BIOMARKERS, VOL. 8, NO. 2 (MARCH–APRIL 2003), 156–161

Taylor & Francis
healthsciences

Comparison of K-*ras* gene mutations in tumour and sputum DNA of patients with lung cancer

LI-FANG ZHANG[1], WEI-MIN GAO[1], ROBERT GEALY[1], JOEL WEISSFELD[2,4], ELAINE ELDER[3], THERESA L. WHITESIDE[3,4] and PHOUTHONE KEOHAVONG[1,4]*

[1] Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA 15260, USA
[2] Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA 15260, USA
[3] Department of Pathology, University of Pittsburgh, Pittsburgh, PA 15260, USA
[4] University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15260, USA

Received 5 June 2002, revised form accepted 26 September 2002

Mutations in the K-*ras* gene are frequently found in lung tumours and are implicated in the development of lung cancer. In order to investigate the clinical usefulness of these mutations in lung cancer, we applied a sensitive method to compare mutations in codon 12 of the K-*ras* gene in DNA extracted from lung tumours and the matched sputum samples obtained from 22 lung cancer patients. K-*ras* mutations were identified in the lung tumours of 12 patients (54.5%) and in the sputum samples of 10 patients (45.5%). Nine patients showed an identical mutation in both the tumour and the matched sputum samples. There was a significant association between the presence of a K-*ras* mutation in a lung tumour and the detection of an identical mutation in the matched sputum sample of the lung cancer patient ($\kappa = 0.64$, 95% confidence interval 0.32–0.95, $p < 0.01$). K-*ras* mutations were detected in sputum samples from cancer patients with all lung tumour grades, and both in the presence and the absence of lymph node metastasis. Therefore, K-*ras* mutations may provide useful diagnostic markers for lung cancer.

Keywords: detection, diagnosis, lung cancer.

Introduction

Lung cancer is currently the leading cause of cancer mortality in the United States. It comprises 14% of new cancer cases in men and 13% in women (Parker *et al*. 1996). Non-small cell lung cancer (NSCLC) comprises 75–80% of all lung cancers, and includes adenocarcinoma (40–50%), squamous cell carcinoma (40–50%) and large cell carcinoma (10%) (Travis *et al*. 1995). In spite of improvements in surgery, radiotherapy, and chemotherapy, the 5 year survival rate for NSCLC is approximately 13%, which has improved only marginally during the last two decades (Naruke *et al*. 1988, Mountain 1989, Lippman *et al*. 1990).

These facts motivate the search for new ways to detect, stage, predict and monitor lung cancer (Schatzkin *et al*. 1990, Birrer and Brown 1992, Mao *et al*. 1994, Mills *et al*. 1995, Keohavong *et al*. 1996). One approach recognizes that cancer is a multi-step process involving key cancer-related genes (Harris 1991, Stanley 1995). The K-*ras* gene has been found to be mutated in many types of

* Corresponding author: Phouthone Keohavong, Department of Environmental and Occupational Health, University of Pittsburgh, 3343 Forbes Avenue, Pittsburgh, PA 15260, USA. Tel: (+1) 412 383 2087; Fax: (+1) 412 383 2123

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2003 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/1354750021000046589

cancers. In lung cancer, K-*ras* mutations are found in 30–50% of lung tumour cases, mostly in adenocarcinomas but also less frequently in squamous cell carcinomas (Barbacid 1987, Rodenhuis and Slebos 1992, Keohavong *et al*. 1996), and about 92% of them occur in codon 12 (Keohavong *et al*. 1996). This fact greatly facilitates the development of sensitive and specific methods for detecting K-*ras* mutations in clinical specimens. We have previously developed a sensitive method for detecting mutations in paraffin-embedded lung (Keohavong *et al*. 1997) and colorectal (Zhu *et al*. 1997) tissue sections obtained from patients with lung or colorectal cancer. For instance, K-*ras* mutations have been detected in lung tumours, tumour-adjacent and normal-appearing lung tissues (Keohavong *et al*. 1997).

K-*ras* mutations have been previously identified in sputum samples of lung cancer patients (Mao *et al.* 1994, Yakubovskaya *et al.* 1995). However, the relationships between the detection of these mutations in sputum samples and the presence of such mutations in the tumours of the same patients remain poorly understood. In this study, we applied a sensitive method to analyse K-*ras* mutations in tumour and matched sputum samples obtained from 22 lung cancer patients (15 adenocarcinomas, five squamous cell carcinomas and two large cell carcinoma), and propose specific detection of K-*ras* mutations in sputum as a potential molecular approach for detecting or monitoring lung cancer.

