Cellular protooncogenes are infrequently amplified in untreated non-small cell lung cancer

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Summary To examine a potential contribution of protooncogene abnormalities other than point-mutational activation of the K-ras protooncogene in the classification of non-small cell lung cancer, amplification of cellular protooncogenes was studied in 47 lung tumour specimens obtained at thoracotomy and in four lung tumour cell lines. The primary tumours included 21 adenocarcinomas, nine large-cell carcinomas, 13 epidermoid carcinomas, one carcinoid and three metastases of primaries outside the lung. The copy numbers per haploid genome of 11 protooncogenes in every tumour sample were determined: H-ras, K-ras, N-ras, c-myc, N-myc, L-myc, erbB, mos, myb, neu (erbB-2) and raf amplifications. The c-myc gene was amplified 5-7-fold in two adenocarcinomas, the H-ras gene 3-5-fold in one adenocarcinoma, while the K-ras and the neu gene were amplified in lung metastases from a colorectal and a breast cancer primary respectively. None of the tumours with an amplified protoonogene simultaneously harboured a mutationally activated K-ras gene. We conclude that amplification of the investigated protooncogenes is a rare event in non-small cell lung cancer. In view of the two c-myc amplifications detected, a systematic study of c-myc expression levels in non-small cell lung cancers appears worthwhile.

Lung cancer is usually divided in four major types: small cell carcinoma (representing 25%), epidermoid carcinoma (30%), adenocarcinoma (25%) and large-cell carcinoma (15%), with uncommon or mixed types making up the remaining 5% (Sobin, 1982). The distinction between small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) is clinically important because SCLC is treated mostly with chemotherapy while the treatment of choice in NSCLC is surgery. NSCLC, in turn, comprises a heterogeneous group of tumours, the classification of which might possibly be improved by determining the activation of protooncogenes in the tumours. It is hoped that elucidation of the genetic mechanisms in lung cancer will eventually result in the design of improved treatment strategies.

The primary candidates in the search for the pathogenic mechanisms in cancer are the known cellular protooncogenes, which have been shown to be important in the transformation of cells in several model systems (for review see Varmus, 1984). Activation of these oncogenes can result from several mechanisms, including gene amplification, allelic deletion, gene rearrangement or certain (point-) mutation(s) in the protooncogene sequences (for reviews see Nishimura & Sekiya, 1987; Altalio & Schwab, 1986). In some cases protooncogene activation correlates well with tumour type or with biological behaviour of the tumour (Yokota et al., 1986b; Seeger et al., 1986; Slamon et al., 1987). Of the known protooncogenes, only a few have been found to be consistently altered in human lung carcinoma (for review see Rodenhuis, 1988). Amplification of the cellular protooncogenes of the myc family has been observed in primary SCLC tumours in several studies (Nau et al., 1985; Wong et al., 1986; Brooks et al., 1987), and has in some cases been correlated with clinical behaviour (Johnson et al., 1987; Funa et al., 1987). In NSCLC, several protooncogene aberrations have been described, including amplification of c-myc (Yokota et al., 1986b; Cline & Battifora, 1987), N-myc (Saksela et al., 1987), (Pulci et al., 1985; Vosden et al., 1986), erbB (Yokota et al., 1986a; Cline & Battifora, 1987; Berger et al., 1987), and neu (Cline & Battifora, 1987) genes. Other genetic abnormalities, such as loss of H-ras and myb alleles (Cline & Battifora, 1987) or chromosomal deletion of an unknown gene on the short arm of chromosome 3 (Naylor et al., 1987; Kok et al., 1987) have also been reported.

We have previously shown that the K-ras protooncogene is activated by point-mutation in about a third of all adenocarcinomas of the lung (Rodenhuis et al., 1987, 1988). In addition to screening our NSCLC DNAs for this type of oncogene activation, we subjected the samples to Southern blotting analysis to detect possible oncogene amplification. A total of 11 genes were examined, concentrating on those which have been described amplified in human primary (lung) tumours. Any consistent protooncogene amplifications might thus be correlated with the clinical data available on the patient donating the tumour.

