Original Article

Relations of the c-myc Gene and Chromosome 8 in Non-small Cell Lung Cancer: Analysis by Fluorescence in situ Hybridization

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Background: Amplification of the c-myc gene has been reported in non-small cell lung cancer (NSCLC). We investigated the c-myc gene amplification and the numerical aberration of chromosome 8 by dual color fluorescence in situ hybridization (FISH) to evaluate the relation between possible genetic abnormalities, pathological factors and prognosis.

Methods: Tumor tissue samples were obtained from 31 patients with NSCLC who underwent lobectomy with mediastinal lymph node dissection. Samples were analyzed by FISH using 8 alpha satellite DNA probe and c-myc gene cosmid probe. The relation between genetic abnormalities, pathological factors (T factor, tumor size, and N factor), and prognostic factors was evaluated by univariate and multivariate analysis, and by the Kaplan-Meier method and log-rank analysis.

Results: Chromosome 8 aberrations were T1 (n=3), 44.0%; T2 (n=18), 35.7%; T3 (n=7), 40.0%; T4 (n=3), 39.7% (p=NS). The c-myc gene amplifications were T1, 54.3%; T2, 51.1%; T3, 51.0%; T4, 66.3% (p=NS). There was no difference between patients whose tumor was more than 5 cm (n=16), and 5 cm or less (n=15) in the rate of chromosome 8 aberration (39.3%: 36.3%), or the rate of the c-myc gene amplification (52.1%: 53.7%). N factors for chromosome 8 aberrations were N0 (n=18), 35.9%; and N2 (n=11), 44.9% (p=NS). In the c-myc gene amplification, there was a significant difference between N0 and N2 (48.6%, 61.3%, p=0.040). In univariate and multivariate analysis, chromosome 8 aberrations correlated with a poor prognosis (p=0.037 and p=0.041). The 5-year survival rate was 15.4% in patients whose rate of chromosome 8 aberrations was 40% or more (n=13), which was significantly less than that in patients with an aberration rate of less than 40% (n=19, 57.9%, p=0.014).

Conclusion: The c-myc gene amplification correlates with lymph node metastasis. Although there was no significant link between the amplification of the c-myc gene and clinical outcome, the numerical chromosome 8 aberrations was considered to be a factor for survival. (Ann Thorac Cardiovasc Surg 2001; 7: 197–203)

Key words: FISH, c-myc, chromosome 8, non-small cell lung cancer, prognostic factor

Introduction

Various genetic alterations and chromosomal aberrations have been observed in non-small cell lung cancer (NSCLC).[1-5] The c-myc gene, localized to 8q24, may play an important role in cell proliferation and differentiation and may induce apoptosis under certain conditions.[6] The c-myc gene amplification in various solid tumors including lung cancer has been evaluated using Southern blotting, polymerase chain reaction (PCR), or immunohistochemical procedures.[1,4,5,7-10] The c-myc gene amplification seemed to be associated with tumor progression,[7,8] and an over expression of the c-myc gene protein may be related to metastasis of lung cancer.[10] The development of fluorescence in situ hybridization (FISH) has facilitated direct examination of cell nuclei for chromosomal aberrations under a fluorescence mi-

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Table 1. Clinicopathological findings and results

