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Analysis of c-*myc* DNA amplification in non-small cell lung carcinoma in comparison with small cell lung carcinoma using polymerase chain reaction

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Abstract Previous studies of c-*myc* DNA amplification in lung cancer have focused primarily on analysis of small cell carcinoma or its tumor cell lines. There are few data about c-*myc* DNA amplification in histological types of lung cancer other than small cell carcinoma. Therefore the present study was conducted to investigate c-*myc* oncogene amplification in non-small cell lung carcinoma. We studied 46 lung tumor specimens for c-*myc* DNA amplification (15 adenocarcinomas, 15 squamous cell carcinomas, 6 large cell carcinomas, and 10 small cell carcinomas). Polymerase chain reaction, digoxigenin DNA labeling, and electrophoresis were utilized to investigate the c-*myc* copy number in the lung tumor specimens. The c-*myc* copy number of non-small cell carcinoma ranged from 1.5 to more than 20.0 in adenocarcinoma and squamous cell carcinoma, and from 6.0 to 12.0 in large cell carcinoma. That of small cell carcinoma ranged from 1.8 to 12.0. The c-*myc* copy number of non-small cell carcinoma was significantly higher than that of small cell carcinoma (Wilcoxon rank sum test, $Z=2.06$ $P=0.040$). However, the differences in c-*myc* copy number among these four histological types were not statistically significant. Amplification of c-*myc* (more than 4 copies) was observed not only in small cell carcinoma but also in non-small cell carcinoma at similarly high frequency (12/15 in adenocarcinoma and squamous cell carcinoma, 6/6 in large cell carcinoma, and 9/10 in small cell carcinoma).

Key words c-*myc* amplification • Lung cancer • Histological type • Polymerase chain reaction • Digoxigenin-labeled probe

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Introduction

The c-*myc* oncogene is located on chromosome 8q24 and encodes a nuclear transcription factor. Its protein product is localized in the nucleus and regulates gene transcription to promote cell growth. The *myc* gene family can cooperate with other oncogenes, such as the activated *ras* or *bcl*-2 gene, in multistage carcinogenesis [1–5]. c-*myc* is activated generally by gene amplification, acceleration of transcription, or translocation. In small cell lung carcinoma (SCLC) or cell lines derived from it, *myc* DNA amplification and high levels of its expression are reported frequently [6–13]. Amplification

of c-*myc* in tumor cell lines established from patients with relapsed SCLC has been associated with shortened survival [14]. The more-aggressive variant phenotype in SCLC cell lines is closely associated with c-*myc* amplification [12, 15]. Most of these previous studies have focused on SCLC and its cell lines, and there are few reports of amplification of the c-*myc* oncogene in non-small cell lung cancer (NSCLC). In the present study, therefore, we examined the c-*myc* DNA copy number in formalin-fixed paraffin-embedded lung tumor specimens from 46 different patients, and correlated the data with the histological types of the lung cancers.

Materials and methods

Sample collection

Formalin-fixed, paraffin-embedded surgical specimens of 46 lung tumors and autopsy specimens of 2 interstitial pneumonias were obtained from the pathology archives of the Department of Pathology, University of Tokyo, and the Japanese Red Cross Medical Center. SK-OV-3 (HTB-77) [16], an ovarian adenocarcinoma cell line, was obtained from the American Type Culture Collection. These 46 lung tumors comprised 15 adenocarcinomas, 15 squamous cell carcinomas, 6 large cell carcinomas, and 10 SCLCs. Each tumor was reviewed histologically, and the proportion of the solid component relative to the total tumor mass was assessed. The 2 interstitial pneumonias and SK-OV-3 cell line were used as controls.

