplan-Meier method was used to estimate the probability of overall survival as a function of time, and differences in the survival of subgroups of patients were compared with Mantel's log-rank test.^{30,31} Multivariate analyses were performed using the Cox regression model to study the effects of different variables on survival.³² All *P* values were based on two-tailed statistical analysis, and a *P*

value <0.05 was considered to indicate statistical significance.

Results

Expression of bcl-2

Of the 203 tumors we studied, 79 carcinomas (38.9%) were bcl-2 positive, and bcl-2 expression was always diffuse in the cytoplasm (Table 1 and Figure 1, A and B). The HSCORE of bcl-2 was 30.6 ± 61.8 in adenocarcinomas, 65.1 ± 81.4 in squamous cell carcinomas, and 95.9 ± 134.2 in large-cell carcinomas. The HSCORE of bcl-2 in adenocarcinomas was significantly lower than that in squamous cell carcinomas and that in large-cell carcinomas ($P = 0.0011$ and $P = 0.0037$, respectively). Of the 121 adenocarcinomas, 35 tumors (28.9%) were bcl-2 positive. Of the 71 squamous cell carcinomas, 39 tumors (54.9%) were bcl-2 positive. Of the 11 large-cell carcinomas, 5 tumors (45.5%) were bcl-2 positive (Table 1). The frequency of bcl-2-positive tumors in squamous cell carcinomas was significantly higher than that in adenocarcinomas ($P = 0.0016$). However, there was no significant correlation between bcl-2 staining and other patient prognostic factors such as tumor status, nodal status, smoking habits, or tumor differentiation.

Table 1. Distribution of bcl-2 and bax Status in 203 Patients with NSCLC According to Clinicopathological Characteristics

Characteristics	n	bcl-2 Status			bax Status			bcl-2/bax Ratio		
		Positive	Negative	P value	Positive	Negative	P value	≥1	<1	P value
Smoking										
Nonsmoker	57	18	39	0.2379	44	13	0.2963	11	46	0.1397
Smoker	146	61	85		102	44		45	101	
Age at surgery (years)										
<60	72	29	43	0.8845	55	17	0.3747	20	52	>0.9999
≥60	131	50	81		91	40		36	95	
Sex										
Male	152	61	91	0.6542	107	45	0.5116	45	107	0.3518
Female	51	18	33		39	12		11	40	
Tumor status										
T1	53	21	32	0.5735	39	14	0.6347	12	41	0.4037
T2	91	36	55		62	29		26	65	
T3	42	18	24		31	11		15	27	
T4	17	4	13		14	3		3	14	
Nodal status										
N0	121	54	67	0.1022	82	39	0.1559	37	84	0.4863
N1	31	12	19		21	10		9	22	
N2	43	12	31		36	7		9	34	
N3	8	1	7		7	1		1	7	
Pathological stage										
Stage I	95	39	56	0.1106	65	30	0.0800	25	70	0.0878
Stage II	43	23	20		27	16		18	25	
Stage IIIA	41	12	29		33	8		9	32	
Stage IIIB	24	5	19		21	3		4	20	
Differentiation										
Well	36	11	25	0.3140	31	5	0.1950	10	26	0.1195
Moderately	101	38	63		75	26		22	79	
Poorly	66	30	36		40	26		24	42	
Histology										
Adenocarcinoma	121	35	86	0.0016	91	30	0.0231	25	96	0.0273
Squamous cell carcinoma	71	39	32		51	20		27	44	
Large-cell carcinoma	11	5	6		4	7		4	7	
Total number of patients	203	79	124		146	57		56	147	

Expression of bax

One hundred and forty-six carcinomas (71.9%) had positive expression of bax, and bax expression was also always diffuse in the cytoplasm (Table 1 and Figure 1, C and D). The HSCORE of bax was 108.6 ± 77.7 in adenocarcinomas, 83.9 ± 53.7 in squamous cell carcinomas, and 57.3 ± 66.3 in large-cell carcinomas. The HSCORE of bax in adenocarcinomas was significantly higher than that in squamous cell carcinomas and that in large-cell carcinomas ($P = 0.0189$ and $P = 0.0360$, respectively). Ninety-one tumors (75.2%) were bax positive in adenocarcinomas, fifty-one tumors (71.8%) were bax positive in squamous cell carcinomas, and four tumors (36.4%) were bax positive in large-cell carcinomas (Table 1). The frequency of bcl-2-positive tumors in large-cell carcinomas was significantly lowest ($P = 0.0231$), whereas no difference in bax expression was found between adenocarcinomas and squamous cell carcinomas. There was no significant correlation between bax expression and other patient prognostic factors such as tumor status, nodal status, smoking habits, or tumor differentiation.

Mutations of p53

Of the 203 patients with NSCLC studied, 75 carcinomas (36.9%) had mutations of p53, including 72 carcinomas

with missense mutations and 3 carcinomas with non-missense mutations (Table 2). With respect to the mutant region of the p53 gene of these 72 carcinomas with missense mutations, 42 carcinomas had structural mutations, 15 carcinomas had mutations of the L3-loop, and 15 carcinomas had mutations of the loop-sheet-helix motif.

Expression of bcl-2 and bax in Relation to Mutant Region of p53

The HSCORE of bcl-2 was 42.8 ± 71.8 in tumors with wild-type p53, 53.9 ± 87.1 in tumors with structural mutations, 57.3 ± 91.1 in tumors with mutations of the L3-loop, and 42.7 ± 76.7 in tumors with mutations of the loop-sheet-helix motif. There was no difference in bcl-2 expression in relation to the mutant region of p53.