Materials and methods

Lung carcinoma specimens were obtained at surgical resection in The Netherlands Cancer Institute, The Antonius Hospital in Nieuwegein or at one of the regional hospitals collaborating in the study. After transportation to the pathology department, a representative part of the tumour was snap-frozen and stored at -70 °C until analysis. Before isolation of cellular DNA, cryostat sections were obtained and the percentage of tumour cells in the specimen was estimated. Non-neoplastic regions were carefully removed so that the final tumour specimens contained 50% or more neoplastic cells.

Of 48 patients, 21 had adenocarcinomas, 13 epidermoid carcinomas, and 9 had large-cell carcinomas according to the World Health Organization classification (Sobin, 1982). The four remaining tumours were a carcinoid and three lung metastases, three from colorectal and one from a breast primary (Table I). Classification of the tumour specimens was done independently by two diagnostic histopathologists (S.S.W. and W.J. Mooi). Clinical parameters such as tumour size, tumour spread, smoking habits, interval to recurrence, etc., were obtained by reviewing the hospital charts. The cell lines A549 and NCI-H23 have been described previously (Liebert et al., 1976; Carney et al., 1985). The GLC-A1 and GLC-A2 cell lines are human lung adenocarcinoma cell lines and were a gift from Dr L.F.H.M. de Leij from the University of Groningen, The Netherlands.

DNA was isolated using standard techniques and used for dot-blotting and for Southern blotting as described previously (Rodenhuis et al., 1987). For Southern blotting the samples were digested with the indicated restriction enzymes (obtained from Boehringer Mannheim, Mannheim, Federal Republic of Germany), separated on 0.8% agarose gels and

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blotted onto Hybond membranes (Amersham International, Amersham, UK) using procedures as indicated by the manufacturer. Protooncogene probes were nick-translated and hybridised to the filters using standard techniques (Janssen et al., 1985). The following molecular probes were used: H-ras 'pEJ' (Goldfarb et al., 1982), K-ras 'pLC3' (Shimizu et al., 1983), N-ras 'clone B' (Hall et al., 1983), c-myc 'pMC-413RC' (Dalla-Favera et al., 1982b), N-myc 'pNB-1' (Schwab et al., 1983), L-myc 'pLmuc10' (Nau et al., 1985), erbB 'fragment III' (Ullrich et al., 1984), mos 'pHBl' (Watson et al., 1982), myb 'pHM2.8' (Leprince et al., 1983), and neu 0.4 kb BamHI cDNA fragment (Bargmann et al., 1986). After hybridisation the blots were washed at a final stringency of 0.1 x SSC (SSC is 0.15 mol sodium chloride and 0.015 mol trisodium citrate per litre) at 65°C. Control hybridisations were done with actin encoding sequences from the pACT-1 plasmid (Dodemont et al., 1982), with a final wash done in 2 x SSC, and 0.5% SDS at 55°C. Autoradiography was performed with Kodak XAR-5 of XS-1 films at −70°C using an intensifying screen. Amplifications were quantified using dot-blot dilution series of the DNAs of tumours containing protooncogene amplifications.

Results

A total of 52 DNAs from human non-small cell lung carcinomas were studied. The histology data of the tumours from which the DNA was isolated are presented in Table I. Each DNA sample was analysed by Southern blotting after digestion with the restriction enzymes HindIII or EcoRI. Initially, using the dot-blot technique, we screened all tumour DNAs for amplifications of the cellular protooncogenes erbB, c-myc and neu. Using this technique we detected a 10-15-fold amplification of the neu protooncogene in a solitary metastasis of a breast carcinoma but not in any of the primary lung carcinomas (results not shown).

