Amplification of the c-myc Oncogene in a Subpopulation of Human Small Cell Lung Cancer

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ABSTRACT

We have examined a panel of human lung cancer cell lines for amplification and expression of the c-myc, N-myc, and c-myb oncogenes. The cell lines analyzed represent various histopathological types of lung cancer: small cell carcinoma with neuroendocrine properties; squamous cell carcinoma with epithelial markers; and large cell carcinoma with a mixed neuroendocrine-epithelial phenotype. Two of six cell lines, both of which were small cell carcinomas, showed about a 20-fold amplification of the c-myc oncogene. In both cell lines, the amplification is accompanied by an enhanced expression of c-myc. The N-myc or c-myb genes were not amplified in any of the cell lines, nor were they expressed in detectable amounts. The results confirm and extend earlier findings on c-myc amplification in small cell lung cancer.

INTRODUCTION

SCLC accounts for about 25% of all lung cancers. The clinical behavior of SCLC tumors is quite variable. This heterogeneity is also reflected by its histopathology, which recognizes 3 subtypes of SCLC: oat cell; intermediate cell; and mixed cell (16). There is also a wide phenotypic variability of cell lines established in vitro from the tumors. The oat cell type is obtained from about two-thirds of the tumors and appears to represent a more differentiated cell than does the intermediate type, which grows more rapidly in culture and is more resistant to therapy in vivo (13, 16).

Chromosomal abnormalities have been described in SCLC. Deletion of a portion of the short arm of chromosome 3 (3p−) has been reported to occur in a number of SCLC tumors (27–29). In addition, both homogeneously staining chromosomal regions and dmins have been found in SCLC cells (27–29). These cytogenetic abnormalities have been reported only in cells from malignant tumors, and they generally are considered to represent amplified DNA (6). The evolution of dmins occurs apparently in malignant tumors, and they generally are considered to represent amplified DNA (6). The evolution of dmins occurs apparently in malignant tumors, and they generally are considered to represent amplified DNA (6).

MATERIALS AND METHODS

Cell Lines. The lung cancer cell lines have been established in Uppsala (designation U). The biological nature of these cell lines, based on their derivation and detailed phenotypic studies, is as follows (Table 1): SCLC, U-1285, U-1690, U-1906, U-2020 (7, 8); large cell carcinoma, U-1810 (9); squamous cell carcinoma, U-1752 (10). The cell lines U-1285 and U-1752 have been described in detail elsewhere (8, 10). U-1690 and U-2020 were both established from pleural fluid from a Caucasian male (U-1690) and a female (U-2020) with bronchial neoplasms, which were diagnosed as SCLC (7). U-1906 was established from a cerebral metastasis from a patient who had a lung tumor with a histopathological picture of the intermediate subtype of SCLC. None of the SCLC tumors demonstrated signs of keratinization or formation of glandular structures. Transmission electron microscopy revealed the presence of dense core granules in all 3 SCLC cell lines, while no prominent tonofilament bundles or signs of mucous production were noticed (7). U-1810 was established from pleural fluid from a Caucasian male presenting with a bronchial primary tumor and mediastinal and supraclavicular lymph node metastases. Sections from tumor and mediastinal metastases showed an epithelial tumor consisting of large cells; no signs of keratinization or glandular formations were present. The nuclei demonstrated distinct nucleoli, and the histopathological diagnosis was LCC (7). The amount of neuron-specific enolase has been analyzed using homogenized material from the cell lines (U-1285, U-1690, U-1906, U-1752, U-1810) and the pleural effusate (U-2020) and was found to be consistent with the reported levels of neuron-specific enolase in different types of lung cancers (see Table 1). In addition, the SCLC cell line H82 (kindly provided by Dr. John Minna, National Cancer Institute, Bethesda, MD) was used as a positive control for c-myc amplification (20). Other cell lines were purchased from the American Type Culture Collection: A-427 and A-549 (19), lung cancer cell lines; JEG, a chorionic carcinoma cell line; and SW 1417 and COLO 205 (5), colon carcinoma cell lines. Human amniotic cells were prepared from placenta (8). The cells were grown in RPMI 1640 containing 10% fetal calf serum and antibiotics. The SCLC lines had a typical in vitro morphology with growth in clusters of variable size. Cultures were usually split twice a week at a 1:3 ratio and were regularly checked for Mycoplasma contamination, with negative results.

