Amplification and Expression of Protooncogenes in Human Small Cell Lung Cancer Cell Lines

P. E. Kiefer, G. Bepler, M. Kubasch, and K. Havemann

Institute of Cancer Research and Molecular Biology [P. E. K., M. K.], and Department of Internal Medicine, Division of Hematology/Oncology, [G. P., K. H.], Philipps-University Marburg, 3550 Marburg, West Germany

ABSTRACT

Amplification and expression of 16 protooncogenes were examined in 12 established small cell lung cancer (SCLC) cell lines. Seven of 12 cell lines showed a 20- to 35-fold amplification of the c-myc oncogene, 3 cell lines showed an 80- to 130-fold amplification of N-myc oncogene, and one cell line had a simultaneous amplification of the c-myc and N-myc oncogene. In this cell line both oncogenes were transcriptionally highly active at the same time. A variant subpopulation of SCLC expressed an 8.5-kilobase v-fms homologous transcript at high levels but without amplification of the c-fms gene. All cell lines examined had similar RNA levels of the N-ras, Ki-ras, Ha-ras, and c-rasH oncogenes. DNA amplification, however, was undetectable. The protooncogenes c-fes, c-fos, and c-erbB were expressed very weakly and the transcripts of the oncogenes c-myc, c-sis, c-erbA, c-arc, and c-adr were not observed in any of the 12 SCLC-cell lines. From these data we conclude that beyond the oncogenes myc and myb, oncogenes whose gene products are GTP binding proteins and phosphodiesterases may also be necessary to develop and keep the malignant state of SCLC. The v-fes homologous transcript found may be involved in the transition of the classic cell type to the variant cell type of SCLC.

INTRODUCTION

Lung cancer has been classified clinically into SCLC1 and non-SCLC, based on differences among tumor spread at the time of diagnosis, response to applied treatment modalities, and overall survival. SCLC accounts for about 20-25% of all new cases of human lung cancer, and 2-year survival rates are still within a 10-20% range, despite intensive clinical research. Based on the expression of DDC, a key enzyme of the diffuse neuroendocrine system (1), established SCLC cell lines can be subclassified in vitro into a classic cell type with measurable activity of DDC and a variant cell type without enzyme activity. Beyond this feature other parameters differ between both subclasses of SCLC cell lines: the classic cell type expresses bombesin-like immunoreactivity, has higher levels of neuron-specific enolase, a longer population doubling time in suspension culture, a lower cloning efficiency in soft agarose, and a longer specific enolase, a longer population doubling time in suspension culture, a lower cloning efficiency in soft agarose, and a longer latency phase of nude mouse heterotransplants than the variant cell type (2, 3).

As a rule, variant cell lines have a higher degree of DNA amplification and a higher level of RNA expression of the c-myc oncogene than do classic cell lines (4). This suggests a possible function of the c-myc oncogene and probably other cellular oncogenes in the transition of the classic to the variant cell type and the malignant behavior of variant cell lines (4-6).

Cellular oncogenes, originally identified by their structural similarity to oncogene sequences in the genome of acutely transforming retroviruses and/or by their capability to transform NIH-3T3 cells in transfection assays, may play an essential role in controlling normal cell growth and development (7-12). In activated forms, which one can find in many human and animal tumor cells, cellular oncogenes may be involved in neoplastic transformation of cells and tumor establishment. Point mutations, deletions, translocations, and gene amplification could be mechanisms that give quiescent protooncogenes a transforming potency (13-22).

Beyond c-myc amplification, L-myc and N-myc amplifications were found in SCLC cell lines with increased RNA expression almost proportional to the corresponding gene. Amplifications of L-myc and N-myc oncogenes have no constant association with both cell types of SCLC. In all SCLC cell lines examined, if at all, only one gene out of three myc-related oncogenes was amplified (23, 24).

