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c-K-*ras* and p53 Mutations Occur Very Early in Adenocarcinoma of the Lung

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The topographical distribution of a mutation provides insight into past patterns of tumor evolution. This approach was applied to two loci commonly mutated in adenocarcinoma of the lung—p53 and c-K-ras. In 41 primary adenocarcinomas, c-K-ras codon 12 point mutations were detected in 8 (19.5%) tumors and p53 point mutations were detected in 10 (24.4%) tumors, with one tumor harboring both mutations. These mutations were only detected in malignant cells and with a homogeneous topographical distribution throughout 16 tumors, including metastasis. Intratumor heterogeneity was detected in only one tumor in which a small portion lacked the specific p53 mutation. Based on this topographical analysis, it is likely that when these mutations occur in adenocarcinoma of the lung, they are usually acquired during the very earliest phases of tumor formation before the bulk of clonal expansion, and in very small precursor lesions. (Am J Pathol 1994, 144:303–309)

Lung carcinoma is caused by cigarette smoking and characterized by poor long term survival. The majority of these tumors are non-small cell lung carcinomas, of which adenocarcinoma is the most common histological type.[1] The best survival in non-small cell lung carcinoma is with surgical resection at early stage of the disease.[2] However, even with apparent curative resection, the 5-year survival rate of stage I disease is approximately 50%.[2]

Neoplasia results from the sequential accumulation of multiple mutations.[3] Recent genetic studies have revealed that c-K-*ras* oncogene or p53 tumor suppressor gene alterations commonly occur in adenocarcinoma of the lung.[4–8] Adenocarcinomas containing these alterations have been significantly associated with a poorer prognosis compared with similar tumors lacking these mutations,[9–12] although few studies have directly examined both mutations.[13,14] The mechanism by which these mutations confer a poorer prognosis is unclear.

It is estimated that approximately 10 to 20 mutations are present in lung cancer.[1] The relative acquisitions of these mutations can be established by determining their topographic distributions. New mutations must result in tumor heterogeneity until clonal dominance is established, since a mutation is likely acquired in a single cell. Therefore, a homogeneous distribution implies that the mutation occurred before clonal expansion, whereas a heterogeneous distribution implies the mutation occurred relatively late during tumor progression. In this study, adenocarcinomas of the lung were analyzed for c-K-*ras* and p53 mutations to establish their frequencies and to determine when they were acquired during tumor progression.

Materials and Methods

Patients and Tissues

The fixed tissues from 41 patients with resected adenocarcinoma of the lung were analyzed. These cases were randomly selected based on the availability of paraffin blocks from the City of Hope National Medical Center (n = 35) and the Los Angeles County-University of Southern California Medical Center (n = 6) from the years 1980 to 1990. The adenocarcinomas were classified by established criteria[15] and included four bronchioloalveolar carcinomas (n = 4). Clinical data and patient follow-up were extracted from the pathology reports, medical records, and the City of Hope Tumor Registry. Sta-

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tistical analysis was performed with Fisher's exact test and log rank test, with $P < 0.05$ considered significant. Survival was measured from the time of surgical resection.

Polymerase Chain Reaction

DNA was extracted from stained tissue sections after histological examination to ensure at least 50% tumor cells. These composite tumor sections were screened for c-K-*ras* mutations by polymerase chain reaction (PCR) as previously described,[16] except that primers (5'TCAAAGAATGGTCCTGGACC, GACTGAATATAAACTTGTGG) which yield a 158-bp first exon k-*ras* product were used. The PCR products were dot blotted and screened for codon 12 or 13 point mutations with radiolabeled allelic specific oligomer (ASO) probes.[17] The wild-type *ras* allele was detected from all specimens, indicating their suitability for PCR.

