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K-*ras* gene mutations: an unfavorable prognostic marker in stage I lung adenocarcinoma

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Abstract Activation of K-*ras* gene by point mutations, a common finding in lung adenocarcinomas, has been suggested to decrease patient survival. We investigated 109 lung adenocarcinomas, mostly small, peripheral, stage I tumours (81/109) for presence of K-*ras* gene mutations at codons 12 and 13. Mutations were detected by denaturing gradient gel electrophoresis analysis of specific sequences amplified by polymerase chain reaction from DNA extracted from archival pathological material. Thirty-three of 109 (30.3%) tumours showed mutations at codon 12 (28/33, 84.8%) or 13 (5/33, 15.2%) of the gene. Mutations and type of nucleotide substitutions were differently distributed among cytological subtypes, being more prevalent among less differentiated (G2 and G3) tumours and among bronchial than bronchiolo-alveolar type adenocarcinomas. Survival analysis showed an adverse effect of K-*ras* mutation on survival, restricted to stage I tumours. Median survival for 81 stage I patients was 30 months for non-mutated tumours versus 20 months for mutated tumours ($p = 0.016$). Multivariate analysis showed that age of patient ($p = 0.001$) and K-*ras* mutation status ($p = 0.04$) were the only independent factors influencing survival significantly. These data strengthen the hypothesis that K-*ras* gene mutations may be useful in identifying a subgroup of patients with poor outcome.

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Introduction

Lung cancer is the most common cancer in developed countries, with estimated age standardized rates of incidence in men of 52.5 per 100,000 in Europe (Parkin et al. 1993). Non-small cell lung carcinomas (NSCLCs) are a heterogeneous group of tumours that comprise over 75% of lung neoplasms (Hammar 1988). Adenocarcinoma accounts for over 30% of all NSCLCs and epidemiological trends indicate that it is rapidly becoming the most frequent histotype (Hammar 1988; Minna et al. 1989). Despite significant advances in diagnosis and surgical treatment, mortality from lung adenocarcinoma remains high with an overall survival rate of about 12% at 5 years and does not exceed 27% at 5 years even for surgically resectable tumours (Minna et al. 1989; Naruke et al. 1988). Thus, additional prognostic indicators of long term biological behaviour are needed to improve the data from clinico-pathological staging by the TNM system (Mountain 1986) that presently determines therapeutic decisions. Pathological features of tumours such as histological subtype or grade lack major prognostic impact on prolonged survival and their predictive value is restricted to the disease-free interval or 1 year survival (Sørensen et al. 1988).

Ras genes (H-*ras*, K-*ras* and N-*ras*) belong to a family of small monomeric GTPases bound to the inner side of the cell membrane which are involved in cytoplasmic signal transduction networks operating in cell proliferation control, cell to cell communication and cell response to external stimuli (Lowry and Willumsen 1993). Point mutations occurring at specific codons of *ras* genes may alter the intrinsic GTPase activity of their encoded proteins that acquire oncogenic potential. *Ras* genes activated by mutations occurring specifically at codons 12, 13 and 61 are frequently found in a variety of human tumours (Bos 1989). K-*ras* activation is particularly asso-

ciated with adenocarcinomas, mainly of the gastrointestinal tract (Bos et al. 1987), pancreas (Almoguera et al. 1988; Pellegata et al. 1992) and lung (Kobayashi et al. 1990; Mitsudomi et al. 1991a; Rodenhuis et al. 1988; Rosell et al. 1993; Sugio et al. 1992).

Previous studies have reported that K-*ras* point mutations of codons 12 and 13 are frequently found in lung adenocarcinomas and they have shown that they may be associated with decreased survival irrespective of disease extent (Mitsudomi et al. 1991b; Slebos et al. 1990). Thus, K-*ras* gene mutations might be useful in identifying a subgroup of patients with dismal prognosis despite potentially curative resection for localized disease.

In the present study we examined K-*ras* gene point mutations on codons 12 and 13 in a group of 109 primary lung adenocarcinomas, mainly small, peripheral, stage I tumours, which underwent radical surgery and that were followed for a median period of 55 months. A novel denaturing gradient gel electrophoresis (DGGE) technique (Myers et al. 1987) was used to detect mutations in polymerase chain reaction (PCR)-amplified DNA extracted from archival pathological material. The aim of the study was to correlate K-*ras* mutation with patients survival as well as with stage and histological features of the tumours, in order to validate the application of the technique in routine diagnostic practice.