Preparation of archival specimens for polymerase chain reaction

Sections of each tumor were first reviewed histologically to identify any areas containing large amounts of non-neoplastic stroma, and such areas were removed from the specimens with scalpel blades. The specimens containing mostly tumor tissue were subjected to polymerase chain reaction (PCR) studies. The extraction of DNA from formalin-fixed paraffin-embedded specimens was performed using the technique of Shibata et al. [17], with minor modifications. Briefly, single 4-μm-thick tissue sections were obtained with a microtome and deparaffinized by two consecutive centrifugations in xylene. The sections were washed twice in 100% ethanol and resuspended in 50 μl of 50 mM TRIS (pH 7.5), 10 mM EDTA, 1% sodium dodecyl sulfate, and 500 μg/ml proteinase K. The preparations were mixed gently for 2 h at 50°C, and the DNA was then extracted with phenol-chloroform. The extracted DNA was diluted to 0.1 μg/μl with double-distilled water, and 10-μl aliquots were added to the PCR mixture.

PCR assay

One microgram of each sample DNA was placed in 100 μl of 1x PCR buffer (10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, dNTPs at 200 μM each, with each primer at 1 μM) containing 2.5 units of Taq DNA polymerase (Perkin-Elmer-Cetus), and the reaction mixture was overlaid with 100 μl of paraffin oil. Two sets of primers, one for the c-*myc* oncogene and the other for the alpha-lactalbumin gene, were present simultaneously in each reaction mixture. The reaction mixtures were cycled on a Perkin-Elmer-Cetus thermal cycler using the following cycling parameters: 5 min at 94°C, 40 cycles of 30 s at 94°C, 1 min at 55°C, and 30 s at 72°C. Synthetic primers used for enzymatic amplification of c-*myc* sequences were 5'-CTCGGAAGGACTATCCTGCTGCCAA-3' and 5'-GGCGC-TCCAAGACGTTGTGTGTTCG-3'. The former corresponds to nucleotides 6840–6864 of the published c-*myc* sequence [18] and the latter is complementary to nucleotides 6989–6965 of the same sequence. Primers used for enzymatic amplification of alpha-lactalbumin sequences were 5'-GGTTCTTGGGGGTAGCCAAAATGAG-3' and 5'-CAGGCAAAGCGATGCCTCCATAACC-3', which correspond respectively to positions 7–31 and 132–156 of the published alpha-lactalbumin sequence [19]. Each of the above sets of primers resulted in enzymatic amplification of sequences within a length of 150 bases. The primers and probes were synthesized on an Applied Biosystems 381A synthesizer using standard techniques, and were purified by high-performance reverse-phase liquid chromatography.

Electrophoresis, Southern blotting, and quantitation of PCR products

A 10-μl portion of the PCR product was electrophoresed in 3.0% NuSieve GTG (FMC USA) agarose gels in duplicate for 50 min at 100 V. After electrophoresis, the gels were blotted onto Hybond-N+ nylon membranes (Amersham, Tokyo), under alkaline conditions (0.4 M sodium hydroxide). One of two membranes was then hybridized with the digoxigenin (DIG)-labeled c-*myc* probe (5'-TCAA-GTTGGACAGTGTCAGAGTCCTGAGAC-3'), corresponding to nucleotides 6870–6899 of the published c-*myc* sequence [18]. The other membrane was hybridized with the DIG-labeled alpha-lactalbumin probe (5'-TTGTCCCTCTGTTCCTGGTGGGCATCCTGT-3'), corresponding to nucleotides 37–66 of the published alpha-lactalbumin sequence [19], overnight at 42° C, using a DIG DNA labeling and detection kit [20] (Boehringer Mannheim, Germany). The resulting bands were detected and quantified using a Bio Image Whole Band Analyzer [21, 22], mode 110 (Bio Image, Ann Arbor, Mich., USA).

Statistical analysis

The difference between two groups, i.e., NSCLC and SCLC, for c-*myc* copy number was assessed using Wilcoxon rank sum test. Differences in c-*myc* copy number among the four histological types of lung cancer were assessed using the Kruskal-Wallis rank sum test.