p53 mutations in exons 5 to 8 were detected using SSCP as previously described.[18] Exon 5 was divided into two segments with two additional internal primers (5' CTGTGACTGCTTGTAGATGG and GGGTTGATTCCACACCCCCG). Electrophoresis of the labeled PCR products was performed at 12 W for 14 to 20 hours on a 6% non-denaturing acrylamide gel with 20% glycerol at room temperature. Bands of altered mobility were excised from the gel, reamplified with the same primers, and then cloned (TA Cloning Kit, Invitrogen, San Diego, CA). Clones were screened for p53 sequences by low stringency hybridization with complementary oligomer probes. Five to 10 positive clones were sequenced (Sequenase 2.0, US Biochemicals, Cleveland, OH) with at least two clones harboring the same mutation. ASO hybridization probes (17 mers) homologous to the corresponding wild-type and mutant sequences were synthesized to distinguish between alleles. False positive mutant p53 sequences were detected in three tumors and were considered amplification artifacts, since sequence-specific oligomer probes failed to hybridize to subsequent PCR products from the entire tumor section or multiple tumor regions isolated by selective ultraviolet radiation fractionation (SURF).

SURF

The topographic distribution of each p53 or *ras* mutation was determined using SURF.[19] Multiple paraffin blocks including at least two with tumor were analyzed to cover all possible cellular phenotypes. Multiple ink dots (5–30/slide) were placed directly over the desired cells either manually or with a micromanipulator and micropipettes. The dotting was documented with a video camera and recorder. The dots covered 50 to 300 cells (usually 100–200 cells) with tumor fractions at least 70% pure and normal fractions not contaminated by tumor cells. After dotting, the DNA in the unprotected cells was inactivated with short wave ultraviolet (254 nm) radiation directly exposed on a transilluminator (Model TS20, UVP, San Gabriel, CA) for 3 to 4 hours.

Table 1. *Adenocarcinomas with c-K-*ras *or p53 Mutations*

Tumor case	Size (*cm*)	Stage	Outcome*	c-K-*ras*⁺	p53
A	2.5 × 2.0	III	59 NED‡	CYS	WT
B	2.0 × 2.0	I	34 NED	VAL	WT
C	4.0 × 4.0	I	53 DWD	VAL	WT
D	3.5 × 3.2	I	7 DWD	CYS	WT
E	2.0 × 1.5	I	10 DWD	CYS	WT
F	4.5 × 4.0	I	1 DWD	CYS	WT
G	1.4	I	23 DWD	ALA	WT
H	6.5 × 5.5	I	112 NED	CYS	204 GAG to TAG (GLU to STOP)
I	3.3 × 2.5	I	30 NED	WT	174 AGG to TGG (ARG to TRP)
J	6.0 × 6.0	III	16 DWD	WT	175 CGC to CAC (ARG to HIS)
K	2.5 × 2.0	I	31 DWD	WT	194 CTT to CGT (LEU to ARG)
L	13 × 9.0	III	12 DWD	WT	214 CAT to CGT (HIS to ARG)
M	6.0 × 4.5	I	24 NED	WT	238 TGT to TAT (CYS to TYR)
N	3.5 × 2.7	I	50 DWD	WT	245 GGC to TGC (GLY to CYS)
O	1.5 × 1.5	II	115 AWD	WT	249 AGG to AGT (ARG to SER)
P	10	I	NFU	WT	266 GGA to CGA (GLY to ARG)
Q	4.2 × 3.5	I	27 DWD	WT	273 CGT to CCT (ARG to PRO)

Summary: 8/41 (19.5%) c-K-*ras*; 10/41 (24.4%) p53; 17/41 (41.5%) c-K-*ras* or p53; 1/41 (2.4%) c-K-*ras* and p53.
* Outcome in months from the time of surgery.
‡ All mutations were in codon 12.
‡ NED, alive with no evidence of disease; DWD, dead with disease; AWD, alive with disease; WT, no mutation detected; NFU, no follow-up available.