With regard to these findings and the results of Slamon et al. (25), who found a number of transcriptionally active protooncogenes in 20 fresh human cancer specimens of different histological types including lung cancer, we performed similar analyses of 16 oncogenes in 12 permanent SCLC cell lines.

MATERIALS AND METHODS

Cell Lines. The SCLC cell lines used were established in our laboratory (SCLC-16H, SCLC-21H, SCLC-22H, SCLC-24H, SCLC-86M) or were donated by Drs. Carney and Gazdar, NCI, Bethesda, MD (NICI-H69, NCI-H69, NICI-H146, NICI-N417, NICI-H526, NICI-N592) and Drs. Pettengil and Sorenson, Dartmouth Medical School, Hanover, NH (DMS-79). All 12 cell lines grew as floating cell aggregates in liquid culture, formed colonies in soft agarose, were tumorigenic in athymic nude mice, were free of Mycoplasma contamination, and expressed human isoenzymes distinct from HeLa cells. Their morphological, biochemical, and chromosomal features have been described in detail (2, 3, 26-28). Cell lines SCLC-22H, SCLC-24H, SCLC-86M, NCI-H69, NCI-H146, and NCI-N592 expressed DDC activity and thus belonged to the classic cell type of SCLC; all other cell lines (SCLC-16H, SCLC-21H, NICI-H82, NICI-N417, NICI-H526, DMS-79) had undetectable DDC activity and were subclassified as variant SCLC cell lines (2, 3). The RNA expression and DNA amplification of c-myc and N-myc were previously analyzed by Little et al. (4) and Nau et al. (23) for the NCI cell lines H69, H82, H146, N417, H526, and N592. Griffin and Baylin (5) could demonstrate c-myc transcripts in the cell lines H69 and H82 but not in N417. These cell lines were included as positive controls for these protooncogenes. All cell lines were kept continuously growing in RPMI 1640 medium supplemented with 10% v/v fetal bovine serum, designated R10 medium (both purchased from GIBCO, Paisley, United Kingdom) in a well humidified atmosphere of 5% CO2/95% air at 37°C.

Purification of DNA. High-molecular weight cellular DNA was prepared from 1-2×106 cells obtained from log-phase growing cell cultures. The cells were washed with phosphate-buffered saline and resuspended in 10 ml lysis buffer (0.5% SDS, 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4), 0.001 M EDTA, and proteinase K, 100 μg/ml. This solution was incubated overnight at 37°C and extracted once with phenol and twice with chloroform:isoamylalcohol (24:1). DNA was precipitated with 0.15 M sodium acetate (pH 5.2) and 2 volumes of cold 100% ethanol pooled off and dried under vacuum. DNA was dissolved and stored in water at 4°C.

Preparation of RNA. Logarithmically growing cells (106) were lysed in 5 ml cold lysis buffer, briefly vortexed, and cytoplasmic RNA was dissolved and stored in water at 4°C.
separated from nuclei by a sucrose cushion. Cellular proteins were hydrolyzed by proteinase K digestion (200 μg/ml) for 30 min at 37°C. The solution was extracted once with phenol:chloroform:isooamylicol (25:24:1), and the RNA was precipitated with 2.5 volumes ethanol and tested for integrity in a 1% glyoxal-agarose gel. For preparation of polyadenylated RNA, cytoplasmic RNA was purified by chromatography on oligo(dT)cellulose as described by Aviv and Leder (29).

Southern Hybridization. Restriction endonuclease-digested DNA was electrophoresed in 1% agarose gel, transferred to nitrocellulose by the method of Smith and Summers (30), prehybridized (30-50% formamide (dependent on homology between probe and DNA))-5x SSC-5x Denhardt's solution-50 mM sodium phosphate buffer, pH 6.5-250 μg/ml denatured salmon DNA) and hybridized (30-50% formamide-5x SSC-1× Denhardt's solution-20 mM sodium phosphate buffer, pH 6.5-10% dextran sulfate-100 μg/ml denatured salmon DNA) to nick-translated probes (Table 1). After hybridization the filters were washed initially 4 times for 5 min in 2× SSC-0.1% SDS, at room temperature, then 3 times for 20 min in 0.1× SSC-0.1% SDS at 50°C and exposed to Kodak XAR-5 film for various periods.