Case	Sex	Age	Type	Stage	TNM	Tumor size (cm)	Survival time (M)	Outcome	Chr. 8 aberration (%)	c-myc amplification (%)
1	F	53	Ad	IA	100	2.3	38	death	51	56
2	F	69	Ad	IA	100	2.2	78	alive	26	47
3	M	72	Ad	IA	100	0.2	92	alive	55	60
4	M	68	Ad	IB	200	5.5	4	death	70	41
5	F	69	Ad	IB	200	3.5	79	alive	8	84
6	M	61	Ad	IB	200	3.5	96	alive	23	46
7	M	47	Ad	IB	200	6.0	93	alive	65	59
8	F	67	Ad	IIIA	220	3.8	54	death	24	63
9	M	76	Ad	IIIA	220	4.6	42	death	34	52
10	F	47	Ad	IIIA	320	3.2	14	death	40	66
11	F	59	Ad	IIIA	220	3.4	64	death	35	47
12	F	41	Ad	IIIA	320	6.2	49	death	31	58
13	F	68	Ad	IIIA	220	3.4	10	death	92	87
14	F	58	Ad	IIIA	220	9.8	22	death	17	53
15	M	69	Ad	IIIB	420	5.5	9	death	61	88
16	M	45	Ad	IIIB	420	9.0	7	death	16	72
17	M	63	Sq	IB	200	4.2	100	alive	12	25
18	M	62	Sq	IB	200	5.1	98	alive	13	27
19	F	60	Sq	IB	200	4.5	104	alive	24	45
20	M	64	Sq	IB	200	5.5	9	death	60	66
21	M	66	Sq	IB	200	5.0	102	alive	30	51
22	M	67	Sq	IIIB	400	8.0	37	death	42	39
23	M	68	Sq	IIIA	320	5.5	7	death	81	34
24	M	43	Sq	IIB	300	6.2	85	alive	34	45
25	M	68	Sq	IIIA	310	7.5	4	death	14	34
26	M	80	Sq	IIIB	230	7.7	2	death	19	56
27	M	72	La	IB	200	4.6	17	death	49	35
28	F	81	La	IB	200	4.5	7	death	41	41
29	F	44	La	IB	200	5.7	95	alive	26	42
30	M	68	La	IIB	300	6.5	82	death	17	66
31	F	70	La	IIIA	320	6.2	24	death	63	54

M: male, F: female, Ad: adenocarcinoma, Sq: squamous cell carcinoma, La: large cell carcinoma

croscope. In addition, dual color FISH allows measurement and comparison of gene deletions and amplifications, and chromosomal aberration rates.[11-16)]

We performed dual color FISH to evaluate the relationship between the c-myc gene amplification, the numerical chromosome 8 aberrations and pathological factors (T factors, tumor size and N factors). We also investigated whether these abnormalities could provide an additional prognostic factor.

Materials and Methods

Tumor tissue samples were obtained from 31 patients with NSCLC who underwent lobectomy at Saitama Cancer Center, Saitama, Japan, between 1988 and 1990. Patients were excluded from the study if they died from other diseases or an unknown cause of death, or if no mediastinal lymph node dissection was performed. The pathological stage was determined according to the TNM classification of the International Union Against Cancer.

The patients consisted of 18 men and 13 women, ranging in age from 41 to 81 (mean 62.7) years. A histological study revealed that 16 had adenocarcinoma (Ad), 10 had squamous cell carcinoma (Sq), and 5 had large cell carcinoma (La). Fifteen patients were in Stage I (IA-3, IB-12), 2 were in Stage IIB, and 14 were in Stage III (IIIA-10, IIIB-4). Regarding T classification, 3 patients were T1; 18 T2; 7 T3; and 3 T4 (all cases were pulmonary metastasis of the same lobe). The maximum tumor size of these patients ranged from 1.2 to 9.8 cm in diameter (mean 5.2 cm). Regarding N classification, 18 were N0, 11 were N2, and one each for N1 and N3 (contra lateral mediastinal lymph node) disease (Table 1).

Sample preparation
Each specimen was minced and put into phosphate buffer saline (PBS). The solution was filtered through a gauze mesh, and centrifuged (1,000 rpm, 5 min.). The sediment thus obtained was suspended in 0.75 M KCl solution, and the suspension was centrifuged. The sediment was finally suspended in Carnoy's solution (methanol : acetate=3:1) and stored at –20°C until hybridization.

Fluorescence in situ hybridization
Before hybridization, a drop of the suspension was placed onto an ethanol- cleaned glass slide. Each slide preparation was denatured in 70% formamide plus 4×SSC (1×SSC: 0.15M NaCl, 15 mM sodium citrate) (pH 7.0) at 75°C for 2 min. After denaturing, the slide preparation was dehydrated in a series of ethanol (70, 80, and 100%) solutions at room temperature for 2 min each. A probe mixture containing 10 μl of hybridization buffer (dextran sulfate, 70% formamide, 2×SSC, pH 7.0), 1 μl of 8 alpha satellite repeat DNA probe (labeled with Spectrum Green to 8 alpha satellite centromere [Vysis, Inc., Chicago, IL]), and 1 μl of c-myc gene cosmid probe (labeled with Spectrum Orange to the 8q24.2-q24.3 region [Vysis, Inc.]) was denatured at 75°C for 5 min then immediately denatured at 40°C for 5 min. The probe mixture was applied to the target area of the preparation. The target area was covered with an 18×18-mm glass coverslip.

