Journal of Cellular Biochemistry 31:297–304 (1986)
Cellular and Molecular Biology of Tumors and
Potential Clinical Applications 9–16

Amplification of the N-*myc* Oncogene in an Adenocarcinoma of the Lung

Kalle Saksela, Jonas Bergh, and Kenneth Nilsson

Department of Virology, University of Helsinki, 00290 Helsinki, Finland (K.S.) and Departments of Pathology (J.B., K.N.) and Oncology (J.B.), University of Uppsala, University Hospital, S-751 85 Uppsala, Sweden

c-*myc* oncogene is the most extensively studied member of the *myc* gene family, which now consists of three characterized members, namely the c-*myc*, N-*myc*, and L-*myc* genes. Deregulation owing to amplification and/or rearrangements of the c-*myc* gene have been described in a variety of human malignancies. Several neuroblastomas have amplifications of the N-*myc* genes. The c-*myc*, N-*myc*, or L-*myc* oncogenes are also found amplified in different cell lines from small cell carcinomas of the lung. In this study, we have examined the c-*myc*, N-*myc*, and c-*erb*B oncogenes in 34 clinical and autopsy tumor specimens representing various histopathological types of human lung cancer, including nine small cell lung cancers. A 30-fold amplification of the N-*myc* gene was found in a tumor histopathologically and histochemically verified as a typical adenocarcinoma. No amplifications of the c-*myc* or c-*erb*B oncogenes were seen in any of the tumors. In the DNA of one small cell carcinoma, an extra c-*myc* and N-*myc* cross-hybridizing restriction fragment was observed, possibly owing to an amplification of a yet uncharacterized *myc*-related gene.

Key words: small cell lung cancer, c-*erb*B oncogene, EGF receptor, c-*myc* oncogene, *myc* gene family, squamous cell carcinoma, gene amplification, neuroblastoma, glioblastoma, neuron-specific enolase, cytokeratin, neuroendochine markers, variant form of small cell lung cancer

Amplifications of the c-*myc* oncogene have been described in many types of tumor cell lines, but they appear to be especially common in the cell lines of the variant form of small cell lung cancer [1–3]. In comparison with the classic small cell lung cancer (SCLC) cell lines, the cell lines of the variant form of SCLC have a faster doubling time and a higher cloning efficiency in culture [2,4]. The SCLC tumors with variant features have also been reported to behave more aggressively in vivo [5]. Since oncogene amplifications are typically found in tumors from patients with an advanced disease, a role for chemotherapy in the generation of c-*myc* amplifications has been discussed.

Received March 5, 1986; accepted April 22, 1986.

There may exist a sizable family of c-*myc* related genes. N-*myc* [6] and L-*myc* [7] are two other members of this gene family. Both of these genes have also been found amplified in different SCLC cell lines [7]. In neuroblastomas, N-*myc* amplification seems to correlate with an advanced clinical stage and a poor prognosis of the disease [8].

In all cases studied, the amplification of the c-*myc* gene has been accompanied by elevation of the levels of c-*myc* RNA and protein. However, very little is known of the cellular functions of the *myc* gene products. Homologies of the deduced amino acid sequences and functional similarities in transformation assays suggest that the protein products of c-*myc* and N-*myc* genes might have similar functions [9–11]. No c-*myc* protein or RNA has been found in cells having an amplified N-*myc* gene, nor have amplifications of more than one *myc*-related gene been observed in the same cells.

Induction of the c-*myc* protein is associated with the G0/G1 transition occurring after growth factor stimulation, but subsequent expression appears to be stable throughout the cell cycle [12]. The level of c-*myc* expression of normal cells has been correlated with mitogenic activity of the corresponding tissues [13], and elevated levels of c-*myc* transcripts have been found also in many tumor cells lacking chromosomal abnormalities that typically deregulate c-*myc* expression [14,15]. In culture, primary embryonic cells transfected with a constitutively highly expressed c-*myc* gene become susceptible to transformation by a complementing oncogene such as c-*ras* [16].

