The Korean Journal of Internal Medicine
Vol. 11, No. 2, June, 1996

DNA Ploidy and Proliferative Activity in bcl-2 Expressed Non-small Cell lung Cancer

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Objectives: *The expressions of bcl-2 have been reported recently in non-small cell lung carcinoma (NSCLC*). As oncogensis is believed to involve a number of genetic alterations, there can be differences in DNA ploidy or proliferative activity even in bcl-2 positive cases according to the superimposed genetic events.*

Subjects and Methods: *On the assumption that we might further discern the biologic behavior of bcl-2 positive NSCLC according to the status of DNA ploidy and proliferative activity, we conducted a study for bcl-2 expression with immunohistochemical staining and DNA analysis on 52 surgical specimens of NSCLC.*

Results: *The bcl-2 was positive in 52% (27/52) of specimens, According to the status of bcl-2 expression, there were no significant differences in tumor stages, performance status score and survival time. Among bcl-2 positive NSCLC, aneuploidy and high proliferative activity were noted in 40% and 44%, respectively. In cases with squamous cell carcinoma (SQC**), the proportion of aneuploidy was significantly higher in bcl-2 positive group compared to bcl-2 negative group ($p<0.01$), which could not be explained with the sole effect of bcl-2. In bcl-2 positive NSCLC, there was no significant survival difference by the status of DNA analysis results. With a Coxproportional hazard model, only T stage was an independent prognostic factor.*

Conclusion: *In bcl-2 expressed NSCLC, proliferative activity and DNA ploidy were not homogeneous, suggesting other genetic alterations. This may explain our results which showed no differences in survival according to the status of the bcl-2 expression.*

Key Words: *bcl-2 oncogene, DNA ploidy, proliferative acivity, non-small cell lung carcinoma*

INTRODUCTION

The bcl-2 protein is known to have a functon to block the cell death pathway (apoptosis) and offer a survival advantage to the cells[1]. The bcl-2 proto-oncogene is normally expressed in hematopoietic stem cells, endocrine cells, neurons and basal cells in intestinal and bronchial epithelium[2]. However it is no longer expressed in more differentiated cells to prevent the accumulation of cells. If there is continued expression of bcl-2 proto-oncogene in differentiated cells, they may accumulate with survival advantage of bcl-2 protein and, with the additional genetic events, may grow to a malignancy[3].

Although the expression of bcl-2 proto-oncogene has been known to be implicated in the oncogenesis of follicular lymphoma and diffuse B cell lymphoma, it is normally expressed both in hematopoietic stem cells and in epithelial basal cells. Recently, there have been studies about bcl-2 expression in bronchogenic malignancy. Pezzella et al.[4] reported that the survival of bcl-2 expressed group of non-small cell lung carcinoma (NSCLC) was better than the bcl-2 negative group and they suggested that the reason for the good prognosis

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Part of this manuscript was presented at the 1995 American Thoracic Society (ATS) International Conference. (Am J Respir Crit Care Med 1995; 151:A277)

* *NSCLC; Non-Small Cell lung carcinoma,*

** *SQC: Squamous cell carcinoma, ADC: Adenocarcinoma*

of the bcl-2 expressed group might be the effect of bcl-2 protein which gives rather a survival advantage than an effect on proliferation. However, oncogenesis is considered a result of multiple genetic insults[5], so there should be additional genetic events in bcl-2 expressed tumors, and survival could be different according to the other superimposed genetic events even in bcl-2 expressed patients.

An animal study of mice transfected with a bcl-2-Ig minigene showed polyclonal B cell accumulation which was mostly in the resting phase (97% was in G0/G1 phase)[6,7]. To our knowledge, there is no report that the overexpression of bcl-2 has resulted from insults causing abnormal cellular DNA contents. Therefore, we presumed an abnormal DNA content or a high proliferative activity as a marker for genetic changes other than bcl-2.

