Widely Dispersed *p53* Mutation in Respiratory Epithelium

A Novel Mechanism for Field Carcinogenesis

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Abstract

Individuals with one aerodigestive tract malignancy have a high incidence of second primary aerodigestive tumors. The mechanism for this field effect has not been determined. We studied an individual with widespread dysplastic changes in the respiratory epithelium but no overt carcinoma. The entire tracheobronchial tree obtained at autopsy was embedded in paraffin, and bronchial epithelial cells were isolated by microdissection. DNA extracted from the microdissected cells was analyzed for point mutations in the *p53* tumor suppressor gene. A single, identical point mutation consisting of a G:C to T:A transversion in codon 245 was identified in bronchial epithelium from 7 of 10 sites in both lungs. Epithelium at sites containing the *p53* mutation was morphologically abnormal, exhibiting squamous metaplasia and mild to moderate atypia. No invasive tumor was found in the tracheobronchial tree or any other location. Cells from peripheral blood, kidney, liver, and lymph node exhibited no abnormality in the *p53* gene. The widespread presence of a single somatic *p53* point mutation in the bronchi of a smoker suggests that a single progenitor bronchial epithelial clone may expand to populate broad areas of the bronchial mucosa—a novel mechanism for field carcinogenesis in the respiratory epithelium that may be of importance in assessing individuals for risk of a second primary tumor as well as in devising effective strategies for chemoprevention of lung cancer. (*J. Clin. Invest.* 1997. 100:2133–2137.) Key words: lung neoplasms • precancerous conditions • smoking • tumor suppressor genes • field cancerization

Introduction

Individuals with one aerodigestive carcinoma are at high risk for developing a second primary tumor. The term "field cancerization" was introduced in 1953 by Slaughter et al. to describe the frequent occurrence of multiple primary tumors in patients with oral squamous carcinoma (1). More recently, it has become clear that multiple carcinomas occur frequently in the lower respiratory tract as well. The incidence of synchronous primary lung cancers is estimated at 0.26–2.3% (2–9). The incidence of metachronous second primary lung carcinoma increases with time after treatment of the original tumor (10–13), reaching 12.6% per patient per year 10 yr after treatment for limited stage small cell carcinoma (10).

Little is known of the cellular and molecular mechanisms through which field cancerization takes place in the lung. One hypothesis is that high exposure of respiratory epithelium to multiple carcinogens in tobacco smoke leads to multiple different genetic mutations at dispersed sites in the airways. Alternatively or concurrently, a single mutant progenitor epithelial cell clone might expand to populate widespread areas of the respiratory tract. In the latter case, a common mutation would be expected at multiple sites in the tracheobronchial tree. Origin from a single mutant progenitor cell clone has been proposed to explain identical allelic losses in multiple bladder tumors (14), but in lung, a study of a limited number of multiple primary tumors has indicated separate genetic profiles (15). Common specific mutations such as identical *p53* mutations have not been reported, challenging the validity of the single mutant clone hypothesis. However, it is possible that genes which commonly mutate in the early stages of lung carcinogenesis have not been characterized, resulting in a lack of specific molecular markers with which to identify an expanded mutant progenitor cell clone.

In this report, we describe a patient with a history of smoking, chronic obstructive pulmonary disease, and widespread dysplastic lesions in his bronchial epithelium. A *p53* codon 245 transversion was found in company with wild-type *p53* in 7 of 10 widely dispersed sites of the bronchial epithelium, but not in multiple other tissues. The most plausible explanation is the expansion and dispersion of a mutant progenitor epithelial cell clone throughout the airways, documenting a novel mechanism for field cancerization in the respiratory epithelium.

Methods

The patient was a 66-yr-old white male smoker (50-pack-yr) with chronic obstructive pulmonary disease (forced expiratory volume in 1s [FEV1]/forced vital capacity [FVC] = 1.4/2.91 = 0.49) and sputum cytology that revealed moderate atypia (16). He was admitted to the hospital with nausea, vomiting, and abdominal distention and was diagnosed with small bowel obstruction. An exploratory laparotomy revealed a kinked small bowel due to adhesions at the site of an appendectomy many years in the past. Adhesions were lysed, and the kinked bowel loop was reduced. Unexpectedly, 4 d after surgery, the patient was found cyanotic and apneic with a bradycardia of 30 beats per minute. Attempts at resuscitation were unsuccessful.