Materials and methods

One hundred and nine consecutive patients who underwent surgical resection for lung adenocarcinoma between September 1984 and December 1991 at the IRCCS Policlinico S.Matteo of Pavia were included in the study. Only patients with peripherally located tumours, accurate pathological staging and potentially curative resection were considered. Patient ages ranged from 38 to 83 years (mean age 63.5 years), 87 were men and 22 were women. Since smoking history was not avaiable for several patients, this was not further considered. Seventy two patients underwent lobectomy or pneumonectomy with staging lymphadenectomy; 24 had simple lobectomy and 13 had atypical pulmonary resection.Tumour stage was assessed according to the TNM system (Mountain 1986).

Pathological classification of adenocarcinomas (Hammar 1988) was performed according to WHO (1981), subdividing tumours into 4 subtypes (acinar, papillary, bronchiolo-alveolar and solid with mucus formation) mainly according to architectural features and growth patterns. They were further classified according to the criteria proposed by Shimosato et al. (1982) into 5 cytological subtypes suggestive of origin from different respiratory cell types (bronchial superficial epithelium, Clara cells, type II pneumocytes, goblet cells, bronchial gland cells). A category of non-classified tumours was included for each classification. Histological grade of tumours was established according to WHO (1981). For each patient, sections from at least 2 tissue blocks were stained with haematoxilin and eosin and alcian blue-periodic acid Schiff (PAS).

All tumour samples used in the study were from routine formalin-fixed, paraffin-embedded material. Tissue blocks were chosen which consisted of at least 75% neoplastic tissue. Alternatively, tumour areas were dissected from surrounding normal tissue. Two or three 10 μm thick tissue sections were cut from each block depending on sample size, collected into microcentrifuge tubes and incubated overnight at 58°C in 50 mM potassium chloride, 10 mM TRIS-HCl pH 8.3, 2.5 mM magnesium chloride, 0.45% Tween 20, 0.45% NP40 and 0.5 mg/ml Proteinase K. Proteinase K was inactivated at 95°C for 15 min and 5–10 μl of the digested material were directly used for enzymatic amplification by PCR.

Oligonucleotide primers used for amplification of K-*ras* gene exon 1 sequences were as follows:

5' primer: 5'-ATGACTGAATATAAACTTGT-3';
3' primer: 5'-CTCTATTGTTGGATCATATT-3';
3' GC-clamp primer: 5'-CGCCCGCCGCGCCCCGCGCCCGT-CCCGCCGCCCCCGCCCCCTCTATTGTTGGATCATATT, used to obtain amplified sequences suitable for DGGE analysis.

Amplification reaction was performed in a volume of 50 μl in 50 mM potassium chloride, 10 mM TRIS-HCl pH 8.3, 1.5 mM magnesium chloride, 200 mM dNTPs, 25 pM of each primer and 0.5 units of AmpliTaq (Perkin-Elmer, Norwalk, Conn., USA). Cycling parameters were 95°C 1 min, 55°C 1 min and 72°C 2 min for 5 cycles and 95°C 45 s, 55°C 45 s and 72°C 1 min for 35 cycles. To avoid contamination by PCR product carry-over and false-positive results, samples were analysed in a separate laboratory from that in which amplification reactions were performed. Aliquoted reagents, aerosol-free tips and all other recommended precautions were also used.

DGGE analysis was performed essentially as described by Myers et al. (1987) and following the conditions we previously reported (Pellegata et al. 1992). K-*ras* exon 1 specific PCR products obtained with the GC-clamp primer were loaded on a 8% polyacrylamide gel in TAE 1X buffer with a linearly increasing gradient from 45% to 75% (vol/vol) denaturant (7M urea/40% formamide vol/vol) and run at 60°C at 50 V for 15h. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV light. Amplification products from human cell lines containing known point mutations of K-*ras* gene were used as controls (Pellegata et al. 1992). Mutated DNAs with electrophoretic patterns different from those of control DNAs were characterized by direct enzymatic sequencing of PCR products eluted from gels by using a thermostable DNA polymerase based system (CircumVent sequencing kit, New England Biolabs, Beverly, Mass., USA) according to the manufacturer's instructions.

Statistical analysis was performed by χ^2 test or Fisher's exact test as appropriate. Survival curves were constructed by the Kaplan-Meier method and the log rank test was used to probe statistical significance between pairs of survival probabilities. Multivariate analysis of independent factors influencing survival was performed by the Cox proportional hazards model, using a group of variables that included patient sex and age, stage of the disease and K-*ras* mutation. Two-sided p values <0.05 were considered to be significant.