Materials and methods

Lung tissue and sputum samples

Fresh-frozen lung tumours and tissues were collected and stored between 1990 and 1999 from informed and consenting lung cancer patients who underwent lung resection at the University of Pittsburgh Medical Center under an IRB approval protocol. Matched sputum samples were also collected from the same patients over this same period. Subjects were instructed to take a deep breath, cough deeply and expectorate into a plastic cup. Each sputum sample was stored in 40 ml of Saccomanno's solution (39% ethanol, 3% polyoxyethylene and 2% isopropanol; Lerner Laboratories, Pittsburgh, Pennsylvania, USA) to fix and preserve the cells. To collect cells, each sputum sample in Saccomanno's solution was blended for 8–15 s in a blender to break the mucus and free the cells. The sample was then centrifuged at 600 *g* for 10 min. The supernatant was discarded and the cell pellet was embedded in oxytetracycline (OTC) solution and stored at −85°C until further study. A smear was prepared on microscope slides for cytological analysis. The diagnosis of carcinoma was confirmed by consulting surgical pathology reports for each patient. The smoking history and family history of cancer were determined by questionnaire. A total of 44 specimens, comprising 22 lung carcinomas and 22 matched sputum samples, were obtained from 22 lung cancer patients and analysed for K-*ras* mutations. These 22 lung cancer patients were the first cases with both tumour and matched sputum samples available for study, and included 15 adenocarcinomas, five squamous cell carcinomas and two large cell carcinomas. Information regarding gender, age, smoking and history of cancer for each patient, and clinical data regarding the histology, grade and stage of the lung carcinoma are shown in Table 1.

DNA preparation

Genomic DNA was isolated by treating each tissue in a lysis buffer consisting of 10 mM Tris, 10 mM ethylene diamine tetra-acetic acid (EDTA), 150 mM NaCl and 2% sodium dodecyl sulphate (SDS) with 10 μg RNase, followed by 20 μg ml^{-1} proteinase K, and extraction with phenol and chloroform and precipitation with ethanol. Each DNA sample was dissolved in Tris-HCl 10 mM, pH 7.5 and 2 mM EDTA, and then divided into small aliquots that were analysed for mutations independently. To extract DNA from sputum samples, material from each sputum sample was first recovered by centrifugation, washed twice with phosphate buffered saline (PBS), and DNA was extracted using the same extraction method as above.

Table 1. K-*ras* mutations in lung cancer patients.

						K-*ras* mutations	
ID	Age (years)	Sex	Histological type	Metastatic	Grade	Tumour	Sputum
67	77	F	Adenocarcinoma	No	2	GAT	GAT
77	63	M	Squamous cell	No	2	GTT	
101	70	M	Adenocarcinoma	Yes	2		
121	65	M	Large cell	No	3		
148	61	M	Adenocarcinoma	No	3		
162	76	F	Adenocarcinoma	Yes	3	TGT	TGT
214	67	M	Adenocarcinoma	No	2	TGT	TGT
236	59	F	Squamous cell	Yes	3	GAT	GAT
293	68	F	Adenocarcinoma	No	2	GTT	GTT
297	53	F	Large cell	Yes	3		
298	71	M	Adenocarcinoma	Yes	3	TGT	TGT
302	56	M	Adenocarcinoma	Yes	3		
313	66	F	Adenocarcinoma	Yes	1	TGT	
335	67	M	Squamous cell	Yes	1		
343	77	M	Adenocarcinoma	No	1	CGT	CGT
415	67	F	Adenocarcinoma	No	2		
419	71	F	Adenocarcinoma	No	1	GTT	GTT
425	92	M	Squamous cell	No	2		
477	39	M	Adenocarcinoma	Yes	2	GTT	GTT
478	44	M	Squamous cell	No	2		GTT
497	74	F	Adenocarcinoma	Yes	2–3		
586	68	F	Adenocarcinoma	No	1	GTT	

F, female; M, male. The wild type K-*ras* codon 12 is GGT.

*K-*ras *mutation detection*

A method combining mutation allele enrichment (MAE) and denaturing gradient gel electrophoresis (DGGE) was used for the detection of infrequent K-*ras* gene codon 12 mutant alleles. It includes two steps of polymerase chain reaction (PCR) and two steps of restriction enzyme digestion (Keohavong *et al*. 1997). It has been used for the analysis of K-*ras* mutations in various areas of lung and colorectal tumours and in tissues adjacent to and distant from these tumours (Keohavong *et al*. 1997, Zhu *et al*. 1997). This method is highly sensitive and can detect one mutant allele present among an excess of 10^4–10^5 wild-type alleles (Keohavong *et al*. 1997). This sensitivity is achieved by two steps of restriction enzyme digestion to eliminate the excess of wild-type alleles and thereby enrich the alleles that contain mutations in codon 12 of the K-*ras* gene, and by subsequent DGGE analysis to identify the mutant patterns in each DNA sample.