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An autopsy was performed within 12 h of death. At autopsy, the only significant anatomic abnormality detected was severe coronary arteriosclerosis with an old left ventricular myocardial infarct. Death was attributed to cardiac arrhythmia secondary to advanced coronary artery disease. No tumor was detected in any organ, including the lungs.

Morphologic analysis

The lungs were fixed for microscopic study by infusion of 10% buffered formalin through the trachea. Serial cross-sections of the entire tracheobronchial tree were mapped according to anatomic location, embedded in paraffin, and sectioned at 6 μm. Hematoxylin and eosin–stained bronchial cross-sections exhibited squamous metaplasia with low grade (mild to moderate) atypia at multiple locations in the tracheobronchial tree. Areas of moderate dysplasia, squamous metaplasia, and normal epithelium were dissected precisely under microscopic visualization (17). Control cells from hilar lymph node, peripheral lung, kidney, and liver were scraped from hematoxylin and eosin–stained sections. Approximately 1,000 cells were isolated from the same anatomical site in multiple serial sections. DNA was extracted from microdissected cells by proteinase K digestion (17). Microdissections and DNA extraction were performed de novo in both the Texas and Colorado laboratories to minimize the possibility of spurious results due to contamination of reagents with mutant DNA.

Analysis of p53 mutation

DNA from ~ 100 cells (~ 1 ng) was analyzed by two methods, single-strand conformation polymorphism (SSCP)[1] with direct sequencing, and restriction fragment–length polymorphism (RFLP).

SSCP with direct sequencing. Initially, DNA was amplified for SSCP (18) directly from proteinase K digestion products by PCR using six nested primer sets encompassing exons 5–9. The sequences of the primer sets used for PCR are as follows: 5A sense external, 5′-TTCAACTCTGTCTCCTTCCT-3′, and internal, 5′-TTCCTCTTC-CTGCAGTACTC-3′; 5A antisense external, 5′-CACAACCTCCGT-CATGTGCT-3′, and internal, 5′-ACTGCTTGTAGATGGCCATG-3′; 5B sense external, 5′-GTGCAGCTGTGGGTTGATTC-3′, and internal, 5′-GTTGATTCCACACCCCCG-3′; 5B antisense external, 5′-ACCCTGGGCAACCAGCCCTGT-3′, and internal, 5′-CAGC-CCTGTCGTCTCTCCAG-3′; 6 sense external, 5′-ACAGGGCT-GGTTGCCCAGGGT-3′, and internal, 5′-GCCTCTGATTCCTCA-CTGAT-3′; 6 antisense external and internal, 5′-AGTTGCAA-ACAAGACCTCAG-3′; 7 sense external and internal, 5′-GTGT-TGTCTCCTAGGTTGGC-3′; 7 antisense external, 5′-GTCAGAG-GCAAGCAGAGGCT-3′, and internal, 5′-CAGTGTGCAGGGTG-GCAAGT-3′; 8 sense external and internal, 5′-TATCCTGAGT-AGTGGTAA-3′; 8 antisense external, 5′-AAGTGAATCTGA-GGCATAAC-3′, and internal, 5′-CACCGCTTCTTGTCCTGCTT-3′; 9 sense external, 5′-GCAGTTATGCCTCAGATTCAC-3′, and internal, 5′-TTATCACCTTTCCTTGCCTC-3′; 9 antisense external and internal, 5′-AAGACTTAGTACCTGAAGGGT-3′.

A water blank control was included with each PCR. Amplification products were screened for *p53* mutation by SSCP. DNA from suspected sites of mutation was amplified using nonlabeled internal primers, gel-purified, and used as template for direct sequencing. Amplified DNA was then sequenced using a femtomolar thermocycle sequencing kit (Promega Corp., Madison, WI) with autoradiography.