Results

Although selection of tumours was performed according to criteria that favoured localized disease, final clinico-pathological staging by the TNM system revealed that 81 patients had a stage I tumour (53 T_1N_0, 28 T_2N_0), 11 had stage II, 15 stage III and 2 stage IV disease. The median follow up time was 55 months. According to the WHO criteria (1981) 17 tumours were classified as acinar type, 48 as papillary, 5 as solid with mucous production, 4 as bronchiolo-alveolar and 36 as mixed type. Twenty-six tumours were well-differentiated (G1), 51 moderately (G2) and 32 poorly differentiated (G3). Cytological subtyping (Shimosato et al. 1982) revealed that 35 were of bronchial superficial type, 41 of bronchiolo-alveolar type, 8 of mixed type and 25 were unclassified (Fig. 1). Most grade 3 tumours fell into the unclassified group since distinctive cytological features could be ascertained unambiguously only in well or moderately differentiated tumours.

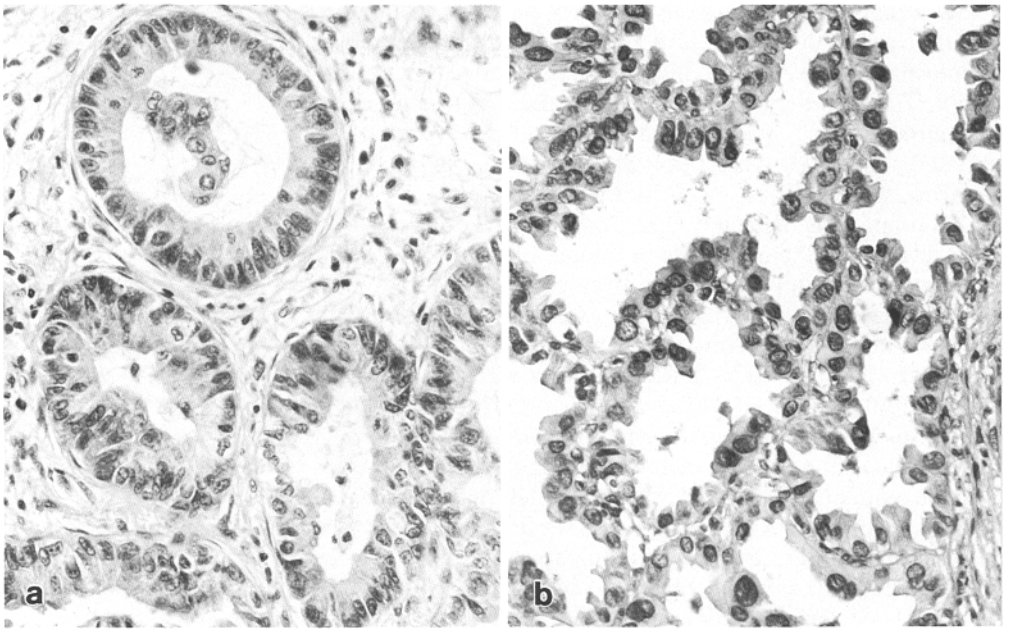

Fig. 1a K-*ras* mutated bronchial surface epithelium type adenocarcinoma. Tall columnar cells with linear surface are arranged in tubules. EEx250. **b** K-*ras* non-mutated bronchiolo-alveolar type adenocarcinoma. Tumour cells with an irregular surface profile proliferate along alveolar septa. EE x250

Thirty-three of 109 (30.3%) adenocarcinomas showed point mutations in K-*ras* gene codons 12 or 13 by DGGE analysis of PCR amplified specific DNA fragments of the first exon of the gene. All mutated samples showed a DGGE band corresponding to the wild type allele (Fig. 2) indicating either maintenance of normal allele in cancer cells or contamination of tumour tissue by non-neoplastic cells. The sensitivity of the method, as previously determined by 'in vitro' reconstruction experiments (Pel-