Besides SCLC, no amplifications of c-*myc* or related genes have been described in other types of human lung cancer tumors. We analyzed a series of lung tumors representing various histopathological types. Cloned DNA fragments of c-*myc* and N-*myc* oncogenes were used as probes in low- and high-stringency hybridization conditions. Because elevated levels of the EGF receptor have been described in several human squamous cell carcinoma cell lines and tumors [19], we found it also of interest to look for c-*erb*B amplifications in these tumor DNAs using cloned c-*erb*B cDNA as a probe. The c-*erb*B oncogene has been shown to code for the EGF receptor [17] and has been found amplified in human glioblastomas [18].

MATERIALS AND METHODS

Tumor Material

Fresh material was received from the Departments of Thorax Surgery and Otolaryngology, University of Uppsala, consisting of lung lobes (23 cases) or biopsies (6 cases) from 29 randomly selected and untreated patients with lung tumors. Parts of the tumors were removed and immediately frozen at −70°C. Material was also taken for routine histopathological examination. All tumor material was examined by two pathologists. The lung tumor that contained an amplified N-*myc* gene was also investigated with monoclonal antibodies against cytokeratin [20] and a sheep antiserum against neuron-specific enolase (NSE) [21], and part of the tumor was homogenized for radioimmunological determination of the NSE content [22]. The protein content was determined according to Lowry et al [23].

In addition, we received autopsy tumor material from five histopathologically verified SCLC patients from the Department of Pathology, University of Helsinki. In three out of these five cases metastatic material was also included in the study.

Isolation of DNA

High molecular weight DNA was isolated from the tumors. The tumors were first powdered with a microdismembranator (B-Braun, Melsungen AB, West Germany) at −70°C and then dissolved in 0.5% sodium dodecyl sulfate (SDS), 0.1 M NaCl, 20 mM ethylamediamine tetracetic acid (EDTA), 50 mM Tris-HCl, pH 8.1. The cellular proteins were hydrolysed by incubating the lysates with 200 μg/ml proteinase K (Merck) for 1 hr at 37°C. The solution was extracted twice with phenol, and twice with an equal volume of butanol-propanol (7:3). Nucleic acids were precipitated with 3 volumes of ethanol, washed in absolute ethanol, and dried in a vacuum. The nucleic acids were then redissolved in 1 mM EDTA, 10 mM Tris-HCl, pH 8 (TE), and RNA was hydrolysed with 100 μg/ml pancreatic ribonuclease A at 37°C for 1 hr. Treatment with proteinase K, extraction with phenol and with butanol-propanol, and ethanol precipitation were performed as above. The DNA was dissolved and stored at 4°C in TE. The DNA concentrations of the preparations were estimated by their light absorbances at 260 nm.

Electrophoresis, Blotting, and Hybridizations

Aliquots of DNA were digested with restriction endonucleases, fractionated by electrophoresis through a 1% agarose gel, and transferred to nitrocellulose paper in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 sodium citrate). Fragment sizes were calculated using lambda phage DNA cleaved with restriction endonuclease Hind III as a standard. Hybridization analyses were performed as described earlier [3].

RESULTS

DNA was extracted from the tumors of 34 lung cancer patients. Twenty-nine of the samples were clinical material, five of the samples were autopsy specimens, and all represented SCLC. Table I shows the histologic types and the origin of the tumors analyzed with radiolabeled probes detailed in the figure legends. In all but one of the tumor DNAs, the analysis revealed only the normal restriction fragments of the germ line proto-oncogenes. In one tumor (patient 18), an increased signal intensity was obtained from the 2-kbp Eco RI fragment of the N-*myc* oncogene (Fig. 1A, lanes Ad). This suggested that N-*myc* was amplified in the tumor DNA. DNA from normal lymphocytes of the patient and other tumors had a single-copy N-*myc* hybridization signal (Fig. 1A, lane C). The degree of amplification of N-*myc* was estimated from diluted DNA and found to be about 30-fold. The copy number of the gene for ornithine decarboxylase (ODC) was chosen for comparison, because it maps to the same chromosomal region (2p23-25) as N-*myc* [24]. The structure of amplified N-*myc* appeared normal in restriction endonuclease analysis of 13 kbp of DNA from the gene and its flanking sequences (Fig. 1B).