The HSCORE of bax was 106.5 ± 70.0 in tumors with wild-type p53, 70.1 ± 57.1 in tumors with structural mutations, 38.3 ± 39.9 in tumors with mutations of the L3-loop, and 150.7 ± 78.6 in tumors with mutations of the loop-sheet-helix motif (Figure 2). Tumors with structural mutations had lower levels of bax expression than those with wild-type p53 ($P = 0.0026$), and tumors with mutations of the L3-loop also had lower levels of bax expression than those with wild-type p53 ($P = 0.0003$). In con-

Table 2. Characteristics of the Patients with Mutations of *p53* in NSCLC

Case	Sex	Age (years)	Histology	Differentiation	T	N	Stage	Smoking	Mutation of <i>p53</i>		HSCORE of bcl-2	HSCORE of bax	Prognosis (months)	
									Codon	Base change				
1	M	69	LA	Poorly	2	0	IB	+	132	AAG → AAT	0	20	74.3	Alive
2	F	60	AD	Moderately	2	0	IB	—	132	AAG → AGG	0	40	44.0	Dead
3	M	67	SQ	Poorly	3	0	IIB	+	132	AAG → AGG	70	80	65.9	Alive
4	M	55	SQ	Moderately	2	1	IIB	+	133	ATG → ATT	90	20	74.3	Alive
5	M	64	AD	Moderately	3	3	IIIB	+	133	ATG → ACG	0	180	14.4	Dead
6	M	50	LA	Poorly	2	0	IB	+	137	CTG → ATG	300	0	41.9	Alive
7	M	64	AD	Moderately	2	0	IB	+	137	CTG → ATG	0	40	38.2	Alive
8	M	74	SQ	Moderately	2	0	IB	+	143	GTG → GCG	90	80	66.9	Alive
9	M	62	AD	Well	2	2	IIIA	—	148	GAT → TAT	0	120	12.1	Dead
10	M	72	SQ	Poorly	2	0	IB	+	150	ACA → AAA	60	30	61.2	Alive
11	M	73	LA	Poorly	2	0	IB	+	150	ACA → AAA	0	80	40.2	Alive
12	M	55	AD	Moderately	4	3	IIIB	+	153	CCC → TCC	0	20	42.9	Dead
13	M	69	SQ	Poorly	2	0	IB	—	153	CCC → ACC	0	0	25.9	Dead
14	F	75	AD	Poorly	3	0	IIB	—	157	GTC → ATC	180	50	35.3	Alive
15	F	70	AD	Moderately	1	0	IA	—	157	GTC → ATC	0	150	75.7	Alive
16	M	70	LA	Poorly	2	0	IB	+	157	GTC → TTC	300	0	75.1	Dead
17	M	61	SQ	Moderately	1	3	IIIB	+	157	GTC → TTC	0	70	5.9	Dead
18	M	71	SQ	Moderately	3	2	IIIA	+	157	GTC → GAC	0	140	4.9	Dead
19	M	66	SQ	Moderately	1	0	IA	+	158	CGC → CAC	0	30	55.7	Alive
20	M	65	LA	Poorly	3	0	IIB	+	158	CGC → CTC	70	0	52.1	Alive
21	M	70	SQ	Poorly	3	0	IIB	+	158	CGC → CTC	160	5	78.9	Alive
22	F	58	AD	Moderately	1	0	IA	—	158	CGC → GGC	60	160	53.7	Alive
23	F	65	AD	Moderately	1	2	IIIA	—	161	GCC → GAC	0	100	42.1	Dead
24	M	63	SQ	Poorly	2	1	IIB	+	163	TAC → TAG	300	80	11.4	Dead
25	M	53	AD	Well	1	0	IA	+	163	TAC → TAT	0	200	61.7	Alive
26	M	68	LA	Moderately	2	0	IB	—	169	ATG → ATA	5	40	56.6	Alive
27	M	55	AD	Well	2	0	IB	+	175	CGC → AGC	100	80	45.8	Alive
28	M	69	SQ	Moderately	2	0	IB	+	175	CGC → AGC	0	40	70.8	Alive
29	F	60	SQ	Moderately	3	1	IIIA	+	175	CGC → AGC	50	30	56.1	Alive
30	F	59	AD	Poorly	1	2	IIIA	—	175	CGC → CAC	60	50	44.9	Alive
31	M	57	SQ	Moderately	2	0	IB	+	175	CGC → CAC	0	120	28.0	Alive
32	M	63	SQ	Moderately	2	0	IB	+	175	CGC → CAC	0	20	52.9	Alive
33	M	61	SQ	Poorly	3	2	IIIA	+	175	CGC → GGC	0	140	58.9	Alive
34	M	43	LA	Poorly	2	3	IIIB	—	213	CGA → TGA	0	70	5.8	Dead
35	M	65	SQ	Moderately	1	0	IA	+	228	GAC → AAC	0	0	16.1	Dead
36	F	61	AD	Well	1	1	IIA	—	228	GAC → GGC	0	120	69.9	Alive
37	M	56	SQ	Moderately	1	0	IA	+	235	AAC → TAC	200	60	39.3	Dead
38	F	68	SQ	Well	2	0	IB	+	239	AAC → ACC	0	45	14.2	Dead
39	M	69	SQ	Moderately	3	0	IIB	+	245	GGC → CGC	0	40	36.7	Alive
40	M	69	AD	Poorly	4	0	IIIB	+	245	GGC → CGC	100	10	14.8	Dead
41	M	50	SQ	Moderately	3	1	IIIA	+	245	GGC → TGC	0	40	15.9	Dead
42	M	70	AD	Moderately	3	2	IIIA	+	245	GGC → GGA	100	80	40.9	Alive
43	M	71	AD	Moderately	2	1	IIB	+	246	ATG → CTG	0	20	30.3	Dead
44	M	57	AD	Poorly	3	2	IIIA	+	248	CGG → CAG	0	0	10.2	Dead
45	M	60	AD	Poorly	1	0	IA	+	248	CGG → CAG	0	0	7.1	Dead
46	M	52	SQ	Moderately	2	1	IIB	+	248	CGG → CTG	160	30	86.0	Alive
47	M	72	AD	Well	2	3	IIIB	+	248	CGG → TGG	0	100	11.3	Dead
48	M	68	AD	Poorly	3	0	IIB	+	248	CGG → TGG	300	30	70.1	Alive
49	M	72	AD	Moderately	3	0	IIB	+	248	CGG → TGG	0	20	11.7	Dead
50	M	46	SQ	Poorly	2	1	IIB	+	248	CGG → TGG	70	0	36.7	Alive
51	F	66	AD	Poorly	3	0	IIB	+	249	AGG → AAG	0	80	35.1	Alive
52	M	82	AD	Moderately	1	0	IA	+	249	AGG → ATG	50	20	36.1	Alive
53	F	69	SQ	Moderately	2	0	IB	+	249	AGG → ATG	180	140	71.1	Alive
54	M	68	SQ	Moderately	2	0	IB	+	251	ATC → ACC	60	30	43.3	Alive
55	M	58	SQ	Moderately	1	0	IA	+	255	ATC → TTC	110	100	72.7	Alive
56	F	69	AD	Moderately	2	0	IB	—	265	CTG → TTG	0	200	60.3	Alive
57	F	70	LA	Poorly	2	1	IIB	+	266	GGA → GAA	0	20	30.6	Dead
58	M	74	SQ	Moderately	1	0	IA	+	270	TTT → TGT	0	180	68.0	Alive
59	M	50	SQ	Moderately	4	2	IIIB	—	270	TTT → TGT	0	150	11.3	Dead
60	M	71	SQ	Moderately	4	0	IIIB	+	273	CGT → CAT	50	180	11.7	Dead
61	M	78	SQ	Moderately	2	0	IB	+	273	CGT → CAT	70	150	12.1	Dead
62	M	48	SQ	Moderately	2	0	IB	—	273	CGT → TGT	60	80	77.5	Alive
63	M	55	AD	Moderately	2	1	IIB	+	273	CGT → TGT	0	180	22.7	Dead
64	F	61	AD	Poorly	2	2	IIIA	+	273	CGT → CTT	0	160	22.0	Dead
65	M	49	AD	Poorly	3	1	IIIA	+	273	CGT → CTT	0	300	8.1	Dead
66	M	69	AD	Moderately	1	2	IIIA	+	273	CGT → CGC	0	200	40.2	Alive
67	F	59	AD	Moderately	3	2	IIIA	—	275	TGT → TCT	0	300	38.8	Alive
68	M	71	LA	Poorly	1	2	IIIA	+	280	AGA → GGA	300	40	15.6	Dead
69	M	81	SQ	Moderately	2	0	IB	+	280	AGA → ACA	50	100	20.2	Dead
70	M	83	SQ	Well	4	0	IIIB	+	281	GAC → GAA	0	120	5.3	Dead
71	M	68	SQ	Poorly	2	0	IB	+	281	GAC → GAA	0	200	13.2	Dead
72	M	69	AD	Moderately	2	0	IB	+	283	CGC → CCC	0	100	11.2	Dead
73	M	68	SQ	Moderately	2	0	IB	+	283	CGC → CCC	50	60	66.8	Alive
74	M	54	AD	Poorly	1	2	IIIA	—	283	CGC → TGC	60	90	9.0	Dead
75	F	71	AD	Moderately	1	2	IIIA	—	285	GAG → AAG	0	200	43.1	Alive

