

# Detection of Oncogene Mutations in Sputum Precedes Diagnosis of Lung Cancer<sup>1</sup>

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## Abstract

The Johns Hopkins Lung Project developed an archive of sputum specimens during a randomized trial of lung cancer screening (1974–1982). We identified 15 patients from that trial who later developed adenocarcinoma of the lung. The primary lung carcinomas from 10 of these 15 patients contained either a *ras* or a *p53* gene mutation. Using a polymerase chain reaction-based assay, stored sputum samples obtained prior to clinical diagnosis were examined for the presence of these same oncogene mutations. In 8 of 10 patients, the identical mutation identified in the primary tumor was also detected in at least one sputum sample. The earliest detection of a clonal population of cancer cells in sputum was in a sample obtained more than 1 year prior to clinical diagnosis. These results provide the basis of a novel approach for detection of lung cancer based on the evolving molecular genetics of this disease.

## Introduction

Lung cancer is the leading cause of cancer-related deaths in western countries (1, 2). The prognosis for patients with lung cancer is primarily dependent on the stage of the tumor at the time of clinical diagnosis. Currently, only 25–40% of all lung tumors are considered resectable at the time of initial assessment and only 20% are found to have limited disease at the time of surgery. Patients diagnosed early with stage I tumors have a 40–70% survival following surgical resection (1, 3, 4). An attempt at lung cancer screening through the use of triannual sputum cytology and annual chest X-ray was found to be inadequate in the JHLP<sup>3</sup> study for the early detection of lung cancer (5, 6). There was no significant difference in mortality of more than 5000 participants screened by sputum cytology plus chest X-ray compared to those screened by radiographs alone. Many of these original cytological sputum samples are still available in the JHLP repository for the development of novel lung cancer screening techniques (7).

Tumors are now known to progress through a series of specific genetic changes in protooncogenes and tumor suppressor genes (8–10). These oncogene mutations can serve as specific markers for cancers because they are retained as tumors continue to grow. Detection of oncogene mutations in urine and stool obtained at surgery has successfully identified patients with bladder and colon cancer, respectively (11, 12). The JHLP repository provided the opportunity to test sputum samples obtained before a clinical diagnosis of lung cancer was established. We chose patients from that trial with cytological samples that were not diagnostic for cancer but who later developed lung cancer. Our goal was to determine whether molecular tests could augment conventional cytological techniques. In this pilot study, we

demonstrate the ability to detect rare lung cancer cells in cytologically “negative” sputum samples obtained months before clinical detection of lung cancer.

## Materials and Methods

**Patients.** We identified 15 patients from the JHLP trial who went on to develop adenocarcinoma or large cell carcinoma of the lung. These patients were chosen simply on the availability of paraffin-embedded tissue following surgical resection and a negative sputum cytology prior to definitive diagnosis. These histological types of lung cancer were selected because they have a higher incidence of *K-ras* mutations than other lung tumors (13, 14).

**Tissue Samples.** We collected paraffin-embedded primary lung tumor samples from the 15 patients selected. DNA was extracted from these paraffin samples and the first exon of the *K-ras* or exons 5–8 of the *p53* gene were amplified by PCR. These PCR products were then cloned and the *K-ras* gene was sequenced to detect mutations. Tumors not containing *K-ras* mutations were sequenced for *p53* mutations to identify tumor-specific markers (11, 12).

Stored, nebulized sputum samples from the JHLP archives containing 2% Carbowax/50% alcohol (Saccomanno's fixative) preservative solution were located (details on collection of samples in Refs. 5–7). From each sample, 5 ml were removed (approximately 50,000–500,000 cells), spun at 1000 × g for 5 min., and rehydrated in 5 ml of normal saline. Cells were then respun at 1000 × g, resuspended in 1 ml of 1% sodium dodecyl sulfate/proteinase K (5 mg/ml), and then incubated at 60°C for 6 h. DNA was extracted twice with saturated phenol-chloroform and ethanol precipitated. Dried DNA was resuspended in 50 μl, and 2 μl were used for each PCR reaction performed in a separate PCR-dedicated room to eliminate the possibility of contamination.

