—Originals—

Chromosome 8 copy numbers and the c-myc gene amplification in non-small cell lung cancer

Analysis by interphase cytogenetics

Hirotoshi Kubokura[1], Kiyoshi Koizumi [1], Mitsunobu Yamamoto [2], Shigeo Tanaka [1]

[1] The Department of Surgery Second, Nippon Medical School
[2] The Department of Thoracic Surgery, Saitama Cancer Center

Abstract

Amplification of the c-myc gene has been reported in non-small cell lung cancer (NSCLC). We performed dual color fluorescence in situ hybridization (FISH) to detect amplifications of the c-myc gene on chromosome 8 to evaluate the relationship between these possible abnormalities and pathological stage. Tumor tissue samples were obtained from 29 patients of NSCLC in Stage I (n=15) and III (n=14) who underwent lobectomy at Saitama Cancer Center. Samples were analyzed for chromosome 8 centromere and c-myc gene by dual color FISH. The numerical aberration rate of chromosome 8 was 36.8 ± 20.3% in Stage I and 40.6 ± 24.8% in Stage III. The amplification rate of c-myc gene was 48.3 ± 15.2% in Stage I and 57.4 ± 17.0% in Stage III. There was a significant difference in the numerical aberration rate of chromosome 8 between patients who survived for 5 years or more (28.8 ± 17.5%) and those who survived less than 5 years (44.7 ± 23.1%). The amplification rate of c-myc gene was not different between patients who survived more and less than 5 years survival, and who survived more and less than 3 years. The 5 year-survival rate in patients who showed 40% or more of chromosome 8 aberrations (n=13) was 15.4%, which revealed significantly less than that of patients who showed less than 40% of aberrations (n=16) (56.3%). There was no difference between the 5 year-survival rate in patients whose amplification rates of c-myc gene were equal or more than 50% (n=16) and less than 50% (n=13) (25.0 % and 53.9%). The rate of chromosome 8 aberrations and the c-myc gene amplification rate were not correlated with pathological stage. However, the rate of chromosome 8 aberration showed correlation in terms of longevity of survival rate, therefore we considered the rate of chromosome 8 aberration to be an additional prognostic factor of patient with NSCLC. (J Nippon Med Sch 1999 ; 66 : 107—112)

Key words : non-small cell lung cancer, dual color FISH, chromosome 8 copy number, the c-myc gene amplification

Introduction

Various genetic alterations and chromosomal aberrations have been observed in patient with non-small-cell lung cancer (NSCLC)[1-6]. The c-myc gene, localized to 8q24, may play an important role in cell proliferation and differentiation and may induce apoptosis of cells under certain condition [7]. Amplification of c-myc gene might be related to metastasis of lung cancer [8].

Correspondence to Hirotoshi Kubokura, The Department of Surgery Second, Nippon Medical School, 1–1–5 Sendagi, Bunkyo-ku, Tokyo 113–8603, Japan

Table 1 Clinicopathological findings and results

Case	Sex	Age	Type	Stage	TNM	Survival Time (M)	Outcome	Chr. 8 aberration (%)	c-myc amplification (%)
1	F	53	Ad	IA	100	38	Death	51	56
2	F	69	Ad	IA	100	78	Alive	26	47
3	M	72	Ad	IA	100	92	Alive	55	60
4	M	68	Ad	IB	200	4	Death	70	41
5	F	69	Ad	IB	200	79	Alive	8	84
6	M	61	Ad	IB	200	96	Alive	23	46
7	M	47	Ad	IB	200	93	Alive	65	59
8	M	63	Sq	IB	200	100	Alive	12	25
9	M	62	Sq	IB	200	98	Alive	13	27
10	F	60	Sq	IB	200	104	Alive	24	45
11	M	64	Sq	IB	200	9	Death	60	66
12	M	66	Sq	IB	200	102	Alive	30	51
13	M	72	La	IB	200	17	Death	49	35
14	F	81	La	IB	200	7	Death	41	41
15	F	44	La	IB	200	95	Death	26	42
16	F	67	Ad	IIIA	220	54	Death	24	63
17	M	76	Ad	IIIA	220	42	Death	34	52
18	F	47	Ad	IIIA	320	14	Death	40	66
19	F	59	Ad	IIIA	220	64	Death	35	47
20	F	41	Ad	IIIA	320	49	Death	31	58
21	F	68	Ad	IIIA	220	10	Death	92	87
22	F	58	Ad	IIIA	220	22	Death	17	53
23	M	68	Sq	IIIA	320	7	Death	81	34
24	M	68	Sq	IIIA	310	4	Death	14	34
25	F	70	La	IIIA	320	24	Death	63	54
26	M	69	Ad	IIIB	420	9	Death	61	88
27	M	45	Ad	IIIB	420	7	Death	16	72
28	M	67	Sq	IIIB	400	37	Death	42	39
29	M	80	Sq	IIIB	230	2	Death	19	56