Cases 42, 56, and 66 are non-missense mutations. T, tumor status; N, nodal status; M, male; F, female; LA, large-cell carcinoma; AD, adenocarcinoma; SQ, squamous cell carcinoma.

Figure 2. HSCORE of bax in 203 patients with non-small-cell lung cancer in relation to *p53* status. Bars indicate the standard error of each group.

trast, tumors with mutations of the loop-sheet-helix motif had higher levels of bax expression than those with wild-type *p53* ($P = 0.0236$).

With respect to the *bcl-2*/*bax* ratio in tumors, the frequency of a *bcl-2*/*bax* ratio of ≥ 1 in relation to the mutant region of *p53* was analyzed. Of 131 tumors (26.0%) with wild-type *p53*, 34 had a *bcl-2*/*bax* ratio ≥ 1 , 15 of 42 tumors (35.7%) with structural mutations had a *bcl-2*/*bax* ratio ≥ 1 , 6 of 15 tumors (40.0%) with mutations of the L3-loop had a *bcl-2*/*bax* ratio ≥ 1 , and only 1 of 15 tumors (6.7%) with mutations of the loop-sheet-helix motif had a *bcl-2*/*bax* ratio ≥ 1 (Figure 3A). The frequency of a *bcl-2*/*bax* ratio ≥ 1 in tumors with mutations of the loop-sheet-helix was significantly lower than that in any other subgroups ($P = 0.0240$).