All slide preparations were incubated in a wet chamber overnight at 40°C. On completion of hybridization, each slide was washed three times in 50% formamide plus 4×SSC (pH 7.0) at 40°C for 5 min, three times in 4×SSC(pH7.0) at 40°C for 3 min, once in 4×SSC plus 1% Triton X solution at 40°C for 5 min, and finally distilled water.

The nuclei were counterstained with 125 μg/ml of DAPI (diamidino-phenylndole, [Vysis, Inc.]) solution.

Detection of signals
Using a fluorescence microscope with a three-band pass filter, 100 nuclei on each slide were scored for the number of signals from the chromosome 8 centromeres (centromere 8) and the 8q24 locus. The copy number category (monosomic, disomic, trisomic, or above) for a given preparation was determined according to the number of 8 alpha centromeres. We defined two signals of Spectrum Green in a nucleus as a normal copy number of centromere 8, and the others (monosomic, trisomic, and above) as numerical aberrations. The c-myc gene amplification was defined as more c-myc signals than centromere 8 signals.

Statistical analysis
Statistical analysis was performed with a SPSS 10.0 software package [SPSS inc. Chicago, IL]. The comparison between the two groups (tumor size and N factors) was analyzed by the unpaired Student's t-test. One-way ANOVA test was used for analysis of four groups (T factors) and then further analysis was performed with Tukey HSD test for multiple pairwise comparisons. A univariate analysis for 5-year survival was performed with the logistic regression analysis (age, tumor size, the numerical aberration rate of chromosome 8 and the amplification rate of c-myc gene). The chi-square test by Fisher's direct method was used for comparison of categorical data (sex, T factor, N factor and histology). Multivariate analyses were performed with the logistic regression procedure, using the back-stepping selection method with maximum-likelihood estimates and default criteria. Actual survival curves were calculated by the Kaplan-Meier method and log-rank test. Data are expressed as the mean ± standard deviation (SD). A p-value of less than 0.05 was considered significant for the group comparison.

Results

The signals for centromere 8 and the c-myc gene were clearly seen in the NSCLC cells subjected to dual color FISH (Fig. 1a, b).

The 31 patients were followed up until May 1998 at our outpatient clinic. At that time, 20 had died of cancer (5 in Stage I, 1 in Stage II, and 14 in Stage III), and the mean survival time was 49.2±38.1 months (ranging from 2 to 104 months) (Table 1). Actual 3-year and 5-year survival rates were 58.1% and 41.9%, respectively for all cases.

Regarding T classification, the rate of chromosome 8 aberration was 44.0±15.7% in T1, 35.7±23.1% in T2, 40.0±24.3% in T3, and 39.7±22.6% in T4. The rate of c-myc gene amplification was 54.3±6.7% in T1, 51.1±16.7% in T2, 51.0±13.7% in T3, and 66.3±25.0% in T4. There were no differences in the T factor in each group.

In sixteen patients the tumor was more than 5 cm in maximum diameter, and in fifteen patients it was 5 cm or less. There was no difference in the rate of chromosome 8 aberration between patients whose tumor size was more than 5 cm, and 5 cm or less (39.3±23.6% and

