Gene Amplification of c-*myc* and N-*myc* in Small Cell Carcinoma of the Lung

ALBERT J. WONG, JOHN M. RUPPERT, JOSEPH EGGLESTON, STANLEY R. HAMILTON, STEPHEN B. BAYLIN, BERT VOGELSTEIN*

The relationship of the copy numbers of the c-*myc* and N-*myc* oncogenes to tumor formation and progression was studied in small cell carcinoma of the lung. When 96 neoplastic lesions from 45 patients were examined, these lesions could be grouped into three categories: high copy (tumors with greater than 3 copies of the N-*myc* or c-*myc* gene per haploid genome), middle copy (1.5 to 3 copies per genome), and normal copy. Fourteen of the patients had middle copy tumors, but this was almost always a result of chromosome duplication rather than the amplification of a small genetic locus. In contrast, five patients had high copy tumors, with the increased copy number in each case due to gene amplification. The amplification did not occur in a heterogeneous fashion within individual patients, since all metastatic lesions from patients with high copy lung tumors were also high copy, while none of 41 metastatic lesions from the other patients were high copy. These data suggest that gene amplification is an important step in neoplastic growth in a subset of patients with small cell carcinoma of the lung and that this genetic event occurs relatively early (before metastasis) in this subset.

THE ACTIVATION OF ONCOGENES IN tumors can occur by two general mechanisms: by a mutation in the DNA that results in a functionally altered protein, or by deregulation resulting in overexpression of the normal cellular product (*1*). The latter can occur through a variety of mechanisms such as chromosomal translocation (*2*), insertion of strong promoter elements (*3*), or gene amplification (*4*). Oncogenes are amplified in a variety of human tumors including neuroblastomas (*5*), where amplification of N-*myc* occurs particularly in aggressive tumors (*6*), retinoblastomas (*7*), glioblastomas (*8*), leukemias (*9*), and carcinomas (*10*). Amplification of the c-*myc* or N-*myc* oncogene has also been observed in small cell carcinoma of the lung (SCCL), (*11–14*); amplification was commonly found in cell lines displaying variant morphologies in vitro (*11–13*). These variant cell lines were thought to be derived from tumors with unusual morphological features in vivo (often involving mixtures of small cell and large cell types within the same tumor), aggressive biological behavior, and poor response to therapy (*11, 13, 15*). Hence, the presence of gene amplification could have important implications for the diagnosis and treatment of patients with SCCL.

In this communication, the amplification of the N-*myc* and c-*myc* genes was studied in human tumors rather than cell lines. These studies were made possible by the feasibility of analyzing DNA prepared from paraffin-embedded samples stored in the pathology archives (*16*). We report here our findings on c-*myc* and N-*myc* amplification in 96 tumor samples from 45 different patients.

The tumors studied were chosen on the basis of availability of tissue from pathology archives and so represent a random sampling of patients with SCCL. To establish the copy number of c-*myc* and N-*myc* in each tumor specimen, DNA was bound to nitrocellulose in a "slot blot" apparatus and hybridized to either the c-*myc* or N-*myc* probe (*17*). Autoradiographic results from each filter were obtained and quantitated by densitometry. The filters were then treated with alkali to remove the previously hybridized probe and successively reannealed with two other probes: (i) pAW101, which contains noncoding sequences on chromosome 14 and served as a control for DNA content; and (ii) either a probe for chromosome 2 (for N-*myc* analysis) or chromosome 8 (for c-*myc* analysis), which was used to control for specific chromosome content (*17*).