RFLP analysis of p53 gene mutation at codon 245. Because direct sequencing revealed a mutation at codon 245 (see below), we designed nested primer pairs for the *p53* gene which result in an amplicon of 152 bp that contains a restriction site (GCGG, nucleotides 730–733) at codon 245 for the enzyme AciI (New England Biolabs, Inc., Beverly, MA). The wild-type sequence was cut by the enzyme AciI, resulting in two fragments 71 and 81 bp in length. The mutation G→T at nucleotide 733 abolishes this restriction site. As a positive control, we used a lung cancer line (NCI-H596) that has a mutation at codon 245, GGC→TGC (19), identical to the mutation identified in the respiratory epithelium of the patient. A lymphoblastoid cell line (BL1963) was used as control for the presence of the wild-type sequence (negative control).

The nested primer sequences used for exon 7 amplification were as follows: sense external, 5′-AAGGCGCACTGGCTTCATCTT-3′, and internal, 5′-TGGGCCTGTGTTATCTCCT-3′; antisense external, 5′-GCACAGCAGGCCAGTGTCAG-3′, and internal, 5′-GGG-TGGCAAGTGGCTCCTG-3′.

Results

10 sites in the tracheobronchial tree were tested for the presence of *p53* mutation by SSCP and direct sequencing. Seven sites exhibited an abnormality in exon 7 by SSCP analysis, which on sequencing proved to represent mutation (G→T transversion) in codon 245 (Fig. 1). The mutation was found at widespread sites within the tracheobronchial tree, as shown in Fig. 2. This mutation was identical at all the sites exhibiting an SSCP abnormality. In all sites, the mutation was confirmed by repeat testing, from microdissection through sequence analy-

1. *Abbreviations used in this paper:* RFLP, restriction fragment–length polymorphism; SSCP, single-strand conformation polymorphism.

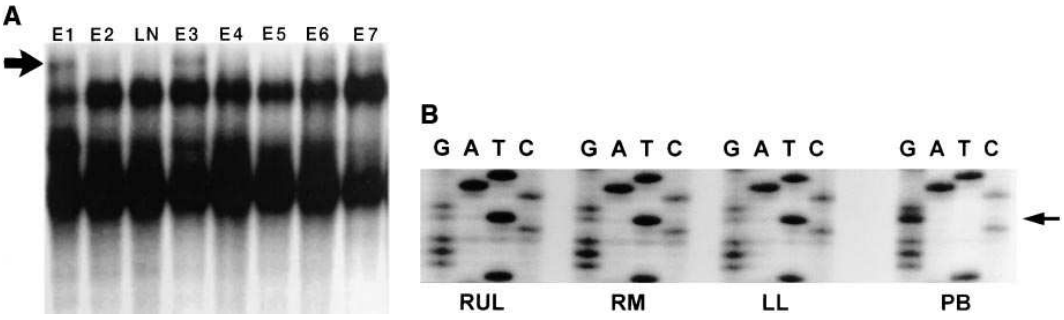

Figure 1. (*A*) Scanned image of SSCP gel radiograph of exon 7 PCR products. Various sites from which cells were obtained for DNA extraction and amplification are represented in gel lanes labeled as follows: *E1*, epithelium, left upper lobar bronchus; *E2*, epithelium, left upper lobar bronchus; *LN*, lymphocytes, left upper lobe; *E3*, right posterior basal segmental bronchus; *E4*, right upper lobar bronchus; *E5*, right upper lobar bronchus; *E6*, right lower lobar bronchus; *E7*, right middle lobar bronchus. *Arrow*, Mutant band of variable intensity in amplification products of epithelial DNA from various sites. Lane *LN* containing lymphocyte DNA exhibits no mutant band. (*B*) Sequencing gel showing mutation at codon 245 and indicating presence of G:C→T:A transversion and replacement of a glycine codon (GGC) with a cysteine codon (TGC) in the mutant *p53* gene. The mutation is present in DNA extracted from bronchial epithelial cells microdissected from the upper lobe right lung (*RUL*), middle lobe right lung (*RM*), and lower lobe left lung (*LL*), but not in DNA from the peripheral blood (*PB*).

