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ras gene mutations in patients with non-small cell lung carcinoma

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ABSTRACT

BACKGROUND: *Lung cancer is the leading cause of cancer mortality in most countries, with every year's increasing incidence. At present, surgical resection of early stage disease presents the only treatment associated with a high likelihood of 5-year survival. On the other hand, patients with advanced disease have 5-year survival less than 5%. This poor prognosis is attributable largely to lack of efficient diagnostic methods for early detection and the inability to cure metastatic disease. Therefore, efforts aimed at early identification and interventions in lung cancer are of the highest importance. Mutations in ras oncogenes appear to play a significant role in the development of non-small cell lung carcinoma (NSCLC). Thus, the aim of our study was to determine the incidence of H-ras and K-ras mutations in patients with NSCLC of different histological subtypes: adenocarcinomas (AC), squamous cell carcinomas (SCC), large cell carcinomas (LCC), and adeno-squamous carcinomas (AC-SCC).*

METHODS: *We analyzed 41 patients with stage I, II and III of histologically confirmed NSCLC (histological grade 2 and 3). DNA was isolated from frozen tumors by standard phenol-chloroform extraction. Mutations in exon 1 H-ras and K-ras gene were detected by PCR-SSCP method.*

RESULTS: *Mutations in the H-ras gene were found in only 2 of 41 analyzed tumors (4.9%). The both mutations were found in SCC making the overall incidence in this histological subtype 10.5% (2 of 19). K-ras mutations were detected in 31.7% (13 out of 41) of tumors, with higher incidence in tumors of clinical stage I - 45% (9 out of 20).*

CONCLUSION: *Our results indicate that H-ras mutations apparently play an inferior role in lung carcinogenesis. However, mutations in K-ras gene probably present an early event in genesis of NSCLC, and not only in adenocarcinomas, as the majority of previous reports indicate, but also in squamous cell carcinomas as well.*

KEY WORDS: *Carcinoma, Non-Small-Cell Lung; Genes, ras; Mutation; Polymerase Chain Reaction; Polymorphism, Single Stranded Conformational*

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INTRODUCTION

Lung cancer is one of the leading causes of cancer-related deaths in the world, causing over 1 million deaths worldwide each year (1). Despite advances in clinical management, mortality and incidence have shown similar increasing trends over the last two decades. Only 13% of patients diagnosed with lung cancer survive 5 years which emphasis the need for improved therapeutic interventions (2). The therapy of non-small cell lung carcinoma (NSCLC) which accounts for ~75-80% of all lung cancer cases, remains a clinical challenge. Radical treatment still gives the best results, but it depends primarily on early detection, since patients with advanced and metastatic disease have 5-year survival rates less than 5% (3). The poor survival following lung cancer may be ascribed to its biological aggressiveness and a relative lack of symptoms attributable to early disease, combined with the rather poor sensitivity of the classical approaches for early detection (4). Thus, the identification of early molecular events in lung carcinogenesis could have a major impact on early detection of this disease (5). Proto-oncogenes of *ras* family (H-*ras*, K-*ras* and N-*ras*) encode for 21 kD proteins, which are located on the inner surface of the membrane where they act as signal transducers. Ras proteins normally respond to growth stimuli, such as epidermal and platelet-derived growth factors by exchanging guanosine triphosphate (GTP) for constitutively bound guanosine diphosphate (GDP) thereby triggering cell division. The signal is terminated when the Ras protein hydrolyzes its bound GTP to GDP in a reaction that is stimulated by a guanosine triphosphatase (GTPase) activating protein (GAP) (6). Hyperexpression of *ras* genes results in growth stimulation, whereas point mutations at codons 12, 13 and 61 alter their structure. This prevents inactivation, which results in inappropriate prolonged signaling for continued cell division, which ultimately leading to cell transformation (7). The frequency of *ras* mutations in human cancers depends on the organ of origin, being rare in squamous cell carcinomas and adenocarcinomas of the breast, stomach and ovary and occurring in 30% to 50% of adenocarcinomas of the lung and the colon and almost all pancreatic cancers (8). Mutations in colon and pancreatic cancers are found only in the K-*ras* gene. In cancers of the urinary tract and bladder, mutations are primarily in the H-*ras* gene and mutations in N-*ras* gene are found in leukemia. Thyroid carcinomas are unique in having mutations in all three *ras* genes (9). Unlike H-*ras* gene, the incidence and prognostic significance of K-*ras* mutations in lung cancer have been largely stud-