Ad : adenocarcinoma Sq : squamous cell carcinoma La : large cell carcinoma

While many studies concerning the c-myc gene amplification in various solid tumors have been evaluated using Southern blotting or polymerase chain reaction (PCR)[1,5,9,10], by means of development of fluorescence in situ hybridization (FISH) has facilitated direct examination of cell nuclei for chromosomal aberrations under a fluorescence microscope. In addition, dual color FISH method allows measurement and comparison of gene deletions and gene amplifications, and chromosomal aberration rates[11–17].

We performed dual color FISH to evaluate the relationship between the c-myc gene amplification and pathological stage. We also investigated whether these abnormalities could provide an additional prognostic factor.

Materials and Methods

Tumor tissue samples were obtained from 29 patients with NSCLC in stages I and III who underwent lobectomy at Saitama Cancer Center, Saitama, Japan, between 1988 to 1990. Following patients were excluded in this study, i. e., died of other diseases, unknown cause of death, and no mediastinal lymph node dissection. The pathological stage was determined according to the TMN classification of the International Union Against Cancer.

The patients consisted of 16 men and 13 women, ranging in age from 41 to 80 years (mean 63.2 years). A histological study revealed that 16 had adenocarcinoma (Ad) (stage I-7, stage III-9), 9 had squamous cell carcinoma (Sq) (stage I-5, stage III-4), and 4 had large cell carcinoma (La) (stage I-3, stage III-1) (**Table 1**).

(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 resulting sediment 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℃ until hybridization.

(2) *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 : 0.15 M NaCl, 15 mM sodium citrate) at 75℃ for 2 min. After this denaturing, the slide preparation was dehydrated in the ethanol series (70, 80 and 100%) at room temperature for 2 min each. A probe mixture containing of 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 the c-myc gene cosmid probe (labeled with Spectrum Orange to 8 q 24.2 − q 24.3 region [Vysis, Inc.]) was denatured at 75℃ for 5 min and immediately denatured at 40℃ for 5 min. And then a 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℃. On completion of hybridization, each slide was washed three times in a 50% formamide plus 4 × SSC (pH 7.0) at 40℃ for 5 min, and three times in a 4 × SSC (pH 7.0) at 40℃ for 3 min, and washing followed in a 4 × SSC plus 1% Triton X solution at 40℃ at 5 min, finally washing in distributed water.

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

(3) *Detection of signals*

Using a Zeiss fluorescence microscope (Germany) with a three-band pass filter, 100 nuclei on each slide was scored for number of signals from the chromosome 8 centromere (centromere 8) and the 8 q 24 locus. The copy number category (monosomic, disomic, trisomic, and above) for each given preparation was determined according to the number of 8 alpha centromeres, as described by Waldman et al.[18]. We defined two signals of Spectrum Green in a nuclei as a normal copy number of centromere 8, and the others (monosomic, trisomic, and above) were defined as a numerical aberration. The c-myc gene amplification was defined as more c-myc signals than centromere 8 signals.