Isolation of DNA and Preparation of Blots. High-molecular-weight DNA was isolated from 107 to 108 cells obtained from logarithmically growing stock cultures as follows. The cells were dissolved in 0.5% SDS:0.1 M NaCl:20 mm EDTA:50 mm Tris-HCl (pH 8.1), and the cellular proteins were hydrolyzed with protease K (500 µg/ml; Merck) for 1 hr at 37°. 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
AMPLICATED c-myc IN LUNG CANCER

Table 1
Phenotypic properties of lung cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histopathological diagnosis of original tumor (7, 8)</th>
<th>Karyotypic features (29)</th>
<th>In vitro properties</th>
<th>Transmission electron microscopy</th>
<th>Intermediate filaments</th>
<th>Relative values (%)</th>
<th>Absolute values (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-1285</td>
<td>SCLC</td>
<td>3 q+</td>
<td>Suspended</td>
<td>Dense core granules</td>
<td>NF* (9)</td>
<td>2.5</td>
<td>380</td>
</tr>
<tr>
<td>U-1690</td>
<td>SCLC</td>
<td>3p-, 3q−</td>
<td>Suspended/attached</td>
<td>Dense core granules</td>
<td>NF</td>
<td>21</td>
<td>2100</td>
</tr>
<tr>
<td>U-1906</td>
<td>SCLC</td>
<td>dmins</td>
<td>Attached/suspended</td>
<td>Dense core granules</td>
<td>NF</td>
<td>8</td>
<td>340</td>
</tr>
<tr>
<td>U-2020</td>
<td>SCLC</td>
<td>3q−, dmins</td>
<td>Attached/suspended</td>
<td>Dense core granules</td>
<td>NF</td>
<td>17</td>
<td>1130</td>
</tr>
<tr>
<td>H-82*</td>
<td>SCLC-large cell variant</td>
<td>3p−, HSR on 15 p−</td>
<td>Suspended</td>
<td>No dense core granules</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>U-1752</td>
<td>SQC</td>
<td>Amplification of 12</td>
<td>Attached</td>
<td>Desmosomes, tonofilaments</td>
<td>K</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>U-1810</td>
<td>LCC</td>
<td>HSR on a B-chromosome</td>
<td>Attached</td>
<td>Tonofilaments, cellular junctions, microvilli, intercellular lumina</td>
<td>K, NF</td>
<td>1</td>
<td>17</td>
</tr>
</tbody>
</table>

* SOC, squamous cell carcinoma; LCC, large cell carcinoma; HSR, homogeneously staining regions; ND, not determined; NF, neurofilament; K, keratin.

Generous gift from Drs. D. N. Carney, A. F. Gazdar, and J. D. Minna, NIH, Bethesda, MD.

RESULTS

c-myc DNA Amplified in SCLC Cells. We first studied the copy number of the c-myc gene in the lung cancer cells by "Southern blotting" DNA analysis. DNA was extracted from the cells, cleaved with restriction endonucleases, electrophoresed, blotted to nitrocellulose, and hybridized with a radiolabelled plasmid containing a 360-base pair Alul-Cla insert from the third exon of human c-myc (3, 14). DNAs from 2 cell lines, U-1285 and U-2020, and the positive control SCLC cell line H82 (20) gave an intense signal from a characteristic 2.7-kilobase pair 3'SS-fl-c-myc fragment (Fig. 1). The other DNAs on the filter were labeled by the probe at intensities corresponding to single-copy sequences in control cells. Other oncogene probes, e.g., N-myc and c-myc probes, gave a uniform labeling of all lung cancer samples on the same filters (shown for c-myc in Fig. 1). This suggested that the c-myc gene was amplified in DNA of both U-1285 and U-2020 cells.

The degree of c-myc amplification was estimated from diluted DNA (Fig. 2). Signals from different amounts of amplified DNA were compared with those from 15 µg of DNA from H82 and U-1906 cells, assumed to have 50 and 2 copies of the c-myc gene, respectively. As can be seen from Fig. 2, the 2.7-kilobase pair c-myc signals are comparable in 0.6 to 1.7 µg of U-1285 DNA and 15 µg of U-1906 DNA. Thus, c-myc is about 10- to 20-fold amplified in U-1285 cells. A similar analysis of U-2020 cell DNA indicated a degree of c-myc amplification of about 8- to 16-fold.