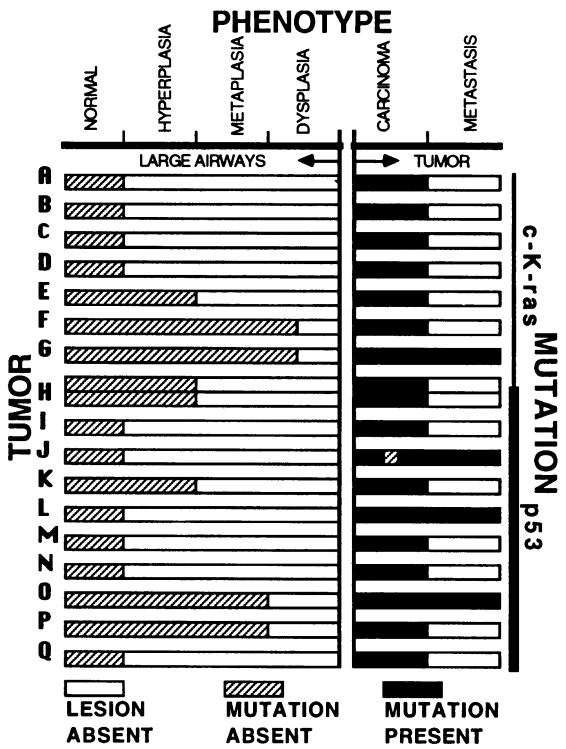

Figure 1. *Graphic representation of the topographic distributions of the c-K-ras and p53 mutations. These mutations were only detected in malignant cells and with homogeneous distributions, except for case J (see Figure 3). Hyperplastic, metaplastic, and dysplastic epithelium were only present in some of the large airways and direct physical relationships between the peripheral adenocarcinomas and these more proximal lesions were absent.*

PCR was performed with the same primers used to screen the composite tumor sections for 36 to 50 (usually 44) cycles. Approximately one-third of the DNA extracted from each dot was amplified. The PCR products were dot blotted and analyzed with labeled ASO probes specific for the wild-type or mutant p53 or c-K-*ras* alleles. An inactivation or "no dot" control consisting of irradiated but unprotected tissue was included with each PCR and demonstrated no detectable amplification. The entire SURF and PCR procedures were repeated at least twice on mirror sections to verify the distribution of the mutations.

Results

Point mutations in c-K-*ras* were detected in 8 (19.5%) of the 41 adenocarcinomas (Table 1). All were in codon 12 with seven G to T transversions (5 CYS, 2 VAL) and one G to C transition (ALA). The topographies of the mutations were determined by SURF followed by PCR. Small cell groups (50–300 cells) of relatively pure composition of normal and at least 20 different tumor regions were analyzed for the mutation. The results are summarized in Figure 1. c-K-*ras* mutations were not detected in stroma, alveoli, and normal, hyperplastic, metaplastic, or dysplastic bronchial epithelium. There were no direct histological transitions between the peripheral tumors and the more proximal dysplastic or metaplastic epithelium of the large airways. c-K-*ras* mutations were only detected in the malignant cells and then with a homogeneous distribution. In less than 5% of the tumor dots, only the wild-type allele could be detected. However, a second SURF and PCR of a mirror section failed to confirm this heterogeneity.

p53 mutations in exons 5 to 8 were detected from 10 (24.4%) of the 41 adenocarcinomas (Table 1). Loss of heterozygosity (LOH) at the p53 locus, as determined by amplification of only the mutant allele, was sometimes detected. Unfortunately, LOH determinations were inconsistent and extremely dependent on the number of PCR cycles and amounts of added DNA. Therefore, LOH data is not reported here. In one tumor, both c-K-*ras* and p53 mutations were present and had identical topographic distributions (Figure 2). All of the p53 mutations were point mutations with a range in the specific types of changes. Although G to T transversions were the most common mutation, they represented only 3 of the 10 mutations. Similar to the c-K-*ras* topography, the p53 mutations were absent in nonmalignant tissues and uniformly present in the carcinoma tissues, except for case J (Figure 1). In this case, the mutation was present in the majority of the tumor including the metastasis, but absent in a small portion (less than 10% of the total tumor) at the margin of the tumor (Figure 3). The majority of the tumor with the p53 mutation was well differentiated with small glands whereas the small portion without the mutation was poorly differentiated.