Subsequently all tumour DNAs were digested with the restriction enzyme EcoRI, blotted onto nylon membranes and hybridised with probes for the three ras, the ral and myb genes. In Figure 1 an example of such a hybridisation is shown. After separate hybridisations of these Southern blots, no abnormal copy numbers of the N-ras, ral or myb genes could be detected. The H-ras gene hybridisation is described below. Hybridisation with the K-ras probe revealed as 15-20-fold amplification in a solitary lung metastasis of colorectal origin which we described previously (Rodenhuis et al., 1987), but no K-ras gene amplifications in the other tumour specimens.

All tumour DNAs were analysed on Southern blots after digestion with HindIII. These blots were hybridised with the probes for the three myc, the erbB, mos and H-ras genes. As shown in Figure 2, the H-ras gene was found amplified 3-5-fold in a single adenocarcinoma (lane 1) but not in any other of the investigated NSCLCs. In addition, a 2-3-fold amplification of the H-ras gene was detected in the GLC-A1 cell line (results not shown). In Figures 2 and 3 examples of hybridisations with the mos probe are shown, which did not reveal a single amplification in any of the DNAs. In lane 3 of Figure 2 an amplification of the c-myc gene in an adenocarcinoma is presented.

![Figure 1 Southern blot hybridisations of EcoRI digested NSCLC DNAs with probes for H-ras, N-ras, ral, myb and K-ras. Lanes 1 and 3, adenocarcinomas; lane 2, epidermoid carcinoma; lane 4, large-cell carcinoma. In these four DNAs none of the hybridisations revealed amplifications of the indicated genes.](image)

| Table 1: Histological classification of the investigated tumours |
|---------------------------------------------------------------|
| Cancer | No. |
|--------------|-----|
| Adenocarcinoma | 21 |
| Epidermoid carcinoma | 13 |
| Large-cell carcinoma | 9 |
| Carcinoid | 1 |
| Solitary metastases | 3 |
| Breast | 1 |
| Colorectal | 4 |
| Adenocarcinoma cell line | |
| Total | 52 |

![Figure 2 Southern blot hybridisations of HindIII digested NSCLC DNAs with probes for c-myc, H-ras and c-mos, and actin control. Lanes 1, 2 and 3 are from adenocarcinomas. In lane 1 an amplification of the H-ras gene is present, while the sample in lane 3 shows an amplification of the c-myc gene.](image)

In Figure 3 the Southern blot hybridisations are shown of six representative, HindIII digested tumour DNA samples hybridised with probes for c-myc, L-myc and N-myc as well as for erbB and mos. In lane 4 the probe for c-myc detects a 5-7-fold amplification of this gene. This stronger signal is not seen with either the N- or L-myc protooncogene probes. Amplification of a c-myc gene could be detected in two of 48 tumours, both of which were adenocarcinomas (Table II). The N- and L-myc genes were not amplified in any of the DNAs we investigated, and neither was the erbB gene.

Discussion

Activation of protooncogenes by amplification is a well studied mechanism that has been reported for several primary human tumours. These include c-myc in SCLC and NSCLC and in other cancers, N-myc in neuroblastoma, retinoblastoma and chronic myeloid leukaemia, c-myc in acute myeloid leukaemia, H-ras and K-ras in bladder carcinoma, and neu in adenocarcinomas of the stomach and breast. Amplifications have also been found in many cell
lines derived from human tumours (for reviews see
Nishimura & Sekiya, 1987; Alitalo & Schwab, 1986). These
data should be interpreted with caution, however, since
somatic amplification of genes has been implicated in several
adaptive responses of cells to environmental stresses. Selec-
tion for drug resistant subpopulations of cells may give rise
to double-minute chromosomes (DMs) or to homogeneously
staining regions (HSRs), which represent large regions of
amplified DNA. Serial passage of cells in culture may also
select for cells with gene amplifications (Alitalo & Schwab,
1986). Thus it is conceivable that some amplifications of
protooncogenes in human tumours may only reflect the
presence of a specific selection pressure, such as chemo-
therapy, rather than play a role in the pathogenesis of the
tumour. For this reason we concentrated on lung tumours
from patients with limited disease, who had not received
chemotherapy before surgery.