ied. These mutations occur primarily in codon 12 and are found more often in adenocarcinomas obtained from smokers than nonsmokers. Besides that, a number of studies have suggested that such mutations represent a possible biomarker of poor survival, particularly in early stage disease (10). This finding, however, is not universal and demands further investigations. The aim of our study was to determine the incidence of H-*ras* and K-*ras* mutations in patients with NSCLC, who undergone a surgical resection. We also correlated the incidence of ras gene mutations with prognostic feature - stage of disease, together with histological subtype and grade of tumors as indices further describing prognosis of NSCLC patients.

PATIENTS AND METHODS

Patients

Fresh tumor samples were obtained from 41 patients with histologically confirmed NSCLC. All patients underwent curative operations at the Military Medical Academy, Belgrade, from October 2000 to December 2002. The patients' ages ranged from 43 to 69 years (median 58) and there were 34 men and 7 women. All patients' were smokers or former smokers and all cases were classified into stages I (n=20), II (n=4) and III (n=17) according to the *TNM Classification of Malignant Tumors* and as 19 squamous cell carcinomas, 17 adenocarcinomas, 3 large cell carcinomas and 2 adeno-squamous cell carcinomas, according to the *Histological Typing of Lung Tumors* (11). Among them, there were 19 tumors G2 grade and 22 of G3 grade (Table 1).

Table 1. Patient characteristics

	No.	%
Patients	41	
Men	34	82.9
Women	7	17.1
Age, years		
Median	58	
Range	43 - 69	
Smoking history		
Smokers	29	70.1
Ex-smokers	12	29.3
Histology		
SCC	19	46.3
AC	17	41.5
LCC	3	7.3
AC-SCC	2	4.9
TNM stage		
I	20	48.8
II	4	9.8
III	17	41.5
Grade		
G2	19	46.3
G3	22	53.7

Tumor tissue was frozen in liquid nitrogen and stored at -80˚C until DNA extraction. Genomic DNA was isolated by conventional proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation (12) and was finally dissolved in 100ml of sterile distilled water.

PCR-SSCP analysis

DNA fragments covering the coding regions of exon I H-*ras* and K-*ras* genes were amplified by PCR, using specific oligonucleotide primers for both genes. PCR reactions were designed to generate fragments 123 bp and 111 bp in length. The sequences of primers used for PCR amplification of exon I H-*ras* and K-*ras* genes were as follows:

H-*ras*, sense:	5' - ATG ACG GAA TAT AAG CTG GT - 3'	(123 bp)
H-*ras*, antisense:	5' - CGC CAG GCT CAC CTC TAT A - 3'	
K-*ras*, sense:	5' - ATG ACT GAA TAT AAA CTT GT - 3'	(111 bp)
K-*ras*, antisense:	5' - CTC TAT TGT TGG ATC ATA TT - 3'	

For PCR amplification exon I of both genes was used an optimized PCR protocol. We optimized PCR conditions according to Taq polymerase, amounts of primers, magnesium concentrations and cycling parameters to obtain efficient amplification of DNA with little or no background. We varied the amount of polymerase from 1 to 2.5 units; primers amounts for final concentration ranging from 0.2 mM to 0.8 mM and $MgCl_2$ concentrations were varied in a range of 1.5 to 2.5 mM (data not shown). The following conditions were found to provide the best sensitivity and specificity: DNA (500 ng) was amplified in 50 ml volumes in a reaction containing 0.6 mM of each primer, 0.2 mM dNTP mix, 10 mM Tris-HCl buffer (pH 8.4), 2.0 mM $MgCl_2$ and 1.5 U of Taq polymerase. PCR was carried out by initial denaturation at 95˚C for 5 minutes, followed by 35 amplification cycles of 950C (60 seconds) for denaturation, 58˚C (H-*ras*) or 51˚C (K-*ras*) (60 seconds) for annealing, 72˚C (60 seconds) for extension, followed by 10 minutes extension at 72˚C.