The morphology of tumor with the amplified N-*myc* gene was typical for an adenocarcinoma (Fig. 2) with prominent tubular structures, some of them containing mucin. Furthermore, the tumor cells contained cytokeratin in immunohistochemical staining. Scattered tumor cells were also strongly stained with the NSE antiserum (data not shown). The NSE value determined by radioimmunoassay (RIA) was found to be 0.31 μg/mg protein, a value in agreement with the histopathological classification of the tumor as an adenocarcinoma [22].

TABLE I. Lung Cancer Tumors Analyzed for *myc* Oncogenes*

Patient no.	Origin	Histopathologic diagnosis
1	B	SCLC
2	B	SCLC
3	L	SCLC
4	L	SCLC
5	L	SQC (poorly differentiated), SCLC?
6	L	SQC (minor SCLC component?)
7	B	SQC
8	L	SQC
9	L	SQC
10	L	SQC
11	L	SQC
12	L	SQC
13	L	SQC
14	L	LCC (poorly differentiated), SQC?
15	B	LCC
16	L	LCC (clear cell type)
17	L	ADC
18	L	ADC
19	B	ADC
20	L	ADC
21	L	ADC
22	L	ADC
23	L	ADC
24	L	BC (atypical)
25	L	BC
26	L	BC
27	L	BC
28	L	Neurilemmoma
29	B	Poorly differentiated lung cancer
30	A	SCLC
31	A	SCLC
32	A†	SCLC
33	A†	SCLC
34	A†	SCLC

*B, biopsy; L, lobectomy sample, A, autopsy sample; A†, metastatic material also analysed; SCLC, small cell lung cancer; SQC, squamous cell carcinoma; LCC, large cell carcinoma; ADC, adenocarcinoma; BC, bronchial carcinoid.

In one tumor DNA from an autopsy SCLC specimen (patient 33) we found extra restriction fragments cross-hybridizing with both the c-*myc* and N-*myc* probes (Fig. 3, and data not shown). The sizes of these restriction fragments were different from those described for the L-*myc* gene [7]. The same bands could be seen also in the DNA from the liver metastasis of the same patient, but more faintly. An extensive restriction enzyme study of the c-*myc* or N-*myc* genes of this DNA did not reveal any rearrangements. Different cross-hybridizing fragments were seen with both c-*myc* I exon and III exon-specific probes. No cross-hybridization was observed using plasmid DNA as a probe (data not shown). We are at the present studying the possibility that these abnormal bands emerged owing to the amplification of a still unknown *myc*-related gene.