**Molecular Analysis of Sputum.** Sputum DNA was amplified by PCR with primers for *K-ras* and *p53* that contained *EcoRI* sites to facilitate cloning. Following 35 cycles of amplification, products were cleaved with *EcoRI* and ligated to Lambda Zap II (Stratagene, La Jolla, CA) (11, 12). XLI-blue cells infected with bacteriophage were plated on L-Agar at a density of 500–3000 plaques/plate, transferred to nylon membranes, and hybridized with oligonucleotides specific for wild type or mutant *K-ras* and *p53*. The oligonucleotides used for hybridizations were labeled with [<sup>32</sup>P]ATP and hybridized according to the method of Sidransky *et al.* (11, 12). Oligonucleotides used for detection included

WT *ras*: 5'-GGAGCTGGTGGCGTAGGCAA-3'  
 Val<sup>12</sup> mutant: 5'-GGAGCTGTTGGCGTAGGCAA-3'  
 Asp<sup>12</sup> mutant: 5'-GGAGCTGATGGCGTAGGCAA-3'  
 Ser<sup>12</sup> mutant: 5'-GGAGCTAGTGGCGTAGGCAA-3'  
 Cys<sup>12</sup> mutant: 5'-GGAGCTTGTGGCGTAGGCAA-3'  
 WT *p53*: 5'-ATGGGCGCCATGAACCGG-3'  
 His<sup>273</sup> mutant: 5'-TTGAGGTGCATGTTTGTG-3'  
 Gly<sup>281</sup> mutant: 5'-TGGGAGAGGCCGGCGCA-3'

## Results

We sought to develop a method for the early detection of lung cancer that could augment current cytological detection based on the emerging molecular biology of this neoplasm. Adenocarcinomas were chosen because these tumors have a higher incidence of *K-ras* mutations (30%) than other lung tumors (13, 14). Moreover, we looked for *p53* gene mutations because these are the most common genetic alterations found in these tumors and a variety of other cancers (15–17). Sequence analysis of the PCR products from the two target genes in 15 patients identified 10 primary tumors which contained

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<sup>3</sup> The abbreviations used are: JHLP, Johns Hopkins Lung Project; PCR, polymerase chain reaction; WT, wild type.

Table 1 Lung cancer patients with gene mutation analysis of sputum

Patient no.	Age (yr)/sex	Tumor location	Tumor type/ stage <sup>a</sup>	Tumor mutations	Mutations found in sputum		
					Pre-Dx <sup>b</sup> (mo.)	Diagnosis (M/T)	Postoperation
L1	65/M	RUL	A/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	K-ras-12Ser	3	1/270	Negative
L2	57/M	LUL	A/T <sub>2</sub> N <sub>1</sub> M <sub>0</sub>	K-ras-12Asp	1	1/300	N/A
L3	63/M	RUL	A/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	K-ras-12Val	4	1/100	Negative
L4	51/M	LUL	A/T <sub>3</sub> N <sub>0</sub> M <sub>0</sub>	p53-273His	13	1/320	N/A
L5	67/M	LUL	A/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	K-ras-12Cys	1	1/1200	Negative
L6	67/M	RUL	A/T <sub>3</sub> N <sub>0</sub> M <sub>0</sub>	p53-281Gly	Negative	0	Negative
L7	70/M	RUL	A/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	K-ras-12Cys	1	1/220	Negative
L8	59/M	RUL	A/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	K-ras-12Cys	1	1/170	Negative
L9	48/M	RUL	L/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	K-ras-12Val	Negative	0	Negative
L10	63/M	RLL	A/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	K-ras-12Cys	1	1/330	Negative
L11	60/M	RUL	A/T <sub>3</sub> N <sub>1</sub> M <sub>0</sub>	None	Negative	0	ND
L12	56/M	LLL	A/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	None	Negative	0	ND
L13	65/M	RUL	A/T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	None	Negative	0	ND
L14	61/F	RUL	A/T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	None	Negative	0	ND
L15	62/M	LUL	A/T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	None	Negative	0	ND

<sup>a</sup> Staging system is according to the ASCC (35), 1986. All sputum samples for patients L1 to L10 were reamplified and recloned, and the assay was repeated for verification. Control patients without cancer were negative by this assay.

<sup>b</sup> Pre-Dx, months prior to actual clinical diagnosis; M/T, number of mutant alleles/total clones with target gene insert; RUL, right upper lobe; LUL, left upper lobe; RLL, right lower lobe; LLL, left lower lobe; A, adenocarcinoma; L, large cell carcinoma; N/A, not available; ND, not done.

either a K-ras or a p53 gene mutation (Table 1). The K-ras mutations were those commonly associated with adenocarcinoma of the lung and occurred predominantly at codon 12 (13, 14). The two p53 mutations occurred at codons 273 and 281, and both were described previously in lung cancers (15–17). Many tumor samples were small (<1 cm) and DNA was insufficient for extensive sequence analysis of p53 (exons 5–8) in all tumors.