Mutated *ras* genes were identified using single strand conformation polymorphism (SSCP) analysis. We used this method because it is rapid, well controlled and reproducible and, according to some researchers, more sensitive in detecting mutations than sequencing (13). We used optimized electrophoretic conditions for exon I of H-*ras* and K-*ras* genes: 200V at 4˚C for 2 hours.

Statistical analysis

The differences in the incidence of *ras* mutations among tumor type, tumor grade and TMN value were calculated by the χ^2 test.

RESULTS

The purpose of our investigation was to evaluate mutation status of two members of ras gene family (H-*ras* and K-*ras*) in non-small cell lung carcinoma of 41 patients who underwent a surgical resection. The obtained results are summarized in Table 2.

Table 2. Relationship between H-*ras* and K-*ras* mutation positivity and clinical features of patients with NSCLC

	Patients n	H-*ras* mutations %	K-*ras* mutations %
Patients	41	2 (4.9)	13 (31.7)
Men	34	2 (5.9)	13 (38.2)
Women	7	-	-
Age rang (median)	43-69 (58)		
Smoking history			
Smokers	29	1 (3.5)	8 (27.6)
Ex-smokers	12	1 (8.3)	5 (41.7)
Histology			
SCC	19	2 (10.5)	7 (36.8)
AC	17	-	6 (35.3)
LCC	3	-	-
AC-SCC	2	-	-
TNM stage			
I	20	1 (5.0)	9 (45.0)
II	4	1 (25.0)	-
III	17	-	4 (23.6)
Grade			
G2	19	1 (5.3)	9 (47.4)
G3	22	1 (4.6)	4 (18.2)

Status of H-ras gene

Mutations in codon 12, 13 H-*ras* gene were detected by SSCP method (Figure 1) in 2 of 41 analyzed tumors and, since both mutations were detected in squamous cell carcinoma, the incidence of H-*ras* mutations in this histological type of NSCLC is 10.5% (2 of 19). One mutation was detected in SCC stage I (1 of 12) and the other one in SCC stage II (1 of 2) (Figure 2). One H-*ras* mutation was found in carcinoma of histological grade G2 (1 of 19) and one mutation was found in carcinoma of histological grade G3 (1 of 22) (Figure 3).

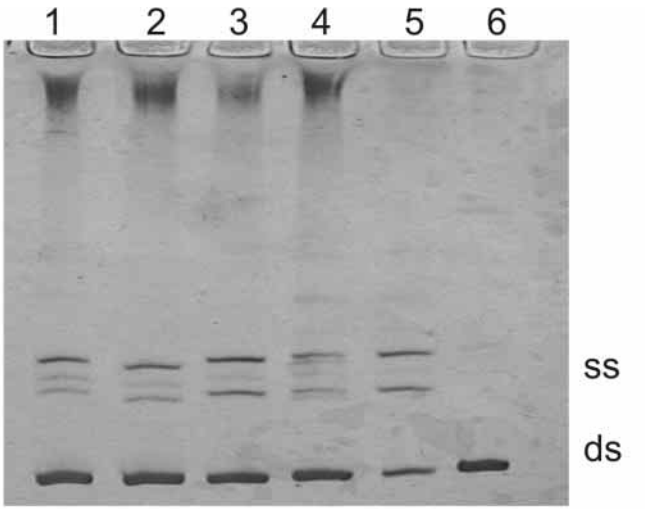

Figure 1. PCR-SSCP analysis of exon I H-*ras* gene using 10% polyacrylamide gel stained with silver nitrate. Lane 2, mutant H-*ras* gene from NSCLC; lane 5, the wild-type H-*ras* gene; lane 6 non-denaturated wild-type H-*ras* gene