The clinical data for the 41 adenocarcinomas are presented in Table 2. The patients with c-K-*ras* or p53 mutations were similar to the patients without the mutations. A greater fraction of the tumors with mutations were in stage I (76%) compared with the tumors without mutations (67%), but this difference was not significant. Mutations were not detected from all four bronchioloalveolar carcinomas, although three were from cigarette smokers. Survival was generally shorter for the patients whose tumors contained mutations. However, with the small numbers of patients, this difference was not significant

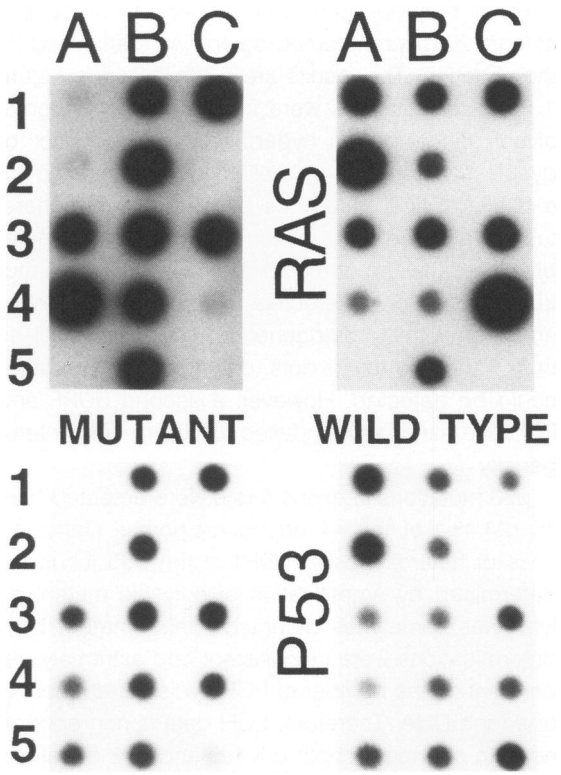

Figure 2. *Dot blots of the c-K-*ras *and p53 PCR products of case H. Illustrated is the DNA and their subsequent PCR products isolated from 11 different "dotted" cell groups after SURF. An aliquot of each fraction (A1–5, B1–5, C1) was amplified for c-K-*ras *or p53. Mutations were absent in the normal tissue fractions (A1–2) but homogeneously present in the tumor fractions (A3-C1). No amplification was detected from an unprotected tissue region (C2), indicating complete inactivation by the ultraviolet radiation. The controls for c-K-*ras *were a composite or unfractionated section (C3), normal DNA (C4), and a water blank (C5). The controls for p53 were two composite sections (C3–4) and normal DNA (C5). Note that the PCR for the A5 tumor fraction was successful for p53 but not for c-K-*ras*. The etiology for this discrepancy is unclear but likely reflects the small amounts of DNA protected by each dot.*

for c-K-*ras* or p53 when considered together or separately or when only stage I tumors were compared.

Discussion

Mutations in p53 and c-K-*ras* are thought to be among the most frequent alterations in lung cancer. Previous studies have suggested that p53 or c-K-*ras* mutations occur early during transformation, since they have been detected in all stages of lung cancer.[4,5,7] The goal of this study was to determine more precisely when these mutations are acquired by examining their topographic distributions. The distribution of a mutation depends on whether it was acquired early or late during clonal expansion. The homogenous tumor distributions of the c-K-*ras* and p53 mutations, even in the smallest adenocarcinomas, indicate that they were acquired before the bulk of the present tumor growth. In one tumor, there was a homogeneous and identical distribution of both its p53 and c-K-*ras* mutations, suggesting that they were acquired in the same cell before clonal expansion.

Detection of these mutations in surrounding normal, hyperplastic, metaplastic, or dysplastic epithelium would further extend the timing of mutation to before transformation. Mutations were not present in such epithelium, although unfortunately, most adenocarcinomas, as in this study, develop distal to the large airways and do not display obvious histological transitions.[1] The absence of identical c-K-*ras* or p53 mutations in the large airways is not surprising, since this is the site of squamous cell carcinomas. Mutant p53 alleles have been detected in the dysplasia adjacent to squamous cell lung cancers[20–22] and by the SURF technique (O. Shibata, unpublished observations). The absence of mutations in proximal airways suggests that it will be difficult to screen for adenocarcinomas by molecular methods.