Standard curve of c-*myc* copy number

A reference DNA series was made by adding serially diluted cloned human c-*myc* exon 3 DNA to human placental DNA to make a series of placental DNA containing 2, 4, 6, 8, 12, 20, and 24 copies of the c-*myc* gene exon 3 per single diploid cell DNA. Sequences from the c-*myc* oncogene and from the alpha-lactalbumin gene used as a control were amplified simultaneously by PCR

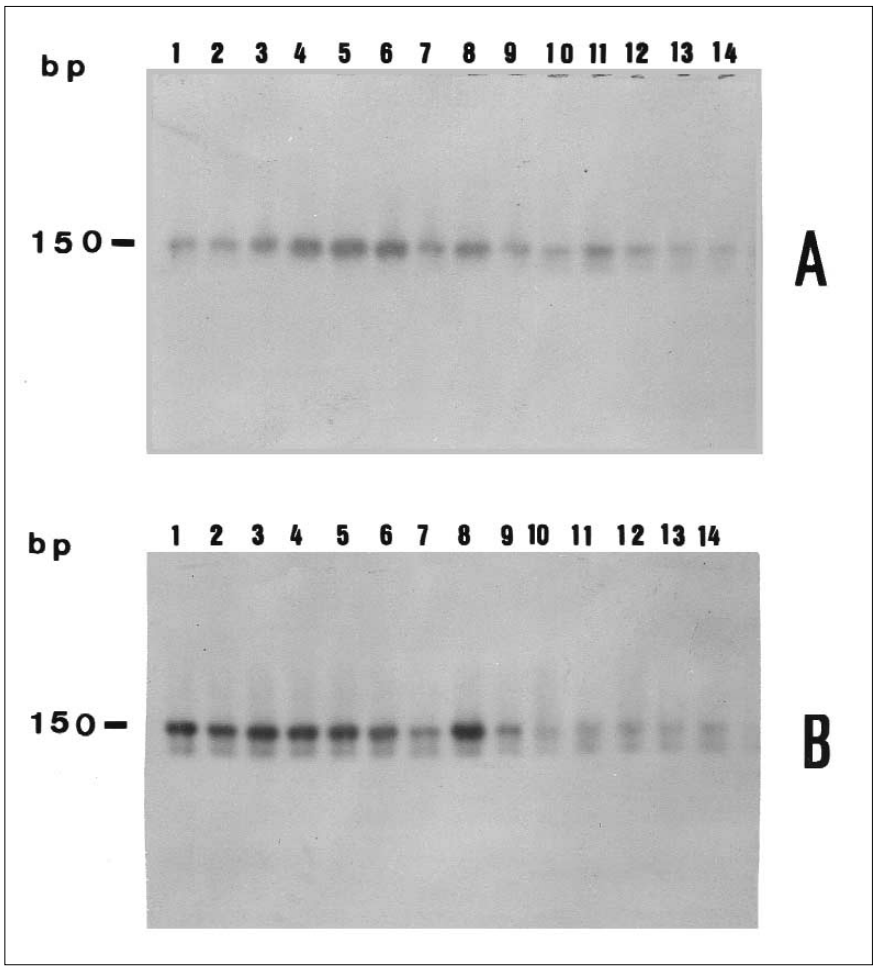

Fig. 1 Southern blot analysis of polymerase chain reaction (PCR) products amplified simultaneously using two sets of primers for the c-*myc* oncogene and the alpha-lactalbumin gene. **A** indicates hybridization with the digoxigenin-labeled c-*myc* probe. **B** indicates hybridization with the digoxigenin-labeled alpha-lactalbumin probe. Lane 1: PCR product of human placental DNA; lane 2: PCR product of placental DNA supplemented with 2 copies of c-*myc* exon 3 DNA per diploid cell DNA; lane 3: PCR product of placental DNA supplemented with 4 copies of c-*myc* exon 3 DNA per diploid cell DNA; lane 4: PCR product of placental DNA supplemented with 6 copies of c-*myc* exon 3 DNA per diploid cell DNA; lane 5: PCR product of placental DNA supplemented with 10 copies of c-*myc* exon 3 DNA per diploid cell DNA; lane 6: PCR product of placental DNA supplemented with 18 copies of c-*myc* exon 3 DNA per diploid cell DNA; lanes 7, 10, 14: PCR product of small cell carcinoma; lanes 8,12,13: PCR product of adenocarcinoma ; lane 9: PCR product of interstitial pneumonia; lane 11: PCR product of squamous cell carcinoma