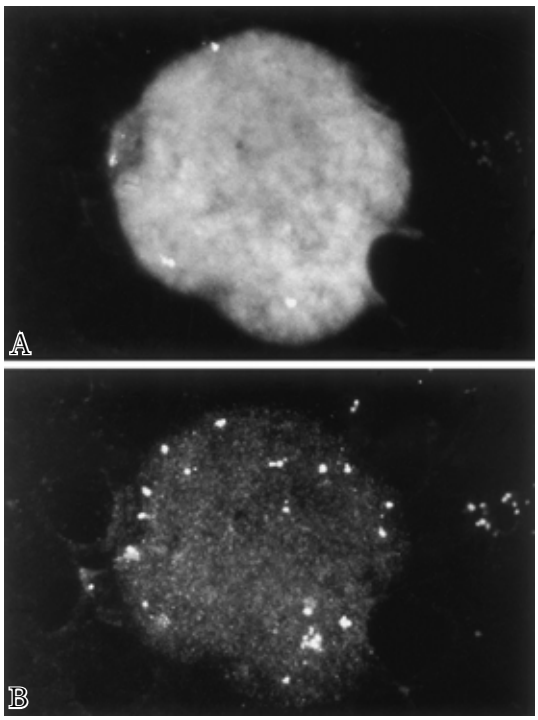


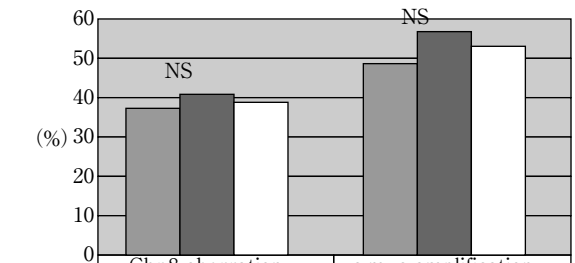
Fig. 1 Photographs of the nucleus in the same patient (Case 21, × 400). A : Four signals (green spot) can be seen from hybridized chromosome 8 alpha centromeric probe in the nucleus. B : More than 10 signals (red spot) can be seen from hybridized 8 q 24 cosmid probe distributed diffusely in the nucleus.

The results were described as mean ± standard deviation (1 SD). Student's t-test was used for statistical analysis. Actuarial survival curves were calculated by the Kaplan-Meier method and log-rank test was used. A P-value less than 0.05 was considered significant.

Results

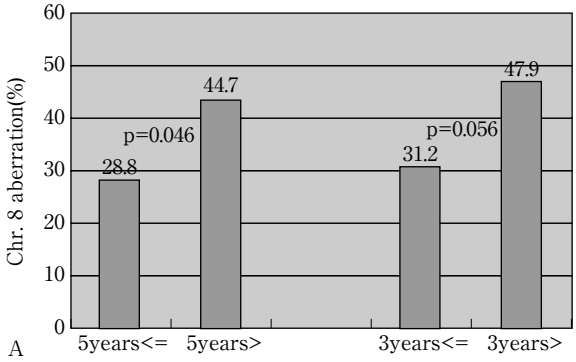
The signals for centromere 8 and c-myc gene were recognized clearly seen in the NSCLC cells subjected to dual color FISH (**Fig. 1**). The 29 patients were followed up until May 1998 at our outpatient clinic, i. e., 19 died of cancer (5 in stage I and 14 in stage III), and the mean survival time was 46.8 ± 38.3 months (ranging from 2 to 104 months) (**Table 1**). Actual 3-year and 5-year survival rates were 66.7%, 66.7%, respectively in stage I, 35.7%, 17.1%, respectively in stage III, and 55.2%, 38.0%, respectively in all cases.

The numerical aberration rate of chromosome 8



	Chr.8 aberration	c-myc amplification
□Stage I	36.9	48.3
□StageⅢ	40.6	56.3
□Overall	38.7	52.7

Fig. 2 Abnormalities and pathological stage. Left : The numerical aberration rate of chromosome 8 was 36.9% in stage I, 40.6% in stage III and 38.7% in overall. Right : The amplification rate of c-myc gene was 48.3% in stage I, 56.3% in stage III and 52.7% in overall. The rate of chromosome 8 aberrations and the c-myc gene amplification rate were not correlated with pathological stage.