The objective of this study is to analyze the DNA contents and proliferative activity in cases with bcl-2 expressed NSCLC. We assumed that we might further discern the biologic behavior of NSCLC according to the status of DNA ploidy and proliferative activity in bcl-2 expressed cases.

MATERIALS AND METHODS

We collected 52 cases of NSCLC who underwent surgical resection from March, 1986 to January, 1993 in Chonnam university hospital, Kwangju, Korea. Those patients who died within a month after surgery were excluded. Anatomic staging was recorded by the postoperative findings with the TNM staging system for NSCLC[8] and performance staus of partients at the time of diagnosis was recorded as Karnofsky scale[9]. Most of the patients were within stage IIIa (Stage I: 22, II: 11, IIIa: 16, IIIb: 1, IV: 2) and adjuvant radiation therapy was done for 3 patients. There were 41 cases with squamous cell carcinoma (SQC) and 11 cases with adenocarcinoma (ADC). Forty-nine of 52 cases with NSCLC and 38 of 41 SQC were elgible for DNA analysis.

1. Immunohistochemical Stain

We used paraffin blocks made from surgical specimens which had been fixed in 10% neutral-buffered formalin. They were cut in 3 μm thickness and put on the ProbeOn Plus slides (Fisher Scientific). All steps for staining procedure were done in Microprobe system (Fisher Scientific) by the principle of capillary action gap[10], which is produced by two approximated ProbeOn Plus slides. After dewaxing and hydration, antigen retrieval system was used by boiling the slides with an 0.01 M citrate buffer (pH 6.0) in a microwave oven (Samsung, 630W) for 10 minutes. Then they were reacted with anti-bcl-2 primary antibody (DAKO-bcl-2, 124, monoclonal mouse, amino acid 51, 54 of bcl-2 protein) for 20 minutes. For the detection of antigen and antibody complexes, we reacted them with secondary antibody (anti-mouse IgG) for 10 minutes, in Redusol (Biomeda) for 5 minutes to block the endogenous alkaline phosphatase and then they were incubated in avidin alkaline phosphatase[11] for 12 minutes. The tissue sections were then ready for the chromogen reaction (Fast Red TR Salt for 20 minutes). They were counterstained with 30 second applications of hematoxylin and mounted in Universal Mount (Research Genetics). All reactions were incubated at 50℃. For positive and negative control reactions, normal human spleen tissue sections were routinely stained with and without primary antibody.

2. DNA Ploidy and Cell Cycle Analysis

After meticulously collecting the paraffin blocks which contained tumor tissues, we cut the paraffin blocks with the thickness of 50 μm, dewaxed them in test tubes with xylene for 1 hour in room temperature and then hydrated them with graded concentration of alcohol solutions (100%, 90%, 70%, 50%, 30% and 10%). The specimens were reacted in citrate buffer for 1 hour, then centrifuged. After removing the supernatants, 2ml of 0.25% trypsin was added and overnighted at 37℃. Next morning, tissue suspension was filtered through a nylon mesh of 40 nm pore size, stained for DNA with propidium iodide and lastly, using FACScan (Fluorescent Activated Cell Scanner, Becton Dickinson Immunocytometry System, USA), stimulated with 488 nm argon laser and expressed the signal strength of fluoresence with the number of channels[12-14]. Samples were considered as aneuploid when the G0/G1 peak was detected in more than 2 peaks of DNA histogram or when the ratio of G2M/G0G1 channel was less than 1.85[15]. For cell cycle analysis, we used Cellfit software (Becton Dickinson Immunocytometry systems) for diploid specimens and Multicycle software (Phoenix flow systems) for the aneuploid specimens to eliminate tissue

fragment fractions and background noise. Because of the difference in software between aneuploid and diploid group, the cell cycle fractions were not eligible for analysis with their actual values, so we divided them into high and low proliferative activity groups by the median values of S phase fraction in each ploid group (aneuploidy: 3.5~24.8%, median: 16.4% / diploidy: 4.9~48.4%, median 28.3%).