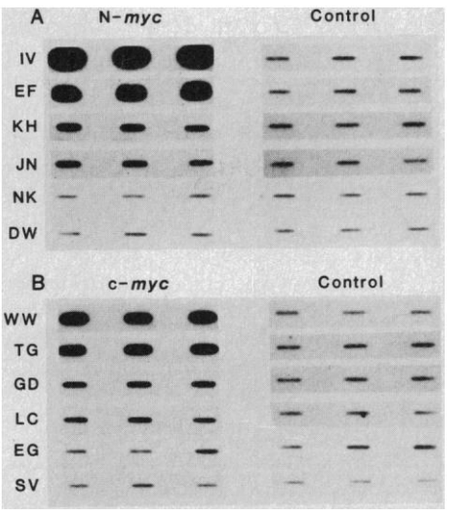

Fig. 1. Slot-blot analysis of DNA from patients with SCCL. DNA was extracted from the paraffin-embedded tissues by the method of Goelz *et al.* (*16*). After alkaline hydrolysis of residual RNA, samples were applied to nitrocellulose filters in triplicate with a Minifold-II apparatus (Schleicher and Schuell, Keene, NH). The filter was hybridized with a *myc* probe [N-*myc* in (A), c-*myc* in (B)] and analyzed by autoradiography. The probes were then removed with 0.1*M* NaOH and 0.5*M* NaCl for 6 minutes and the filter was rehybridized with the control probe (pAW101). Patients IV, EF, WW, and TG had HC tumors; patients KH, JN, GD, and LC had MC tumors; and NK, DW, EG, and SV had NC tumors.

From analysis of tumors (Fig. 1 and Table 1) three patterns emerged: high copy (HC) tumors (which contained more than 3 copies of either N-*myc* or c-*myc* per haploid genome when compared to either the chromosome 14 or homologous chromosome control probes), middle copy (MC) tumors (between 1.5 and 3 copies per genome when compared to the chromosome 14 probe), and normal copy (NC) tumors. In Fig. 2, the results are displayed graphically for the five patients with HC tumors and for representative tumors from five patients with MC and NC tumors. There was no significant cross-hybridization between the N-*myc* and the c-*myc* probes under the conditions used in these experiments.

No patient displayed both N-*myc* and c-*myc* amplification in the same tumor. (One patient had an increase in copy number of the N-*myc* gene as well as amplification of the c-*myc* gene, but this increase in N-*myc* resulted from a chromosomal duplication rather than gene amplification per se, as shown later.) Metastatic lesions from the patients of the HC group were examined for amplification. Metastases were obtained at the same time as the lung lesion for all of the patients except WW. We reasoned that if the amplification event occurred in a heterogeneous fashion, then some cells without amplification might exist in the tumor and go on to form metastases. However, examination of metastases showed that HC amplification of the *myc* genes was present in all ten of the metastases examined from these patients (Fig. 3 and Table 1). The degree of amplification was similar in the lung tumors and in the metastases of each patient.

We compared these results to metastatic lesions from patients whose pulmonary lesions did not contain a high number of c-*myc* or N-*myc* copies. If amplifications of

A. J. Wong, J. M. Ruppert, B. Vogelstein, Oncology Center, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
J. Eggleston and S. R. Hamilton, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
S. B. Baylin, Oncology Center and Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

*To whom correspondence should be addressed.

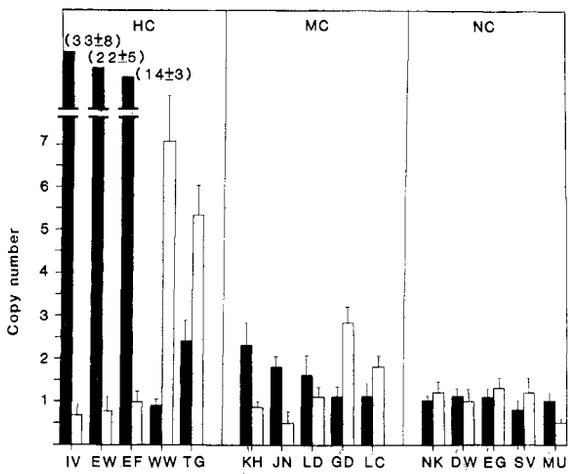

Fig. 2. Comparison of N-*myc* and c-*myc* copy numbers. Copy numbers of the N-*myc* (black bars) and c-*myc* (open bars) genes are shown for the five patients with HC lesions and for representative patients with MC lesions and NC lesions. The lines represent the standard deviation calculated from at least six different determinations. The numbers in parentheses above the bars refer to the mean and standard deviations of the copy numbers for the HC samples. Copy numbers were determined as described (*29*).

these genes could occur randomly or heterogeneously within SCCL tumors, then some of the metastatic lesions in these patients would be expected to exhibit this specific genetic change. A total of 41 metastatic lesions from 17 different patients were evaluated; 11 of these patients had been treated with chemotherapy. None of these metastases contained high copies of N-*myc* or c-*myc* (Fig. 3 and Table 1). The results indicated that the amplification occurred relatively early in tumor development (before metastasis). In addition, those tumors whose development did not involve HC amplification as a crucial step were unlikely to evolve such amplification during the process of metastasis.