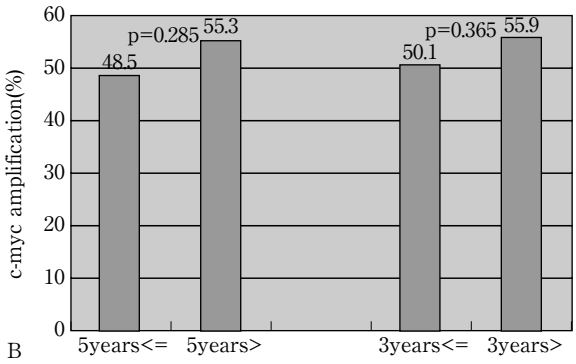


Fig. 3 A Survival time and Chr. 8 aberration. Left : The mean chromosome 8 aberrations was 28.8% in patients who survived 5 years (n=11), significantly less than those who died within 5 years (n=18, 44.7%, p=0.046). Right : There was no significant difference between patients who survived more and less than 3 years (31.2% and 47.9% ; p=0.056).

Fig. 3 B Survival time and c-myc amplification. Left : There was no significant difference between patients who survived more and less than 5 years in the amplification rates of c-myc gene (48.5% and 55.3% ; p=0.285). Right : There was also no significant difference between patients who survived more and less than 3 years (50.1% and 55.9% ; p=0.365).

was 36.8 ± 20.3% in stage I and 40.6 ± 24.8% in stage III (p=NS). The amplification rate of c-myc gene was 48.3 ± 15.2% in stage I and 57.4 ± 17.0% in stage III (p=NS) (**Fig. 2**).

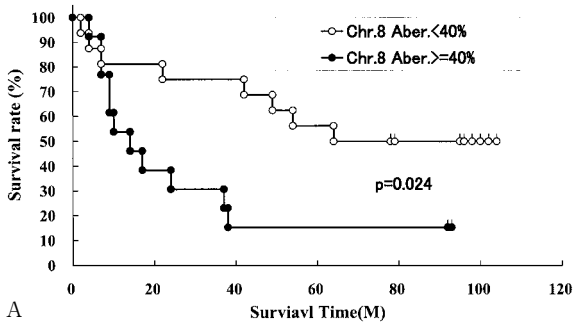
Eleven patients survived for 5 years or more (10 in stage I, 1 in stage III). Sixteen patients survived for 3 years or more (11 in stage I, 5 in stage III). The numerical aberration rate of chromosome 8 was 28.8 ± 17.5% in patients who survived 5 years, significantly less than those who died within 5 years (44.7 ± 23.1% ; p=0.046). In the numerical aberration rate of chromosome 8, there was no significant difference between patients who survived more and less than 3 years (31.2 ± 15.7% and 47.9 ± 26.0% ; p=NS) (**Fig. 3 A**). The amplification rates of c-myc gene in patients who survived 5 years or more and who died within 5 years were 48.5 ± 16.1% and 55.3 ± 16.6%, respectively (p=NS). And those who survived 3 years or more and who died within 3 years were 50.1 ± 14.1% and 55.9 ± 19.0%, respectively (p=NS) (**Fig. 3 B**).

We also evaluated the relationship between these abnormalities and the patient outcome using the Kaplan-Meier method and log-rank analysis. The mean chromosome 8 aberration rate was 38.7 ± 22.2% and the mean c-myc gene amplification rate was 52.7 ± 16.4%. The 5 year-survival rate was 15.4% in patients whose chromosome 8 aberrations were 40% or more (n=13), which showed significantly less than 56.3% in patients whose aberrations less than 40% (n=16) (p=0.024) (**Fig. 4 A**). The 5 year-survival rate in patients whose amplification rates of c-myc gene were 50% or more (n=16) and less than 50% (n=13) were 25.0% and 53.9%, respectively (p=NS) (**Fig. 4 B**).