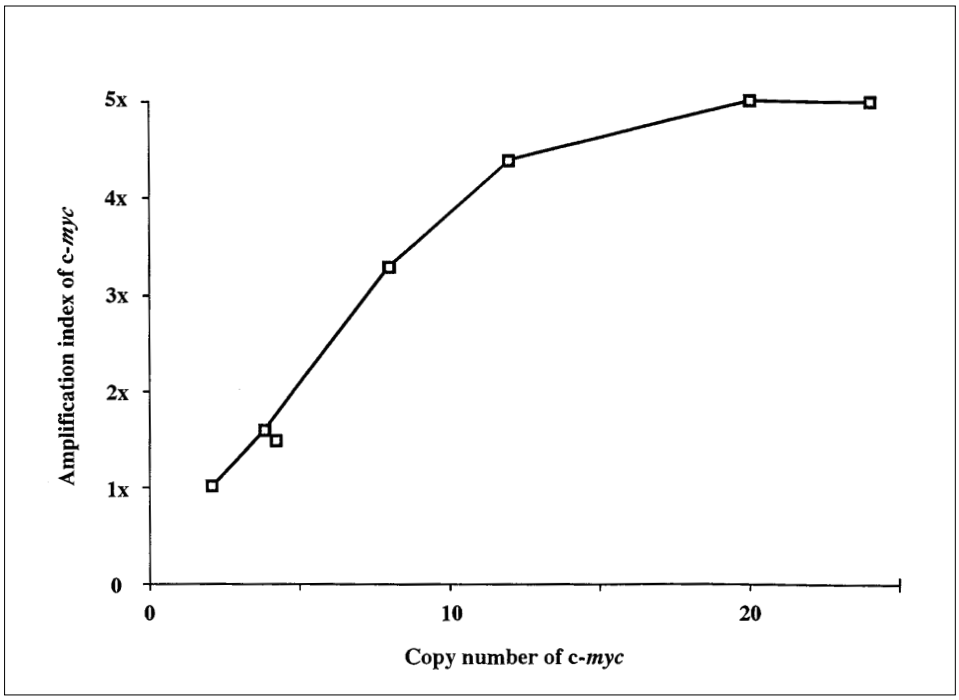

Fig. 2 Standard curve of c-*myc* copy number. c-*myc* amplification index = (c-*myc* band IOD/alpha-lactalbumin band IOD) of sample/(c-*myc* band IOD/alpha-lactalbumin band IOD) of placenta. *IOD* Integrated optical density

in the reference DNA series and the specimens DNAs. Then 10 µl of PCR product was electrophoresed in 3% NuSieve GTG agarose gel in duplicate and transferred to the nylon membranes. One of the two membranes was hybridized with the DIG-labeled c-*myc* probe and visualized by immuno enzymatic reaction according to the manufacturer's instructions (Fig. 1A). The other filter was hybridized with the DIG-labeled alpha-lactalbumin probe and processed in the same way (Fig. 1B). The total integrated optical density within the visualized c-*myc* or alpha-lactalbumin band boundary was quantified by the Bio Image Analyzer (mode 110). Then the c-*myc* amplification indices of the reference DNA series and all the specimens were calculated by dividing the ratio of the c-*myc* band integrated optical density (IOD) to the corresponding alpha-lactalbumin band IOD of the reference DNA series, or specimens by the ratio of the c-*myc* band IOD to the alpha-lactalbumin band IOD of the control placental DNA lacking the cloned c-*myc* exon 3 DNA.

c-*myc* amplification index = (c-*myc* band IOD/alpha-lactalbumin band IOD) of sample/(c-*myc* band IOD/alpha-lactalbumin band IOD) of placenta

A standard curve of the reference DNA containing the copy number of c-*myc* was constructed by plotting the c-*myc* amplification index on the *y* axis against the c-*myc* copy number on the *x* axis (Fig. 2). For each PCR the standard curve was constructed. The standard curve was linear at less than 20 copies, and reached a plateau at more than 20 copies. The c-*myc* copy number for each specimen was obtained by extrapolating the calculated c-*myc* amplification index of the specimen against the standard curve generated with the reference DNA series.