In addition, 79 tumors with *bcl-2*-positive expression were classified into two groups in relation to the *bcl-2*/*bax* ratio. Of these 79 *bcl-2*-positive tumors, 34 of 49 tumors (69.4%) with wild-type *p53* had a *bcl-2*/*bax* ratio ≥ 1 , 15 of 17 tumors (88.2%) with structural mutations had a *bcl-2*/*bax* ratio ≥ 1 , all of 6 tumors with mutations of the L3-loop had a *bcl-2*/*bax* ratio ≥ 1 , and only 1 of 7 tumors (14.3%) with mutations of the loop-sheet-helix motif had a *bcl-2*/*bax* ratio ≥ 1 (Figure 3B). Of the *bcl-2*-positive tumors, the frequency of a *bcl-2*/*bax* ratio ≥ 1 in tumors with mutations of the loop-sheet-helix was significantly lower than that in tumors with wild-type *p53* ($P = 0.0087$).

Association of *bcl-2* and *bax* Expression with Survival of NSCLC Patients

The survival of 203 patients with NSCLC stratified according to *bcl-2*, *bax*, and *bcl-2*/*bax* ratio status is shown in Table 3 and Figure 4. The 5-year survival rate of patients with *bcl-2*-positive tumors was significantly better than that with *bcl-2*-negative tumors (74.5% versus 46.6%, $P = 0.0006$; Table 3 and Figure 4A). Especially, of the 121

Figure 3. A: *bcl-2*/*bax* ratio of 203 patients with non-small-cell lung cancer in relation to *p53* status. B: *bcl-2*/*bax* ratio of 79 patients with *bcl-2*-positive non-small-cell lung cancer in relation to *p53* status.

adenocarcinomas, patients with *bcl-2*-positive tumors had a better prognosis than those with *bcl-2*-negative tumors (74.1% versus 43.4%, 5-year survival, $P = 0.0039$). On the other hand, there was no difference in survival in relation to *bax* expression (56.5% versus 59.8%, 5-year survival; Table 3 and Figure 4B).

The survival of 203 patients with NSCLC was then analyzed in relation to *bcl-2* and *bax* status (Figure 4C); 56 patients had tumors positive for both *bcl-2* and *bax*, 23 patients had *bcl-2*-positive but *bax*-negative tumors; 90 patients had *bcl-2*-negative and *bax*-positive tumors; and 34 patients had tumors negative for both *bcl-2* and *bax*. Overall survival of patients with *bcl-2*-positive and *bax*-negative tumors was significantly better than that with tumors positive for both (91.3% versus 67.4%, 5-year survival, $P = 0.0417$). In addition, overall survival of patients with tumors positive for both was significantly better than that of patients with *bcl-2*-negative and *bax*-positive tumors (67.4% versus 49.9%, 5-year survival, $P = 0.0341$) and was also significantly better than that with tumors negative for both (67.4% versus 39.7%, 5-year survival, $P = 0.0201$). Furthermore, of the *bcl-2*-negative tumors, there was no difference in survival of patients in relation to *bax* expression.

With respect to the *bcl-2*/*bax* ratio, overall survival of patients with tumors having a *bcl-2*/*bax* ratio of ≥ 1 was significantly better than that of patients with tumors with a *bcl-2*/*bax* ratio of < 1 (81.0% versus 48.7%, 5-year survival, $P = 0.0004$; Table 3 and Figure 4D). Of the 121

Table 3. Five-Year Survival of 203 Patients with NSCLC According to Clinicopathological Characteristics and Gene Status

