Differential Expression of the c-erbB-2 Gene in Human Small Cell and Non-Small Cell Lung Cancer

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ABSTRACT

We studied non-small cell lung cancers (NSCLC) from 60 patients for abnormalities in the c-erbB-2 gene. Eleven human lung cancer cell lines, including four derived from small cell lung cancer (SCLC) and seven derived from NSCLC were also examined for altered c-erbB-2 gene expression. Southern blot analysis of paired tumor and normal lung samples demonstrated that amplification of the c-erbB-2 gene is rare in NSCLC (2/60) and not restricted to adenocarcinomas. One patient showed an EcoRI restriction fragment length polymorphism for the c-erbB-2 locus. Normal lung c-erbB-2 cell lines demonstrated minimal or nondetectable expression of c-erbB-2 mRNA compared to high levels of expression by seven of seven NSCLC lines. The highest expression levels were seen in four of four adenocarcinomas. We conclude that c-erbB-2 expression is different in SCLC compared to NSCLC and high expression of c-erbB-2 is consistently present in lung adenocarcinomas.

INTRODUCTION

Various alterations in protooncogenes have been implicated in the pathogenesis of malignant human and animal tumors (1). One group of protooncogenes encodes cellular growth factors or their receptors. The c-erbB-1 gene encodes the epidermal growth factor receptor (2), c-sis the B-chain of the platelet-derived growth factor (3), and c-fms a related or identical molecule for the receptor of the granulocyte-macrophage colony stimulating factor (4). A fourth member of this group of protooncogenes called neu was identified in ethylnitrosourea-induced rat neuroblastomas (5). The human counterpart of neu, called HER-2/neu or c-erbB-2, has been sequenced and mapped to the chromosomal locus 17q21 (6–8).

The protein product of c-erbB-2 is a membrane protein of 185,000 molecular weight with tyrosine kinase activity and extensive sequence homology to EGFR* (6, 8, 9). The c-erbB-2 gene has been shown to be amplified in certain human tumors (10, 11). Amplification of the c-erbB-2 gene was reported to correlate with spread of disease (12, 13) and relapse and survival (14) in human breast cancer.

We conducted a prospective study for abnormalities in the c-erbB-2 gene in 60 patients with NSCLC. Microscopically uninvolved lung tissue was available from each patient for comparison with tumor tissue. In addition, 11 human lung cancer cell lines including four derived from SCLC and seven derived from NSCLC were examined for potential differences in c-erbB-2 gene expression.

MATERIALS AND METHODS

Patient Specimens. Fresh tumor and paired uninvolved lung tissue was obtained from 60 patients with NSCLC immediately following surgery. Lymph node metastases were obtained from seven patients. None of the patients received chemotherapy or radiation therapy prior to surgery. All specimens were reviewed by a pathologist and only tumor samples that contained at least 60% malignant cells were used in the study. Tissue samples were stored in the gas phase of a liquid nitrogen freezer until further processing.

Human Tumor Cell Lines. Six NSCLC lines (NCI-H1157, NCI-H226, NCI-H460A, NCI-H322, NCI-H522, and NCI-H596) and four SCLC lines (N417, H209, H82, and H69) were kindly provided by J. Minna and A. Gazdar, NCI, Bethesda, MD. A431 (human vulva carcinoma) was a gift from G. Gallick, M. D. Anderson, Houston, TX, and BT474 (human breast cancer) and A549 (bronchioalveolar carcinoma) were obtained from ATCC, Rockville, MD. All the lines were grown in RPMI 1640 (Irvine, Santa Ana, CA) supplemented with 10% heat-inactivated fetal calf serum (Irvine, Santa Ana, CA).

DNA Extraction and Analysis. DNA was extracted from frozen tissue samples after pulverization in liquid nitrogen and from tumor cell lines after lysis by a method of Davis, et al. (15). Routinely, 10 μg DNA was digested with restriction endonuclease EcoRI (10 units/μg DNA) according to the manufacturer (Boehringer-Mannheim, Indianapolis, IN). Digested samples were electrophoresed on 0.8% agarose gels and transferred after alkali denaturation to gene screen plus membranes (New England Nuclear, Boston, MA). A 3-kilobase HindIII-KpnI fragment of human neu cDNA clone pCER204 (12) was labeled with [32P]CTP (New England Nuclear, Boston, MA) using the random multiprime labeling system (Amersham, Arlington Heights, IL). Hybridization was carried out at 65°C in 1 M NaCl, 10% dextran sulfate, 1% SDS, 100 μg/ml salmon sperm DNA, and 106 cpm specific activity for 36 h. Washing was done in 0.5x SSC, 1% SDS at 65°C for 1 h and 0.1x SSC at room temperature for 1 h.