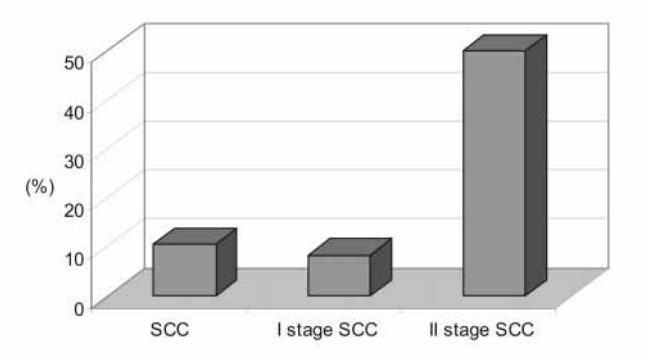

Figure 2. The incidence of H-*ras* mutations found in different stages of SCC

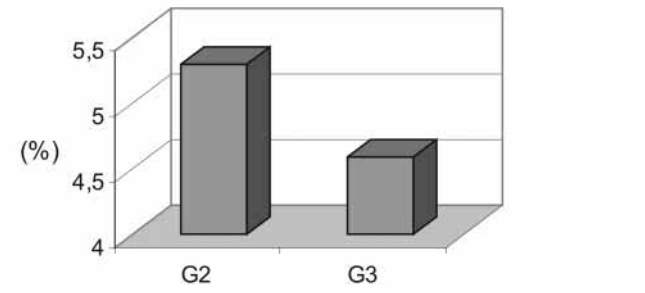

Figure 3. The incidence of H-*ras* mutations found in NSCLC of different histological grades

Status of K-ras gene

Mutations in codon 12, 13 K-*ras* gene were detected in 13 of 41 (31.7%) analyzed tumors (Figure 4) and found incidence varied in different histological subtypes, grades and TNM stages of tumor. Mutations were found in 45% (9 of 20) stage I carcinomas and 23.6% (4 of 17) stage III carcinomas (Figure 5). The overall incidence of mutations in K-*ras* gene in SCC was 36.8% (7 of 19) and in AC 35.3% (6 of 17) (Figure 6). Mutations were not found in carcinomas stage II and in AC-SCC and LCC. While 47.4% (9 of 19) carcinomas with histological grade G2 carried mutations in K-*ras* gene, only 18.2% (4 of 22) G3 carcinomas carried such a mutation (Figure 7). The difference in number of mutations founded in these G2 and G3 carcinomas was statistically significant (χ^2=4.01; p(0.05).

DISCUSSION

A number of published data showed that mutations in oncogenes of *ras* family represent one of the most frequent abnormalities of dominant oncogenes in human tumors. These

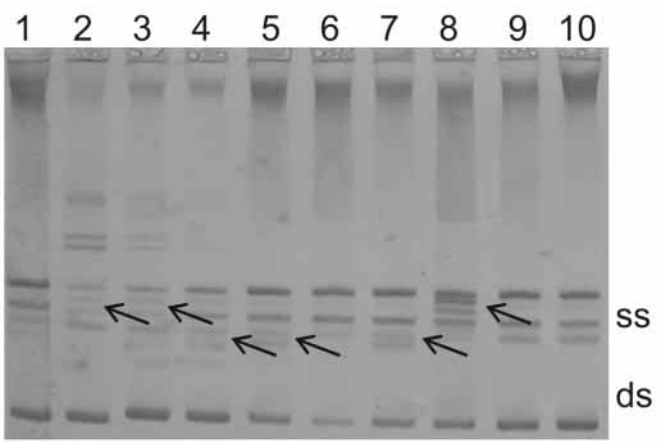

Figure 4. PCR-SSCP analysis of exon I K-*ras* gene using 10% polyacrylamide gel stained with silver nitrate. Extra bands are seen in Lanes 2,3, 4, 5, 7 and 8; Lane 10, sample from peripheral blood leukocytes as a control

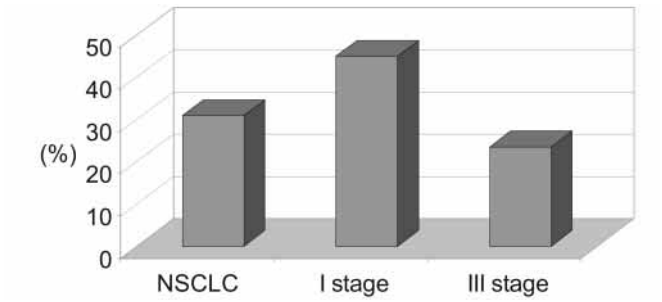

Figure 5. The incidence of K-*ras* mutations found indifferent stages of NSCLC

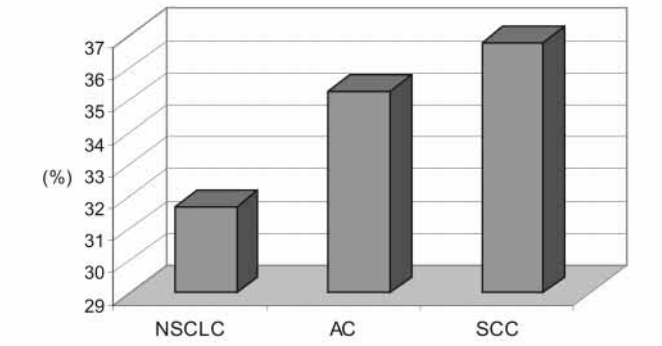

Figure 6. The incidence of K-*ras* mutations found in different histological subtypes of NSCLC