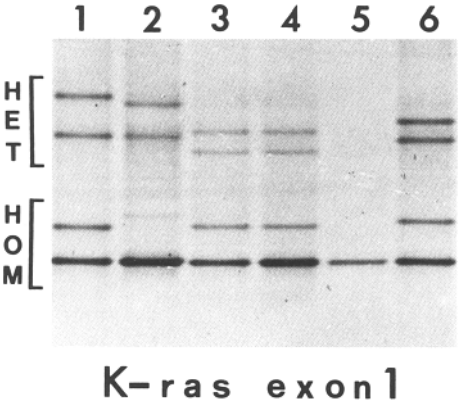

Fig. 2 Negative image of ethidium bromide stained denaturing gradient gel showing patterns from 6 different samples. Tumour DNAs were amplified with 5' and GC-3' ampliprimers specific for the first exon of the K-*ras* gene and the polymerase chain reaction products were loaded onto a 45% to 75% gradient of denaturants. The following mutations are shown: lane 1: GGC>TGC at codon 13; lane 2: GGC<GAC at codon 13; lane 3: GGT>TGT at codon 12; lane 4: GGT>TGT at codon 12; lane 5: wild type sequence GGT at codon 12; lane 6: GGT>GAT at codon 12. On the left hand of the figure the heteroduplex (*HET*) and homoduplex (*HOM*) molecules are indicated

Table 1 Distribution of K-*ras* gene mutations in 32 lung adenocarcinomas (one of the 33 mutations cited in the text was not typed due to technical problems).

Mutated codon	Type of mutation	Frequency
Codon 12 GGT (Gly)	TGT (Cys)	12 (37.5)*
	GTT (Val)	7 (21.8)
	GCT (Ala)	5 (15.6)
	GAT (Asp)	3 (9.3)
Codon 13 GGC (Gly)	TGC (Cys)	3 (9.3)
	GAC (Asp)	2 (6.2)

* Numbers in parentheses, percentage

Table 2 K-*ras* mutated versus non-mutated lung adenocarcinomas according to histological grade (classification according to WHO) and cytological subtype (classification according to Shimosato et al.)

K-*ras* gene codon 12–13		Grade 1	Grade 2	Grade 3
Mutated	33	4	17	12
Non-mutated	76	22	34	20

K-*ras* gene codon 12-13		Bronchial superficial type	Bronchiolo alveolar type	Mixed type	Unclassified type
Mutated	33	15	8	1	9
Non-mutated	76	20	33	7	16

legata et al. 1992), enabled us to detect mutated sequences up to a DNA dilution corresponding to a 1/25 ratio of neoplastic versus non-neoplastic cells. Moreover, all samples used in the present study contained at least 75% of tumour tissue. Therefore, no significant proportion of mutated sequences were likely to be overlooked in the experimental conditions used for the study.

All mutations detected were base substitutions, 28 were at codon 12 and 5 were at codon 13. The types of nucleotide substitution observed, along with the corresponding amino acid variations, are summarized in Table 1. One mutation at codon 12 was not classifiable due to technical problems. G to T transversions were most frequently found (22/32, 68.7%), then G to C transversions and G to A transitions were equally represented (5/32, 15.6%). *Ras* gene mutations tended to be more numerous in male patients (29/87, 33.3%) than in females (3/22, 13.6%), without reaching statistical significance. Mutations occurred more frequently in adenocarcinomas with cytological features suggestive of origin from bronchial superficial epithelium (15/35, 42.8%) rather than in those from terminal airway cells (Clara cells and type II pneumocytes) (8/41, 19.5%) (Table 2); this difference was also not statistically significant. Types of nucleotide substitutions also differred between the two groups, the bronchial superficial type showing a great excess of G to T tranversion and few G to A transitions or G to C transversions (13 versus 3) as compared with the bronchiolo-alveolar type (3 versus 5).

No significant differences were observed in K-*ras* mutation positive and negative groups according to patient age, tumour size and grade and disease stage, however non-mutated tumours showed a trend toward a better differentiation – 4 of 26 (15%) G1 tumours were mutated when compared with 17 of 51 (33%) G2 and 12 of 32 (38%) G3 tumours – (Table 2).

The 5 year survival rate for the whole group of 109 patients examined was 49.6%, with significant dependence from disease stage ($p = 0.018$). K-*ras* mutation presence showed a trend toward negative correlations with survival in the whole group of 109 patients ($p = 0.07$), without reaching statistical significance (Fig. 3a). No significant correlation of K-*ras* mutation with survival was observed in 28 patients (10 mutated) with stage above I. However, when only 81 stage I patients (23 mutated) were considered, survival was significantly dependent on K-*ras* mutation status, with median survival of 20 months for 23 K-*ras* positive cases as compared with median survival of 30 months for 58 K-*ras* negative cases ($p = 0.016$) (Fig. 3b). In addition K-*ras* mutation status of the 53 T_1N_0 patients (14 mutated) significantly affected survival ($p = 0.040$) (Fig. 3c)

Multivariate analysis of independent variables affecting survival, performed according to the Cox proportional hazards model showed that, when considering the whole group of 109 patients, those significantly influencing survival were age at diagnosis ($p = 0.04$) and disease stage ($p = 0.001$). Restriction of the analysis to 81 stage I patients indicated that besides age at diagnosis ($p = 0.001$), the only variable significantly influencing survival was K-*ras* mutation status ($p = 0.04$), while no significant effects on survival were observed for pathological findings such as tumour size, grade or cytological type.