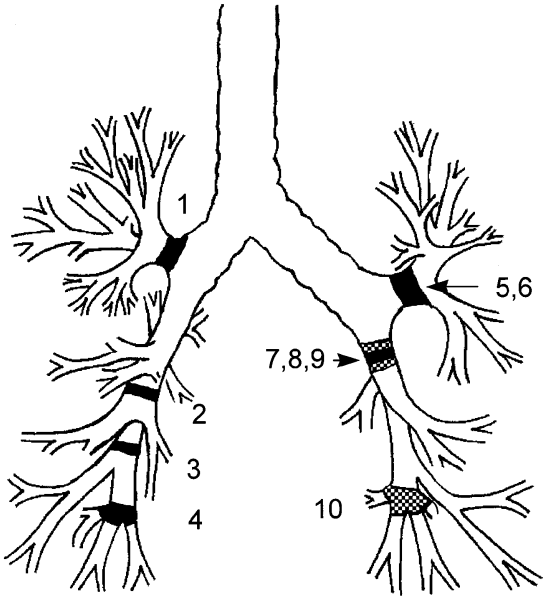

Figure 2. Diagram of tracheobronchial tree showing locations of epithelium harboring only wild-type (*checkered bands*) or wild-type and mutant *p53* (*black bands*) alleles detected by SSCP and sequencing of mutant alleles. Mutation was present in epithelial cells from 7 of 10 widely dispersed sites, including all sites tested in the right lung (*left*), and two upper lobar and one lower lobar site in the left lung (*right*). The following bronchial sites are shown: *1*, Upper lobar; *2*, middle lobar; *3*, lower lobar; *4*, posterior basal segment; *5* and *6*, upper lobar (two sites); *7*, *8*, and *9*, lower lobar (three sites); *10*, posterior basal segment.

sis, and identical results were obtained in each duplicate sample, making contamination of PCR solutions with mutant sequence highly unlikely. Renal tubules, lymph nodes, and liver were analyzed at multiple sites, and no mutation was identified in these specimens or found in peripheral blood cells. In all the sites exhibiting mutation, mild to moderate squamous atypia was observed in the microdissected cell populations.

In the RFLP analysis of codon 245 (exon 7) of the *p53* gene (Fig. 3), the lymph node and microdissected histologically normal epithelium demonstrated only wild-type (71- and 81-bp fragments) *p53* sequence, whereas in the microdissected bronchial squamous metaplasia (left upper lobe) and dysplasia (right upper lobe) specimens, both mutant (152-bp) and wild-type (71- and 81-bp fragments) sequences were detected. Histologically normal peripheral lung demonstrated mainly wild-type sequence, but a faint band corresponding to the mutant sequence was detected.

Discussion

Recent molecular studies have indicated that malignant epithelial tumors result from a stepwise series of mutations that begin in situ before the occurrence of stromal invasion. This sequence of mutations has been especially well-documented in the colon (20), where the advantage of grossly visible adenomatous polyps permits localization of sites of premalignant molecular change. Mutations are most prevalent in tumor suppressor genes (21, 22), and may occur in a preferential order. For example, in colorectal carcinogenesis, 5q loss at the adenomatous polyposis coli locus often precedes loss of the DCC (deleted in colorectal cancer) gene and *p53* mutation (23, 24).

In the lung, one of the most widely studied molecular changes in both pulmonary malignancies and premalignant epithelium is mutation in the *p53* tumor suppressor gene. Missense point mutations are found in the *p53* gene at exons 5–9 in 56% of invasive tumors (25). The most common base substitution observed in lung carcinomas of smokers is G:C→T:A transversion, also found in this patient. It has been suggested that such a transversion occurs in carcinogen-exposed cells when a bulky carcinogen–DNA adduct forms at the G base on the transcribing DNA strand (25, 26). The altered base cannot be read by the cell's replicative machinery, and DNA polymerases preferentially fill in the nontranscribed strand with an adenine on the strand opposite to the adduct (27). This results in G→T substitution in the coding strand during the subsequent round of transcription.