Following identification of tumors with oncogene mutations, we obtained all of the available corresponding sputum samples from these affected patients. None of the patients who we analyzed ever had a positive sputum cytology in the JHLP study. Most samples were read as minimally or moderately dysplastic but were not diagnostic for cancer and were not followed by any clinical intervention. Therefore, all but one of the patients (L4 evaluated because of cough) were diagnosed by chest X-ray.

Those sputum samples that were available (generally one to two samples prior to clinical diagnosis) were analyzed by a PCR-based assay able to detect 1 mutant-containing cell among an excess background of 10,000 normal cells (11, 12). This assay is based on the amplification of sputum DNA, followed by cloning into a phage vector and transfer to nylon membranes. A mutant-specific oligomer is then hybridized to each of the filters to identify specific point mutations in either the K-ras or the p53 gene present in sputum.

Using this assay, we detected neoplastic cells in previous “negative” cytological sputum samples from 8 of the 10 patients who had tumors containing oncogene mutations. Positive sputum samples harboring a clonal population of cancer cells were obtained from 1 to 13 months prior to clinical diagnosis (Table 1).

Detection of one cancer cell among 160 normal cells (1/320 ÷ 2 = 160; each normal cell contributes 2 WT alleles) was detected in sputum from patient L4 13 months prior to his clinical diagnosis (Table 1). The tumor from this patient contained >95% positive plaques when probed with a mutant-specific codon 273 oligomer for p53, and his sputum sample also contained several positive clones (Fig. 1). To verify identification of the codon 273 mutation in the sputum, a positive plaque from the sputum assay was picked and sequenced confirming presence of the same mutation (Fig. 2). In positive sputum samples, the ratio of cancer cells to normal cells ranged from 1:600 (patient L5) to 1:50 (patient L3), with the majority of samples containing approximately 1 cancer cell among 150 normal cells. Five control patients with lung cancer whose tumors did not contain either a K-ras (codons 12 and 13) or a p53 (exons 5–8) gene mutation by sequence analysis were negative by this assay for all 12 K-ras and both p53 mutant-specific probes.

In two patients, both of whom had only a single sputum sample available, we were not able to detect a clonal population of mutant-containing cells despite the presence of mutations in their primary tumors (Table 1). Furthermore, we were not able to trace the first emergence of a clonal population of cancer cells in positive patients by our sputum assay because of missing samples prior to diagnosis. However, in 6 of the 10 patients, sputum samples were available >24 months prior to diagnosis and were negative. This suggests 13–24 months as a limit for molecular diagnosis by our assay. As a further control, prior to clinical presentation, 6 of 8 patients who initially tested positive were found to test negative in sputum samples obtained following complete surgical resection of their tumor.

Discussion

We detected rare cancer cells in the sputum of 8 of 10 patients with oncogene mutations in their primary tumor prior to clinical diagnosis.

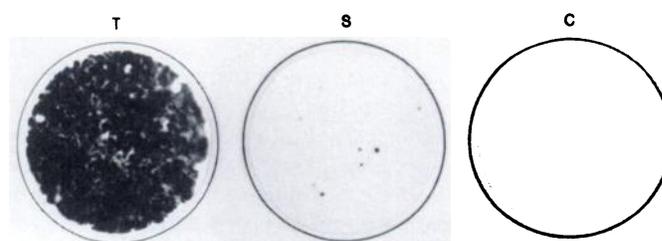


Fig. 1. Gene mutations in sputum. Many clones with a PCR insert from the p53 gene hybridized to an oligomer probe specific for the codon 273 Arg-His mutation in the patient’s (L4) tumor (T). A fewer number of clones hybridized to the same specific probe in the patients sputum (S). There were no hybridizing clones to this probe in a control sputum from a patient (L15) without a p53 gene mutation in his primary lung cancer (C).

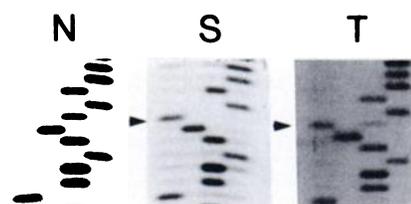


Fig. 2. Confirmation of detected mutations. Sequencing gels of pooled clones for p53 show the WT sequence in a normal (N) control and the 273 CGT-CAT (Arg → His) mutant sequence (arrow) in the tumor (T) with a residual WT band. One positive plaque (clone) depicted in Fig. 1 (S) was picked and sequenced revealing the same mutant band in sputum (S) as that found in the primary tumor. In each panel, the lanes represent A, C, G, and T terminations from left to right.