In 48 primary NSCLC and in four NSCLC derived cell
lines no amplifications of the cellular protooncogenes myb or
mos were detected. Since the c-mos gene is localised on
chromosome 8 in the same region as c-myc, the detected
increased copy numbers of c-myc are likely to result from
gene amplification rather than from chromosome
duplication.

Although several reports indicate that the c-erbB proto-
oncogene, encoding the epidermal growth factor receptor
(EGFR), has a role in the carcinogenesis of human lung
cancer, we did not find any abnormal copy numbers of this
gene. Recently published data on this subject indicate that
enhanced levels of the EGFR frequently occur in epidermoid
lung carcinomas (Berger et al., 1987) and less commonly in
other NSCLC types (Veale et al., 1987). High expression of
erbB does not necessarily require amplification of the gene,
as has been shown in a study including 25 NSCLC (Lee et al.,
1987). Furthermore, high expression of the EGFR has
been observed in several different tumour types and cell lines
without amplification of the gene (Alitalo & Schwab, 1986).
Although the erbB gene has been found amplified in some
NSCLCs (Cline & Battifora, 1987), overexpression of the
EGF receptor without amplification appears to occur much
more frequently. These data combined with our findings
indicate that activation by amplification of the erbB gene
may represent only a minority of the activational mecha-
nisms of increased EGFR expression.

Similar to the apparent involvement of the erbB gene in
the carcinogenesis of epidermoid tumours, the neu
protooncogene may have a role in the pathogenesis of
adenocarcinomas. This gene has been reported to be ampli-
fied in five of 63 glandular tumours, but not in the three
adenocarcinomas of the lung which were also investigated
(Yokota et al., 1986a). In about 20% of breast carcinomas
the neu gene is found amplified (Van De Vijver et al., 1987),
a phenomenon that appears to have clinical implications
(Slamon et al., 1987). This report shows that in 21 lung
adenocarcinomas not a single amplified neu gene was
present. We thus conclude that the gene is not frequently
amplified in NSCLC. The only case of a neu amplification
we did detect was in a solitary lung metastasis of a breast
carcinoma. A similar case of neu amplification in a lung
metastasis from a breast primary has been described for a
case in which the primary tumour had normal copy numbers
of the gene (Yokota et al., 1986b). Thus, amplification of
the neu gene might in some cases aid in distinguishing different
types of adenocarcinoma.

Members of the ras family of protooncogenes are
expressed in many normal and tumour cells (for review see
Bus, 1988). These genes have been described as being altered
in human lung tumours by amplification (Pulciani et al.,
1985; Vousden et al., 1986) as well as by point mutation
(Rodenhuis et al., 1987), both types of activation also being
frequently observed in cell lines (Nishimura & Sekiya, 1987).
In our series of NSCLC specimens we have described the
frequent (about 30%) mutational activation of the K-ras
gene in lung adenocarcinomas, but not in other types of
NSCLC (Rodenhuis et al., 1988). The K-ras and N-ras
protooncogenes were not amplified in any of the primary
lung carcinomas. This result is in agreement with other
studies (Heighway & Hasleton, 1986; Cline & Battifora,
1987). The only ras amplification we found in this study was
of the H-ras gene in a single adenocarcinoma, which was K-
ras mutation negative. This is the first time an H-ras
protooncogene amplification is detected in a fresh tumour
specimen of a NSCLC. In general, however, amplifications of
the ras genes are rare in NSCLC.