Autoradiography was performed at ~70°C with X-Omat AR2 films (Kodak, Rochester, NY). The intensity of the hybridization signal in paired tumor and normal samples was quantitated by soft laser densitometry (Model SL-2D; Biomed, Fullerton, CA). Membranes were then rehybridized with a β-pseudoglobulin gene probe β2-1 (16) which served as an internal control for loading differences between paired samples. Amplification was defined as a threefold or greater increase in the intensity of the hybridization signal in the tumor DNA relative to the paired control DNA.

RNA Extraction and Analysis. RNA was extracted from cell lines at 2/3 confluence according to the procedure of Chirgwin, et al. (17). Fifteen μg of total cellular RNA was size separated on 1.2% formaldehyde gels as described (18) and blotted onto gene screen plus membranes (New England Nuclear, Boston, MA). To quantitate levels of RNA expression, RNA dot blot analysis was performed (Minifold II apparatus; Schleicher and Schuell, Keene, NH) as described (19). For Northern blot hybridization and RNA dot blot analysis, membranes were hybridized as described for Southern blot hybridization except that 2 x 106 cpm specific activity was used and the hybridization and washing temperature was reduced to 60°C. Membranes were rehybridized with a β-pseudoglobulin gene probe β2-1 (16) which served as an internal control for loading differences between paired samples.

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*The abbreviations used are: EGFR, epidermal growth factor receptor; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; cDNA, complementary DNA; SSC, standard saline citrate (0.15 M sodium chloride:0.015 M sodium citrate, pH 7.4); h.p., human placenta; SDS, sodium dodecyl sulfate.
RESULTS

c-erbB-2 Gene Amplification in Primary NSCLC. DNA from 60 paired NSCLC and normal lung tissue samples including seven samples with lymph node metastases were analyzed by Southern blot hybridization with a specific c-erbB-2 cDNA probe. In two of the 60 (3.3%) patient samples, we detected amplification of the c-erbB-2 gene. Fig. 1, A and B, show the results of 14 representative paired tumor (T) and normal lung (N) samples. Amplification was 6-fold in tumor sample 7- and 20-fold in tumor sample 10. The membranes were rehybridized to a β-pseudoglobin gene probe which detects a 7.2-kilobase EcoRI fragment. The intensity of this signal was used to equilibrate loading differences between paired samples.

These results demonstrate that amplification of the c-erbB-2 gene is a rare event in human NSCLC. In addition, amplification of the c-erbB-2 gene in NSCLC is not restricted to the histological subgroup of adenocarcinomas (Patient 7), since the highest level of amplification was observed in a squamous cell carcinoma (Patient 10).

Restriction Fragment Length Polymorphism of the c-erbB-2 Gene in NSCLC. In 1/60 patients we detected an additional 11-kilobase EcoRI fragment which was present in the tumor (T), normal lung (N), and a lymph node metastasis (M). Fig. 2 shows that no additional fragment is present when the DNA samples were digested with restriction enzymes BamHI and HindIII. DNA from a normal h.p. which shows an identical EcoRI restriction fragment pattern to the 59 paired patient samples is included as a control. We conclude that the presence of the additional 11-kilobase EcoRI restriction fragment in this patient sample is due to an EcoRI restriction fragment length polymorphism in the c-erbB-2 gene.

c-erbB-2 Gene Expression in SCLC versus NSCLC. We investigated whether there were quantitative or qualitative changes in c-erbB-2 gene expression. Twelve human tumor cell lines including seven derived from NSCLC (NCI-H157, NCI-H226, NCI-H460A, NCI-H322, NCI-H522, NCI-H596, and A549) and four from SCLC (N417, H209, H82, and H69) were chosen for this analysis. The human breast cancer cell line BT474 which contains multiple copies of the c-erbB-2 gene and shows a high level of c-erbB-2 expression was chosen as a positive control for the sensitivity of the analysis (21). The human vulva carcinoma line A431 which contains an amplified EGFR gene and shows a high level of EGFR expression was included as a specificity control for the analysis because the EGFR and c-erbB-2 genes share sequence homologies. DNA and RNA from a normal h.p. served as controls for normal tissue.

