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# The Relationship between the Presence of Chromosomal Instability and Prognosis of Squamous Cell Carcinoma of the Lung: Fluorescence in situ Hybridization Analysis of Paraffin-embedded Tissue from 47 Korean Patients

To evaluate the prognostic importance of chromosomal instability (CIN) in squamous cell carcinoma (SCC) of the lung, the relationship between CIN detected by fluorescence *in situ* hybridization (FISH) and survival in SCC patients was examined. Forty-seven surgical specimens of lung SCC were analyzed. To identify tumors with CIN, *p16* and multi-target DNA FISH assays for *c-myc*, chromosome 6, *EGFR*, and chromosome 5 (LAVysion, Vysis) were performed on nuclei extracted from paraffin-embedded tumor tissues. Survival rates were compared in terms of age, T factor, N factor, CIN, and smoking status. A sample was defined as CIN-positive if at least four of the five chromosomes were positive. Among the 47 specimens, 9 (19%) were CIN-positive. The overall survival rate was 66%. Overall survival rates were estimated as 33.3% for CIN-positive patients and 76.7% for CIN-negative patients (Hazard ratio 3.47; 95% Confidence interval, 1.25-9.67; *P*=0.017). In multivariate analysis, the presence of CIN was a predictive factor for survival. CIN-positive based on FISH can be prognostic factor of lung SCC.

Key Words : *Chromosomal Instability; In Situ Hybridization, Fluorescence; Lung Neoplasms; Carcinoma, Squamous Cell*



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## INTRODUCTION

Lung cancer is a leading cause of death in both men and women in developed countries. To date, a number of genetic events that promote lung carcinoma tumorigenesis, a multifactorial process, have been identified (1).

Chromosomal abnormality is one of the hallmarks of neoplastic cells, and the persistent chromosomal instability is reported in human cancers (2). Chromosomal instability (CIN) or aneusomy occurs at the chromosomal level, resulting in losses and gains of whole chromosomes (3). CIN can be detected using fluorescence *in situ* hybridization (FISH) to measure fluorescence signals from targeted chromosomes in the interphase nuclei of surgical specimens of non-small cell lung cancer (NSCLC) (4, 5). CIN in NSCLC may be an independent factor for predicting poor prognosis (4). Recent study reported CIN-positive, defined as at least the existence of three chromosome aneusomy in primary adenocarcinoma (AC) of the lung with FISH, was associated with poor prognosis, and thus advocated as an independent prognostic factor in Korean patients (6). However, in squamous cell carcinoma (SCC) of the lung, the second most frequent histologic subtype, the relationship between CIN-positive and prognosis remains to be established. In the present study, we investigated the number of chromosomal aneusomy to define CIN-positive, and analyzed the relationship between CIN-positive and survival using FISH in Korean patients with SCC of the lung.

## MATERIALS AND METHODS

### Participants

We reviewed the clinical characteristics and pathological specimens of 47 patients with squamous cell carcinoma of the lung subjected to resection at the Asan Medical Center, Seoul, from January 2002 to May 2003. This study protocol was approved by the Asan Medical Center Institutional Ethics Review Board (2008-0026). Patients included 46 men and 1 woman with a median of 59 yr. Lobectomy was performed in 40 (85.1%) patients, and pneumonectomy in the remaining 7 (14.9%) cases. All patients were subjected to mediastinal lymph node dissection. No presurgical chemotherapy or radiotherapy was administered. Diagnosis of lung SCC was confirmed by histological examination of the surgical specimens. Tumor-node-metastasis staging of cancers was performed using the latest criteria (7). The pathological stage was classified as I in 22 patients, II in 17 patients, and III in 8 patients. Patients were regularly monitored in the outpatient department for 72 months after tumor resection. The demographic and clinical characteristics of patients are summarized in Table 1.