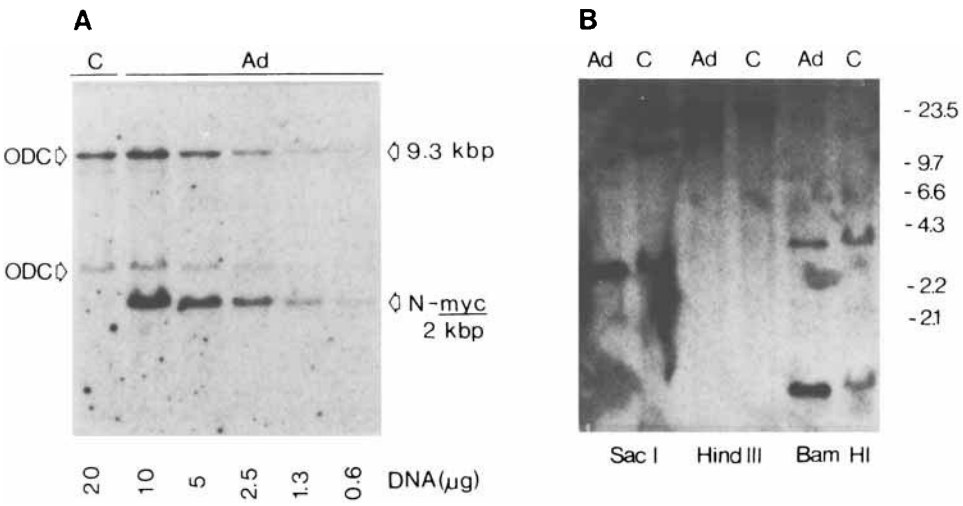

Fig. 1. A) Analysis of the copy number of N-*myc* and ODC genes in tumor DNA. DNA was isolated from the adenocarcinoma tumor of patient 18 (lanes Ad) and from the SCLC tumor of paitent 5 (lane C), digested with Eco RI, electrophoresed, blotted, and hybridized with radioactively labeled DNA of the pNb-1 plasmid containing a 1-kbp Eco RI-Bam HI fragment from the second exon of N-*myc* [6] and with a radioactive insert of the human ornithine decarboxylase cDNA (plasmic pODC 10/2H [24], a kind gift from Dr. Olli Jänne). The blot was washed in high-stringency conditions and autoradiographed. It can be seen from the radioactive signal obtained from different amounts of adenocarcinoma DNA that the N-*myc* oncogene is amplified about 30-fold, whereas the signal from the ODC gene is of similar intensity in 10–20 μg of DNA from both adenocarcinoma and control DNA. B) Mapping of N-*myc* loci by restriction endonuclease analysis. DNA from the adenocarcinoma (Ad) was diluted 20-fold, and analyzed together with undiluted control DNA from the tumor of patient 5 (C). Three different endonuclease digestions are shown for comparison. The fragment lengths obtained with endonucleases Sac I, Hind III, and Bam HI were similar, except for the differences in mobility owing to loading of different amounts of DNA in the sample wells.

DISCUSSION

The c-*myc* oncogene has been found amplified in a subpopulation of in vitro grown cell lines of small cell carcinoma of the lung [1,3]. The cell lines with amplified c-*myc* in general have faster doubling times and fewer neuroendocrine markers [4]. A morphology typical for the SCLC cell lines with a c-*myc* amplification has been reported. These cell lines also seem to originate from SCLC tumors with variant morphological features [2]. Here we report oncogene DNA analysis in a series of lung cancer tumors, including nine SCLSs. None of the investigated tumors, except for one adenocarcinoma sample, disclosed amplifications of the c-*myc* or N-*myc* oncogenes.

The fact that no amplifications of the c-*myc* genes were seen in any of the SCLC tumors suggests that these amplifications are more common in established SCLC cell lines than in the clinical tumor material. The reason for the discrepancy between the in vitro situation and the in vivo situation is as yet unexplained. One alternative could be in vitro growth selection of clones with the amplified c-*myc* gene, already present but not detected in the tumor biopsy. Altered growth properties of the cells with c-*myc* amplifications may adapt them particularly well to culture conditions. On the other hand, no *myc* amplifications have been described to occur during the in vitro

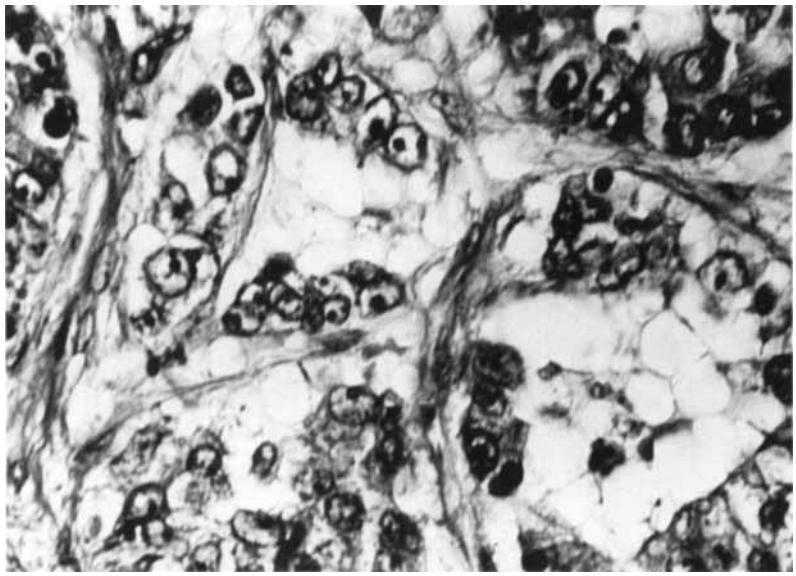

Fig. 2. Histology of the adenocarcinoma tumor with the N-*myc* amplification. The picture shows cells with a large cytoplasm and prominent nucleolae, arranged in tubular structures typical for an adenocarcinoma (van Gieson stain, magnification about ×250).