Results

The c-*myc* copy numbers of the 2 interstitial pneumonias and SK-OV-3 (controls in the present study) were 1.8, 1.9, and 1.7 respectively. The reproducibility of these values was confirmed by repeated examination (at least twice). The same method was applied to tumor specimens.

Although the DNA of interstitial pneumonia tissues was extracted from formalin-fixed paraffin-embedded specimens, we used the ratio of c-*myc* DNA to alpha-lactalbumin DNA to obtain c-*myc* copy number of specimens in our assay system. Therefore the influence of DNA damage in formalin-fixed paraffin-embedded specimens was taken into account. As interstitial pneumonia tissue is composed of diploid cells, its c-*myc* copy number is assumed to be about 2 copies. The DNA of SK-OV-3 was extracted from unfixed fresh cell pellet and its c-*myc* copy number was determined by the same method as formalin-fixed paraffin-embedded specimens. As SK-OV-3 is a hypodiploid cell line, its c-*myc* copy number is assumed to be in the range of 1–2 copies. The c-*myc* copy numbers of interstitial pneumonia and SK-OV-3 obtained in the present study are in the expected range. The expected c-*myc* copy numbers were obtained for both DNA extracted from formalin-fixed paraffin-embedded tissue and from unfixed fresh cell pellet. This demonstrates the validity of our approach for quantitative gene amplification studies.

In the same way, the c-*myc* copy numbers of 46 lung tumors were obtained. The c-*myc* copy number of NSCLC ranged from 1.5 to more than 20.0 and that of SCLC ranged from 1.8 to 12.0 (Fig. 3). The difference between NSCLC and SCLC for c-*myc* copy number was assessed with the Wilcoxon rank sum test. In consequence, the c-*myc* copy number of NSCLC was significantly higher than that of SCLC (Z=2.06, *P*=0.040).

The c-*myc* copy number of individual histological types of NSCLC and SCLC is shown in Fig. 4. The c-*myc* copy number ranged from 1.5 to more than 20.0 in adenocarcinoma and squamous cell carcinoma, from 6.0 to 12.0 in large cell carcinoma, and from 1.8 to 12.0 in SCLC. The median c-*myc* copy number was 8.5 in adenocarcinoma, 9.0 in squamous cell carcinoma, l0.2 in large cell carcinoma, and 6.0 in SCLC. Differences in the c-*myc* copy number among these four histological types were assessed with the Kruskal-Wallis rank sum test. This failed to reveal any significant difference in the c-*myc* copy number between these four histological types (Fig. 4).