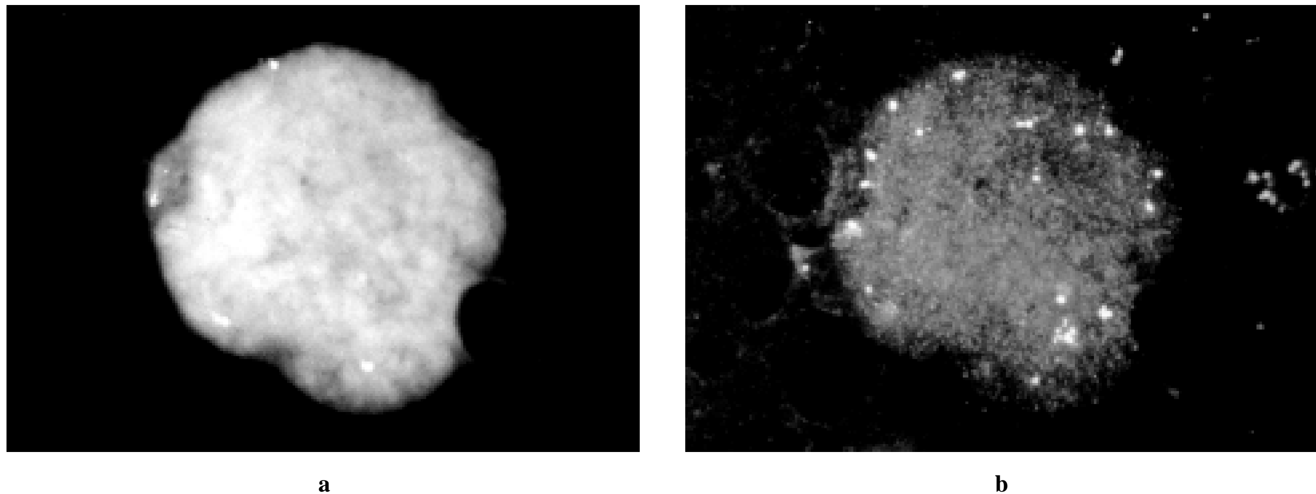

Fig. 1. Photographs of a nucleus in one patient (case 21). (×400)
a: Four signals (green spots) from the hybridized chromosome 8 alpha centromeric probe can be seen in the nucleus.
b: More than 10 signals (red spots) from the hybridized 8q24 cosmid probe are distributed diffusely in the nucleus.

36.3±20.5%). There was no difference in the rate of c-myc gene amplification between patients whose tumor size was more than 5cm, and 5cm or less (52.1±16.1% and 53.7±16.6%).

Regarding N classification, the rate of chromosome 8 aberration was 35.9±19.1% in N0 and 44.9±25.7% in N2. There was a significant difference in the rate of c-myc gene amplification between N0 and N2 (48.6±14.7% and 61.3±16.4%) (Fig. 2). (Patients with N1 and N3 disease were excluded because of insufficient numbers.)

Thirteen patients survived for 5 years or more and 18 died within 5 years. We evaluated the prognostic factors for the long-term (5-years) survival using univariate and multivariate analysis for 8 variables as follows; age, sex, T factor (T1-2 vs. T3-4), tumor size, N factor (N0-1 vs. N2-3), histology (adenocarcinoma vs. non-adenocarcinoma), chromosome 8 aberrations and c-myc gene amplifications.

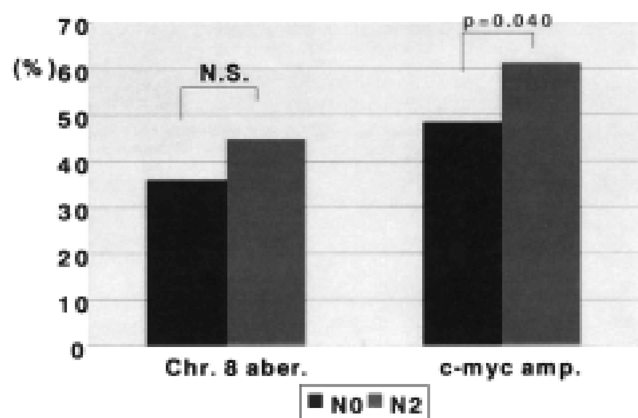

Fig. 2. There was no significant difference in the rate of numerical chromosome 8 aberrations between N0 and N2 (N0: 35.9±19.1 vs. N2: 44.9±25.7%). However, the amplification rate of c-myc gene significantly correlated with lymph node metastasis. (N0: 48.6±14.7% vs. N2: 61.3±16.4%, p=0.040)

Univariate analysis revealed significant differences in N factor and chromosome 8 aberrations for poor prognosis (Table 2).