Briefly, the first PCR was performed in a 25 μl reaction mixture containing 10 ng of the template DNA and 0.2 μM of each primer (KI1-1, 5′-TATTATAAGGCCTGCTGCTGAAA-3′; and the mismatch primer PKB, 5′-AGGCACTCTTGCCTACGGCA-3′), 60 μM of each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 units Gene Amp Taq DNA polymerase (Perkin-Elmer Corp., Branchburgh, New Jersey, USA). The amplification was performed for 15 cycles (1 min at 94°C, 1 min at 54°C and 2 min at 72°C). Subsequently, 1.5 μl of the PCR products were digested in a 6 μl reaction with 4 units of Ban I restriction endonuclease (New England Biolabs, Beverly, Massachusetts, USA) at 37°C for 2 h. The digestion material was used as a template in the second PCR for 40 cycles in a similar reaction mixture, except that 4 μCi of [α-^{32}P]dATP were added, and the primer KI1-1 was replaced by the GC-clamp primer (PKGC) 5′-GCCGCCTGCAGCCCGCGCCCCCCGTGCCCCCGCCCCGCCGCCGGCCCGGCCGCCTA-TAAGGCCTGCTGAAAATG-3′. After amplification, a 10 μl aliquot from each PCR reaction was diluted to 60 μl with BanI buffer and digested with 30 units of BanI restriction enzyme at 37°C for 2 h. The digestion material was recovered by ethanol precipitation, dissolved in 10 mM TE buffer, and electrophoresed through a 10% polyacrylamide gel. The gel was autoradiographed. The position of the DNA fragment in the gel was located by superimposing the autoradiogram on the gel. The portion of the gel containing the digestion-resistant fragment was excised from the gel and migrated through a 12.5% polyacrylamide containing a 35–50% linear gradient of denaturant concentration (100% = 40% urea w/v + 40% formamide v/v). The gel was dried and autoradiographed. Mutant alleles present in the gel were isolated and characterized further by sequencing analysis.

Results and discussion

Primary lung tumours and matched sputum samples from 22 lung cancer patients were examined for mutations in the K-*ras* gene. Detailed characteristics of the individuals under study are shown in the Table 1. Altogether, there were 22 tumour tissues and 22 matched sputum samples from the 22 lung cancer patients. The results of the K-*ras* mutations identified in the tumours and the matched sputum samples from the 22 lung cancer patients are summarized in Table 1. There were 10 female patients (with an average age of 67.9 years), including nine smokers (smoking an average of 43.9 packs/year) and 12 male patients (with an average age of 64.3 years), including 11 smokers (consuming an average of 59.2 packs/year). Twelve patients (54.5%) each showed a mutation in their lung tumour sample. This K-*ras* frequency is higher than that we observed previously (Keohavong *et al*. 1996), but is similar to that reported by another study (Husgavel-Pursiainen *et al*. 1993). However, these high frequencies may be due to the relatively small numbers of patients analysed in these studies. The mutations consist mostly of G to T transversions (75% G to T, 16.7% G to A, and 8.3% G to C), consistent with the fact that these lung cancer cases were smokers and that these mutations may be generated by carcinogens found in tobacco smoke (Husgavel-Pursiainen *et al*. 1993). Ten patients (45.5%) each showed a mutation in their sputum sample (patients 67, 162, 214, 236, 293, 298, 343, 419, 477 and 478). Comparison of mutations between tumour and matched sputum samples showed that nine of these 10 patients also showed an identical mutation in their lung tumour sample. Therefore, K-*ras* mutations are frequently detected in sputum samples of lung cancer patients and this detection is significantly associated with the presence of an identical mutation in the matched tumour sample ($\kappa = 0.64$, 95% confidence interval 0.32–0.95, $p < 0.01$). These data suggest that a sensitive detection of K-*ras* mutations in sputum samples might provide a useful diagnostic marker for lung cancer.