3. Statistical Analysis

Survival times were calculated from the day of surgery. Statistical analysis was performed for the patients with NSCLC and for the group with squamous cell carcinoma, but not for the group with adenocarcinoma since there were only 11 cases of adenocarcinoma. We used SAS statistical analysis package (SAS Institute, Cary, N.C.) for the frequency tables, t-test and univariate analysis. Life tables and analysis of survival functions were done by the Kaplan-Meier product limit method. Comparison of survival functions were done by the method of Breslow, Mantel-Cox, Peto-Prentice, Tarone-Ware. For the calculation of relative risks, we used Cox proportional hazard model with BMDP statistical software. The statistical significance was defined as p value less than 0.05 except Cox proprotional hazard model in which p value limit for entering covariates into the model was set to $p<0.1$. In the hazard model, maximum partial likelihood ratio test was used after screening covariates with Peduzzi-Hardy-Holford statistic[16].

RESULTS

Overall survival time ranged between 40 to 2218 days. As only 27% (14/52) of patients were confirmed dead during the follow-up period, median survival time was not estimated. According to the anatomic stage, the group with a stage of less than IIIa showed a tendency of prolonged survival compared to the group with more advanced stages (Breslow $p=0.06$, Fig. 1). In the immunohistochemical stain for bcl-2 on human spleen tissue, the positive reactions were observed in T and B cell areas (mantle zone) and red pulp, but not in germinal centers[17]. The positive reaction in NSCLC specimens showed as a diffusely or focally positive staining for bcl-2 protein (Fig. 2). the bcl-2 protein was positive in 51.9% (27/52) of NSCLC and no difference was noted in positive rate between SQC

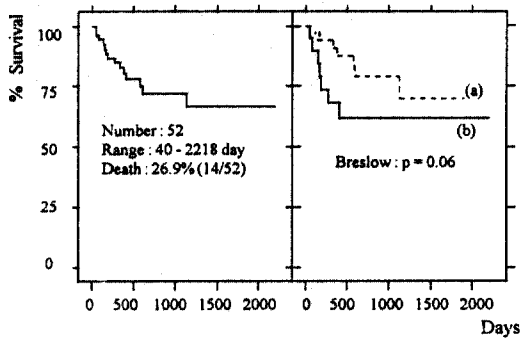

Fig. 1. Survival of 52 patients with non-small cell lung carcinoma (*left*) and comparison of survival according to anatomical stage (*right*)
(a) stage <IIIa, N =33 3/4 survival time: 1125 day
(b) stage≥IIIa, N=19 3/4 survival time: 177 day

(56.1% 23/41) and ADC (36.4% 4/11).

According to the status of bcl-2 expression, the distributions of age, anatomic stages and performance status scores and the proportions of smokers were not different. However, the dose of smoking (pack-year) in smokers (N=40) was significantly higher in bcl-2 negative patients than positive patients (Table 1). The survival difference was not noted in 52 cases with NSCLC and in 41 cases with SQC according to the status of bcl-2 expression (Fig. 3).

Of 49 NSCLC specimens which were eligible for DNA analysis, 28.6% (14/49) showed aneuploidy (SQC: 28.9%(11/38), ADC: 27.3%(3/11)). No significant differences were noted in the age, sex, smoking history, amount of smoking, anatomic stages and performance status scores by either the DNA ploidy (Table 2) or proliferative activity (Table 3). The survival was not different in cases with NSCLC and in cases with SQC according to the DNA ploidy (Fig. 3) or proliferative activities (data not shown).

In twenty-five bcl-2 positive cases, aneuploidy was noted in 40%(10/25) and high proliferative activity in 44%(11/25). Especially in cases with SQC, 47.6% (10/21) of bcl-2 positive group showed aneuploidy as opposed to 5.9%(1/17) of bcl-2 negative group ($p<0.01$) (Table 4). Comparing the survival time according to the ploidy pattern and proliferative activities in bcl-2 positive cases, no significant differences were noted (data not shown).