Adenocarcinoma of the lung lacks an established histological precursor lesion[1] and therefore the earliest stage of multi-step carcinogenesis is likely to occur in a very small and indolent lesion. Our findings also suggest that any antecedent histologically recognizable tumors or preneoplastic lesions lacking these mutations were likely to be much smaller than the current tumors. A maximum estimate of the size of such a precursor lesion is less than 10% of the current tumor, assuming the ability to detect at least 10% intratumor heterogeneity (as with case J) and a static growth rate of the hypothetical preneoplastic lesion. For the six cases with small T1 lesions (tumors less than 3 cm in size), the precursor lesions without p53 or c-K-*ras* mutations are estimated to be less than 0.3 cm. Multiple mutations are likely necessary for transformation[3] and the exact order in which they are acquired cannot be determined in this study. Nevertheless, p53 or c-K-*ras* mutations are typically acquired very early relative to the bulk of clonal expansion and clinical presentation. A recent study also suggests that c-K-*ras* mutations may occur years before clinical presentation.[23]

A heterogeneous distribution was detected for a p53 mutation in only one tumor (case J). This small region of intratumor heterogeneity may theoretically represent the remnants of an earlier lesion, a genetically distinct synchronous tumor, or an advanced lesion which has loss the mutation. The later possibility is favored, since this region was less differen-