Characteristics	bcl-2 Status			bax Status			bcl-2/bax Ratio		
	Positive	Negative	<i>P</i> value	Positive	Negative	<i>P</i> value	≥1	<1	<i>P</i> value
Smoking									
Nonsmoker	79.7	54.7	0.0565	57.5	76.9	0.1597	88.9	56.8	0.0630
Smoker	73.4	43.8	0.0027	57.3	54.9	0.9239	79.5	45.9	0.0014
Age at surgery (years)									
<60	74.4	33.6	0.0015	46.4	56.5	0.3590	77.9	38.8	0.0049
≥60	74.7	54.1	0.0610	62.4	61.2	0.8585	83.3	54.2	0.0240
Sex									
Male	71.1	45.7	0.0064	55.3	57.9	0.6421	76.2	47.4	0.0041
Female	86.6	51.1	0.0169	60.8	66.7	0.6470	100.0	53.3	0.0241
Tumor status									
T1	77.1	65.1	0.2830	74.7	56.2	0.1407	81.5	67.0	0.3775
T2	76.5	46.8	0.0357	56.7	62.8	0.6462	86.5	47.5	0.0063
T3	77.8	31.3	0.0042	44.4	72.7	0.1514	80.0	35.6	0.0086
T4	25.0	28.8	0.5934	35.7	0.0	0.8048	33.3	26.8	0.9101
Nodal status									
N0	81.8	65.8	0.1100	74.4	70.0	0.6588	91.1	65.2	0.0233
N1	81.5	37.9	0.0259	57.3	50.0	0.5671	74.1	46.8	0.1692
N2	41.7	15.3	0.3248	21.3	28.6	0.9542	55.6	13.9	0.0691
N3	0.0	0.0	0.8223	14.3	0.0	0.2092	0.0	0.0	0.8223
Pathological stage									
Stage I	82.2	70.3	0.4214	78.8	67.7	0.1592	95.0	68.6	0.0881
Stage II	85.6	30.0	0.0004	53.5	68.8	0.5087	80.8	45.5	0.0283
Stage IIIA	50.0	23.5	0.2738	29.5	37.5	0.9136	66.7	21.3	0.0390
Stage IIIB	20.0	23.7	0.9037	28.6	0.0	0.9621	25.0	22.5	0.8673
Differentiation									
Well	81.8	67.5	0.4796	69.6	80.0	0.6509	80.0	68.8	0.6211
Moderately	73.2	49.4	0.0107	57.8	61.4	0.4090	83.4	51.5	0.0065
Poorly	73.3	26.9	0.0026	44.5	52.9	0.5095	79.2	30.4	0.0018
Histology									
Adenocarcinoma	74.1	43.4	0.0039	53.6	47.7	0.7856	77.5	46.0	0.0131
Squamous cell carcinoma	73.7	55.9	0.1216	62.3	75.0	0.3966	84.4	54.3	0.0162
Large-cell carcinoma	80.0	50.0	0.3674	50.0	71.4	0.4144	75.0	57.1	0.6242
Total number of patients	74.5	46.6	0.0006	56.5	59.8	0.6103	81.0	48.7	0.0004

adenocarcinomas, overall survival of patients with a bcl-2/bax ratio ≥1 was significantly better than that of patients with tumors with a bcl-2/bax ratio <1 (77.5% *versus* 46.0%, 5-year survival, *P* = 0.0131). In addition, this was similarly true of 71 patients with squamous cell carcinomas (84.4% *versus* 54.3%, 5-year survival, *P* = 0.0162).

Cox regression analysis of prognostic variables for NSCLC, adenocarcinoma, and squamous cell carcinoma, using both bcl-2 status and bcl-2/bax ratio status, is shown in Table 4. The bcl-2/bax ratio status was a significant indicator for survival of patients with NSCLC (Hazard ratio = 3.290, *P* = 0.0083), those with adenocarcinoma (Hazard ratio = 3.242, *P* = 0.0137), and those with squamous cell carcinoma (Hazard ratio = 6.610, *P* = 0.0053), whereas bcl-2 status alone was not a significant factor for survival of these patients.

Association of bcl-2 Expression with Survival of NSCLC Patients According to Mutant Region of *p53*

Of patients with bcl-2-positive tumors, the 5-year survival of patients with mutations of the loop-sheet-helix motif of *p53* was significantly lower than that of patients with wild-type *p53* (28.6% *versus* 75.0%, *P* = 0.0050) and that of patients with structural mutation of *p53* (28.6% *versus* 87.8%, *P* = 0.0082; Figure 5).

On the other hand, of patients with bcl-2-negative tumors, no difference was found between survival of patients with mutations of the loop-sheet-helix motif of *p53* and those with other groups of *p53*.

Discussion

Our study demonstrated that bcl-2/bax ratio status was a significant factor for a good prognosis in patients with NSCLC as described previously.²⁶ Furthermore, mutations of the loop-sheet-helix motif of *p53* were strongly correlated with overexpression of bax, whereas other mutations of *p53* were correlated with lower levels of bax expression.

It becomes clear that many proteins that induce apoptosis can regulate the cell cycle.³³ For example, it has been shown that expression of *c-myc* initiates both proliferation and apoptosis *in vitro*.³⁴ In addition, expression of the tumor suppressor protein retinoblastoma (Rb), a known regulator of cell cycle progression, can repress apoptosis induced by radiation.³⁵ The two-part mechanism of these molecules, involving both cell proliferation and apoptosis, might play an important role in the regulation of cell selection and growth arrest during differentiation into a post-mitotic state in normal organs.³⁶

Initially, it was reported that bcl-2 and bax were regulators of apoptosis.³⁷ Although their precise mechanisms

Figure 4. A: Overall survival of 203 patients with non-small-cell lung cancer in relation to bcl-2 status. B: Overall survival of 203 patients with non-small-cell lung cancer in relation to bax status. C: Overall survival of 203 patients with non-small-cell lung cancer in relation to bcl-2 and bax status. D: Overall survival of 203 patients with non-small-cell lung cancer in relation to bcl-2/bax ratio status.

of action remain unknown, it is widely accepted that these proteins dimerize with themselves or with each other.^{38,39} However, it is still unknown whether bax acts directly in their potential pathway or merely acts as an antagonist of bcl-2. Recently, many studies have demonstrated that bcl-2 and bax also have potential effects on cell proliferation.^{20–24} Increasing the level of bcl-2 has been shown to retard cell proliferation due to prolongation of the G1 phase of the cell cycle, sustaining the level of cyclin-dependent kinase inhibitor p27^{Kip1}.²⁰ In contrast, overexpression of bax has been correlated with accelerated entry into S phase, with decreased levels of p27^{Kip1}.²¹ In addition, bax has been shown to reverse the inhibitory effect of bcl-2 on cell proliferation.^{22,23} This is likely due to neutralization of the bcl-2 activity by heterodimerization with bax as overexpression of bax alone does not accelerate cell proliferation.²² Thus, it is considered that bcl-2 might act as a direct effector in their cell proliferation pathway and that bax could act as an inhibitor of bcl-2 by combining with bcl-2.³⁷