### Extraction of nuclei from paraffin-embedded tissues

We performed FISH on cell nuclei isolated from both tumor and control samples, as described previously (8). To set the normal reference range of the FISH probe, mononuclear cells of peripheral blood from 20 healthy individuals were used as the control. The mean+3 S.D. (standard deviation) value of the normal range was set as the reference. Paraffin-embedded tissue samples and glass pellets 1-mm-diameter were placed in small nylon gauze bags. The material was deparaffinized with xylene for 1.5-3 hr, followed by incubation with decreasing concentrations of ethanol (100%, 90%, 70%, and 35% for 1 hr, respectively). Deparaffinized tissue specimens were transferred into distilled water, and incubated with 0.1% protease (Sigma P8038, St. Louis, MO, USA) in 0.1N Tris-HCl at 37℃ for 3-10 min. Following termination of digestion by incubation on ice and centrifugation (8 min, 500 rpm), the supernatant was removed, and cell pellet resuspended in 200 $\mu$L of Canoy's fixative. The cell suspension (10 $\mu$L) was dropped on a positively charged slide and air-dried. Samples were immediately processed for FISH analysis.

### Fluorescent in situ hybridization study

FISH was performed according to the manufacturer's instructions with minor modifications, as described in a previous report (9). The commercially available multi-target FISH assay (LAVysion; Vysis; Downers Grove, IL, USA) includes directly labeled DNA FISH probes for *EGFR* (7p12,SpectrumRed), *MYC* (8q24,SpectrumGold), chromosome 5 (5p-15.2,SpectrumGreen), and chromosome 6 (centrometric at 6p11.1-q11,SpectrumAqua). FISH analysis of *p16* was conducted using the LSI® p16 (9p21) SpectrumOrange/CEP®9 SpectrumGreen™ Probe (Vysis; IL, USA). After staining, 4′,6-diamidino-2-phenylindole (DAPI II) (Abott/Vysis) was applied to the target areas, and the slides analyzed under a fluorescence microscope using single bandpass filter sets. FISH signals were measured at a magnification of ×630 after automatic relocation of equivocal cells with the appropriate software in the DAPI II single bandpass filter set on the microscope. Nuclear signals were only measured in cells with clearly defined nuclear borders and visible signals. In total, 200 nuclei were counted, and the level of each centromeric signal recorded. *EGFR*, *MYC*, chromosome 5, and chromosome 6 (Fig. 1) were analyzed for increased copy number, and the *p16* gene examined for deletion. Cells displaying gain (>2) or loss (<2) in copy number were determined when they constituted 10% of the total cell population. This cutoff value was based on earlier studies reporting that the mean+3 S.D. value of gain or loss of each chromosome in lymphocytes and normal colorectal epithelium was less than 10% (10).

### Statistical analysis

The chi-square test was used to compare categorical variables, and the Mann-Whitney U-test for continuous nonparametric variables. The Student's t-test was applied to compare continuous parametrical variables. Survival rates were calculated with the Kaplan-Meier method. The significance of survival differences between subgroups was examined by univariate analysis using the log-rank method. Survival was defined as the time from surgery to death or last follow-up. We assessed the discriminatory ability of CIN number to predict survival as previous study. A sample was considered CIN-positive when at least four chromosomal aneusomies out of the five chromosomes, well known for chromosomal

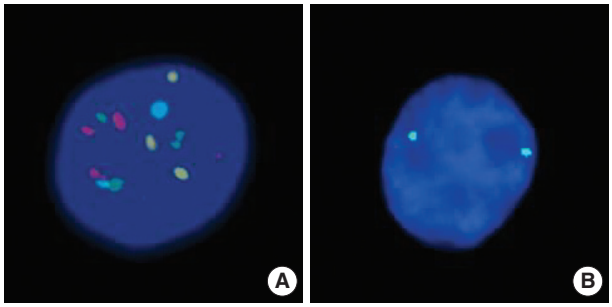


Fig. 1. Image of fluorescent in situ hybridization (FISH) performed on extracted nuclei of lung cancer tissue. (A) LaVysion FISH: cell showing increase copy number of 7p12, chromosome 5 and 8q24 region. A Cell shows five red signals (7p12 region), three Gold signals (8q 24 region), four green signals (5p15.2) and two aqua signals (chromosome 6). (B) p16 FISH: cell with deletion p 16 gene. A cell shows homozygous deletion of p16 gene (no orange signal) on normal number of chromosome 9 (two green signal).