In previous studies with cultured cell lines from SCCL tumors, c-*myc* and N-*myc* amplification were often found in cell lines displaying variant morphologies (*11, 13*). The five tumors with HC levels of N-*myc* or c-*myc* were classified histologically as representing the intermediate subtype of SCCL. This was in contrast to the 40 patients with MC or NC tumors, whose tumors were divided between classic oat cell, intermediate, and mixed small cell–large cell subtypes (Table 1). None of the HC tumors had a mixed small cell–large cell phenotype and the ten HC metastases had morphologies similar to those of the original lesions.

Of the five patients with HC tumors, three underwent radio- and/or chemotherapy and two had complete responses with survival times of over 15 months. The number of patients was too small to determine a statistically significant difference in response to therapy of this group. However, the presence of numerous copies of the *myc* genes did not preclude a good response to standard therapeutic regimens.

Multiple hybridization analyses of normal tissues showed that the c-*myc* and N-*myc* copy number fell within ±20% of the expected value (between 0.8 and 1.2 copies per haploid genome). Hence, we could readily detect an increased copy number of the c-*myc* and N-*myc* genes when that number exceeded an average of 1.5 copies per haploid genome. Of the 45 patients examined, 6 showed 1.5 to 3.0 copies of the c-*myc* gene and 8 showed between 1.5 to 3.0 copies of the N-*myc* gene when compared to the chromosome 14 probe (Table 1).

There were two potential explanations for the increased c-*myc* or N-*myc* copy number in the MC tumors. The first explanation would involve the amplification of a discrete, small (100 to 3000 kb) amplification unit. Such units are found in tumors containing double-minute chromosomes (DM's) or homogeneously staining regions with HC amplification of c-*myc* and N-*myc* oncogenes (*18, 19*), as well as in cells containing amplification of drug resistance genes (*20*). In MC tumors, amplification could involve a small number of DM's or a larger number of DM's present in only a small portion of the cells. A second explanation would simply involve aneuploidy, with tumor cells containing more than two copies of large parts of the relevant chromosomes. These two explanations can be distinguished by hybridization of the tumor DNA samples to probes for sequences on the same chromosomes as N-*myc* or c-*myc*. This analysis was performed for 12 tumors containing 1.5 to 3.0 copies per cell of the c-*myc* or N-*myc* gene. A probe for the carbamyl phosphate synthetase-1 (CPS-1) gene was used to control for chromosome 2 content and a c-*mos* gene probe was used to control for chromosome 8 content. These probes represent sequences that are tens of millions of base pairs removed from the N-*myc* and c-*myc* genes; therefore, they would not be involved in a small amplification unit containing an N-*myc* or a c-*myc* gene. The copy number of the CPS-1 gene and c-*mos* gene in 11 of 12 cases of MC tumors analyzed was the same as the N-*myc* and c-*myc* genes in these tumors. It is unlikely that these tumors had lost one chromosome 14, since the copy number of both the N-*myc* and c-*myc* gene would then be increased, which was not seen in any of the MC tumors. Hence, the increased copy number of the *myc* genes in this group of tumors was probably due to aneuploidy for large parts of chromosome 2 or 8 rather than due to true gene amplification events.

One patient (TG) was found to have MC numbers of N-*myc* as well as HC numbers of c-*myc* (Fig. 2). In this patient, the MC number of N-*myc* was due to chromosomal duplication rather than true gene amplification, since the tumor was shown to have 1 copy of the c-*mos* gene but approximately 2.5 copies of the CPS-1 gene per haploid genome.