cultivation of cells. Another explanation could be that six out of the nine examined SCLC tumor samples were from untreated patients, while the majority of established cell lines used in previous studies were obtained from drug-treated patients. This may have resulted in clonal selection of cells with altered geno- and phenotypes. A third alternative is that the number of examined SCLC tumors (nine) was too small to include variant subtypes. The present tumors could all represent "classic" SCLC, which should be expected to lack amplified c-*myc* oncogenes.

In this paper, we, for the first time, describe the presence of N-*myc* amplification in a primary lung adenocarcinoma. The adenocarcinoma diagnosis was based on a typical morphology with production of mucin, an epithelial phenotype reflected by the positive cytokeratin staining, and relatively low levels of NSE. However a value of 0.31 μg NSE/mg protein may even occur in some SCLC cell lines [25]. It should also be mentioned that NSE cannot be regarded as an ultimate marker for neuroendocrine differentiation, because unrelated human tumors such as lymphoblastoid cell lines, myeloma cell lines, and an Epstein-Barr virus-transformed chronic lymphocytic leukemia cell line have disclosed even higher NSE values [25].

The elevated level of *myc* expression owing to the amplifications of the *myc* genes give cells a growth advantage of as yet uncharacterized nature. This is often acquired by the SCLC cells, but amplification can occur in other tumors as well, as shown by the present results. Also, it may be that adenocarcinomas of the lung are a more heterogenous group of tumors than has been recognized. In part, then, our findings may merely reflect the difficulty in predicting the molecular and cellular biology of tumors on the basis of histopathologic diagnosis. After all, the major types of lung cancer are all histogenetically related [26].

The nature of the novel amplified 5.5 kbp *myc* cross-reacting fragment will be examined by molecular cloning. It may represent an additional gene of the *myc* family

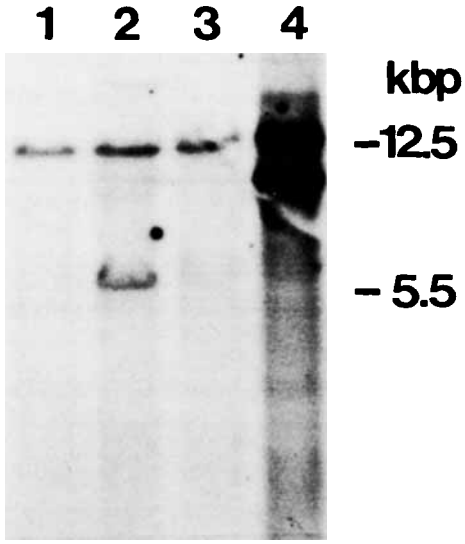

Fig. 3. Analysis of c-*myc*-related DNA sequences in lung cancer and colon carcinoma DNA. DNA from the SCLC tumor of patient 32 (lane 1), from the SCLC tumor of the patient 33 (lane 2), and from the liver metastasis of patient 33 (lane 3) were extracted and subjected to Southern blotting and hybridization analysis with a radioactive Cla I-Alu I-fragment from the III exon of c-*myc*. DNA from the colon carcinoma COLO 320 cells (lane 4) is analyzed as a positive control for c-*myc* amplification and rearrangement. As can be seen from the figure, the normal genomic 12.5 kbp Eco RI fragment of the c-*myc* oncogene is of similar size and intensity in all lung carcinoma DNAs, but there is an extra band hybridizing weakly in the DNA from liver metastasis and more strongly in the lung tumor. The size of the fragment is about 5.5 kbp, as can be interpolated from the mobilities of the Hind III fragments of bacteriophage lambda, indicated on the right.

similar to N-*myc* and L-*myc*, both of which were discovered by cross-hybridization of amplified, homologous DNA [6,7].