Amplified c-myc Expressed at Elevated Levels. For analysis of the levels of c-myc RNA in the lung cancer cells, polyadenylated RNA was isolated from the cells as detailed in "Materials and Methods," diluted serially, spotted and dried on nitrocellulose, and analyzed by hybridization with the c-myc probe. The analysis indicated that expression of c-myc RNA was about 20- to 16-fold enhanced in U-1285 cells and U-2020 cells, respectively, as compared with several other cells (Fig. 3). However, slightly elevated amounts of c-myc mRNA were also found in U-

vacuum. The nucleic acids were then redissolved in 1 mM EDTA:10 mM Tris-HCl (pH 8), and RNA was hydrolyzed with pancreatic RNase A (100 µg/ml; Sigma) at 37° C for 1 hr. Treatment with proteinase K, extraction with phenol, extraction with butanol:propanol, and ethanol precipitation were performed as above. The DNA was dissolved and stored at 4° in 1 mM EDTA:10 mM Tris-HCI (pH 8). The cellular proteins were hydrolyzed with proteinase K (500 ng/ml) for 30 min at 37°. The DNA was sheared with a Virtis homogenizer. The NaCl concentration was raised to 0.5 M, and polyadenylated RNA was bound to oligo(dT)-cellulose (Collaborative Research Type III) for 4 hr under agitation. The oligo(dT)-cellulose was centrifuged (1500 rpm, 3 min) at room temperature, the supernatant was discarded, and the oligo(dT) was resuspended in 0.5 M NaCl in cell lysis buffer. This step was carried out 3 times. The oligo(dT) was then poured into a column, and the polyadenylated RNA was eluted with 10 mM Tris (pH 7.6):1 mM EDTA. The RNA was precipitated in the presence of 0.3 M sodium acetate (pH 8.0) with 3 volumes of ethanol at −70°.

Spot Hybridization Analysis of RNA. Polyadenylated RNA (4 µg) was precipitated, dried, dissolved in 12 × SSC:40% formaldehyde, heated for 5 min at 60°, diluted serially, and spotted on nitrocellulose. The filter was baked, prehybridized, and hybridized as detailed elsewhere (24).

Electrophoresis and Blotting. Aliquots of DNA were digested with restriction endonucleases, fractionated by electrophoresis through a 1% agarose gel, and transferred to nitrocellulose paper in 6 × SSC. Fragment sizes were calculated using λ-phage DNA cleaved with restriction endonuclease HindIII as a standard.

RNA was dissolved in sample buffer containing 20 mM morpholino propane sulfonic acid (pH 7.0), 1 mM EDTA, 5 mM sodium acetate, 50% (v/v) formamide, and 2.2 M formaldehyde. Samples containing amounts of RNA as indicated were heated for 10 min at 60° and electrophoresed in 0.8% agarose gels. Electrophoresis buffer was the same as the sample buffer. The position of rRNA was visualized by ethidium bromide staining, and the RNA was transferred to nitrocellulose paper in 20 × SSC.

Radioactive Labeling of Cloned DNA. Standard nick translation reactions were carried out according to manufacturer’s instructions (Amersham). Specific activities were 5 × 10^6 to 2 × 10^7 cpm/µg DNA template.

Hybridizations and Analysis of Hybrids. Hybridizations were performed in Denhardt’s mixture (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 3 × SSC, 50% formamide, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), salmon sperm DNA (200 µg/ml), and yeast tRNA (150 µg/ml), for 24 hr at 41°. Approximately 10^7 cpm were applied to each filter of about 200 sq cm in 15 ml hybridization mixture. Filters were washed in 1 × SSC:0.1% SDS at 50° and autoradiographed. Filters were reused for up to 6 times without detectable loss of signal. For denaturation of DNA:DNA hybrids, the filters were soaked with slight agitation in 0.2 M NaOH:0.1% SDS at room temperature for 10 min, rinsed with tap water, neutralized for 3 min in 0.5 M Tris-HCl (pH 7.0), and washed in 0.1% SDS. Filters were dried at room temperature on filter paper, preincubated in hybridization mixture, and rehybridized as described above.

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Fig. 1. Analysis of c-myc in tumor cells. DNA from the tumor cells was digested with SstI and analyzed by Southern blotting as described in "Materials and Methods." Left, hybridization with the c-myc probe. The myc hybrids were melted in alkaline, the radioactive signal was washed off, and the filter was rehybridized with a radioactive c-myo probe (pF8, a kind gift from R. C. Gallo; Ref. 15) to confirm that the different samples had similar genomic equivalents of DNA. HUA, human amniotic epithelial cells; SW 1417 and COLO 201, colon carcinoma cell lines. The heavy band at 3.5 kilobase pairs (kbp) results from amplification of c-myc in COLO 201 cells (5).