Documentation of sequential mutations during lung carcinogenesis has been complicated by the absence of macroscopically visible lesions to indicate sites of premalignant change. However, despite this obstacle, an increasing body of evidence suggests that mutations may occur in nonmalignant bronchial epithelium. Several studies have documented *p53* mutations in noncancerous bronchial mucosa of lungs resected

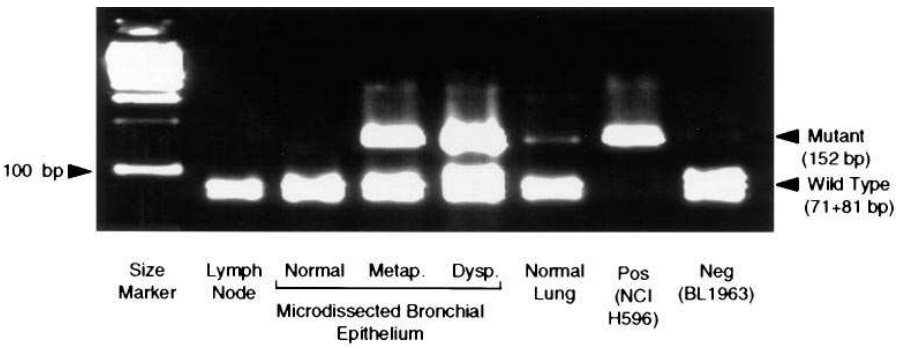

Figure 3. RFLP analysis of codon 345 (exon 7) of the *p53* gene using microdissected bronchial epithelial samples, lymph node, and peripheral lung tissues. The lymph node and microdissected histologically normal epithelium demonstrate only wild-type (71- and 81-bp fragments) p53 sequence, whereas in the microdissected bronchial squamous metaplasia (left upper lung) and dysplasia (right upper lung specimens, both mutant (152-bp) and wild-type (71- and 81-bp fragments) sequences were detected. Only one band is seen in the wild-type sequence after digestion because of the similar length (71- and 81-bp) of the fragments. Histologically normal peripheral lung demonstrates mainly wild-type sequence, but a faint band corresponding to the mutant sequence was detected. *Pos*, Positive control for the presence of mutant sequence (lung carcinoma cell line NCI-H596). *Neg*, Negative control for the presence of mutant sequence (lymphoblastoid cell line BL1963). Size marker, 100-bp ladder.

for invasive carcinoma (28–30). In those reports, four cases are described in which *p53* mutations in nonmalignant bronchial epithelium occurred synchronously with identical mutations in tumor cells. Three of these four mutations are missense transversions involving exons 5 or 7, while the fourth is a missense transition in exon 5. Mutations found in both tumors and accompanying benign bronchial epithelium have also included loss of heterozygosity of chromosomes 3p (17, 28, 31) and 9p (32). All of the studies describing mutations in tumor and benign bronchial mucosa have evaluated limited areas of benign bronchial epithelium near the tumor at the time of surgical resection, but have not examined epithelium distant from the tumor.

Patterns of loss observed to date at widely dispersed bronchial sites in individuals without lung cancer have not provided support for the notion that lung carcinogenesis may be the result of expansion of mutant epithelial cell clones in the respiratory epithelium, as this case does. In a recent study, Mao et al. have reported that apparently random patterns of allelic loss on chromosomes 3p and 9p are more frequent than the shared loss of the same allele at multiple bronchial sites (33). Moreover, allelic loss is a very early event in lung carcinogenesis that appears to correlate with smoking.

Although several reports indicate that p53 protein expression may be increased in bronchial epithelium before the occurrence of invasive carcinoma (34–38), few studies have evaluated genetic abnormalities in bronchial epithelium before and in the absence of invasive cancer. One recent report (30) describes two biopsies obtained sequentially at 9-mo intervals from the same bronchial site in a single patient without detectable tumor. Both biopsies contained the same two *p53* mutations (a stop codon in exon 5, and a codon deletion in exon 7). One of the mutant alleles was present in low copy number in the earlier biopsy but in much higher copy number in the later biopsy, suggesting expansion of a subclone with the double mutation over the 9-mo interval between biopsies. With the exception of this single case, the issue of whether *p53* mutations occur in bronchial mucosa before the appearance of invasive carcinoma has not been addressed, nor has the extent of mutation in the airway been described.