In three patients (patients 77, 313, and 586; Table 1), the mutations found in the tumours were not identified in the matched sputum samples. The detection of mutations in sputum samples from lung cancer patients relies on the assumption that malignant cells are shed from tumour tissues into the surrounding body fluids and provide sufficient DNA for mutation identification by our PCR-based methods. In this context, the results observed in patients 77, 313, and 586 may be due to no or too few tumour cells to be detectable by our method being shed from their tumours into their sputum. One patient (patient 478; Table 1) showed a mutation in the sputum sample but not in the matched lung tumour tissue. This result may be due to several factors, including the possibility of tumour heterogeneity; mutation may occur as a secondary event during lung tumour development and may only be present in a specific area of the tumour mass. Although tumour cells with such a mutation may be found in sputum samples, they may have failed to be detected in the matched tumour sample in this patient since only a small portion of each tumour was used for DNA extraction and mutation analysis. Unlike the mutations found in the other nine sputum samples, the mutation in the sputum sample of patient 478 was detected as only a very low fraction mutation (data not shown), and therefore we cannot exclude the possibility

that it could be due to a technical artefact associated with the limitations of our assay.

As shown in Table 1, various factors, including the histological type of the tumours, the tumour grade and the presence or absence of metastasis, did not seem to impact the detection of K-*ras* mutations in sputum samples. K-*ras* mutations were found in sputum samples of patients with all lung tumour grades, including two of the five patients with a grade 1 tumour, five of the nine patients with a grade 2 tumour, and three of the seven patients with a grade 3 tumour. The 22 patients involved in this study included 15 lung adenocarcinomas, five squamous cell carcinomas and two large cell carcinomas. Ten of the adenocarcinomas had a K-*ras* mutation in the tumours, including eight that had identical mutations in both the tumour and the matched sputum samples. One of the five lung squamous cell carcinomas had an identical mutation in both the tumour and sputum samples (patient 236), while one had a mutation in only the tumour sample (patient 77) and one had a mutation in only the sputum sample (patient 478). Therefore, contrarily to the results of previous reports by us and others (Barbacid 1987, Rodenhuis and Slebos 1992, Keohavong *et al*. 1996), we found here that K-*ras* mutations were frequently detected in samples from patients with lung squamous cell carcinoma. The reason for this discrepancy is not clear, although this result may be due to the small number of samples from this lung cancer type analysed in this study. Finally, 10 of the 22 patients had lymph node metastasis, while 12 did not have. Among the 10 patients with lymph node metastasis, four had an identical K-*ras* mutation in both the tumour and the matched sputum samples. Among the 12 patients without lymph node metastasis, six had a K-*ras* mutation in the sputum samples, including five patients who had identical mutations in both tumour and sputum samples. Therefore, our results show that K-*ras* mutations were detected in sputum samples both in the presence and the absence of lymph node metastasis. The presence of lymph node metastasis would indicate that the lung cancer is at a more advanced stage than in those without lymph node metastasis.

In summary, K-*ras* mutations are frequently found in lung tumours, particularly in lung adenocarcinomas, and it has been widely suggested that the detection of these mutations in DNA from sputum (Mao *et al*. 1994, Yakubovskaya *et al*. 1995) or bronchoalveolar lavage (Mills *et al*. 1995, Scott *et al*. 1997, Ahrendt *et al*. 1999, Oshita *et al*. 1999) samples could provide a specific method for the diagnosis of lung cancer. In this study, we applied a sensitive method to analyse K-*ras* mutations in tumours and matched sputum samples of lung cancer patients. We demonstrated that K-*ras* mutations are frequently found in sputum samples from these patients, and there is a highly significant association between the detection of K-*ras* mutations in sputum samples and the presence of identical mutations in the matched lung tumours. These results suggest that these non-invasive sputum-based assays for genetic analysis might provide a useful diagnostic method for lung cancer, particularly lung adenocarcinoma, to complement existing conventional approaches.

Acknowledgements

This work was supported by American Cancer Society grant RPG-99-161-01-CNE.

References

Ahrendt, S. A., Chow, J. T., Xu, L. H., Yang, S. C., Eisenberger, C. F., Esteller, M., Herman, J. G., Wu, L., Decker, A., Jen, J. and Sidransky, D. 1999, Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early lung cancer. *Journal of the National Cancer Institute*, **91**, 332–339.

Barbacid, M. 1987, *ras* genes. *Annual Review of Biochemistry*, **56**, 779–827.

Birrer, M. J. and Brown, P. H. 1992, Application of molecular genetics to early diagnosis and screening of lung cancer. *Cancer Research*, **52**, 2658s–2664s.

Harris, C. C. 1991, Chemical and physical carcinogenesis: advances and perspectives. *Cancer Research*, **51**, 5023s–5044s.

