**Amplification and overexpression of the cyclin D1 and epidermal growth factor receptor genes in non-small-cell lung cancer**

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**Abstract**  
Purpose: To study the structure and expression of the cyclin D1 and the epidermal growth factor receptor (EGFR) genes in a cohort of 298 non-small-cell lung cancer (NSCLC) specimens.  
Methods: Gene structure was studied by Southern analysis, and gene expression was studied by Northern analysis and immunohistochemical analysis.  
Results: Amplification of the cyclin D1 locus was found in 14/298 (5%) specimens. All 12/12 specimens with amplification of the cyclin D1 gene for which RNA was available were found to express the cyclin D1 transcript, and 11/12 overexpressed the transcript to levels higher than that of uninvolved lung. The EGFR gene was amplified in 17/286 samples of NSCLC tested, and was overexpressed in 22/169 (13%) cases tested, including 12/13 cases with amplification of the gene for which RNA was available. Cyclin D1 gene amplification was associated with advanced lymph node involvement ($P = 0.043$), but not with larger tumor size or adverse outcome. Cyclin D1 gene amplification and overexpression occurred independently of retinoblastoma tumor-suppressor gene (RB) inactivation, but tumors with amplification of the cyclin D1 gene were more likely to have EGFR gene amplification ($P < 0.005$). No correlation of EGFR gene amplification or overexpression with tumor size, lymph node involvement, patient demographic data, or survival was identified.  
Conclusions: These data indicate that the cyclin D1 and EGFR genes are amplified and overexpressed in NSCLC, and amplification of the cyclin D1 gene occurs frequently in conjunction with amplification of the EGFR gene.  

**Key words**  
Cyclin D1 · Epidermal growth factor receptor · Lung cancer · Gene amplification

**Introduction**  
Lung cancer is the leading single cause of cancer-related deaths in the United States for both men and women (Parker et al. 1997). Approximately 75% of lung cancers are classified as non-small-cell lung cancers (NSCLC), a group made up of squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma (Minna et al. 1989). In order to define the molecular events that may drive the development of NSCLC, we have studied the structure and expression of two genes of potential importance, those encoding cyclin D1 and the epidermal growth factor receptor (EGFR). The cyclin D1 gene is a member of a family of genes that encode proteins important to the regulation of the cell cycle (Sherr 1993). It resides on a region of chromosome 11 that was originally identified as the breakpoint of the t(11;14) translocation in B cell lymphomas, termed \textit{bcl1}. Amplification of this region, including co-amplification of two nearby oncogenes, \textit{int2} and \textit{hst1}, was found in cancers of the breast, head and neck, and other sites prior to the cloning of the cyclin D1 gene (Ali et al. 1989; Berenson et al. 1989; Liderauer et al. 1988; Merritt et al. 1990; Somers et al. 1990). But as neither \textit{int2} nor \textit{hst1} was demonstrated to be highly expressed as a consequence of amplification (Liscia et al. 1989), they were not easily implicated in the pathogenesis of these tumors. The cyclin D1 gene was subsequently shown to be co-amplified with these loci, and appears to be the gene that is overexpressed as a consequence of this process (Jiang et al. 1992; Motokura et al. 1991; Schuuring et al. 1992a), and thus appears to be the \textit{bcl1} region oncogene. Amplification and overexpression of the cyclin D1 gene has been reported in...
DNA analysis

High-molecular-mass DNA from tumor and uninvolved lung tissue specimens was studied by Southern blot analysis. DNA was digested to completion with HindIII, BamHI or EcoRI in the presence of 4 mM spermidine. Approximately 10 µg digested DNA was separated in 0.8% agarose gels, transferred to nylon membranes and probed with cDNA fragments of the genes of interest labeled with [32P]dCTP by random priming to a specific activity of 10^7 dpm/µg. Hybridization was carried out in 5×SSPE (NaCl/sodium phosphate/EDTA), 5×Denhardt’s solution, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/ml salmon sperm DNA at 65°C for 24 h. Membranes were then washed sequentially in 2× standard saline citrate (SSC) at 25°C for 20 min, 0.1×SSC, 0.1% SDS at 50°C for 5 min and subjected to autoradiography.

RNA analysis

RNA from tumor and uninvolved lung tissue was studied by Northern blot analysis. A 10-µg sample of total RNA was separated by electrophoresis in formaldehyde gels, transferred to nylon membranes, and probed with cDNA fragments labeled as described above. Hybridization was carried out in 50% formamide, 10% dextran sulfate, 1 M NaCl, 0.4% SDS, 100 µg/ml salmon sperm DNA at 42°C for 24 h. Membranes were washed in 2× SSC, 1% SDS at 65°C for 30 min and subjected to autoradiography.

Immunohistochemical analysis

Fragments of the frozen tissue specimens were cut by cryostat into 6-µm sections in duplicate and examined first by hematoxylin and eosin staining to confirm the histology of the tissue as tumor or uninvolved lung. Serial sections were then fixed in cold acetone and rinsed in phosphate-buffered saline (PBS), and endogenous peroxidase activity was quenched with 0.5% H2O2 for 15 min. Sections were then washed in PBS three times, incubated in 10% rabbit serum for 20 min, and then incubated in primary antibody (Amersham), or irrelevant control antibody for 60 min. Sections were then washed again in PBS, incubated in rabbit anti-(mouse immunoglobulin) secondary antibody for 30 min, washed in PBS, incubated in peroxidase/anti-peroxidase antibody for 30 min, washed in PBS, incubated in 0.5% H2O2 for 15 min, and rinsed in phosphate-buffered saline (PBS). The tissue was incubated in Diaminobenzidine for 3–5 min, washed in PBS, incubated in 0.5% H2O2 for 15 min, and rinsed in phosphate-buffered saline (PBS). The tissue was mounted in Canada balsam and imaged with a light microscope.

Materials and methods

Specimen collection and processing

Patients enrolled on Lung Cancer Study Group (LCSG) protocols who required surgical resection of their primary, untreated non-small-cell cancers were also enrolled on LCSG study 871, which was designed to evaluate molecular events in this disease. Specimens of tumor and, whenever possible, adjacent uninvolved tissue were removed during surgery, quick frozen in liquid nitrogen and stored at −70°C until processed. A fragment of each specimen was homogenized, and DNA and RNA were extracted, quantified by fluorometry, and tested for the presence of high-molecular-mass species by gel electrophoresis as described (Ausubel et al. 1989). Remaining tissue fragments from each specimen were embedded in freezing medium for immunohistochemical analysis. The tumors were classified by histopathology and surgical stage at the participating hospitals throughout North America (Mountain 1986). The cohort analyzed included 116 adenocarcinomas, 33 large-cell carcinomas, 119 squamous cell carcinomas, 9 mixed-histology tumors, and 4 tumors of other histological types. No histological information was available for 17 of the specimens. The tumors were staged according to the classification of Mountain (1986) as stage I, 61%; stage II 14%; and stage III 24%.

Statistical analysis

Tabulated values were analyzed by Pearson’s χ²-test. In those instances where the observed frequencies were too small for the required assumptions, Fisher’s exact test was substituted. Tabulated values were combined in cases where the observed events were scarce, and the biological sense was not changed.

Results

Cyclin D1

The gene for cyclin D1 and the adjacent loci int2, hst1, and the bell breakpoint marker pRe8SS were studied by