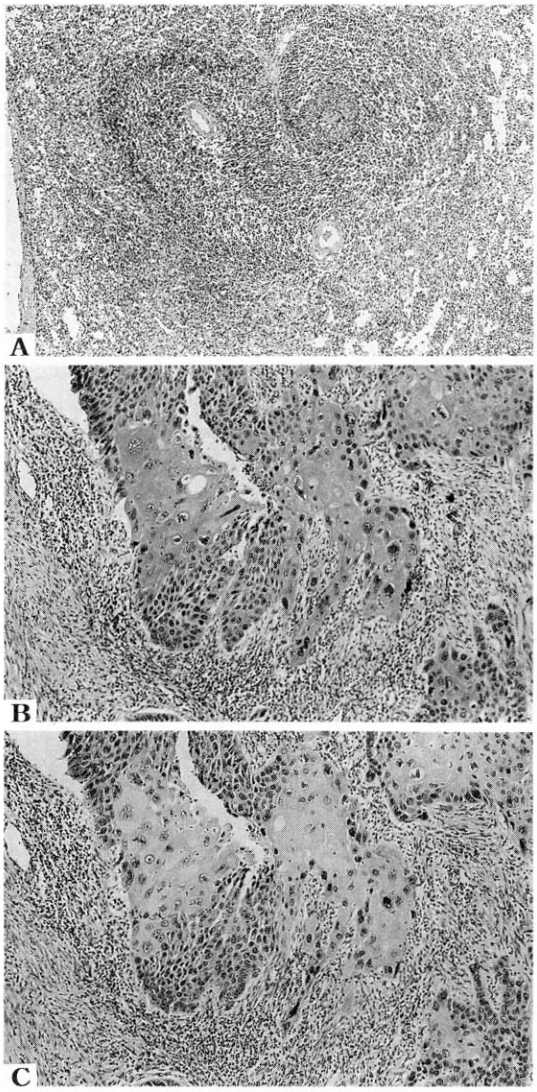

Fig. 2. Immunohistochemical stain of human spleen and squamous cell lung carcinoma tissue with a monoclonal antibody for bcl-2 protein (alkaline phosphatase, avidin alkaline phosphatase method, chromogen: Fast Red TR salt, counterstained with hematoxylin);
A: shows a normal human spleen expressing bcl-2 protein in mantle zone and red pulp but not in germinal center. (LM x 40)
B: shows a squamous cell carcinoma expressing bcl-2 protein in tumor cell nest. (LM x 200)
C: shows a squamous cell carcinoma same as panel B, stained without primary antibody. (LM x 200)

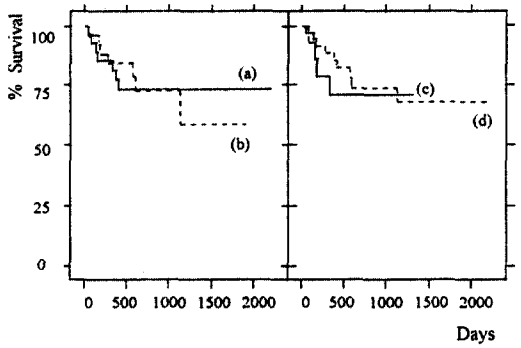

Fig. 3. Survival of 52 patients with non-small cell lung carcinoma according to the status of bcl-2 expression (left) and DNA ploidy (right)
(a) bcl-2 positive (27) 3/4 survival time: 400 day
(b) bcl-2 negative (25) 3/4 survival time: 594 day
(c) Diploidy (35) 3/4 survival time: 594 day
(d) Aneuploidy (14) 3/4 survival time: 334 day

In Cox regression analyses with NSCLC, SQC and bcl-2 positive subsets of subjects, only T stage was entered to the equation as an independent poor prognostic indicator (Table 5). However, histologic types, N stage, status of bcl-2 expression and DNA analysis results were not entered as significant prognostic factors.