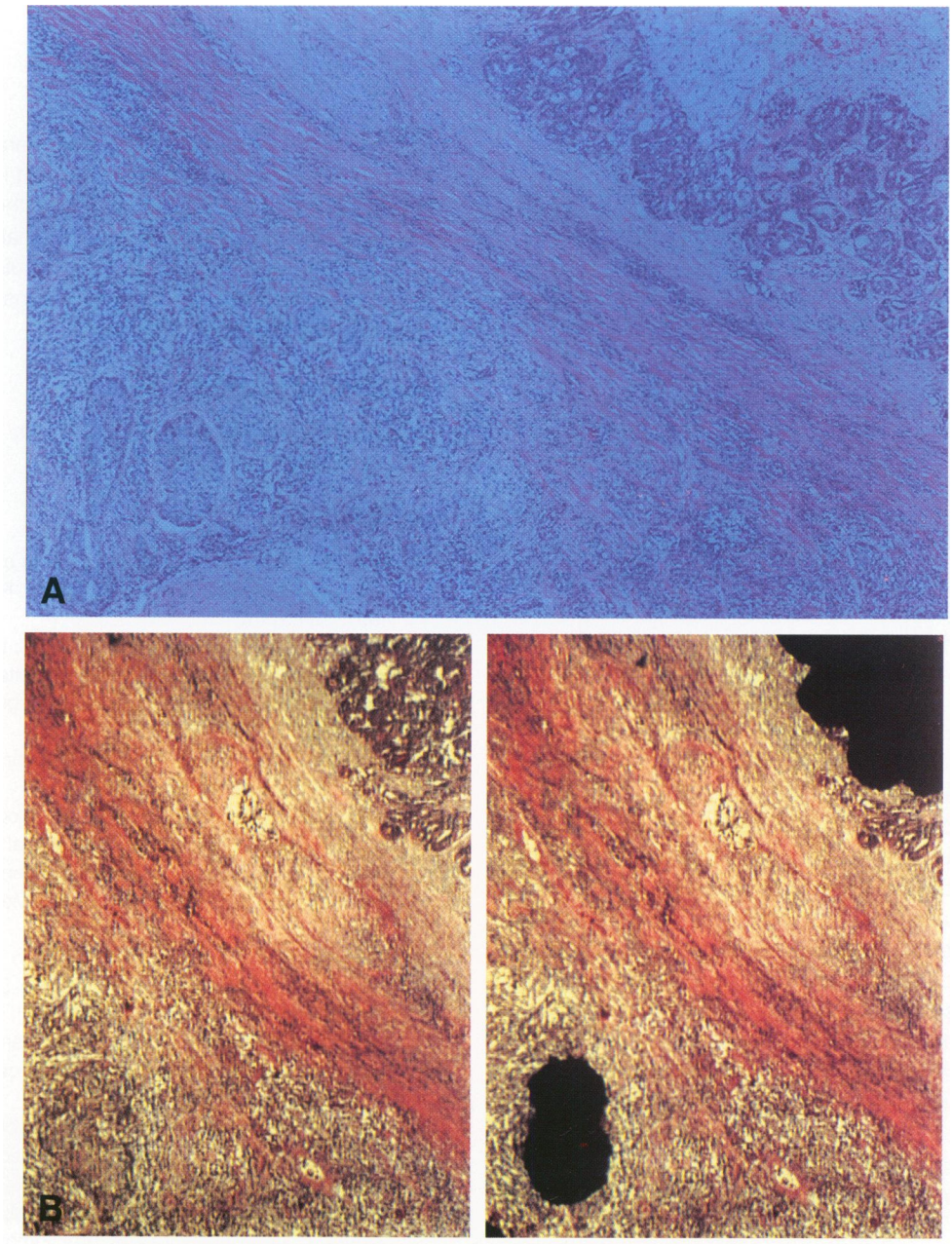

Figure 3. A: *Photomicrograph of the junction between the adenocarcinoma (case J) harboring the specific p53 mutation (right) and the more poorly differentiated adenocarcinoma without the mutation (left). The more poorly differentiated tumor comprised less than 10% of the entire tumor and was located along a margin (H&E, ×40).* B: *Video image of the same tumor before (left) and after (right) the dots (H&E, ×100, no coverslip).* C: *Dot blot of the p53 PCR products from the SURF of the section. The wild-type sequence of exon 5 could be detected throughout the tumor (A1-C2) but the specific point mutation in codon 175 could be only detected from the better differentiated tumor (B3–4, B6-C2) and was absent from the poorly differentiated tumor (A1-B2, B5). Controls consist of a no dot UV inactivation control (C3), a composite section (C4), normal genomic DNA (C5), and a water blank (C6).*

Table 2. *Clinical Data*

	c-K-ras mutation	p53 mutation	c-K-ras or p53 mutation	No mutation
Age	61.1	55.8	58.5	63.2
Sex (M:F)	3:5	7:3	9:8	12:12
Smoker	8/8	9/10	16/17	20/23
Stage				
I	7	7	13	16
>I	1	3	4	8

tiated and was at the margin of the tumor. Despite the loss of the specific p53 mutation, clonal expansion was evident and was likely compensated by other mutations, possibly even at another codon of p53. Another possibility is LOH, with loss of the mutant allele, rather than the wild-type allele, since LOH at the p53 locus has been noted to occur independent of point mutations.[5] Regardless of the specific mechanism, this small region of intratumor heterogeneity did not evidently represent a successful enhancement, since tumor progression, as represented by metastasis, occurred with the better differentiated carcinoma cells containing the specific p53 mutation. The persistence of the c-K-*ras* and p53 mutations in the majority of the tumors and their metastasis suggests that these mutations are essential to maintain the malignant phenotype.

In this study of primary tumors, the frequency of adenocarcinomas with c-K-*ras* mutations was similar to previous studies and confirms the clustering of mutations in codon 12 and G to T transversions.[4,8,12] The frequency of p53 mutation (24.4%) in this study was slightly lower than most other studies of primary tumor specimens (range of 14 to 41%).[5–7,11] Although only exons 5 to 8 of p53 were examined in this study, the majority of p53 mutations are present in these exons.[5,13] Similar to other studies,[7,13,14] the presence of a c-K-*ras* mutation did not significantly correlate with the presence or absence of a p53 mutation. Survival time tended to be shorter for patients with c-K-*ras* or c-K-*ras* and p53 mutations. However, with the small number of patients, we were unable to confirm that patients with tumors harboring these mutations had significantly shorter survival times. Although the exact role of these mutations in lung cancer is unknown, their homogeneous distribution and association with a worse prognosis suggest an inherently more aggressive phenotype from the very earliest phases of tumor formation.

The preneoplastic lesion of adenocarcinoma of the lung is poorly understood and defined and difficult to study because of its peripheral location. Based on this topographical analysis, it is likely that when c-K-*ras* or p53 mutations occur, they are usually acquired in early and relatively small precursor lesions. The mapping of additional mutations onto similar tumors should provide further insight into all phases of lung cancer evolution.

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