Ras gene abnormalities in lung metastases of colorectal
origin seem to occur more frequently: of four studied
samples one had a K-ras amplification, while two others
harboured ras gene mutations, one in N-ras cordon 61 and
one in K-ras codon 12 (Rodenhuis et al., 1988). These
mutations are known to occur with some frequency in colon
cancers (Bus et al., 1987). Thus, three of four colorectal
metastases to the lung (including one we did not test for ras
amplification) contain an activated ras protooncogene. It is

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Table II  Summary of tumours with oncogene amplifications

| Patient no. | Age | Histology               | TNM category | Oncogene amplified | Level of amplification |
|------------|-----|-------------------------|--------------|--------------------|------------------------|
| 18         | 68  | Metastasis of colorectal cancer |              | K-ras             | 15–20 ×                |
| 19         | 53  | Metastasis of breast cancer  |              | c-neu              | 10–15 ×                |
| 42         | 57  | Large-cell               | T1N0M0       | c-myc             | 5–7 ×                  |
| 105        | 65  | Adenocarcinoma           | T1N1M0       | H-ras             | 3–5 ×                  |
| 107        | 71  | Adenocarcinoma           | T2N0M0       | c-myc             | 5–7 ×                  |
| GLC-A1     |     | Adenocarcinoma cell line |              | H-ras             | 2–3 ×                  |
tempting to speculate that ras activations of any type may be associated with the ability of colorectal cancer cells to metastasise to the lungs. In several model systems a correlation between ras expression and metastasis formation has been described, including that of H-ras transfected NIH-3T3 fibroblasts (Egan et al., 1987), and a system of mouse T lymphoma cells (Collard et al., 1987).

The myc family of protooncogenes has frequently been found to be altered in lung cancer, although most of the data focuses on SCLC and SCLC-derived cell lines. The latter tend to have amplified myc genes (Johnson et al., 1987; Kiefer et al., 1987), which is reflected by overexpression at the mRNA level (Ibson et al., 1987). Amplifications of the cellular myc genes in primary SCLC have also been described, but these occur with much lower frequency than in SCLC cell lines (Saksela et al., 1986; Wong et al., 1986). For NSCLC only a few studies report myc gene amplifications in primary tumours. The c-myc gene was observed to be amplified in one of two bronchioloalveolar carcinomas (Yokota et al., 1986a), and in three of 26 NSCLC, all adenocarcinomas (Cline & Battifora, 1987). Our findings confirm that the c-myc protooncogene is amplified in a minority of NSCLCs, in our study two of 48 samples, one large-cell carcinoma and one adenocarcinoma. It thus appears that the c-myc amplifications, though infrequent in NSCLC, do occur in adenocarcinoma. In SCLC myc amplification has been reported to correlate with a poor prognosis (Johnson et al., 1987). Whether or not this is also the case in NSCLC cannot be determined at present. Two other members of the myc gene family, N-myc and L-myc (Schwab et al., 1983; Nau et al., 1985), commonly amplified in SCLC cell lines, have occasionally been described to be amplified in uncultured lung tumours (Minna et al., 1986; Saksela et al., 1986). In our series no N- or L-myc amplifications could be detected. An important role for amplification of these genes in NSCLC is thus unlikely. Future investigations will have to elucidate the occurrence of other myc gene activations such as overexpression or translocation in NSCLC.

Since we previously screened in NSCLC DNAs for mutational activation of the cellular ras genes (Rodenhuis et al., 1987, 1988), the simultaneous presence of an amplified protooncogene with a mutated ras gene could be examined. At least for one lung carcinoma xenograft maintained in nude mice it has been described that an amplified and mutationally activated K-ras gene can occur simultaneously with an amplified c-myc gene (Taya et al., 1984). Despite the relatively high frequency of K-ras codon 12 mutations in our series, we did not detect alterations of more than a single protooncogene. This seems to indicate that unidentifed protooncogenes can complement for K-ras mutations and/or protooncogene amplifications in the pathogenesis of human lung cancer.

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