Detection of activating mutations in the *ras* family genes in cytological specimens from lung tumours

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Abstract. Mutations in the *ras* family genes (K-*ras* mainly) represent a common event in lung tumorigenesis which is frequently associated with poor clinical outcome. In order to investigate whether K-*ras* mutations are detectable in cytological material obtained from patients with lung cancer, 37 cytological specimens (16 fine needle aspiration and 21 bronchoscopy) were assessed for codon 12 point mutations in the H-, K- and N-*ras* genes by combined polymerase chain reaction-restriction fragment length polymorphism. K-*ras* codon 12 point mutations were found in 8 out of 37 (22%) specimens while no mutations were found in the H-*ras* and N-*ras* genes. Mutations were found in 27% (3 out of 11) of adenocarcinomas while in squamous cell carcinomas the incidence of mutations was 18% (3 out of 17). In addition, a K-*ras* codon 12 point mutation was found in one (12%) among 8 small cell carcinomas and in the only Hodgkin's lymphoma with metastasis in the lung. Our results are in agreement with previous results that recognise high incidence of K-*ras* activation in lung carcinomas, and indicate that detection of mutant *ras* alleles is possible in cytological material.

Introduction

The development of neoplasia requires specific genetic events targeting the activation of oncogenes and the inactivation of tumour suppressor genes (TSGs) (1). The *ras* family genes that encode for small GTPases of the plasma membrane, are frequently found activated in various human tumours. Activation of the *ras* family genes usually occurs by point mutations at codons 12, 13 and 61, abolishing from the mutant protein the property to exchange GTP with GDP and to remain constantly activated (2).

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Activated members of the *ras* family have been detected in approximately 90% of pancreatic and 40% of colorectal cancers (3). In lung tumours 30% of the specimens, mainly adenocarcinomas, harbour *ras* mutations. The mutations usually affect the K-*ras* proto-oncogene, but H-*ras* and N-*ras* genes have also been found to harbour activating point mutations. Furthermore, lung tumours harbouring an activated member of the *ras* family, are characterised by poor clinical outcome, indicating an important role for the *ras* genes in the development of the disease (4-8).

A major problem in lung tumorigenesis is the late stage of the disease in which the tumour is detectable. The detection of particular molecular alterations, highly associated with the development of the disease, could serve as a molecular marker for the development of the tumour and help in the early detection of cancer.

In the present study, we evaluated the incidence of activated members of the *ras* family in cytological material obtained from patients with lung cancer. The detection of the mutant *ras* alleles was performed by a sensitive polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay.

Materials and methods

Specimens. 37 cytological specimens (16 fine needle aspiration (FNA) and 21 bronchoscopy), consisting of 11 adenocarcinomas, 17 squamous cell carcinomas (SCC), 8 small cell carcinomas and one Hodgkin's lymphoma with metastasis in lung were obtained from the Department of Cytology, General Hospital of Nikeas, Pireas, Greece.

DNA extraction. Specimens were washed with 1X PBS buffer and DNA was extracted following standard proteinase K and phenol-chloroform extraction (9).

Polymerase chain reaction. Approximately 300 ng of genomic DNA was amplified by the PCR in a 50 µl reaction volume containing 500 µM dNTPs, 30 pM of each forward and reverse primer (10), 0.5 U of *Taq* DNA polymerase and 5 µl of 10X buffer (670 mM Tris.HCl, pH 8.5; 166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100 µM, ß-mercaptoethanol and 1% (w/v) Triton X-100). PCR programs consisted of 95°C for 4 min and 35 cycles of 95°C, 57°C and 72°C for 40 sec each step.

Detection of ras mutations at codon 12 by restriction fragment length polymorphism (RFLP). PCR amplification of the *ras* genes surrounding codon 12 was performed as described above. 30 µl of the PCR product was digested overnight with 20 U of the restriction endonucleases *Msp*I (H-*ras*) and *Bst*NI (K-*ras* and N-*ras*) in conditions recommended by the suppliers, electrophoresed in a 2% agarose gel (or 6% polyacrylamide gel for the N-*ras* codon 12) and stained with ethidium bromide.

Results

In the present study we employed a combined PCR-RFLP assay in order to detect point mutations at codon 12 of H-, K- and N-*ras* genes in cytological material from patients with lung cancer (11 adenocarcinomas, 17 squamous cell carcinomas (SCC), 8 small cell carcinoma and one Hodgkin's lymphoma with metastasis in the lung). Our analysis revealed that 8 among 37 (22%) specimens harboured a mutation at the K-*ras* gene (Table I, Fig. 1) while no mutations were found in the H-*ras* and N-*ras* genes, confirming previous reports (4-8) of higher incidence of mutations in lung cancer occuring in the K-*ras* gene.