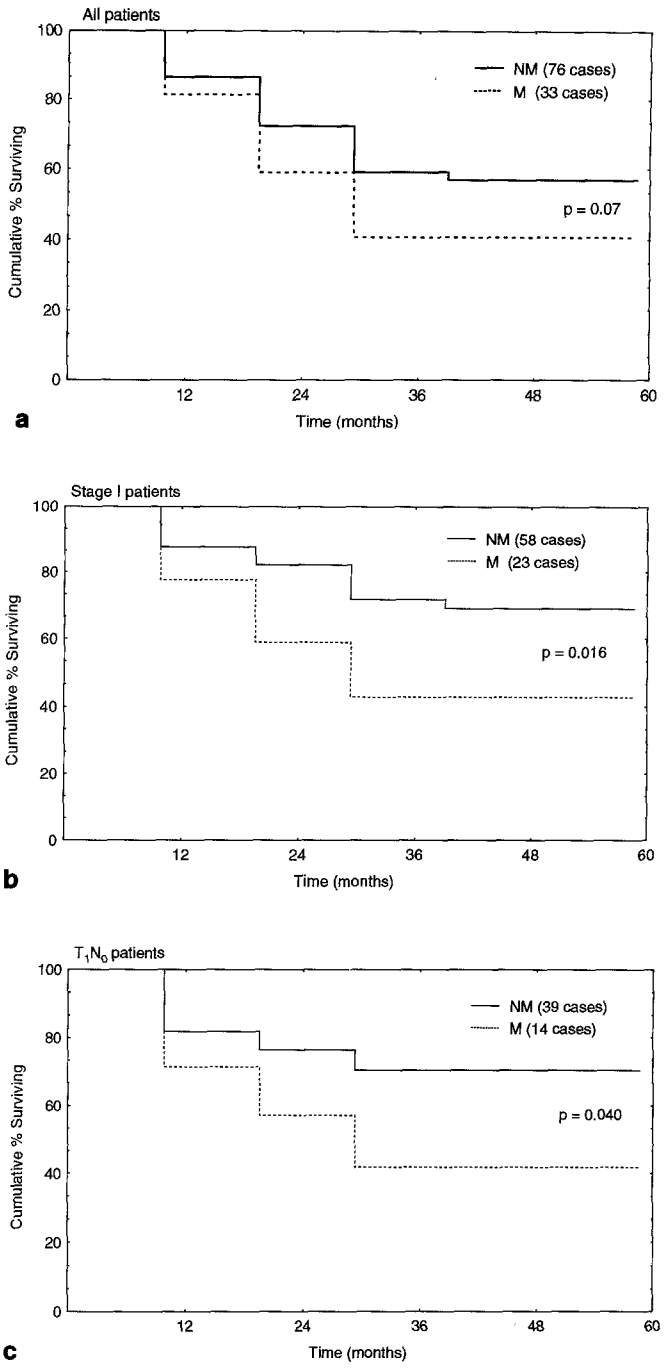

Fig. 3a–c Kaplan-Meier survival curves for lung adenocarcinoma patients with respect to presence (*M*) or absence (*NM*) of K-*ras* gene mutation. Survivals probabilities are compared for **(a)** 109 patients at all stages ($P = 0.07$), **(b)** 81 stage I patients ($P = 0.016$) and **(c)** 51 T_1N_0 patients ($P = 0.04$). P values were calculated with log rank test

Discussion

Recent advances in our understanding of molecular genetics of cancer have led to the development of a model of malignant transformation as a progressive, sequential accumulation of specific mutations involving several genes essential for the control of cell proliferation and differentiation (Weinberg 1989). The sequence of distinct molecular events has been clarified in detail only for some tumour models (Fearon and Vogelstein 1990). For most of the common epithelial tumours the complexity of tumourigenesis and the technical problems involved in gene mutation detection have so far hampered any correlation of specific genetic events with clinical features relevant to therapeutic decisions. However, studies on some hereditary neoplasms of childhood (Haber and Housman 1991) and on lymphoid malignancies (Rabbitts 1991) indicate that despite the large number of gene mutations present in a given tumour type, few critical molecular events actually behave as rate-limiting steps in disease progression. Recent studies suggest that K-*ras* gene activation may represent one of such critical events in the natural history of lung adenocarcinoma (Kobayashi et al. 1990; Mitsudomi et al. 1991a; Rodenhuis et al. 1988; Rosell et al. 1993; Sugio et al. 1992).