instability in lung cancer, were increased copy number or deleted as described above (6). Patients with predicted mortality were distinguished with 66.7% sensitivity and 73.7% specificity with this cutoff point. We analyzed CIN status, age, tumor stage, nodal stage and smoking history using Cox proportional hazards models to determine predictable factor for survival.

A univariate factor with *P* value of less than 0.20 was selected for multivariate analysis. Factors independently associated with event were identified. $P<0.05$ was considered significant. Statistical analyses were performed using the SPSS system for Windows (Version 14.0).

Table 1. Baseline characteristics of study participants*

| Parameters | All (n=47) | CIN (+) (n=9) | CIN (−) (n=38) | *P* value |
|---|---|---|---|---|
| Age (yr) | 59 (42-70) | 59 (42-60) | 59 (55-70) | 0.604† |
| Sex, n (%) | | | | |
| Male | 46 (97.9) | 9 (100) | 37 (97.4) | 1 |
| Female | 1 (2.1) | 0 | 1 (2.6) | |
| T stage, n (%) | | | | |
| T1 | 8 (17) | 1 (11.1) | 7 (18.4) | 0.608‡ |
| T2 | 34 (72.3) | 8 (88.9) | 24 (68.4) | |
| T3 | 3 (6.4) | 0 | 3 (7.9) | |
| T4 | 2 (4.3) | 0 | 2 (5.3) | |
| N stage, n (%) | | | | |
| N0 | 25 (53.2) | 5 (55.6) | 20 (52.6) | 0.982‡ |
| N1 | 16 (38) | 3 (33.3) | 13 (34.2) | |
| N2 | 6 (8.6) | 1 (11.1) | 5 (13.2) | |
| TNM stage, n (%) | | | | |
| I | 22 (46.8) | 5 (55.5) | 17 (44.7) | 0.946‡ |
| II | 17 (36.2) | 3 (33.3) | 14 (36.9) | |
| III | 8 (10.6) | 1 (11.1) | 7 (18.5) | |
| Smoking, n (%) | | | | |
| None | 2 (4.3) | 0 | 2 (5.3) | 0.394‡ |
| Former | 13 (27.7) | 4 (44.4) | 9 (23.7) | |
| Current | 32 (68.1) | 5 (55.6) | 27 (71.1) | |
| Pack year | 40 (0-100) | 38 (15-60) | 40 (0-100) | 0.401† |
| Operation, n (%) | | | | |
| Lobectomy | 40 (85.1) | 9 (100) | 31 (81.6) | 0.318‡ |
| Pneumonectomy | 7 (14.9) | 0 | 7 (18.4) | |

*Data are presented as median (range), number (%); †Mann-Whitney U-test; ‡Fisher's exact test.

## RESULTS

The median age of both CIN-positive group and CIN-negative group was 59 yr. The level of lymph node involvement, tumor stage, and smoking history did not differ significantly between the groups (Table 1). The frequencies of chromosomal aneusomy in SCC specimens are summarized in Table 2. Nine cases met our criteria for CIN positive. As of November 2008, 16 patients had died. The mean survival duration was 52±8 months (95% Confidence interval (CI), 37-67) for CIN-positive group and 71±3 months (95% CI, 65-78). The overall survival rate was 66%. Overall survival rates were estimated as 33.3% for CIN-positive patients and 76.7% for CIN-negative patients (Hazard ratio (HR) 3.47; 95% CI, 1.25-9.67; $P=0.017$). Multivariate analysis using Cox proportional hazards models was used to compare survival between CIN-positive and CIN-negative patients. In relation to CIN-negative patients and after adjusting for sex, pathologic nodal staging, tumor staging, age, and smoking history, the presence of CIN was associated with survival (HR, 3.47; 95% CI, 1.25-9.67; $P=0.017$) (Table 3). The Kaplan-