The observation that bcl-2 diminishes cell proliferation and that bax accelerates cell proliferation by inhibiting bcl-2 activity might reflect the biological behavior of some malignant tumors. Actually, expression of bcl-2 has been believed to be a significant factor for a good prognosis in NSCLC,^{26,40–43} in breast cancer,⁴⁴ and in ovarian cancer.⁴⁵ Previous reports using combined assessment of bcl-2 and bax expression in NSCLC demonstrated the significant difference of survival between patients with

bcl-2-positive and bax-positive tumors and patients with bcl-2-negative and bax-positive tumors in stage 1 NSCLC.²⁶ In our study of patients with NSCLC, patients with bcl-2-positive tumors had a significantly better survival than those with bcl-2-negative tumors, although bax expression alone was not related to a prognosis. In addition, of patients with bcl-2-positive tumors, the overall survival of patients with bax-positive tumors was lower than that of patients with bax-negative tumors. In contrast, of patients with bcl-2-negative tumors, patient survival was not related to bax status. Cox's regression model demonstrated that the bcl-2/bax ratio was a much stronger factor for a prognosis in NSCLC than bcl-2 status alone, which suggests that bax could act merely as an antagonist to bcl-2.

Immunohistochemical results were initially quantitated using both the HSCORE and the percentage of positive cells. Using Cox regression model analysis, the prognostic value of the bcl-2/bax ratio calculated by the percentage of positive cells demonstrated a lower Hazard ratio and lower *P* value compared with that of bcl-2/bax ratio calculated by HSCORE (2.924 versus 3.290, Hazard ratio; 0.0148 versus 0.0083, *P* value, data not shown). Therefore, the semiquantitative HSCORE method likely provides a more accurate reflection of the biological behavior of these tumors than that provided by the percentage of positive cells.

In our study, bcl-2 expression was significantly higher in squamous cell carcinomas, whereas bax expression was significantly higher in adenocarcinomas, which con-

Table 4. Multivariate Regression Analysis in Predicting Survival of 203 Patients with NSCLC

Histology variables	Assigned score	NSCLC			Adenocarcinoma			Squamous cell carcinoma		
		Hazard ratio	(95% CI)	<i>P</i> value	Hazard ratio	(95% CI)	<i>P</i> value	Hazard ratio	(95% CI)	<i>P</i> value
bcl-2 Status										
Positive	0	0.937	(0.451–1.948)	0.8617	1.475	(0.447–2.870)	0.5232	0.404	(0.131–1.250)	0.1158
Negative	1									
bcl-2/bax Ratio status										
≥1	0	3.290	(1.360–7.961)	0.0083	3.242	(1.272–8.262)	0.0137	6.610	(1.750–24.966)	0.0053
<1	1									
<i>p53</i> Status										
Wild type	0	1.255	(0.805–1.954)	0.3162	1.314	(0.687–2.510)	0.4090	1.767	(0.756–4.129)	0.1884
Mutant	1									
Smoking										
Nonsmoker	0	0.812	(0.419–1.574)	0.5378	0.749	(0.318–1.764)	0.5083	0.617	(0.149–2.551)	0.5047
Smoker	1									
Sex										
Male	0	0.496	(0.251–0.979)	0.0431	0.384	(0.169–0.875)	0.0228	1.532	(0.338–6.942)	0.5799
Female	1									
Age at surgery (years)										
<60	0	1.084	(0.674–1.743)	0.7395	1.364	(0.726–2.565)	0.3349	1.605	(0.577–4.466)	0.3645
≥60	1									
Tumor status										
T1	1	1.228	(0.956–1.576)	0.1075	1.370	(0.987–1.902)	0.0600	1.108	(0.683–1.798)	0.6771
T2	2									
T3	3									
T4	4									
Nodal status										
N0	0	1.914	(1.505–2.434)	<0.0001	1.732	(1.290–2.326)	0.0003	3.172	(1.819–5.529)	<0.0001
N1	1									
N2	2									
N3	3									
Differentiation										
Well	0	1.398	(0.987–1.980)	0.0590	1.869	(1.187–2.942)	0.0069	0.756	(0.374–1.528)	0.4361
Moderately	1									
Poorly	2									

firms previous observation.²⁶ Concerning the regulation of *bcl-2* and *bax* expressions, transcriptional dysregulation of the *bcl-2* gene in the majority of non-Hodgkin's B-cell lymphomas is caused by the t(14;18) chromosomal translocation of the gene that brings it into juxtaposition with the immunoglobulin heavy-chain locus.⁴⁶ However, such translocations or other gross structural alterations in the *bcl-2* gene are not found in solid tumors, and the presence of unknown mechanisms of *bcl-2* and *bax* regulation in solid tumors is still suggested.⁴² On the other hand, the significant difference in expression in relation to histological type of NSCLC might reflect different clinical

behaviors related to histological type.⁴⁷ For example, squamous cell carcinomas are likely to relapse locoregionally, whereas adenocarcinomas are likely to have distant metastasis even when the primary tumors are still small.⁴⁷