Southern blot hybridization (Fig. 3) to EcoRI digested DNA samples extracted from the 12 cell lines and human placenta with the c-erbB-2-specific cDNA probe confirms that only cell line BT474 contains multiple copies of c-erbB-2.

To analyze c-erbB-2 expression, tumor cell lines were grown under identical conditions and RNA was extracted from 2/3 confluent cultures. For quantitative analysis of c-erbB-2 expression, RNA dot blot analysis was performed. Fig. 4 shows that three SCLC lines show minimal expression of c-erbB-2. The
Differential expression of c-erbB-2 gene

Fig. 3. Southern blot analysis of DNA isolated from human placenta (h. plac.) and 12 human tumor cell lines. 10 μg DNA was digested with EcoRI and hybridization was performed as in Fig. 1. Only cell line BT474 (breast carcinoma) contained multiple copies of c-erbB-2. Interestingly, the consistently elevated expression in NSCLC lines compared to SCLC differed among the histological subtypes. The lowest comparable elevated level of expression (3–6-fold) was detected in the large cell carcinoma line H460A and the two squamous cell carcinoma lines H226 and H157. The highest levels of expression were observed in 4/4 adenocarcinomas (A549, H322, H522, and H596) with 8–32-fold elevated c-erbB-2 expression levels. To confirm that the observed differences in c-erbB-2 expression were not due to loading differences, we rehybridized the membranes with a 1.8-kilobase BamHI fragment of the human γ-actin cDNA clone pHFl (data not shown).

Fig. 4. RNA dot blot analysis of total cellular RNA (10–1.25 μg) extracted from cell lines and human placenta shown in Fig. 3A. Three SCLC lines (H69, N417, H209) show minimal expression of c-erbB-2 mRNA compared to 7/7 NSCLC lines (H522, H322, H226, H460A, H157, A549, and H596). BT474 which contains multiple copies of c-erbB-2 (Fig. 3A) is shown as positive control.

DISCUSSION

Several quantitative and qualitative abnormalities in protooncogenes, both at the DNA level or at the expression level have been reported with respect to NSCLC and SCLC. Amplification of c-myc, N-myc, and L-myc was observed in SCLC (21, 22) but was rarely observed with NSCLC (23). Brauch et al. (24) demonstrated that deletions in the short arm of chromosome 3 occur predominantly in SCLC. A study by Kok et al. (25) showed deletions at chromosome locus 3p21 in all major types of lung cancer.

Activation of the c-K-ras oncogene by different point mutations was found in a substantial number of patients with H82 cell line was also tested (data not shown). The N417 cell line was chosen as a reference and a relative expression of 1 was assigned. The three other SCLC lines showed the same (H209, H82) or twofold elevated expression (H69). In contrast, 7/7 NSCLC lines demonstrated 4–32-fold elevated expression of c-erbB-2. To confirm that the observed differences in c-erbB-2 expression were not due to loading differences, we rehybridized the membranes with a 1.8-kilobase BamHI fragment of the human γ-actin cDNA clone pHFl (data not shown).

We demonstrated by Northern blot analysis that there were no qualitative differences in c-erbB-2 mRNA transcripts. Representative samples are shown in Fig. 5. In the two SCLC lines N417 and H209 we did not detect the 4.8-kilobase c-erbB-2 transcript by Northern blot analysis (data shown for N417) although we see expression by RNA dot blot analysis. This discrepancy is probably due to differences in sensitivity and specificity of the two test systems.

Fig. 5. Northern blot analysis of representative samples from Fig. 1A. 15 μg of total cellular RNA was electrophoresed on a 1.2% formaldehyde gel and hybridized as described (Fig. 1). A 4.8-kilobase mRNA transcript is not detected in SCLC line N417.
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