ACKNOWLEDGMENTS

We thank Dr. Lena Steinholtz from the Central Hospital in Västerås for providing us with clinical material, the staff of the Departments of Thorax Surgery and Otolaryngology for providing us with the fresh operation specimens, and Sven Påhlman and Ingegärd Hjertson for the RIA analyses. We also wish to thank Kirsi Pylkkänen for excellent technical assistance, Mervi Laukkanen for typing the manuscript, and Dr. Kari Alitalo for critical comments on the manuscript.

This study was supported by the Finnish Medical Foundation, the Finnish Cancer Research Organization, Svenska Nationalföreningen mot hjärt- och lungsjukdomar and The Swedish Cancer Society. Part of the work was carried out under contract with the Finnish Life and Pension Insurance Companies.

REFERENCES

1. Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD: Nature 306:194, 1983.
2. Gazdar AF, Carney DN, Nau MM, Minna JD: Cancer Res 45:2924, 1985.
3. Saksela K, Bergh J, Lehto V-P, Nilsson K, Alitalo K: Cancer Res 45:1823, 1985.

4. Carney DN, Gazdar AF, Bepler G, Guccion JG, Marangos PJ, Moody TW, Zweig MH, Minna JD: Cancer Res 45:2913, 1985.
5. Radice PA, Matthews MJ, Ihde DC, Gazdar AF, Carney DN, Bunn PA, Cohen MH, Fossieck BE, Makuch RW, Minna JD: Cancer 50:2894, 1982.
6. Schwab M, Alitalo K, Klempnauer K-H, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M, Trent J: Nature 305:245, 1983.
7. Nau MM, Brooks BJ, Battey J, Sausville E, Gazdar AF, Kirsch IR, McBride OW, Bertness V, Hollis GF, Minna JD: Nature 318:69, 1985.
8. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D: N. Engl J Med 313:1111, 1985.
9. Schwab M, Varmus HE, Bishop JM: Nature 316:160, 1985.
10. Kohl NE, Legouy E, DePinho RA, Nisen PD, Smith RK, Gee CE, Alt FW: Nature 319:73, 1986.
11. Stanton LW, Schwab M, Bishop JM: Proc Natl Acad Sci USA 83:1772, 1986.
12. Hann SR, Thompson CB, Eisenman RN: Nature 314:366, 1985.
13. Stewart TA, Bellve AR, Leder P: Science 226:707, 1984.
14. Rothberg PG, Erisman MD, Diehl RE, Rovigatti UG, Astrin SM: Mol Cell Biol 4:1096, 1984.
15. Slamon D, de Kernion JB, Verma IM, Cline MJ: Science 224:256, 1984.
16. Land H, Parada LF, Weinberg RA: Nature 304:596, 1983.
17. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD: Nature 307:521, 1984.
18. Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J: Nature 313:114, 1985.
19. Ozanne B, Shum A, Richards CS, Cassells D, Grossman D, Trent J, Gusterson B, Hendler F: In: Feramisco J, Ozanne B, Stiles C, (eds): "Growth Factors and Transformation." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1985, pp 41–49.
20. Holthöfer H, Miettinen A, Lehto V-P, Lehtonen E, Virtanen I: Lab Invest 50:552, 1984.
21. Bergh, J, Esscher T, Steinholtz L, Nilsson K, Påhlman S: Am J Clin Pathol 84:1, 1985.
22. Påhlman S, Esscher T, Bergvall P, Odelstad L: Tumor Biol 5:127, 1984.
23. Lowry OH, Rosebrough NJ, Farr AL, Randell RJ: J Biol Chem 193:265, 1951.
24. Winqvist R, Mäkelä TP, Seppänen P, Jänne OA, Alhonen-Hongisto L, Jänne J, Grzeschik K-H, Alitalo K: Cytogenet Cell Genet (in press), 1986.
25. Påhlman S, Esscher T, Nilsson K: Lab Invest (in press), 1986.
26. Bergh J, Nilsson K, Dahl D, Andersson L, Virtanen I, Lehto V-P: Lab Invest 51:307, 1984.