Furthermore, we demonstrated that mutations of the loop-sheet-helix motif of *p53* were correlated with overexpression of *bax* in patients with NSCLC, whereas mutations of other portions of *p53* were correlated with lower levels of *bax* expression. In contrast, no relationship was detected between mutations of *p53* and *bcl-2* expression in our study. Previously, we reported that the mutation of the loop-sheet-helix motif of *p53* was a significant factor for a poor prognosis in patients with NSCLC.¹² The *bax* gene promoter region contains four motifs with homology to consensus *p53*-binding sites, and *p53* is believed to be a direct activator of the *bax* gene.¹⁵ The overexpression of *bax* associated with mutations of the loop-sheet-helix motif of *p53* might lead to a poor prognosis in patients with this subtype of *bcl-2*-positive tumors, by inhibiting *bcl-2* activity.³⁷

Previous studies of protein-DNA complexes have shown that α -helices are used by most of the major families of DNA-binding proteins, including the zinc finger,⁴⁸ the steroid receptor,⁴⁹ and the leucine zipper motif.⁵⁰ *p53* also uses the H2 α -helix included in the loop-sheet-helix motif, that fits in the major groove of the target

Figure 5. Overall survival of 79 patients with *bcl-2*-positive non-small-cell lung cancer in relation to *p53* status.

DNA to make contact with the edges of the bases.¹¹ Our results also emphasized that the H2 α -helix might play an important role in DNA recognition and that overexpression of bax might be one of the genetic factors associated with new carcinogenesis caused by mutations of the loop-sheet-helix motif of p53.

Using transforming mutants of p53, some mutations of p53 revealed enhancement of target gene expression and might therefore cause new carcinogenesis.^{51,52} Recently, it was reported that the codon 283-mutant p53 could cause overexpression of several target genes, including bax.⁵³ However, there are many kinds of mutations of p53 with possible different functions.^{25,54} Therefore, additional investigations are required to clarify the mechanism of action of mutant p53 in relation to its target genes, including the bcl-2 family.

References

- Cordon-Cardo C: Mutation of cell cycle regulators: biological and clinical implications for human neoplasia. *Am J Pathol* 1995, 147:545-560
- Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancers. *Science* 1991, 253:49-52
- Vogelstein B, Kinzler KW: p53 function and dysfunction. *Cell* 1992, 70:523-526
- Greenblatt MS, Bennett WP, Hollstein M, Harris CC: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994, 54:4855-4878
- Velculescu VE, El-Deiry WS: Biological and clinical importance of the p53 tumor suppressor gene. *Clin Chem* 1996, 42:858-868
- Deb S, Jackson CT, Subler MA, Martin DW: Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. *J Virol* 1992, 66:6164-6170
- Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C, Levine AJ: Gain of function mutations in p53. *Nature Genet* 1993, 4:42-46
- Hann BC, Lane DP: The dominating effect of mutant p53. *Nature Genet* 1995, 9:221-222
- El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B: Definition of a consensus binding site for p53. *Nature Genet* 1992, 1:45-49
- Farmer G, Bargonetti J, Zhu H, Friedman P, Prywes R, Prives C: Wild-type p53 activates transcription in vitro. *Nature* 1992, 358:83-86
- Cho Y, Gorina S, Jeffrey PD, Pavletich NP: Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* 1994, 265:346-355
- Huang C, Taki T, Adachi M, Konishi T, Higashiyama M, Miyake M: Mutations in exon 7 and 8 of p53 as poor prognostic factors in patients with non-small cell lung cancer. *Oncogene* 1998, 16:2469-2477
- Erber R, Conrad C, Homann N, Enders C, Finckh M, Dietz A, Weidauer H, Bosch FX: TP53 DNA contact mutations are selectively associated with allelic loss and have a strong clinical impact in head and neck cancer. *Oncogene* 1998, 16:1671-1679
- Miyashita T, Harigai M, Hanada M, Reed JC: Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res* 1994, 54:3131-3135
- Miyashita T, Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 1995, 80:293-299
- Sandri MI, Isaacs RJ, Ongkeko WM, Harris AL, Hickson ID, Broggini M, Vikhanskaya F: p53 regulates the minimal promoter of the human topoisomerase II α gene. *Nucleic Acids Res* 1996, 24:4464-4470
- Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR, Kley N: Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* 1995, 377:646-649
- Kieser A, Weich HA, Brandner G, Marme D, Kolch W: Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. *Oncogene* 1994, 9:963-969
- Li ZH, Zhu YJ, Lit XT: Wild-type p53 gene increases MDR1 gene expression but decreases drug resistance in an MDR cell line KBV200. *Cancer Lett* 1997, 119:177-184
- Linette GP, Li Y, Roth K, Korsmeyer SJ: Cross talk between cell death and cell cycle progression: BCL-2 regulates NFAT-mediated activation. *Proc Natl Acad Sci USA* 1996, 93:9545-9552
- Brady HJM, Gil-Gomez G, Kirberg J, Berns AJM: Bax α perturbs T cell development and affects cell cycle entry of T cells. *EMBO J* 1996, 15:6991-7001
- Borner C: Diminished cell proliferation associated with the death-protective activity of Bcl-2. *J Biol Chem* 1996, 271:12695-12698
- O'Reilly LA, Huang DCS, Strasser A: The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. *EMBO J* 1996, 15:6979-6990
- Mazel S, Burtrum D, Petrie HT: Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. *J Exp Med* 1996, 183:2219-2226
- Rolley N, Butcher S, Milner J: Specific DNA binding by different classes of human p53 mutants. *Oncogene* 1995, 11:763-770
- Apolinario RM, Valk P, Jong JS, Deville W, Ark-Otte J, Dingemans AC, Mourik JC, Postmus PE, Pinedo HM, Giaccone G: Prognostic value of the expression of p53, bcl-2, and bax oncoproteins, and neovascularization in patients with radically resected non-small-cell lung cancer. *J Clin Oncol* 1997, 15:2456-2466
- Mountain CF: Revisions in the international system for staging lung cancer. *Chest* 1997, 111:1710-1717
- McCarty KS Jr, Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, Konrath J, Soper JT, Budwit DA, Creasman WT, Seigler HF, McCarty KS Sr: Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res* 1986, 46:4244s-4248s
- Brambilla E, Negoescu A, Gazzeri S, Lantuejoul S, Moro D, Brambilla C, Coll JL: Apoptosis-related factors p53, bcl2 and bax in neuroendocrine lung tumors. *Am J Pathol* 1996, 149:1941-1952
- Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958, 53:457-481
- Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* 1966, 50:163-170
- Cox DR: Regression modes and life-tables. *J R Stat Soc B* 1972, 34:187-220
- Rubin L, Philpott KL, Brooks SF: The cell cycle and cell death. *Curr Biol* 1993, 3:391-394
- Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC: Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992, 69:119-128
- Haas-Kogan DA, Kogan SC, Levi D, Dazin P, T'Ang A, Fung YK, Israel MA: Inhibition of apoptosis by the retinoblastoma gene product. *EMBO J* 1995, 14:461-472
- Vairo G, Innes KM, Adams JM: Bcl-2 has a cell cycle inhibitory function separable from its enhancement of cell survival. *Oncogene* 1996, 13:1511-1519
- Strasser A, Huang DCS, Vaux DL: The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumorigenesis and resistance to chemotherapy. *Biochim Biophys Acta* 1997, 1333:F151-F178
- Oltvai ZN, Millman CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. *Cell* 1993, 74:609-619
- Yin XM, Oltvai ZN, Korsmeyer SJ: BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature* 1994, 369:321-323
- Pezzella F, Turley H, Kuzu I, Tungekar MF, Dunnill MS, Pierce CB, Harris A, Gatter KC, Mason DY: bcl-2 protein in non-small-cell lung carcinoma. *N Engl J Med* 1993, 329:690-694
- Fontanini G, Vignati G, Bigini D, Mussi A, Lucchi M, Angeletti CA, Basolo F, Bevilacqua G: Bcl-2 protein: a prognostic factor inversely correlated to p53 in non-small-cell lung cancer. *Br J Cancer* 1995, 71:1003-1007
- Kitagawa Y, Wong F, Lo P, Elliott M, Verburg LM, Hogg JC, Daya M: Overexpression of bcl-2 and mutations in p53 and K-ras in resected human non-small cell lung cancers. *Am J Respir Cell Mol Biol* 1996, 15:45-54