Multivariate analysis revealed that N factor, chromosome 8 aberrations and tumor size correlated with poor prognosis (Table 3).

We also evaluated the relationship between these abnormalities and patient outcome using the Kaplan-Meier method and log-rank analysis. The mean chromosome 8 aberration rate was 36.8±22.2% and the mean c-myc gene amplification rate was 51.7±17.2%. On the basis of these mean ratios, the patients were divided into 2 groups based on aberration rate (≥40%, <40%) and 2 groups based on amplification rate (≥50%, <50%). The 5 year-survival rate was 15.4% in patients whose rate of chromosome 8 aberration was 40% or more (n=13), which was significantly less than that in patients with an aberration rate of less than 40% (n=18, 57.9%) (Fig. 3a). The 5 year-survival rate in patients whose amplification rate for the c-myc gene was 50% or more (n=17) and less than 50% (n=14) was 29.4% and 53.3%, respectively (Fig. 3b).

Discussion

A previous report has shown that the incidence of c-myc gene amplification is 13-23% in small cell lung cancer

Table 2. Results of univariate analysis for prognostic factors

Variable		Survival<5 years (n=18)	Survival 5 years (n=13)	p value (95% CI)
Amp. of c-myc	(years)	64.6 ± 11.4	60.2 ± 9.7	0.279 (–3.69-12.34)
Sex	Male (n)	10	8	0.972
	Female (n)	8	5	
T factor	T1-2 (n)	10	11	0.074
	T3-4 (n)	8	2	
Tumor size	(cm)	5.7 ± 2.1	4.4 ± 1.6	0.061 (–0.06-2.73)
N factor	N0-1 (n)	7	12	0.008*
	N2-3 (n)	11	1	
Histology	Ad (n)	10	6	0.879
	non-Ad (n)	8	7	
Aber. of Chr. 8	(%)	44.7 ± 23.1	28.3 ± 16.4	0.037* (1.09-31.74)
Amp. of c-myc	(%)	55.3 ± 16.6	49.5 ± 15.5	0.337 (–6.28-17.75)

CI: confidence interval, *: statistical significant.

(SCLC), and 5-12% in NSCLC by means of the traditional molecular biochemical procedure.[1] However, in our study, the incidence of the c-myc gene amplification in all specimens was 25% to 88%. It is possible that low-level amplification of the c-myc gene, which can only be detected by the very sensitive FISH method, may take place at a very early stage or even in the precancerous stage of the disease.

Cancer may progress with the accumulation of various genetic abnormalities in a multistep process of carcinogenesis.[17,18] Recently, a number of abnormalities in genes such as K-ras, p53, RB, and c-myc, have been reported, and the characteristics and process of multiple carcinogenetic stages are now being studied in NSCLC by PCR, Southern blotting or immunohistochemical procedure. In multistep carcinogenesis of lung cancer, the c-myc gene is supposed to be associated with tumor progression and metastasis. Volm et al.[10] reported that tumors with an overexpression of proteins encoded by the c-myc gene showed a significantly increased formation of metastasis in primary lung cancer. There was also a significant correlation between the c-myc amplification and lymph node metastasis of NSCLC in our study by FISH analysis.

As a factor prescribing a T classification (especially T3, T4), the location of the tumor is more important than the size of tumor. We thought that tumor size related to a genetic abnormality rate as an index to tumor growth rather than T factors. Bergh and Barr have reported that the c-myc gene seemed to be associated with tumor progression in small cell lung cancer.[7,8] However, neither T factors nor tumor size correlated with these abnormalities. There was no correlation with tumor increase and the c-myc gene amplification or chromosome 8 aberration.

Table 3. Results of multivariate analysis for prognostic factors

Variables	OR (95% CI)	p value
Age (year)	1.12 (0.985-1.268)	0.084
Tumor size (cm)	2.11 (1.002-4.337)	0.043
N factor (N0-1 vs. N2-3)	50.27 (1.964-1286.9)	0.018
Aber. of Chr. 8 (%)	1.09 (1.004-1.176)	0.041

OR: odds ratio, CI: confidence interval.