DISCUSSION

Recent studies[4,18] have suggested the possible role of bcl-2 in the oncogenesis of NSCLC as well as follicular lymphoma. Pezzella et al.[4] studied surgically obtained fresh frozen tissue of 122 NSCLC with their own developed anti-bcl-2 primary antibody. They yielded the positive rate of 25%(20/80) in SQC and 12%(5/42) in ADC. Gaffney et al.[18] examined 51 surgical formalin fixed specimen, using the commercial primary antibody (DAKO) which is the same as the authors, and reported the positive rate of bcl-2 in NSCLC as 25.5% (SQC: 31.7%, ADC: none). In this data, we observed 52% of positive rate which is more than twice that of previous studies. As described in Methods, we applied an antigen retrieval system using a microwave oven in an enhancer solution to obtain the best quality of staining results. The differeence in positive rate of bcl-2 may be due to the actual difference of positive rate between the study populations or may be due to higher sensitivity of our staining methods. The

Table 1. Characteristics of 52 Patients with Non-Small Cell Lung Carcinoma and 41 Patients with Squamous Cell Carcinoma According to Status of bcl-2 Expression

Number	Non-Small Cell 52		Squamous Cell 41	
bcl-2	Positive	Negative	Positive	Negative
Sex (M/F)	24/3	22/3	21/2	18/0
Age (years(SD))	56.8(8.7)	55.7(10.2)	57.9(8.9)	58.6(2.1)
Smoking (Y/N)	21/6	19/5	20/3	16/2
Pack Years (mean(SD))	35.4(15.3)	48.5(21.0)*	35.7(15.6)	50.7(20.9)*
T Stage (1~4)	1/20/5/1	2/18/4/0	0/17/5/1	1/12/4/0
N Stage (0~2)	12/6/8	10/7/7	11/4/7	6/6/5
PS score# (≥80/<80)	21/6	18/7	18/5	12/6

* $p<0.05$, # PS score: performance status score (Karnonfsky Scale)
Note that mathematical sum does not match for all vaiables due to some missing values (Table. 1, 2, 3).

Table 2. Characteristics of 49 Patients with Non-Small Cell Lung Carcinoma and 38 Patients with Squamous Cell Carcinoma according to DNA Ploidy

Number	Non-Small Cell 49		Squamous Cell 38	
DNA	Aneuploidy	Diploidy	Aneuploidy	Diploidy
Sex (M/F)	12/2	32/3	11/0	26/1
Age (years(SD))	56.7(10.9)	56.5(8.6)	60.5(8.3)	57.9(8.5)
Smoking (Y/N)	11/3	27/8	10/1	24/3
Pack Years (mean(SD))	42.3(14.7)	42.4(21.2)*	43.5(14.9)	43.1(21.6)
T Stage (1~4)	1/9/3/0	1/28/6/0	0/7/3/0	0/21/6/0
N Stage (0~2)	7/1/5	15/12/8	6/1/3	11/9/7
PS score# (≥80/<80)	11/3	26/9	9/2	19/8

PS score: performance status score (Karnonfsky Scale) No variable showed significant differences.

Table 3. Characteristics of 49 Patients with Non-Small Cell Lung Carcinoma and 38 Patients with Squamous Cell Carcinoma according to Proliferative Activity

Number	Non-Small Cell 49		Squamous Cell 38	
Proliferative Activity	High	Low*	High	Low*
Sex (M/F)	22/1	22/4	18/0	19/1
Age (years(SD))	57.4(9.7)	55.8(8.8)	59.7(8.1)	57.8(8.7)
Smoking (Y/N)	18/5	20/6	16/2	18/2
Pack Years (mean(SD))	46.4(23.2)	38.7(14.8)*	47.3(23.4)	39.6(15.4)
T Stage (1~4)	1/16/5/0	1/21/4/0	0/12/5/0	0/16/4/0
N Stage (0~2)	12/3/7	10/10/6	7/3/7	10/7/3
PS score# (≥80/<80)	18/4	19/7	13/5	15/5

* Cut off value: median value of S phase fraction # PS score: performance status score (Karnofsky Scale)
No variable showed significant differences.