The higher incidence of mutations was found in adenocarcinomas (3 out of 11, 27%). In SCC 3 out of 17 (18%) specimens were found positive for a K-*ras* point mutation. In addition, a K-*ras* codon 12 point mutation was found in a Hodgkin's lymphoma with metastasis in the lung (Table II). One (12.5%) mutation was also found in 8 small cell carcinomas. No association was found between the presence of mutation and the stage of the disease (Table III). As regards the cytological method of tissue extraction, 5 (31%) FNA samples among 16 were found positive for a K-*ras* mutation while only 3 (14%) among 21 bronchoscopy samples were positive for a K-*ras* mutation (Table IV).

Discussion

Activating mutations in the *ras* family genes represent a relatively common feature of lung tumours. We report that detection of activated *ras* alleles is possible in cytological material from patients with lung cancer. This could be of considerable importance in the clinical practice because the presence of mutant *ras* alleles is associated with poor prognosis in lung tumours (4-8).

The higher incidence of *ras* mutations was found in adenocarcinomas (3/11, 27%). Significant incidence of mutations was also found in SCC (3/17, 18%). Although the incidence of mutations reported in this study is lower than those reported by other investigators, generally our data are in agreement with previous findings. Suzuki *et al* (5) and

Table I. Codon 12 point mutations in the *ras* family genes in 37 cytological specimens from patients with lung cancer.

Sample No.	K-*ras*	H-*ras*	N-*ras*	Cytology[a]	Stage	Cytological method[b]
1	+	-	-	SCC	III	FNA
2	-	-	-	SCC	III	B
3	-	-	-	SCC	III	B
4	-	-	-	SCC	I	B
5	-	-	-	SCC	II	B
6	-	-	-	SCC	III	B
7	-	-	-	SCC	III	FNA
8	-	-	-	SCC	I	B
9	-	-	-	SCC	III	B
10	-	-	-	SCC	III	B
11	-	-	-	SCC	III	FNA
12	-	-	-	SCC	II	B
13	-	-	-	SCC	II	FNA
14	+	-	-	SCC	I	FNA
15	-	-	-	SCC	III	FNA
16	-	-	-	SCC	III	B
17	+	-	-	SCC	II	B
18	-	-	-	AC	III	FNA
19	-	-	-	AC	III	FNA
20	-	-	-	AC	III	B
21	+	-	-	AC	III	FNA
22	-	-	-	AC	III	FNA
23	-	-	-	AC	III	B
24	-	-	-	AC	I	B
25	-	-	-	AC	III	B
26	-	-	-	AC	III	B
27	+	-	-	AC	III	FNA
28	+	-	-	AC	III	B
29	-	-	-	small cell	III	FNA
30	-	-	-	small cell	III	FNA
31	-	-	-	small cell	III	FNA
32	-	-	-	small cell	III	FNA
33	-	-	-	small cell	I	B
34	-	-	-	small cell	II	B
35	-	-	-	small cell	II	B
36	+	-	-	small cell	III	B
37	+	-	-	Hodgkin's lymphoma	II	FNA

[a]AC, adenocarcinoma; SCC, squamous cell carcinoma. [b]B, bronchoscopy; FNA, fine needle aspiration.

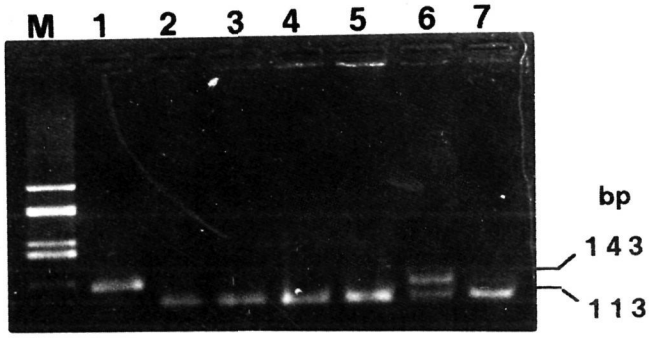

Figure 1. Detection of K-*ras* codon 12 point mutations by a combined PCR-RFLP assay. Lane 1, DNA from the cell line SW 480 (mutant for K-*ras* codon 12); lanes 2-5, normal samples; lanes 6,7, samples with a mutation at K-*ras* codon 12; M, pUC18/HaeIII.

Table II. K-*ras* codon 12 point mutations in association with the cytological diagnosis.

Cytological type	No. of samples	K-*ras* mutations (%)
Adenocarcinoma	11	3 (27)
SCC[a]	17	3 (18)
Small cell	8	1 (13)
Hodgkin's lymphoma	1	1
Total	37	8 (22)

[a]SCC, squamous cell carcinoma.