We previously referred to a complicated relationship between various oncogenes and tumor suppressor genes in the carcinogenesis mechanism. Oncogenes, such as p53, Rb,bcl-2 and HER2/neu, as prognostic factors for lung cancer have been reported.[19-21] We could not find any correlation between the c-myc gene amplification and clinical outcome. However, the numerical chromosome 8 aberrations correlated significantly with a poor prognosis in univariate and multivariate analysis ($p=0.037$, and $p=0.041$, respectively). In multivariate analysis, the odds ratio of the numerical chromosome 8 aberrations was 1.09 per 1%, i.e., 2.28 per 10% of aberrations. A comparison by the Kaplan-Meier method also revealed a significant difference between those with a high (40% or more) rate of chromosome 8 aberration and those with a low (less than 40%) rate ($p=0.014$).

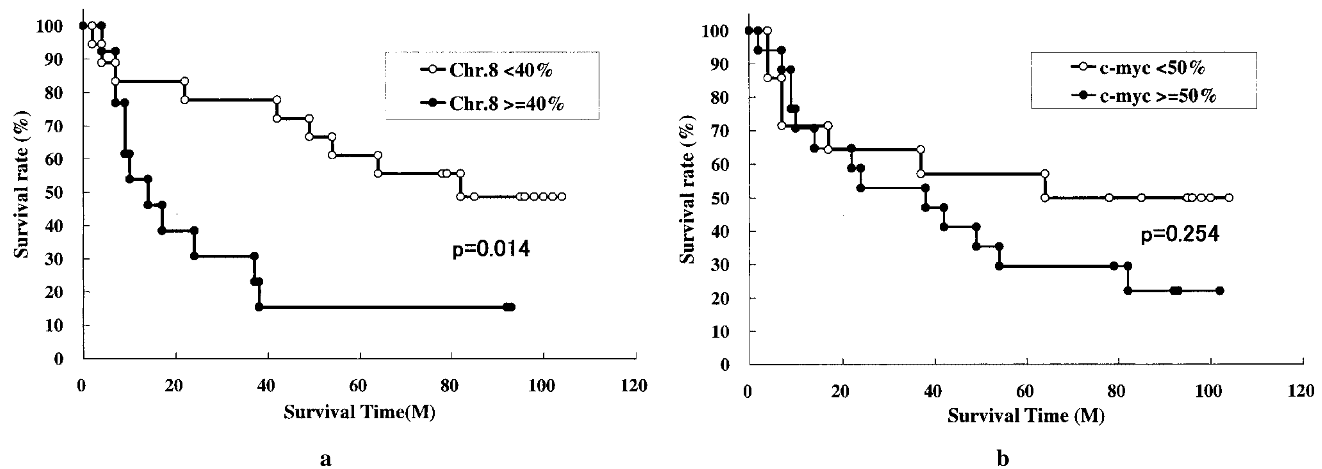

Fig. 3. a: The 5 year-survival rate was 15.4% in patients whose rate of chromosome 8 aberration was 40% or more (n=13), which was significantly less than that in patients with an aberration rate of less than 40% (n=18) (57.9%) (p=0.014). The numerical aberration of chromosome 8 was associated with a longer survival.
b: The 5 year-survival rate in patients whose amplification rate for the c-myc gene was 50% or more (n=17) and less than 50% (n=14) was 29.4% and 53.3%, respectively (p=0.377). There was no significant correlation between the rate of gene amplification and clinical outcome.

This study is limited due to the number of patients, and is the reason why the numerical chromosome aberration influences on clinical outcome could not be explained in this study. So we need further study.
Although it was difficult to make a comprehensive prognosis from an abnormality in one gene, our finding of a relationship between the numerical chromosome 8 aberration and clinical outcome may be applicable for future treatment planning based on further investigation of various chromosomal abnormalities.

In conclusion, we have found that the c-myc gene amplification was detected at a higher rate with FISH analysis than that with Southern blotting or immunohistochemical procedure. The numerical chromosome 8 aberration and the c-myc gene amplification have no correlation with T factor and tumor size, but the c-myc gene amplification correlates with lymph node metastasis. Although there was no significant link between the amplification of the c-myc gene and clinical outcome, the numerical chromosome 8 aberration was considered to be a factor for survival.

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