Table 2. Frequencies of signals per cell for each DNA probe included in the FISH assay in squamous cell carcinoma of the lung*

| | Type of abnormality | Number (%) |
|---|---|---|
| *C-MYC* | Increase copy number (>10%) | 12 (25.5) |
| Chromosome-5 | Increase copy number (>10%) | 15 (31.9) |
| *EGFR* | Increase copy number (>10%) | 18 (38.3) |
| Chromosome-6 | Increase copy number (>10%) | 8 (17) |
| *p16* | Deletion (>10%) | 23 (48.9) |

*Data are presented as mean±standard deviation, number (%), median (range).

Table 3. Results of univariate and multivariate analysis of prognostic factors for survival

| Factors | Univariate analysis Hazard ratio (95% CI) | *P* value | Multivariate analysis Hazard ratio (95% CI) | *P* value |
|---|---|---|---|---|
| Age | 0.90 (0.82-0.99) | 0.041 | | |
| Sex | 3.66 (0.47-28.34) | 0.215 | | |
| T stage | 0.58 (0.19-1.81) | 0.352 | | |
| N stage | 1.23 (0.74-2.03) | 0.417 | | |
| smoking | 2.39 (0.31-18.39) | 0.401 | | |
| *C-MYC* | 2.86 (1.06-7.72) | 0.038 | | |
| Chromosome-5 | 1.95 (0.72-5.26) | 0.188 | | |
| *EGFR* | 1.85 (0.69-4.95) | 0.220 | | |
| Chromosome-6 | 2.69 (0.93-7.83) | 0.069 | | |
| *p16* | 1.70 (0.61-4.75) | 0.312 | | |
| CIN | 3.47 (1.25-9.67) | 0.017 | 3.47 (1.25-9.67) | 0.017 |

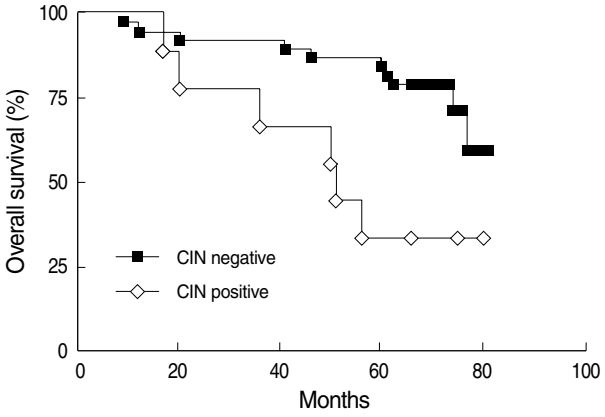


Fig. 2. Overall survival curve according to CIN status ($P$=0.013).

Meier survival curves constructed according to CIN status after resection of lung SCC are shown in Fig. 2 ($P$=0.013).

## DISCUSSION

NSCLC accounts for approximately 80% of lung cancers, and the AC and SCC histologic subtypes represent more than 85% of NSCLC cases (11). Chromosomal instability occurs at the chromosomal level leading to loss or gain of whole chromosomes (3) and, chromosomal aneusomy has been recognized as a marker for lung cancer (12).

FISH is a convenient way to count numbers of specific chromosomes in interphase nuclei (13). FISH is utilized to diagnose malignancy and to detect cytogenetic abnormalities (14-16). FISH has been also used to diagnose lung cancer in clinical specimens in conjunction with conventional morphologic analysis (17, 18). In addition, recent study showed chromosomal aneusomy, based on FISH in bronchial high-grade lesions, was associated with invasive lung cancer (19).