Aneuploidy is frequently present in SCCL tumors (*21*) and it was of interest to determine whether different lesions from the same patient had different copy numbers of the c-*myc* or N-*myc* genes. In 10 of 17 patients with MC or NC tumors, all of the lesions were in the corresponding category of copy number. Seven of the patients, however, had some MC and some NC lesions. Hybridization analyses showed that in each of these seven cases, the increased copy number was due to aneuploidy rather than

Table 1. Distribution of N-*myc* and c-*myc* copy number categories among patients with SCCL.

Copy number	Number of patients				HC metastases
	Total	Histologic subtype*	N-*myc*	c-*myc*	
HC (>3 copies)	5	0 Oat 5 Inter 0 SC-LC	3	2	10/10
MC (1.5 to 3 copies)	14	8 Oat 5 Inter 1 SC-LC	8	6	0/13
NC	26	6 Oat 15 Inter 5 SC-LC	26	26	0/28

*Abbreviations: Oat, classic oat cell subtype of SCCL; Inter, intermediate cell subtype of SCCL; and SC-LC, mixed small cell–large cell carcinoma.

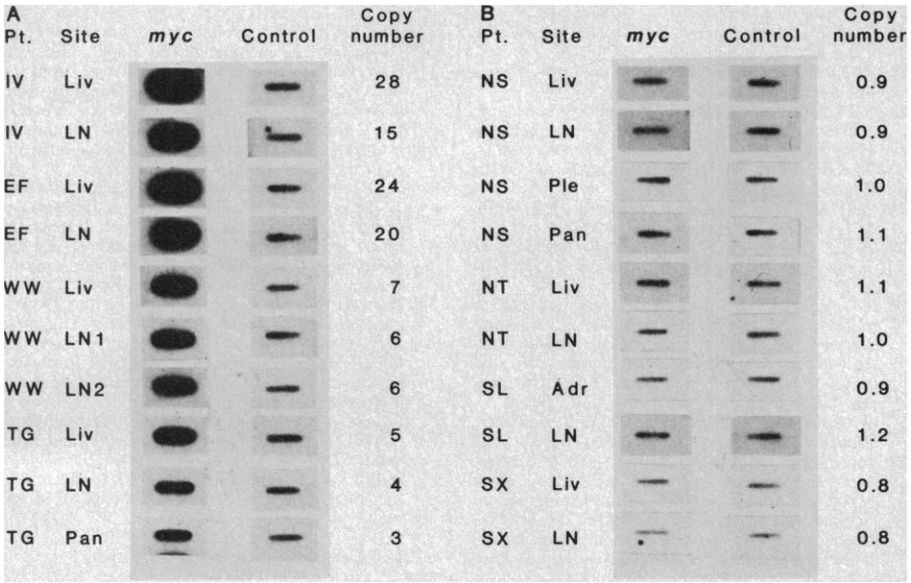

Fig. 3. N-*myc* or c-*myc* copy number in metastases of patients (Pt.) with SCCL. Extrapulmonary metastases were analyzed for copy number as described in Fig. 1. The ten metastases from the patients with HC lung tumors (A) are shown in comparison with ten metastases from patients with NC lung tumors (B). Samples from patients IV, EF, NS, and NT were hybridized with an N-*myc* probe; samples from patients TG, WW, SL, and SX were hybridized with a c-*myc* probe. Abbreviations: Adr, adrenal; Liv, liver; LN, lymph node; Pan, pancreas; and Ple, pleura.

gene amplification per se. This result is consistent with the heterogeneous karyotype patterns often noted in different metastatic lesions of solid tumors (*22*).

Thus, high degrees of amplification of the N-*myc* or c-*myc* gene occur in a subset of SCCL patients. We believe that gene amplification is a significant event in those tumors in which it occurs because gene amplification is generally unstable (*20*). Cells containing amplified drug resistance genes can be selected by growth in the presence of a variety of cytotoxic drugs. The amplified genes are present in drug-resistant cell lines in the form of DM's, while homogeneously staining regions appear upon subsequent culture (*20*). Upon removal of the drug, the DM's and the amplified genes within them are rapidly lost (*20*). Gene amplification in primary tumors is usually present in the form of DM's (*23*). By analogy with the drug resistance systems, the amplified c-*myc* or N-*myc* genes present in SCCL tumors would be rapidly lost during the numerous cell divisions that occur during tumorigenesis unless the amplified genes continuously gave the tumor cells containing them some selective advantage in vivo.