K-*ras* point mutations of codons 12 and 13 are frequently found in lung adenocarcinoma, with prevalence ranging from 15% to 30% according to the population studied. K-*ras* mutation prevalence is strictly correlated with a history of cigarettes consumption (Slebos et al. 1991) and most of the nucleotide substitutions observed are G to T transversions (Rodenhuis and Slebos 1992), that are characteristic of the mutagenic effects of aromatic hydrocarbons present in tobacco smoke (Meuth 1990; Strauss 1992). The high prevalence of mutations observed in tumours with cytoarchitectural features of cells derived from bronchial superficial epithelium (Kobayashi 1990) is also suggestive of the carcinogenic effects of inhaled substances.

Our results are in good agreement with published reports as to the prevalence of mutations observed (33 of 109 tumours analysed, 30.3%) and their characteristic spectrum of nucleotide substitutions (22 G to T transversions observed in 33 cases, 66.6%). Present data confirm the efficacy and reliability of mutation detection based on DGGE analysis of archival material. The method is simple, unexpensive, does not require use of radioisotopes and can be easily adapted to routine use in clinical practice both on fresh tumours (Pellegata et al. 1992) and on standard formalin-fixed, paraffin-embedded material.

Our results also extend previous observations relative to the different distribution of K-*ras* mutations among the various cytological subtypes of adenocarcinomas (Kobayashi 1990). A larger proportion of mutations occurred in tumours with phenotypic characteristics of bronchial epithelial cells compared with those showing cytological features of bronchiolo-alveolar cell types (Clara cells and type II pneumocytes). This association does not reach statistical significance and is not as straightforward as that reported by Japanese authors (Kobayashi 1990), who found an almost complete absence of mutations in tumours with bronchiolo-alveolar cytology (1/23 tumours examined). Nevertheless, it is interesting in the light of the different spectrum of point mutations observed in these two kinds of lung adenocarcinomas. An excess of G to T substitutions rather than G to A or G to C changes was found in the bronchial type tumours when compared with the bronchiolo-alveolar type. This behaviour may suggest the involvement of different mutagenic agents (Meuth 1990), in keeping with the epidemiological differences observed between the two tumour types (Minna et al. 1989). A nucleotide substitution spectrum with prevalence of G to A transitions, significantly different from that observed in commonly occurring lung tumours, has been recently reported for point mutations of the *p53* gene in lung tumours from uranium mine workers (Våhåkangas et al. 1992).

Previous studies on lung adenocarcinoma have pointed out the significant correlation between K-*ras* activation and unfavorable prognosis. In most studies, survival probabilities were not stratified according to different stages of disease (Mitsudomi et al. 1991b; Sugio et al. 1992), although Slebos et al. (1990) stated that K-ras mutations influence on survival was retained in stage I and stage I and II patients. Some authors have distinguished between patients who received radical or palliative treatment and two groups compared survival probabilities in tumours with or without lymph node metastases (Rosell et al. 1993; Sugio et al. 1992). Sugio et al. (1992) found that the prognostic significance of K-*ras* mutations on survival was limited to patients with radically resectable non-metastatic tumours, corresponding to stages I, II and IIIa, whose 5 year survival rate was 53.3% in the *ras*-positive group as compared with 83.6% in the *ras*-negative group.

We concentrated on small, peripheral, localized tumours which were more likely to have had radical surgical resection and we attempted to differentiate patients with different survival by means of K-*ras* mutation status. The initial selection performed for small peripheral tumours accounts for the low number of high stage lesions present in our group which differs from the proportion of cases observed in practice. No significant differences in survival probability were seen between K-*ras* mutation positive and negative cases in patients with disease stage greater than I. This is probably due to the small number of cases considered, as most patients die within a relatively short time after surgery and large numbers of observations are needed to detect small differences in survival. The inclusion of high stage patients is also probably responsible for the lack of statistical significance in survival probabilities observed on the whole group of our 109 patients at all stages, as well as in another study (Sugio et al. 1992). This conclusion is supported by the results obtained in the more homogeneous group of 81 stage I patients, where differences of survival probability between K-*ras* mutated and non-mutated cases were significant ($p = 0.016$) (Fig. 3b). These differ-

ences were also maintained for the 53 patients with tumours at T_1N_0 stage ($p = 0.040$) (Fig. 3c).