Northern Hybridization. For Northern blots, cytoplasmic or polyadenylated RNA was electrophoresed in glyoxal gel, transferred with 20× SSC to nitrocellulose, and hybridized in the presence of 10% dextran sulfate and 50% formamide. Filters were washed essentially as described for Southern hybridization. Twenty μg of cytoplasmic or 5 μg polyadenylated RNA were loaded in each lane (31).

RNA Dot Blot Analysis. Cytoplasmic RNA was denatured in 6x SSC-0.22 m formaldehyde for 15 min at 50°C, diluted serially, and spotted on nitrocellulose equilibrated with 20× SSC. The blots were prehybridized and hybridized as described for Southern hybridization (32).

**RESULTS**

Protooncogene Expression in SCLC Cell Lines. Cytoplasmic RNA was isolated from 12 established SCLC cell lines, the same RNA batches were analyzed by the dot and Northern blot techniques with 32P-labeled oncogene probes listed in Table 1 using a β-actin probe as internal control probe, and the dot blot autoradiographs were quantified by densitometer tracing (DU 6 Beckmann photometer). To simplify the evaluation of data we estimated the intensity of hybridization corresponding to a scale of hybridization signal intensities (Fig. 1A). The scale ranges from 0, i.e., no expression detected, to ++++ for high level expression. A summary on the expression of 9 oncogenes is given in Table 2. The weak hybridization signals of v-fes, v-fos, and v-erbB were negative corresponding to the scale of intensity given in Fig. 1A. We could not detect transcripts homologous to abl, mos, erbA, sis, and src probes. For the protooncogenes N-ras, Ki-ras, Ha-ras, and c-rafl we found similar levels of this transcripts in all SCLC cell lines examined. RNA expression related to the cellular oncogenes c-fms, c-myc, c-myb, and N-myc, however, differed considerably among the SCLC cell lines.

In order to exclude false-positive hybridization signals due to soiling of RNA preparations with genomic DNA, we blotted RNase T1 and RNase A digested probes from all cell lines tested as negative controls. A representative example is shown in Fig. 1C. We also digested RNA probes with DNase I to demonstrate that the intensity of the signal does not change thereby.

The sizes of transcripts homologous to protooncogenes c-fms, c-myc, N-myc, and c-myb were determined by the Northern blot technique (Fig. 2). We found one c-myc homologous transcript of 2.3 kb with the human c-myc C1al/EcoRI probe and one c-myb related transcript of 3.5 kb (Fig. 2, A and B). The transcript sizes correspond to previously reported data from SCLC cell lines (4, 5). The N-myc probe hybridized to a 3.5-kb...
Table 2 Summary of results on the expression of protooncogenes in SCLC cell lines

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x—xxxx, level of expression; 0, no expression detected.

Fig. 2. A, B, C, Northern blot analysis showing the sizes of mRNA transcripts detected in SCLC related to c-myc, N-myc, and c-myb. The c-myb filter was boiled for 10 min in twice-distilled water to remove the c-myb probe and subsequently rehybridized to a β-actin probe. The analysis was performed on 20 μg cytoplasmic RNA. D, Northern blot analysis of an 8.5-kb v-fms-related transcript in cell lines with high level of fms expression. Five μg poly(A)+RNA were loaded in each lane for SCLC cell lines and 30 μg total cellular RNA were used for TPA-induced differentiation HL60 (3.3 × 10^6 M for 30 h).
RNA from cell lines which highly expressed this oncogene (SCLC-22H, NCI-H526, NCI-H69, NCI-N592) and to an additional transcript of 1.9 kb in the variant cell line NCI-H526 as recently described by Nau et al. (23) (Fig. 2C).