Fig. 2. Estimation of c-myc copy number. SstI-digested samples were diluted serially, and similar volumes of the different concentrations of DNA (µg amounts indicated on top abscissa) were analyzed as in Fig. 1. Differences in the migration of the 2.7-kilobase pair (kbp) fragments result from differential loading of the electrophoretic lanes.

1906 and A-549 cells, both of which have single-copy c-myc DNA sequences (Fig. 2 and data not shown). The U-1906 is also a SCLC cell line; the A549 cell line has been reported to express phenotypic traits of pulmonary type II pneumocytes or alveolar epithelial cells (19).

Fig. 3. c-myc RNA analysis by spot blot hybridization. Oligo(dT)-selected sample RNAs were diluted serially and spotted on nitrocellulose to give the µg amounts shown on the top abscissa. The level of c-myc expression was measured by hybridization with a radioactive c-myc probe.

Fig. 4. Northern blotting analysis of a typical 2.3-kilobase (kb) c-myc RNA species in cells with high level of c-myc expression.
(Fig. 4). The intensity of the band, however, varies as expected from the quantitative analysis presented in Fig. 3. By similar analysis, no rearrangements were seen in either the N-myc or the c-myb loci (data not shown).

**DISCUSSION**

Our results confirm and extend the findings of Little *et al.* (20), who found c-myc amplified in cells of the variant form of SCLC. The cell lines used in the present study have been well characterized for several phenotypic properties in culture, including karyotype, production of neuroendocrine enzymes and hormones, and cell type-specific intermediate filaments (Table 1; Ref. 9). The U-1285 and U-2020 cells have a fast doubling rate. The U-1285 cells grow in suspension, while the U-2020 the cells grow both in suspension and attached to culture dishes (7, 8). The U-2020 cells have 30 to 120 dmin chromosomes in all metaphases, and the U-1906 cells also show rare dmins, but only in occasional metaphases (29). In addition, the U-1906 cell line has an extra chromosome 8 in about one-third of all metaphases. This may relate to the slightly elevated expression of single-copy c-myc in U-1906 cells noted in the present study. No structural abnormalities of chromosomal band 8q24, the site of the resident normal c-myc gene (26), are detected in any of the cells.

We assume but have yet to prove that the amplified c-myc copies in both U-1285 and U-2020 cells are contained in the dmins. Two precedents for such findings have been published (4, 24). Experimental studies have shown that retention of dmins in tumor cells in culture requires a continuous selection pressure (21). We cannot identify such a pressure at present because too little is known about the functions of c-myc to implicate its amplification in the growth advantage that some SCLC cells enjoy. However, c-myc expression is coupled to the G1 commitment phase of the cell cycle in growth-stimulated cells (17), and loss of this regulation of expression may occur in at least chemically transformed cells (11). c-myc also encodes a nuclear protein (2). Amounts of the c-myc protein also elevated in the SCLC cells where the c-myc gene is amplified. These features suggest that the functions of c-myc may be responsible for the rapid growth properties of a variant subpopulation of SCLC cells.

Where does c-myc amplification come into play in the development of SCLC? Recent studies have indicated that for oncogenic transformation of normal diploid cells, at least 2 genetic functions are required (18). DNA damage activating 2 complementary oncogenes is found to fall into 3 categories: (a) mutations, e.g., in the c-ras cellular oncogenes, (b) chromosomal translocations, such as seen in Burkitt lymphomas; (c) oncogene amplification (for a review, see Ref. 3). The first of these mechanisms alters the structure of, e.g., the c-ras-encoded protein; the 2 latter ones may merely increase the dosage of an often unaltered oncogene product.

Similarly, in different lung cancer cells, one has found amplifications of the c-myc oncogene (Ref. 20 and the present results) or mutational damage to the c-Ki-ras gene (12, 25). One may speculate that oncogene amplification is not an obligatory tumorigenic lesion but most probably a late development in the progression of some tumors. Similar results have been obtained from studies of the N-myc gene in neuroblastomas (22). About one-half of Grade III and IV neuroblastomas show amplification of N-myc, whereas Grade I and II tumors have the normal diploid complement of N-myc. A bias is inherent in cell culture studies of neuroblastomas, however. Cell lines are obtained only from Grade III and IV tumors; thus, there is an overrepresentation of N-myc amplification in neuroblastoma cell lines. Cell culture studies of SCLC cells should also be interpreted with caution until clinical tumor material has been studied.

**Note Added in Proof**

John Minna and collaborators have recently discovered amplifications of c-myc-related sequences, N-myc (22), and L-myc in SCLC cell lines (personal communication).

**REFERENCES**


* M. Schwab, personal communication.
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