Nakamura and colleagues suggested that the existence of chromosomal instability was associated with poor survival in patients with non-small cell lung cancer, based on FISH analysis of surgical specimens (4). Recently Choi and his colleagues effectively detected CIN in primary adenocarcinoma of the lung using FISH, and showed that CIN-positive, defined as the number of chromosome aneusomy, was associated with poor prognosis in adenocarcinoma patients (6). They reported that the 5-yr overall survival rate was estimated as 68.7% for CIN-positive patients and 93.5% for CIN-negative patients (HR, 2.34; 95% CI, 1.04-5.26; $P$=0.04). CIN-positive status was significantly associated with decreased overall survival using multivariate Cox proportional hazards models. It has been not known whether CIN or chromosomal aneusomy detected by FISH is associated with poor prognosis in patients with squamous cell carcinoma of the lung. We evaluated distribution of chromosomal instability using FISH in SCC and assessed the discriminatory ability of CIN number to predict survival and tried to determine significant association between CIN status and survival. A sample was considered CIN-positive when at least four of chromosomal aneusomy out of the five chromosomes different from that of adenocarcinoma of the lung (6). The overall survival was poor prognosis in CIN-positive patients compared to CIN-negative subjects ($P$=0.013). The multivariate analysis using Cox model showed correlation between CIN-positive and poor prognosis in our results. Our study showed CIN status could be an independent poor prognostic factor in squamous cell carcinoma of the lung. By revealing CIN status of surgical specimen after operation of lung cancer, more treatment modality such as adjuvant chemotherapy or radiotherapy can be applied to patients to gain survival benefit.

Our study has some limitations. The patient number was small and the prognostic effect of CIN in lung SCC needs to be confirmed in a population with larger sample size or in an independent cohort. We did not analyze other genetic mutations. Theoretically, carcinogenesis is initiated with genetic alterations including mutation and changes of copy number. In that manner, we infer that CIN status is related to other genetic alterations in lung cancer, although we did not perform the mutation study. Due to our facility limitation, we cannot add chromosomal instability more specific to lung SCC such as amplification of 3q and 18p or deletion at 2q35, 3p14.1-3p14.3 and so forth (20, 21). To our knowledge, this is the first report of an association between at four number of chromosomal instability detected using FISH and survival of patients with squamous cell carcinoma of the lung.

In summary, chromosomal instability can be detected efficiently using FISH. CIN positivity was independent poor prognostic factor in SCC of the lungs.

## REFERENCES

1. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. *A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer Res 2003; 63: 1727-30.*
2. Masuda A, Takahashi T. *Chromosome instability in human lung cancers: possible underlying mechanisms and potential consequences in the pathogenesis. Oncogene 2002; 21: 6884-97.*
3. Lengauer C, Kinzler KW, Vogelstein B. *Genetic instabilities in human cancers. Nature 1998; 396: 643-9.*
4. Nakamura H, Saji H, Idiris A Kawasaki N, Hosaka M, Ogata A, Saijo T, Kato H. *Chromosomal Instability Detected by Fluorescence in Situ Hybridization in Surgical Specimens of Non-Small Cell Lung Cancer Is Associated with Poor Survival. Clin Cancer Res 2003; 9: 2294-9.*
5. Kang JU, Koo SH, Kwon KC, Park JW, Shin SY, Kim JM, Jung SS. *High frequency of genetic alterations in non-small cell lung cancer detected by multi-target fluorescence in situ hybridization. J Korean Med Sci 2007; 22 Suppl: S47-51.*
6. Choi CM, Seo KW, Jang SJ, Oh YM, Shim TS, Kim WS, Lee DS,

Lee SD. *Chromosomal instability is a risk factor for poor prognosis of adenocarcinoma of the lung: Fluorescence in situ hybridization analysis of paraffin-embedded tissue from Korean patients*. *Lung Cancer 2008; 64: 66-70*.