This is the largest group of stage I tumours analysed so far for K-*ras* mutations. No prognostic markers of long term survival are presently available for this class of patients, whose survival probabilities do not exceed 50% at 5 years despite potentially curative resection (Minna et al. 1989). Our data indicate that presence or absence of K-*ras* mutations may effectively discriminate between two groups of stage I patients with median survival of 20 and 30 months, respectively. Survival differences may be even more evident in T_1N_0 tumours, where survival probabilities at 5 years were respectively 40% and 73% in the two groups. Among long term survivors with follow up longer than 50 months only 2 out of 17 had K-*ras* mutation versus 9 of 17 who died within 2 years from diagnosis.

Multivariate analysis showed that in stage I patients K-*ras* status behaves as an independent variable in determining survival and it confirmed that standard pathological variables are of little value in predicting long term survival (Sørensen et al. 1988). In keeping with other studies (Mitsudomi et al. 1991b; Rodenhuis and Slebos 1992; Sugio et al. 1992), no statistically significant differences in mutation prevalence were seen in relation to disease stage, size, grade or histological pattern of tumour. However, a trend toward a higher prevalence of K-*ras* mutations seems to characterize less differentiated (G2 and G3) and bronchial cell type pulmonary adenocarcinomas.

At the present state of knowledge it is not possible to identify how K-*ras* mutation affects the biological characteristics of tumour growth and progression and how they might be therapeutically modified. Recent studies (Westra et al. 1993) performed on lung tumours from former smokers suggest that K-*ras* mutation might be a very early, initiating event in the natural history of the disease, as already pointed out for other tumour models (Kumar et al. 1990). Thus, clarification of the complex network of interaction that links K-*ras* mutation with other genetic events occurring during lung tumour progression (Mitsudomi et al. 1992; Sundaresan et al. 1992) remains an important goal of future studies. Meanwhile, we believe that available clinico-pathological data should prompt consideration of the use of K-*ras* mutation to identify patients with a potentially poor outcome. The potential benefit of adjuvant treatment for such patients should be evaluated in prospective studies.

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References

Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M (1988) Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. Cell 53: 549–554

Bos JL (1989) *ras* oncogenes in human cancer: a review. Cancer Res 49: 4682-4689

Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, Van Boom JH, Van der Eb AJ, Vogelstein B (1987) Prevalence of *ras* gene mutations in human colorectal cancer. Nature 327: 293–297

Fearon E, Vogelstein B (1990) A genetic model for colorectal tumourigenesis. Cell 61: 759–767

Haber DA, Housman DE (1991) Rate-limiting steps: the genetics of pediatric cancers. Cell 64: 5–8

Hammar SP (1988) Common neoplasms. In: Dail DH, Hammar SP (eds), Pulmonary pathology. Springer, Berlin Heidelberg New York, pp. 727–846

Kobayashi T, Tsuda H, Noguchi M, Hirohashu S, Shimosato Y, Goya T, Hayata Y (1990) Association of point mutation in c-Ki-*ras* oncogene in lung adenocarcinoma with particular reference to cytological subtypes. Cancer 66: 289–294

Kumar R, Sukumar S, Barbacid M (1990) Activation of *ras* oncogenes preceding the onset of neoplasia. Science 248: 1101–1104

Lowry DR, Willumsen BM (1993) Function and regulation of *ras*. Ann Rev Biochem 62: 851–891

Meuth M (1990) The structure of mutation in mammalian cells. Biochem Biophys Acta 1032: 1–17

Minna JD, Pass H, Glatstein E, Ihde DC (1989) Lung cancer. In: DeVita VT, Hellman S, Rosenberg SA (eds), Cancer: principle and practice of oncology. JB Lippincott, Philadelphia, pp. 591–705

Mitsudomi T, Viallet J, Linnoila I, Minna JD, Gazdar AF (1991a) Mutation of *ras* genes distinguish a subset of non-small cell lung cancer cell lines from small cell lung cancer cell lines. Oncogene 6: 1353–1362

Mitsudomi T, Steinberg SM, Oie HK, Mulshine JL, Phelps R, Viallet J, Pass H, Minna JD, Gazdar AF (1991b) *ras* gene mutations in non-small cell lung cancers are associated with shortened survival irrespective of treatment intent. Cancer Res 51: 4999–5002