Those patients whose tumors did not contain oncogene mutations and control patients without cancer were also negative by our assay (data not shown). There did not appear to be any correlation between the site of the tumor and the ability to detect mutations in sputum samples by this assay. "False negative" samples may be due to inappropriate collection, poor cellular content, or the variable presence of tumor cells in sputum. Information gained from cytological diagnosis in sputum through routine light microscopy has previously suggested that examination of two or three samples may improve the diagnostic yield significantly (18, 19), and this likely pertains to molecular diagnosis as well. The percentage of cells identified here is significantly lower than those previously identified in urine (11) and stool (12) using a similar approach. This may be in part due to a large dilution effect from the inflammatory cells present in sputum samples (18, 19) or additionally, from the larger number of normal epithelial cells derived from the large surface area of the bronchopulmonary system.

Our stringent selection bias (negative sputum cytology and surgical resection) provided a group of patients with small lesions and a good outcome. All of these patients were eventually diagnosed by a positive radiograph and only six died because of their disease. Thus, patients with mutations detected in sputum 1–4 months prior to diagnosis still had small tumors amenable to surgical resection and a very favorable survival rate at 5 years (60%). In the JHLP trial, those patients diagnosed by sputum cytology generally had a better survival rate than those diagnosed by chest X-ray. Thus, our molecular screening approach may prove useful as an adjunct to cytological analysis and allow identification of patients who are candidates for surgical resection.

Patient L4 with a large T<sub>3</sub> lesion had the longest interval (13 months) from sputum detection to diagnosis. He also had a negative computed tomography scan-guided transthoracic biopsy 6 months prior to definite diagnosis because a small lesion was noted on chest X-ray. This patient thus had two opportunities for routine cytopathological detection prior to clinical diagnosis. He died of metastatic disease 4 months following surgical resection. This particular case serves to illustrate the limitations of routine cytology and the ability to augment morphological analysis through the use of probes for specific gene mutations.

We chose a laboratory detection method that necessitates cloning because it is quantitative and allows a precise estimation of the number of cancer cells present. Other PCR-based techniques (20–22) or tests using ligation in detection or amplification (23) could also be chosen since they can detect one mutant copy among  $\geq 10^5$  normal copies and are more amenable to automation. Furthermore, we targeted *K-ras* because of alterations previously found in adenocarcinomas, although these mutations are not found in other lung tumors. Although *p53* mutations are found in the majority of epithelial cancers, they usually occur as late events (24, 25). Recent evidence suggested that *p53* mutations may occur somewhat earlier in lung carcinogenesis (26–28) and our early detection of a *p53* mutation in the sputum of patient L4 supports this. However, because *p53* mutations are so varied, a specific oligomer has to be synthesized for identification of each mutation. Despite these limitations our findings suggest that *K-ras* and *p53* may be suitable targets for early detection strategies. As other gene alterations involved in lung cancer progression are identified, it will be possible to incorporate new markers into this novel molecular approach.

The sensitivity of this type of molecular approach raises the very real possibility of identifying cancer before a tumor mass can be seen by radiological methods. Patients thus identified could be candidates for chemoprevention strategies (29, 30). Repeated molecular diagnosis could serve as important intermediate end points in such chemo-

prevention approaches (31). In the absence of visible tumors, improved bronchoscopic assessment detecting intrinsic alterations in autofluorescence between normal and neoplastic cells could be used (32). Following visualization of "affected" mucosa, the laser bronchoscope could be used therapeutically to eliminate the cancer clone and prevent the development of a lung tumor. Novel biological approaches including various types of gene therapy could also be considered (33, 34).

We have demonstrated the ability to detect a clonal population of mutation-containing cells among an excess background of normal cells in sputum cytology samples. Due to the unique nature of these archival samples, we were able to follow the development of clinical lesions after sputum collection, indicating that these gene mutations may be detected with significant lead time prior to clinical diagnosis. Furthermore, those patients identified were amenable to surgical resection and possible cure. Because so many patients die of lung cancer each year, these results have significant implications for the use of emerging molecular techniques as adjuncts to cancer screening. Furthermore, the ability to detect gene mutations more than 1 year prior to clinical diagnosis holds promise for screening of patients at high risk for this deadly disease.

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