43. Ishida H, Irie K, Itoh T, Furukawa T, Tokunaga O: The prognostic significance of p53 and bcl-2 expression in lung adenocarcinoma and its correlation with Ki-67 growth fraction. *Cancer* 1997, 80:1034–1045
44. Elledge RM, Green S, Howes L, Clark GM, Berardo M, Allred DC, Pugh R, Ciocca D, Ravdin P, O'Sullivan J, Rivkin S, Martino S, Osborne CK: bcl-2, p53, and response to tamoxifen in estrogen receptor-positive metastatic breast cancer: a southwest oncology group study. *J Clin Oncol* 1997, 15:1916–1922
45. Marx D, Binder C, Meden H, Lenthe T, Ziemek T, Hiddemann T, Kuhn W, Schauer A: Differential expression of apoptosis associated genes bax and bcl-2 in ovarian cancer. *Anticancer Res* 1997, 17:2233–2240
46. Tsujimoto Y, Croce CM: Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Natl Acad Sci USA* 1986, 83:5214–5218
47. Minna JD: Neoplasms of the lung. *Principles of Internal Medicine*. Edited by Isselbacher KJ. New York, McGraw-Hill, 1991, pp 1102–1110
48. Pavletich NP, Pabo CO: Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science* 1991, 252:809–817
49. Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB: Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 1991, 352:497–505
50. Ellenberger TE, Brandl CJ, Struhl K, Harrison SC: The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted alpha helices: crystal structure of the protein-DNA complex. *Cell* 1992, 71:1223–1237
51. Iwamoto KS, Mizuno T, Ito T, Tsuyama N, Kyoizumi S, Seyama T: Gain-of-function p53 mutations enhance alteration of the T-cell receptor following X-irradiation, independently of the cell cycle and cell survival. *Cancer Res* 1996, 56:3862–3865
52. Park DJ, Chumakov AM, Miller CW, Pham EY, Koeffler HP: p53 transactivation through various p53-responsive elements. *Mol Carcinogen* 1996, 16:101–108
53. Crook T, Parker GA, Rozycka M, Crossland S, Allday MJ: A transforming p53 mutant, which binds DNA, transactivates and induces apoptosis reveals a nuclear:cytoplasmic shuttling defect. *Oncogene* 1998, 16:1429–1441
54. Kawamura M, Yamashita T, Segawa K, Kaneuchi M, Shindoh M, Fujinaga K: The 273rd codon mutants of p53 show growth modulation activities not correlated with p53-specific transactivation activity. *Oncogene* 1996, 12:2361–2367