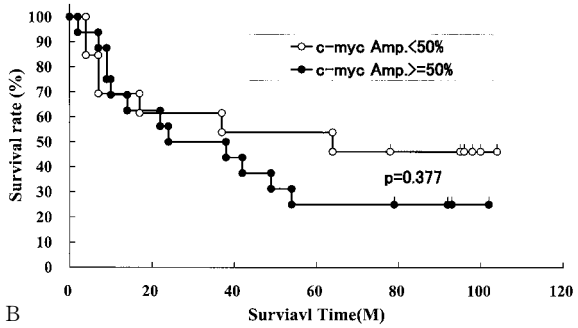
Discussion

Cancer may progress with the accumulation of variety of genetic abnormalities in a multistep process of carcinogenesis[19]. The mechanism of multistep carcinogenesis well characterized in colorectal cancer (adenoma-carcinoma sequence)[20-21].

On the other hand, difficulties in early diagnosis



A



B

Fig. 4 A Survival rate and Chr. 8 aberration. The 5 year-survival rate was 15.4% in patients whose chromosome 8 aberrations were 40% or more (n=13), which showed significantly less than 56.3% in patients whose aberrations less than was 40% (n=16, p=0.024). The numerical aberration of chromosome 8 was associated with a longer survival.

Fig. 4 B Survival rate and c-myc amplification. The 5 year-survival rate in patients whose amplification rates of c-myc gene were 50% or more (n=16) and less than 50% (n=13) were 25.0% and 53.9%, respectively (p=0.377). There was no significant correlation between the rate of c-myc gene amplification and patient clinical outcome.

have meant that the developmental mechanism of lung cancer has not been elucidated. However, in recent years, a number of abnormalities in genes such as K-ras, p 53, RB, and c-myc, have been reported to cause dysplasia and atypical adenomatous hyperplasia in normal lung epithelium. The characteristics and the process of multiple carcinogenetic stages are now being studied assuming precancerous lesions of squamous cell carcinoma or adenocarcinoma.

In multistep carcinogenesis, the amplification of c-myc gene is supposed to be associated with metastasis. But there was no significant correlation between the c-myc amplification and pathological stage of NSCLC in our study by means of FISH analysis.

A previous report has shown that the incidence of c-myc gene amplification is 13～23% in small-cell lung cancer (SCLC), and 5～12% in NSCLC by the traditional molecular biochemical procedure[1]. However, in our study, the amplification of c-myc gene was detected in all specimens, in 25% to 88%. It is possible that low-level amplification of c-myc gene, which can be detected of all cells only by the very sensitive FISH method, may take place at a very early stage or even in the precancerous stage.

We also could not find any correlation between the rate of the c-myc gene amplification and clinical outcome. However, the numerical chromosome 8 aberrations were significantly lower in patients who survived for 5 years or more. In patients who survived for 3 years, an apparent difference was shown (p=0.056). A similar result was observed between the survival period and the aberration ratio using Kaplan-Meier method evaluated by log-rank analysis (p=0.024).

We previously reported a complicated relationship between various oncogenes and tumor suppressor genes in a carcinogenesis mechanism. 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 aberrations and clinical outcome may be applicable in future treatment planning based on further investigation of various chromosomal abnormalities.

In conclusion, we have found that amplification of c-myc gene was detected at a higher rate with FISH analysis than that with Southern blotting or that with PCR. Furthermore, the numerical chromosome 8 aberration and the c-myc gene amplification have no correlation with pathological stage. This result suggests that the low-level amplification of c-myc gene was not only evident at the onset of lung cancer, but also the amplification ratio showed no correlation with lymph metastasis. In fact, the c-myc gene alteration was recognized only at the onset of carcinogenesis.

Therefore, although there was no significant correlation between the rate of c-myc gene amplification and patient clinical outcome, the numerical aberration of chromosome 8 was considered to be a factor expected longevity of survival rate.

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