Table III. K-*ras* codon 12 point mutations in association with the stage of the disease.

Stage	No. of samples	K-*ras* mutations (%)
Stage I+II	12	3 (25)
Stage III	25	5 (20)

Table IV. Codon 12 point mutations in the K-*ras* gene in association with the cytological method.

Cytological method	No. of samples	K-*ras* mutations (%)
FNA[a]	16	5 (31)
Bronchoscopy	21	3 (14)

[a]FNA, fine needle aspiration.

Rodenhuis and Slebos (8) detected mutations at K-*ras* codon 12 in approximately 30% of lung tumours, while the incidence of H-*ras* and N-*ras* point mutations was limited to 1.5% and 4.5% respectively. The majority of the mutations were found in adenocarcinomas, however, activating mutations were also detected in large cell carcinomas (LCC, 14%) and SCC (5.5%) (5). The high incidence of K-*ras* mutations in adenocarcinomas as compared to other histological entities of lung tumours (such as LCC and SCC), was not confirmed by a Spanish group (11) who demonstrated the presence of activating mutations of the K-*ras* gene in 21% of the SCC, while in adenocarcinomas the incidence of mutations was 14% of the specimens. The discrepancy may be due to the different epidemiological factors and is common to studies on the *ras* genes (9,12).

A major problem in the detection of mutant *ras* alleles in cytological material is the presence of DNA from the adjacent normal tissue, in significant ratio as compared to the DNA from the tumour cells. In the case of FNA, normal DNA is derived from the peripheral blood which is present during the aspiration, while in the case of bronchoscopy normal epithelial cells may be present. In the case of a heterozygous mutation, a ratio of 1:1 between tumour versus normal cells results in a ratio of 1:3 between mutant versus normal *ras* alleles. In this case, a competition occurs during the *in vitro* amplification reaction, which results in significant decrease of the signal derived by the mutant allele. This may explain the slightly lower incidence of mutations reported in our study, as compared to that reported by others. However, the present study indicates that the detection of mutant K-*ras* alleles is possible in DNA extracted from cytological material and can be successfully applied in the clinical practice, at least in a subset of cases. Most of the mutations (Table IV) were detected in the FNA material, indicating that this is the more suitable technique for the detection of *ras* mutations.

Future investigations, aiming to increase the specificity of the cytological techniques and the sensitivity of the PCR amplification should be performed in order to make possible the application of molecular techniques in routine clinical practice.

References

1. Spandidos DA: A unified theory for the development of cancer. Biosci Rep 6: 691-708, 1986.
2. Spandidos DA (ed): *Ras* oncogenes. Plenum Press, New York, London pp1-323, 1989.
3. Bos JL: *ras* oncogenes in human cancer (review). Cancer Res 49: 4682-4689, 1989.
4. Rodenhuis S and Slebos RJ: The *ras* oncogenes in human lung cancer. Am Rev Respir Dis 142: 27-30, 1990.
5. Suzuki Y, Orita M, Shiraishi M, Hayashi K and Sekiya T: Detection of *ras* gene mutations in human lung cancers by single-strand conformation polymorphism analysis of polymerase chain reaction products. Oncogene 5: 1037-1043, 1990.
6. Kratzke RA, Shimizu E and Kaye FJ: Oncogenes in human lung cancer. Cancer Treat Res 63: 61-85, 1992.
7. Slebos RJ and Rodenhuis S: The *ras* gene family in human non-small cell lung cancer. Monogr Natl Cancer Inst 13: 23-29, 1992.
8. Rodenhuis S and Slebos RJ: Clinical significance of *ras* oncogene activation in human lung cancer. Cancer Res 53 (Suppl): 2665-2669, 1992.
9. Kiaris H, Eliopoulos AG, Sivridis E, Ergazaki M and Spandidos DA: Activating mutations of *ras* family genes in prostatic cancer. Oncol Rep 2: 427-430, 1995.
10. Kiaris H and Spandidos DA: Analysis of H-*ras*, K-*ras* and N-*ras* genes for expression, mutations and amplification in laryngeal tumours. Int J Oncol 7: 75-80, 1995.
11. Rosell R, Li S, Skacel Z, Mate J-L, Maestre J, Canela M, Tolosa E, Armengol P, Barnadas A and Ariza A: Prognostic impact of mutated K-*ras* gene in surgically resected non-small cell lung cancer patients. Oncogene 8: 2407-2412, 1993.
12. Saranath D, Chang SE, Bhoite LT, Panchal RG, Kerr IB, Mehta AR, Johnson NW and Deo MG: High frequency of mutation in codon 12 and 61 of H-*ras* oncogene in chewing tobacco-related human oral carcinoma in India. Br J Cancer 63: 573-578, 1991.