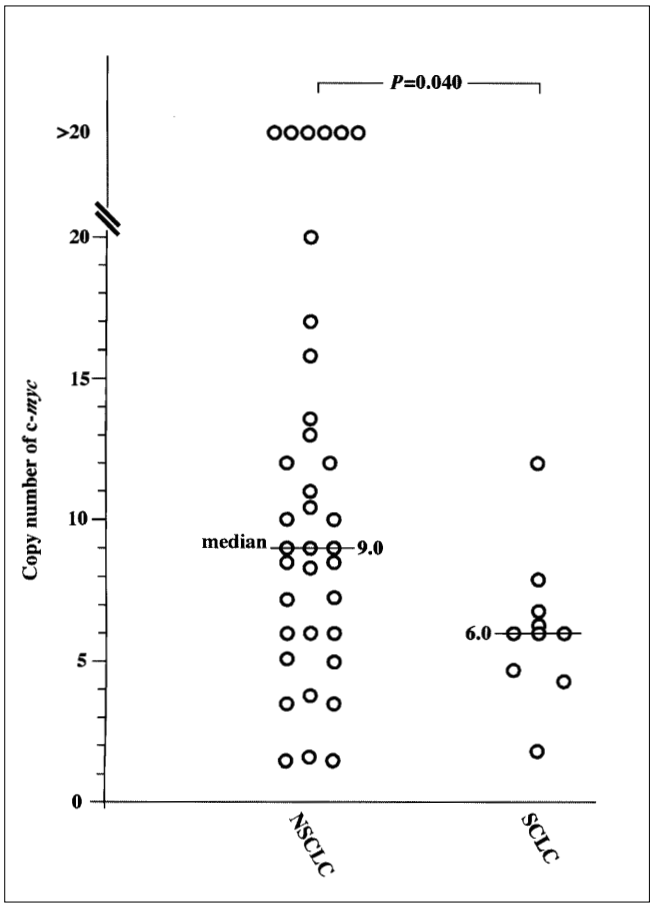

Fig. 3 Comparison of c-*myc* copy number between non-small cell lung carcinoma (*NSCLC*) and small cell lung carcinoma (*SCLC*). The c-*myc* copy number of NSCLC was significantly higher than that of SCLC

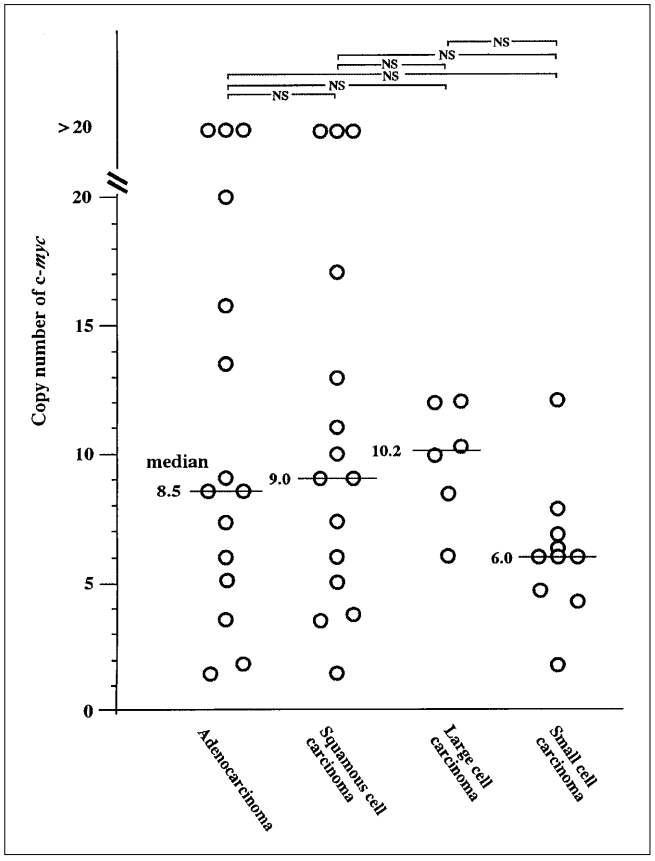

Fig. 4 c-*myc* copy number of individual histological types of NSCLC and SCLC. There was no significant difference in the c-*myc* copy number between individual histological types.
NS Not significant

c-*myc* amplification (more than 4.0 copies) was observed in 30 (83%) of the 36 NSCLCs, i.e., 12 (80%) of the 15 adenocarcinomas, 12 (80%) of the 15 squamous cell carcinomas, 6 (100%) of the 6 large cell carcinomas, and 9 (90%) of the 10 SCLCs. Of these c-*myc*-amplified cases, high gene amplification (more than 20.0 copies in the range over the linearity of the assay) was observed in 3 (20%) of the 15 adenocarcinomas, 3 (20%) of the 15 squamous cell carcinomas, 0 (0%) of the 6 large cell carcinomas, and 0 (0%) of the 10 SCLCs.

Adenocarcinoma with high gene amplification of c-*myc* included 2 (50%) of 4 poorly differentiated cases, 1 (16.7%) of 6 moderately differentiated cases, and 0 (0%) of 5 well-differentiated cases. Adenocarcinomas without amplification of c-*myc* included 0 (0%) of 4 poorly differentiated cases, 0 (0%) of 6 moderately differentiated cases, and 3 (60%) of 5 well-differentiated cases.