In our studies on human tumors, amplification of c-*myc* or N-*myc* genes was not associated with variant morphologies in vivo, and conversely, none of the tumors we examined with small cell–large cell phenotypes displayed such amplification. Previous studies (*13*) in which amplification of c-*myc* or N-*myc* was associated with variant morphologies were largely done with cell lines. Perhaps amplification of c-*myc* or N-*myc* in SCCL tumors causes the appearance of unusual morphological properties upon growth of these tumors in vitro. Such variant morphologies may be discernible in primary tumors in vivo before tissue culture, but often are not.

Albino *et al.* (*24*) noted a mutation of N-*ras* in only one of five cell lines derived from different hepatic metastases of a patient with melanoma. It was suggested that the mutation was probably a manifestation of tumor heterogeneity and that it was unnecessary for growth or metastasis of the cancer in that patient. However, amplification of either c-*myc* or N-*myc* in SCCL was invariably present in metastatic lesions whenever the original lung lesion contained it and was never present in metastatic lesions when the pulmonary lesion did not contain it. Hence, the amplification was not a characteristic that could readily be explained by the concept of tumor heterogeneity (*25*). Although it could be argued that the amplification in each of the metastatic lesions occurred de novo at the site of metastasis because of host or environmental factors operative in a particular subgroup of patients, it is more likely that the amplification occurred prior to metastasis and that cells containing this genetic event eventually metastasized.

Finally, one must ask why all SCCL tumors do not contain high levels of c-*myc* or N-*myc* gene amplification. For reasons argued above, it would appear that in those SCCL tumors containing high copies of the c-*myc* or N-*myc* genes, cells with the amplification had a selective growth advantage over any cells in the tumor that had lost the amplified genes. Genetically engineered enhanced N-*myc* and c-*myc* expression can result in a growth advantage for cells (*26*). In addition, studies with drug resistance genes show that gene amplification can occur relatively frequently in cell lines (*20*), especially tumorigenic cell lines (*27*), when cells with such amplification can be selected for by a relative growth advantage. One might thus expect that any SCCL tumor would, when it became large enough, develop a gene amplification in one of its component cells that would ultimately allow the selective outgrowth of the cell with the amplification. Possible reasons why this does not occur in the majority of SCCL tumors include the following: (i) Tumors without c-*myc* or N-*myc* gene amplification may be expressing these genes at a high level as a result of other genetic or epigenetic mechanisms. (ii) The effect of elevated c-*myc* or N-*myc* gene expression may be replaced by activation of other oncogenes acting in similar fashion [for example, p53 and *fos* (*1*)]. A related *myc* gene, L-*myc*, is amplified in some SCCL cell lines and in at least one SCCL tumor (*28*). (iii) Finally, it is possible that *myc* amplification only provides a selective advantage to tumors under very defined and unusual conditions such as those that may be mediated by immune responses or specific microenvironments.

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29. Slot blots were exposed to Kodak XAR-5 film at −70°C and intensifying screens for varying periods of time so that the signal intensities obtained between two different probes were comparable and fell within the linear response range of the film. Intensities were quantitated with a Hoeffer model 1650 scanning densitometer interfaced with a Hewlett-Packard 3390a integrator. The amplification was computed by dividing the signal intensity from the oncogene probe by the signal intensity from pAW101. Placental DNA was used to normalize signal intensities between the two films. The amplification reported is the average of three samples. In most cases, the samples were analyzed on at least two filters so that the average reported is from 6 to 12 different determinations. This procedure resulted in highly reproducible values for copy numbers of specific genes. Statistical analysis indicated that the values were reproducible with an average standard deviation of 20% (see error bars in Fig. 2).
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The E5 Transforming Gene of Bovine Papillomavirus Encodes a Small, Hydrophobic Polypeptide