The most striking and surprising finding in this case is the widespread distribution of bronchial cells bearing a single point mutation in *p53* codon 245, a codon which is frequently mutated in lung cancer (25). A thorough autopsy, including microscopic examination of the entire tracheobronchial tree and examination of all organs including the brain, showed no evidence of tumor. The widespread *p53* mutation in the tracheobronchial tree was verified by repetitive testing at all sites, from the point of microdissection with consistent results by SSCP and directed sequencing. Tissue sections were also microdissected at the laboratory of one of the co-authors (A.F. Gazdar), and results were confirmed by an entirely different RFLP technique. In SSCP, sequencing, and RFLP gels, the mutant allele in the *p53* gene was always accompanied by the normal allele, suggesting that the mutation in this case was heterozygous. The absence of this mutation in the peripheral blood and nonpulmonary parenchymal organs indicates that the mutation in this case is somatic, not germ-line.

That mutant cells may have had a proliferative advantage in this patient is consistent with current understanding of the function of p53. The *p53* gene product is regarded as a cell cycle checkpoint, arresting progression through the G1 phase of the mitotic cycle in response to cellular injury and allowing time for repair of replication errors (39–41). Loss of p53 function is postulated to speed passage through the mitotic cycle, and to render cells prone to replication error, further mutation, and malignant transformation. In invasive tumors, *p53* is inactivated by a combination of mutation in one allele and deletion or mutation in the second (40). However, it is probable that the two mutations do not occur simultaneously, but that heterozygously mutated epithelium is present in the airways for some time before complete inactivation of the *p53* tumor suppressor gene occurs.

What effect heterozygous mutation might have had on the phenotype of respiratory epithelium is suggested by the mechanism of action of p53. Functional p53 forms a tetramer through a domain at the COOH terminus of the protein (42–45), the tetramerization domain. Inactivation of p53 occurs predominantly through mutation in exons coding for a DNA-binding domain (25), leaving the tetramerization domain unaffected. Therefore, mutant p53 monomer may be incorporated with wild-type monomer into a tetrameric complex, but incorporation of the mutant monomer inactivates the entire complex, exerting a dominant negative effect (46, 47).

However, dominance of the mutant form may be incomplete. Using a bicistronic vector in which mutant and wild-type *p53* are expressed equally, Frebourg et al. (48) found that p53 transcriptional activity was reduced but not abolished, and that growth of the Saos-2 osteosarcoma cells, which lack *p53*, was suppressed by stable transfection with the vector. It is of interest that one of the p53 mutants tested in this system contained the same G:C to T:A transversion in codon 245 found in this patient, and that p53 transcriptional activity of the bicistronic vector containing this vector was $\sim$ 40% of the activity of the wild-type protein transfected alone. While coexpression of mutant and wild-type p53 may result in enough p53 transcriptional activity to suppress tumor cell growth, transcriptional activity may be reduced sufficiently to provide a proliferative advantage to nonmalignant epithelial cells.

The presence of an identical *p53* point mutation at multiple foci in the lower respiratory tract in this case suggests broad expansion of a single mutant bronchial epithelial clone. This finding strongly supports this novel mechanism of field carcinogenesis. Additional studies will be necessary to determine the frequency with which expansion of clones of premalignant bronchial epithelium containing mutations of *p53* and other genes involved in carcinogenesis occurs. To date, no additional genetic abnormalities (loss of heterozygosity involving a limited number of chromosome 3p and 9p alleles) have been defined in these foci. Prospective studies will be necessary to establish the prognostic significance and chronology of further mutational change in premalignant airway epithelium. However, we may already infer that interventional strategies designed to interrupt premalignant progression will have to take into account the possibility of widespread distribution of mutant epithelial cells. Finally, a promising prospect raised by this study is that common shared mutations may provide an opportunity to target a single mutant clone.

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