Husgavel-Pursiainen, K., Hackman, P., Ridanpaa, M., Anttila, S., Karjalainen, A., Partanen, T., Taikina-Aho, O., Heikkila, L. and Vaino, H. 1993, K-*ras* mutations in human adenocarcinoma of the lung: association with smoking and occupational exposure to asbestos. *International Journal of Cancer*, **53**, 250–256.

Keohavong, P., De Michele, M. A. A., Melacrinos, A. C., Landreneau, R. J., Weyant, R. J. and Siegfried, J. M. 1996, Detection of K-*ras* mutations in lung carcinomas: relationships to prognosis. *Clinical Cancer Research*, **2**, 411–418.

Keohavong, P., Zhu, D., Whiteside, T. L., Swalsky, P., Bakker, A., Elder, E. M., Siegfried, J. M., Srivastava, S. and Finkelstein, S. D. 1997, Detection of infrequent and multiple K-*ras* mutations in human tumors and tumor-adjacent tissues. *Analytical Biochemistry*, **247**, 394–403.

Lippman, S. M., Lee, J. S., Lotan, R., Hittelman, W., Wargovich, M. J. and Hong, W. K. 1990, Biomarkers as intermediate end points in chemoprevention trials. *Journal of the National Cancer Institute*, **82**, 555–560.

Mao, L., Hruban, R. H., Boyle, J. O., Tockman, M. and Sidransky, D. 1994, Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. *Cancer Research*, **54**, 1634–1637.

Mills, N. E., Fishman, C. L., Scholes, J., Anderson, S. E., Rom, W. N. and Jacobson, D. R. 1995, Detection of K-*ras* oncogene mutations in bronchoalveolar lavage fluid for lung cancer diagnosis. *Journal of the National Cancer Institute*, **87**, 1056–1060.

Mountain, C. F. 1989, Value of the new TNM staging system for lung cancer. *Chest*, **96**, 47s–49s.

Naruke, T., Goya, T., Tsuchiya, R. and Suemasu, K. 1988, Prognosis and survival in resected lung carcinoma based on the new international staging system. *Journal of Thoracic and Cardivascular Surgery*, **96**, 440–447.

Oshita, F., Nomura, I., Yamada, K., Kato, Y., Tanaka, G. and Noda, K. 1999, Detection of K-ras mutations of bronchoalveolar lavage fluid cells aids the diagnosis of lung cancer in small pulmonary lesions. *Clinical Cancer Research*, **5**, 617–620.

Parker, S. L., Tong, T., Bolden, S. and Wingo, P. A. 1996, Cancer statistics, 1996. *CA: A Cancer Journal for Clinicians*, **46**, 5–27.

Rodenhuis, S. and Slebos, R. J. 1992, Clinical significance of *ras* oncogene activation in human lung cancer. *Cancer Research*, **52**, 2665s–2669s.

Schatzkin, A., Freedman, L. S., Schiffman, M. H. and Dawsey, S. M. 1990, Validation of intermediate end points in cancer research. *Journal of the National Cancer Institute*, **82**, 1746–1752.

Scott, F. M., Modali, R., Lerman, T. A., Seddon, M., Kelly, K., Dempsey, E. C., Wilson, V., Tackman, M. S. and Mulshine, J. L. 1997, High frequency of K-*ras* codon 12 mutations in bronchoalveolar lavage fluid of patients at high risk for second primary lung cancer. *Clinical Cancer Research*, **3**, 479–482.

Stanley, L. A. 1995, Molecular aspects of chemical carcinogenesis: the roles of oncogenes and tumor suppressor genes. *Toxicology*, **96**, 173–194.

Travis, W. D., Travis, L. B. and Devesa, S. S. 1995, Lung cancer. *Cancer*, **75**, 191–202.

Yakubovskaya, M. S., Spiegelman, V., Luo, F. C., Malaev, S., Salnev, A., Zborovskaya, I., Gasparyan, A., Polotsky, B., Machaladze, Z., Trachtenberg, A. C., Belitsky, G. A. and Ronai, Z. 1995, High frequency of K-*ras* mutations in normal appearing lung tissues and sputum of patients with lung cancer. *International Journal of Cancer*, **63**, 810–814.

Zhu, D., Keohavong, P., Finkelstein, S. D., Swalsky, P., Bakker, A., Weissfeld, J., Srivastava, D. and Whiteside, T. L. 1997, K-ras gene mutations in normal colorectal tissues from K-*ras* mutation-positive colorectal cancer patients. *Cancer Research*, **57**, 2485–2492.