Table 4. Distribution of 49 Patients with Non-Small Cell Lung Carcinoma and 38 Patients with Squamous Cell Carcinoma according to Status of bcl-2 Expression and DNA ploidy, Proliferative Activity

Number	Non-Small Cell 49		Squamous Cell 38	
bcl-2	Positive	Negative	Positive	Negative
DNA ploidy				
Aneuploidy	10	4	10	1*
Diploidy	15	20	11	16
Proliferative Activity				
High	11	12	10	8
Low	14	12	11	9

* $p<0.01$, # Cutoff value: median value of S phase fraction

Table 5. List of Variables Predicting Survival According to Cox Proportional hazard Model (A) in 52 Patients with Non-Small Cell Lung Carcinoma (B) in 41 Patients with Squamous Cell Carcinoma (C) in 27 Patients with bcl-2 Expressed Tumors

Variables	Score		Regression Coefficients*	Significance	Relative Risk
(A) T stage	1(≤T2)	2(≥T3)	1.157	0.055	3.18
(B) T stage	1(≤T2)	2(≥T3)	1.385	0.036	3.99
(C) T stage	1(≤T2)	2(≥T3)	2.012	0.011	7.48

* Coefficient estimated by Cox proportional hazard model.
p value limit for entering covariates into the model is $p<0.1$

expression of bcl-2 is not limited to NSCLC. Ikegaki et al.[19] observed the bcl-2 expression in 5 of 6 cell lines of small cell lung carcinoma.

Considering the relationship of the prognosis of NSCLC and the expression of bcl-2 oncogene, Pezzella et al.[4] reported that the survival in bcl-2 positive group was better than the negative group. However, Gaffney et al.[18] reported no difference in survival according to the status of bcl-2 expression, and implied that their finding was more rational since the apoptosis could be regulated by other genes and modulators of bcl-2 such as bcl-x[20] or Bax[21]. Moreover, there should be other superimposed genetic alterations other than bcl-2 which could make the DNA pattern and survival variable.

Although we lacked enough numbers in the study population to observe statistically significant survival differences, we found that there was no survival difference according to the status of bcl-2 expression. In the DNA analysis, aneuploidy and high proliferative activity were seen in about half of bcl-2 positive cases. Especially in cases with SQC, the incidence of aneuploidy was significantly higher in the bcl-2 positive group than the negative group, which could not be explained with the sole effect of bcl-2. Although we expected that there should be survival differences according to the proliferative activity or DNA ploidy even in the bcl-2 positive group, there were no statistically significant differences. However, this may be a consequence of a small number of our subjects in obtaining a clear statistical data or due to a possible bias so that we could not observe the natural course of disease because the majority of our subjects were treated surgically in their early stage.

However, there are several areas of potential concern with this study. Firstly, reflecting the higher incidence of bcl-2 expression in NSCLC, we are more concerned about the dysregulation of bcl-2 in the oncogenesis of NSCLC. Secondly, we do not know how to explain the higher cumulative dose of cigarette smoking in the bcl-2 negative group than the positive group. Although the associations of smoking with mutations of K-ras[22] and p53[23] have been reported, that with bcl-2 is not known to us. As results from DNA analysis suggest, examining other concurrent genetic alterations would give us a more clear idea about the biologic behavior of NSCLC.

In conclusion, with an immunohistochemical stain for bcl-2 protein on surgically obtained NSCLC

specimens, we obtained 52% of positive rate. The heterogeneous DNA ploidy and proliferative activity patterns in the bcl-2 positive tumors suggested the addition of other genetic alterations. This may explain our results which showed no difference in survival according to the status of the bcl-2 expression.

ACKNOWLEDGMENTS

This manuscript was supported by the Research Institute of Clinical Medicine, Chonnam University Hospital, Kwangju, Korea.

The authors thank F. Pezzella, M.D. for advice and suggestions and also Y.I. Go, M.D. and A.S. Jang, M.D. for faithful assistance.

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