Squamous cell carcinomas with high gene amplification of c-*myc* included 1 (25%) of 4 poorly differentiated cases, 1 (12.5%) of 8 moderately differentiated cases, and 1 case (33%) showing marked nuclear atypism among 3 well-differentiated cases. Squamous cell carcinomas without amplification of c-*myc* included 0 (0%) of 4 poorly differentiated cases, 2 (25%) of 8 moderately differentiated cases, and 1 (33%) of 3 well-differentiated cases.

Discussion

We extracted DNA from paraffin-embedded specimens of lung cancer, including four histological types, and examined it for the c-*myc* oncogene. Kinoshita et al. [23] used DNA from normal human lymphocytes as a c-*myc* non-amplified control and found that the cut-off index for c-*myc* amplification (median of controls+3 SD) was 2.2-fold. They considered that signals 2.2-fold greater than that of the normal human lymphocyte represented abnormal amplification. Brennan et al. [6] considered that the copy number of the *myc* gene was amplified if the signals were fourfold greater than the single-copy gene controls. Although the values obtained by our method are semi-quantitative, the c-*myc* copy number was 1.8 and 1.9 in two diploid cell controls, and 1.7 in the hypodiploid cell control, and hence a c-*myc* copy number of more than 4 copies was considered to be amplified.

We observed c-*myc* amplification not only in SCLC but also in adenocarcinoma, squamous cell carcinoma, and large cell carcinoma at high frequencies. The frequency of c-*myc* amplification observed in our study is higher than that reported by other investigators [7, 15, 24]. This may be partly due to the use of PCR in our assay system to detect c-*myc* amplification. Recently, Barr et al. [25] reported that c-*myc* expression suppressed the formation of tumors by SCLC cells in athymic nude mice by downregulating vascular endothelial growth factor. They hypothesized that the amplification of c-*myc* in cells grown from lung tumors with a poor prognosis [10–12, 14] was an artifact of selection for growth in vitro. However, as Barr et al. [25] pointed out, c-*myc* might play a role in metastasis, invasion, and resistance to chemotherapy, rather than the development of tumors [25–28]. Our material may have contained many advanced lesions. It is also possible that some of the amplification detected in our material may not have been associated with actual overexpression of the c-*myc* gene.

High gene amplification (more than 20 copies), which is off the scale in our assay system, was observed in 3 (20%) of 15 adenocarcinomas and 3 (20%) of 15 squamous cell carcinomas. These cases with high gene amplification of c-*myc* were observed at a higher frequency in poorly differentiated types than moderately differentiated types.

In well-differentiated types, cases with marked nuclear atypia also showed 20 copies or more of c-*myc*. Non-amplified cases of c-*myc* were found most frequently in well-differentiated types and not in poorly differentiated types.

Although we did not find any instances of high gene amplification of c-*myc* in large cell carcinoma, this may be partly due to the low number of available samples.

c-*myc* copy number in NSCLC was higher than SCLC, with a statistically significant difference (Wilcoxon rank sum test. Z=2.06, *P*=0.040), although the *P* value was marginal and c-*myc* copy numbers between the two groups overlapped. Moreover, high gene amplification of more than 20.0 copies was observed in 6 (16.7%) of 36 NSCLCs, but none (0%) of 10 SCLCs. As all of the SCLCs in this study corresponded to the pure small cell carcinoma subtype, according to the histopathological classification of small cell lung cancer by The International Association for the Study of Lung Cancer [29], this relatively low c-*myc* amplification in SCLC may reflect differences in the cell nature between NSCLC and SCLC, such as bronchoepithelial carcinoma versus neuroendocrine cell carcinoma. However, to clarify this it would be necessary to study neuroendocrine cell markers, such as neuron-specific enolase, chromogranin A, serotonin, and synaptophysin.

In conclusion, our data indicate that amplification of the c-*myc* oncogene was present not only in SCLC but also in NSCLC, i.e., adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, at a high frequency, and c-*myc* copy number in NSCLC was significantly higher than in SCLC.

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