RICHARD SCHLEGEL, MARTA WADE-GLASS, MICHAEL S. RABSON, YU-CHUNG YANG

Bovine papillomavirus (BPV-1) contains two independent transforming genes that have been mapped to the E5 and E6 open reading frames (ORF's). The E5 transforming protein was identified by means of an antiserum against a synthetic peptide corresponding to the 20 COOH-terminal amino acids of the E5 ORF. The E5 polypeptide is the smallest viral transforming protein yet characterized; it had an apparent size of 7 kilodaltons. The transforming polypeptide is encoded entirely within the second half of the E5 ORF and its predicted amino acid composition is very unusual; 68% of the amino acids are strongly hydrophobic and 34% are leucine. Cell fractionation studies localized this polypeptide predominantly to cellular membranes.

PAPILLOMAVIRUSES ARE THE CAUSative agents of benign papillomas in many vertebrate species and, in humans, are responsible for benign warts or papillomas of a variety of squamous epithelial surfaces including skin, larynx, and genitalia (*1*). Some papillomaviruses are associated with lesions that can progress to malignant carcinomas. In animals, this progression has been best studied with the Shope papillomavirus (CRPV) and the bovine alimentary tract papillomavirus (BPV-4) (*2*). In humans, a number of cancers, including cervical carcinoma and cutaneous carcinoma in patients with epidermodysplasia verruciformis, have been associated with specific human papillomaviruses (*3*). Among a subgroup of papillomaviruses that can readily transform mouse cells in vitro, the bovine papillomavirus (BPV-1) has been characterized most extensively. This prototype virus induces fibropapillomas in cattle (*4*) and fibroblastic tumors in hamsters (*5*) and transforms mouse cells efficiently in vitro (*6*). BPV-1 contains two genetically independent transforming functions. One of these transforming genes maps to the E6 open reading frame (ORF) (*7, 8*), which has recently been shown to encode a 15.5-kD cysteine-rich molecule that is present in both the cell nucleus and cellular membranes (*9*). The second transforming function of BPV-1 is located in the E5 ORF. More specifically, frameshift mutations (*10–12*) or the insertion of a translation termination codon (*13*) at the Bst XI site (nucleotide 3889) within the second half of the E5 ORF dramatically inhibit cell transformation activity. Correction of an E5 ORF frameshift mutation by a second mutation can restore transforming activity, further suggesting that translation of the 3′ end of the E5 ORF is essential for transformation (*12*). This report identifies the E5 transforming protein of BPV-1 as a small, membrane-associated hydrophobic polypeptide.

The organization of the segment of the BPV-1 genome containing the E5 ORF is shown in Fig. 1A. The full E5 ORF consists of 297 base pairs (3714 to 4010), with a maximum coding capacity of 99 amino acids (*14*). If the first initiation codon at amino acid position 56 (base 3879) is used, the resultant protein should consist of 44 amino acids and have a molecular size of 5978 daltons. Although the E2 ORF overlaps a portion of the E5 ORF, it terminates immediately prior to the first methionine residue of the E5 protein (Fig. 1).

To identify and characterize the E5 protein, E5-specific antibodies were prepared by means of a synthetic peptide (Fig. 1B) corresponding to the COOH-terminus of the E5 ORF as an immunogen in rabbits. The synthetic peptide was solubilized in 1% sodium dodecyl sulfate (SDS) and mixed with complete Freund's adjuvant prior to subcutaneous injection. The techniques for antiserum production and titration of antibodies to peptides by enzyme-linked immunosorbent assay (ELISA) have been described (*15*).

The utility of this antiserum in recognizing the BPV-1 E5 protein was demonstrated by the immunoprecipitation of a 7-kD protein from a mouse cell line (C127) that had been transformed by the BPV-1 complementary DNA (cDNA)–containing plasmid C88 (Fig. 2A). This cDNA plasmid contains the BPV-1 E2, E3, E4, and E5 ORF's expressed by the SV40 early promoter (*8*); the derivative cell line has been designated YC-C88-A. The 7-kD protein was not present in the parent C127 cell line. The specific-

Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, MD 20892.