A \textit{v-fms} related transcript of 8.5 kb was found in polyadenyl acid RNA of the variant cell lines NCI-N417, SCLC-16H, and SCLC-21H. The TPA-induced 5.0-kb \textit{v-fms} homologous transcript of the promyelocytic cell line HL 60 was detected by the same \textit{v-fms} probe (33) (Fig. 2D).

We also hybridized the RNA from TPA-induced HL60 cells and the RNA from the SCLC cell line NCI-N417 to an EcoRI/ HindIII fragment of p-cfms 104 (5' end probe) obtained from A. Ulrich which represents the extracellular domain of the \textit{c-fms} product (34). With this probe we could demonstrate the 5.0-kb \textit{c-fms} RNA from HL60 cells but not the 8.5-kb transcript from SCLC cells (data not shown).

**Amplification of Protooncogenes in SCLC Cell Lines.** We analyzed the copy number of cellular oncogenes which were transcribed at elevated levels in SCLC cell lines by Southern blot technique. Genomic DNA was extracted, digested with restriction endonucleases, electrophoresed in 1% agarose gel, blotted on nitrocellulose filters, and hybridized with the radio-labeled oncogene probes listed in Table 1. DNA from cell lines SCLC-16H, SCLC-21H, SCLC-22H, SCLC-24H, SCLC-86M, NCI-H82, and NCI-N417 showed a more intense \textit{c-myc} signal in the autoradiographs than the remaining cell lines (Fig. 3). With the \textit{c-myc} probe, the variant cell line NCI-H526 had a multiple stronger hybridization signal than in the other cell lines. As described above, this cell line expressed the \textit{c-myc} transcript at high levels. Cell lines NCI-H69, NCI-H526, and NCI-N592 showed an intense characteristic 16.0-kb HindIII digested N-myc fragment. Other oncogene probes revealed a uniform labeling of all cell lines. These results suggest amplification of the c-myc protooncogene in the cell lines SCLC-16H, SCLC-21H, SCLC-22H, SCLC-24H, SCLC-86M, NCI-H82, and NCI-N417, an amplification of the N-myc gene in cell lines NCI-H69, NCI-H526, NCI-N592, and a c-myc amplification in cell line NCI-H526. After EcoRI and HindIII digestion, we found an accessory amplified N-myc hybridization band in NCI-H526, as described by Nau et al. (23). NCI-H69 also had an unamplified 4.0-kb EcoRI fragment (Fig. 4). With other oncogene probes we detected only the characteristic restriction endonuclease fragments.

In order to estimate the degree of amplification, DNA was cleaved by restriction endonucleases, diluted serially, electrophoresed, blotted, and hybridized as described. Hybridization signals were compared with those from cell lines with a known degree of amplification (Fig. 3B). A 25- and 47-fold DNA amplification for \textit{c-myc} of the cell lines NCI-H82 and NCI-N417 and a \textit{N-myc} amplification of the cell lines NCI-H69, NCI-H526, and NCI-N592 has been recently reported. The degrees of \textit{N-myc} amplification were specified at 85-fold in NCI-H69 and at 115- (5.0-kb band) and 135- (2.0 kb) fold, respectively, in NCI-H526 (4, 23).

The 32P-labeled hybridization bands were cut out and measured in a scintillation counter. The cell lines SCLC-16H and SCLC-86M had a 30- to 35-fold and the cell lines SCLC-21H, SCLC-22H, and SCLC-24H a 20- to 25-fold amplification of the \textit{c-myc} oncogene. Intensity of signals was compared with NCI-H82 and NCI-H146 which is not amplified for \textit{c-myc} (4). The \textit{c-myc} oncogene of NCI-H526 was amplified 20- to 25-fold as compared to the unamplified cell line NCI-H82. In order to estimate the degree of \textit{N-myc} amplification in NCI-N592, we used NCI-H69, which is supposed to have 85-fold amplification (23). Our analyses showed 130- to 140-fold amplification of \textit{N-myc} oncogene in NCI-N592.