Mitsudomi T, Steinberg SM, Nau MM, Carbone D, D'Amico D, Bodner S, Oie HK, Linnoila RI, Mulshine JL, Minna JD, Gazdar AF (1992) *p53* gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of *ras* mutations and clinical features. Oncogene 7: 171–180

Mountain CF (1986) A new international staging system for lung cancer. Chest 89 [Suppl 4]:225S–233S

Myers RM, Maniatis T, Lerman LS (1987) Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol 155: 501–527

Naruke T, Goya T, Tsuchiya R, Suemasu K (1988) Prognosis and survival in resected lung carcinoma based on the new international staging system. J Thorac Cardiovasc Surg 96: 440–447

Parkin DM, Pisani P, Ferlay J (1993) Estimates of the wordlwide incidence of eighteen major cancers in 1985. Int J Cancer 54: 594–606

Pellegata NS, Losekoot M, Fodde R, Pugliese V, Saccomanno S, Renault B, Bernini LF, Ranzani GN (1992) Detection of K-*ras* mutations by denaturing gradient gel electrophoresis (DGGE); a study on pancreatic cancer. Anticancer Res 12: 1731–1736

Rabbitts TH (1991) Translocations, master genes and differences between the origins of acute and chronic leukemias. Cell 67: 641–644

Rodenhuis S, Slebos RJC, Boot AJ, Evers SG, Mooi WJ, Wagenaar SS, van Bodegom PC, Bos JL (1988) Incidence and possible clinical significance of K-*ras* oncogene activation in adenocarcinoma of the human lung. Cancer Res 48: 5738–5741

Rodenhuis S, Slebos RJ (1992) Clinical significance of *ras* oncogene activation in human lung cancer. Cancer Res [suppl] 52: 2665s–2669s

Rosell R, Li S, Skacel Z, Mate JL, Maestre J, Canela M, Tolosa E, Armengol P, Barnadas A, Ariza A (1993) Prognostic impact of mutated K-*ras* gene in surgically resected non-small cell lung cancer patients. Oncogene 8: 2407–2412

Shimosato Y, Kodama T, Kameya T (1982) Morphogenesis of peripheral type adenocarcinoma of the lung. In: Shimosato Y, Melamed MR, Nettesheim P, (eds) Morphogenesis of lung cancer, vol 1. FL:CRC Press, Boca Raton pp. 65–89

Slebos RJC, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJ, Wagenaar SS, Vanderschueren RG, van Zandwijk N, Mooi WJ, Bos JL, Rodenhuis S (1990) K-*ras* oncogene activation as a prognostic marker in adenocarcinoma of the lung. N Engl J Med 323: 561–565

Slebos RJC, Hruban RH, Dalesio O, Mooi WJ, Offerhaus JA, Rodenhuis S (1991) Relationship between K-*ras* oncogene activation and smoking in adenocarcinoma of the human lung. J Natl Cancer Inst 83: 1024–1027

Sørensen JB, Hirsch FR, Olsen J (1988) The prognostic implication of histopathological subtyping of pulmonary adenocarcinoma according to the classification of the World Health Organization. An analysis of 259 consecutive patients with advanced disease. Cancer 62: 361–367

Strauss BS (1992) The origin of point mutations in human tumour cells. Cancer Res 52: 249–253

Sugio K, Ishida T, Yokoyama H, Inoue T, Sugimachi K, Sasazuki T (1992) *ras* gene mutations as a prognostic marker in adenocarcinoma of the human lung without lymph node metastasis. Cancer Res 52: 2903–2906

Sundaresan V, Ganly P, Hasleton P, Rudd R, Sinha G, Bleehen NM, Rabbits P (1992) *p53* and chromosome 3 abnormalities, characteristic of malignant lung tumours, are detectable in preinvasive lesions of the bronchus. Oncogene 7: 1989–1997

Våhåkangas KH, Samet JM, Metcalf RA, Welsh JA, Bennet WP, Lane DP, Harris CC (1992) Mutations of *p53* and *ras* genes in radon-associated lung cancer from uranium miners. Lancet 339: 576–580

Weinberg RA (1989) Oncogenes, antioncogenes and the molecular basis for multistep carcinogenesis. Cancer Res 49: 3713–3721

Westra WH, Slebos RJ, Offerhaus GJ, Goodman SN, Evers SG, Kensler TW, Askin FB, Rodenhuis S, Hruban RH (1993) K-*ras* oncogene activation in lung adenocarcinomas from former smokers. Cancer 72: 432–438

World Health Organization (1981) Histologic typing of lung tumours. 2nd edn World Health Organization, Geneva