7. Mountain CF. *Revisions in the International System for Staging Lung Cancer*. *Chest 1997; 111: 1710-7*.
8. Stock C, Ambros IM, Lion T, Haas OA, Zoubek A, Gadner H, Ambros PF. *Detection of numerical and structural chromosome abnormalities in pediatric germ cell tumors by means of interphase cytogenetics*. *Genes Chromosomes Cancer 1994; 11: 40-50*.
9. Lee DY, Cho HI, Kang YH, Yun SS, Park SY, Lee YS, Kim Y, Lee DS. *The role of fluorescence in situ hybridization (FISH) for monitoring hematologic malignancies with BCR/ABL or ETO/AML1 rearrangement: a comparative study with FISH and G-banding on 919 consecutive specimens of hematologic malignancies*. *Cancer Genet Cytogenet 2004; 152: 1-7*.
10. Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel PL, Chesi M, Davis FE, Drach J, Greipp PR, Kirsch IR, Kuehl WM, Hernandez JM, Minvielle S, Pilarski LM, Shaughnessy JD Jr, Stewart AK, Avet-Loiseau H. *Genetics and cytogenetics of multiple myeloma: a workshop report*. *Cancer Res 2004; 64: 1546-58*.
11. Pirozynski M. *100 years of lung cancer*. *Respir Med 2006; 100: 2073-84*.
12. Taguchi T, Zhou JY, Feder M, Litwin S, Klein-Szanto AJ, Testa JR. *Detection of aneuploidy in interphase nuclei from non-small cell lung carcinomas by fluorescence in situ hybridization using chromosome-specific repetitive DNA probes*. *Cancer Genet Cytogenet 1996; 89: 120-5*.
13. Mendelin J, Grayson M, Wallis T, Visscher DW. *Analysis of chromosome aneuploidy in breast carcinoma progression by using fluorescence in situ hybridization*. *Lab Invest 1999; 79: 387-93*.
14. Kwak KW, Kim SH, Lee HM. *The utility of fluorescence in situ hybridization for detection of bladder urothelial carcinoma in routine clinical practice*. *J Korean Med Sci 2009; 24: 1139-44*.
15. Kim SR, Kim HJ, Kim SH. *Clinical utility of fluorescence in-situ hybridization profile test in detecting genetic aberrations in acute leukemia*. *Korean J Lab Med 2009; 29: 371-8*.
16. Yoon YS, Lee DS, Min HC, Jang JY, Lee SE, Hwang DW, Han HS, Kim SW. *Analysis of molecular cytogenetics alteration of pancreatic cancer identified by fluorescent in situ hybridization (FISH) and its clinical significance*. *Korean J Hepatobiliary Pancreat Surg 2008; 12: 75-85*.
17. Bubendorf L, Müller P, Joos L, Grilli B, Vogel S, Herzog M, Barascud A, Feichter G, Dalquen P, Tamm M. *Multitarget FISH analysis in the diagnosis of lung cancer*. *Am J Clin Pathol 2005; 123: 516-23*.
18. Halling KC, Rickman OB, Kipp BR, Harwood AR, Doerr CH, Jett JR. *A comparison of cytology and fluorescence in situ hybridization for the detection of lung cancer in bronchoscopic specimens*. *Chest 2006; 130: 694-701*.
19. Jonsson S, Varella-Garcia M, Miller YE, Wolf HJ, Byers T, Braudrick S, Kiatsimkul P, Lewis M, Kennedy TC, Keith RL, Bjornsson J, McWilliams A, Lam S, Hirsch FR, Franklin WA. *Chromosomal Aneusomy in Bronchial High-Grade Lesions Is Associated with Invasive Lung Cancer*. *Am J Respir Crit Care Med 2008; 177: 342-7*.
20. Tseng RC, Hsieh FJ, Hsu HS, Wang YC. *Minimal deletion regions in lung squamous cell carcinoma: Association with abnormality of the DNA double-strand break repair genes and their applications on gene identification and prognostic biomarkers*. *Lung Cancer 2008; 59: 332-9*.
21. Yakut T, Schulten HJ, Demir A, Frank D, Danner B, Egeli U, Gebitekin C, Kahler E, Gunawan B, Urer N, Oztürk H, Füzesi L. *Assessment of molecular events in squamous and non-squamous cell lung carcinoma*. *Lung Cancer 2006; 54: 293-301*.