**DISCUSSION**

We could demonstrate a number of transcriptionally active oncogenes in SCLC cell lines for which 4 patterns of expression were observed: (a) five protooncogenes, c-myc, c-rap, N-ras, Ha-ras, and Ki-ras were expressed in all cell lines examined. c-myc expression differed considerably in level from cell line to cell line. The variant cell lines, excluding NCI-H526 and DMS-79, expressed the \textit{c-myc} oncogene at higher levels than did the classic cell lines. Some classic SCLC cell lines (all established in Marburg; SCLC-22H, SCLC-24H, and SCLC-86M) also had a high degree of \textit{c-myc} amplification. A uniform expression pattern of Ki-ras, Ha-ras, N-ras, and c-rap1 was found in all cell lines; (b) the oncogenes \textit{c-myc} and \textit{N-myc} were expressed at different levels in some cell lines without correlation between the pattern of expression and the phenotype of SCLC; (c) 3 of
6 variant SCLC cell lines expressed a strong 8.5-kb \(v\)-fms homologous transcript; (d) for the oncogenes \(c\)-erb\(A\), \(c\)-mos, \(c\)-sis, \(c\)-ab1, and \(c\)-src, we did not observe transcripts in SCLC. The expressions of \(c\)-fos, \(c\)-fes, and \(c\)-erb\(B\) were weak because of insufficient contrast with the background and thus were valued as negative.

We also found a simultaneous amplification of 2 oncogenes, \(c\)-myb and \(N\)-myc, in a single SCLC cell line (NCI-H526), both of which were transcriptionally highly active. Nine of 12 cell lines revealed amplification of one of the 2 myc-related oncogenes, \(N\)-myc and \(c\)-myc. \(L\)-myc was not amplified in these cell lines.²

Our data on \(N\)-myc and \(c\)-myc agree with those of Nau et al.

² B. Johnson, personal communication.
gene family but is not identical to the CSF-1 receptor encoding gene.

The mechanism of the v-fms-related gene activation in a subpopulation of variant cell lines remains obscure. Neither the Southern blot analyses of the 3 cell lines showed an amplification nor could we detect a rearrangement with the restriction endonucleases used. Rearrangements that could possibly be picked up by other restriction endonucleases cannot be excluded. Of interest is that only those variant cell lines expressed this v-fms homologous transcript which at an earlier point had been classic cell lines. This holds true for SCLC-16H which altered its phenotype after prolonged in vitro cultivation (28), for SCLC-21H which supposedly altered its phenotype in vivo (40), and for NCI-N417 which altered its phenotype after nude mouse xenotransplantation (3, 40).

As the first step, the coherence between v-fms-related expression and the phenotypic transition of SCLC in vivo and in vitro needs to be proved through other studies. In the next step the gene product of the v-fms homologous transcript of SCLC cell lines should be characterized, and experiments with in vitro inductions of such a transition should be performed.

ACKNOWLEDGMENTS

We thank Rolf Müller for giving us the oncogene probes (v-myc, v-myb, v-fos, v-erb/b, N-ras, Ki-ras, Ha-ras, v-fes, v-fms, v-mos, v-src), Axel Ulrich for the c-fms probe, Ulf Rapp for the ras1 probe, Hermann Herr for the abl and v-abl probes, Dieter Gallwitz for the β-actin probe, Juergen Niessing for communicating results before publication, Herbst for the abl and v-sis probes, Dieter Gallwitz for the 0-actin (myb, v-fos, -erb/t, N-ras, Ki-ras, Ha-ros, v-fes, v-fms, v-mos, -src), and Sigrid Kaschuba for technical and Silke Harnisch for secretarial assistance.

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As the first step, the coherence between v-fms-related expression and the phenotypic transition of SCLC in vivo and in vitro needs to be proved through other studies. In the next step the gene product of the v-fms homologous transcript of SCLC cell lines should be characterized, and experiments with in vitro inductions of such a transition should be performed.


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