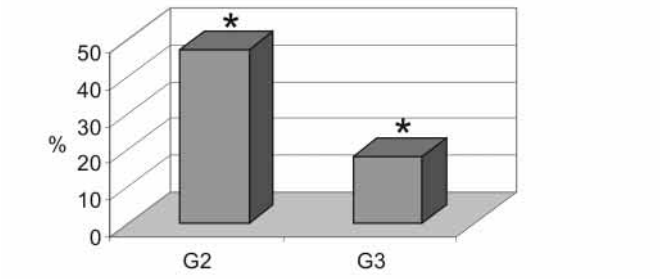

Figure 7. The incidence of K-*ras* mutations found inNSCLC of different histological grades. * indicates statistically significant difference of mutation incidence found in grades G2 and G3 carcinomas

mutations were detected in about 30% of all human cancers and in all cases they were found in codons 12, 13 and 61 (14). The consequence of such mutations is synthesis of structurally and functionally changed Ras protein.

Activation of *ras* is thought to represent only one step in the 'genetic cascade' of events leading to malignant transformation. *In vitro* work demonstrates that transfection of an activated *ras* gene can transform normal mouse fibroblasts (15). In human tumors, frequency

of found activated *ras* genes is dependent on tissue and tumor type. Mutations of H-*ras* gene were, for instance, rarely detected in NSCLC. But, considering the fact that v-H-*ras* is capable of transforming normal human bronchial epithelial cells in culture (16), we were interested in determining if there are any mutations in exon I of H-*ras* gene in our samples. We observed only two mutations among 41 NSCLC analyzed samples, which is more than the incidence found by Vachtenheim and his associates (17). They analyzed 141 samples of NSCLC and found only one mutation of H-*ras* gene in SCC. Published data shows that H-ras mutations were found in SCC and by Suzuki and his associates (18). They analyzed 36 samples of SCC and detected two mutations in H-*ras* gene. The frequency of H-*ras* mutations in SCC that we obtained was 10.5%, since both mutations in this gene that we observed were in SCC. But, H-*ras* mutations were not detected only in SCC. Isobe found mutation in this gene in one of 30 analyzed adenocarcinomas of stage I, which makes 3.3% (19). The difference between our and previously published data could be a consequence of a different number of analyzed samples and a different methodological approach.

According to our results, unlike H-*ras* gene, mutations in K-*ras* are frequent in NSCLC, even more frequent than the majority of other researchers have found. For example, Vachtenheim has found K-*ras* mutations in 12% of analyzed tumors (17), Nelson in 14.3% (20), Graziano in 16.4% (10) and Schiller in 24% (8). We analyzed 41 samples of NSCLC and found K-*ras* gene mutated in 13 carcinomas, which makes the incidence of 31.7%. However, NSCLC represents a heterogeneous group of carcinomas, consisting mainly of squamous cell carcinomas, adenocarcinomas, large cell carcinomas, and adeno-squamous cell carcinomas; the frequency of mutation in K-*ras* gene detected in these different histological subtypes is not the same. Unlike some other authors (20,10), we did not find any mutation in this gene in analyzed large cell carcinomas and adeno-squamous cell carcinomas. But, considering the fact that there were only two AC-SCCs and three LCCs, we need to analyze more samples to make a valid conclusion.

The frequency of mutations in K-*ras* gene found in AC varied from 15-60% (17). Siegfried found this gene mutated in 31.5% of analyzed AC (21) and Vachtenheim in 37% (17), which is consistent with the incidence obtained in our study - 35.3%. The most recent data show the increasing incidence of adenocarcinomas of the lung, which now represent the most frequent histological subtype of NSCLC (22). These findings are very concerning because adenocarcinoma has the worst prognosis of all histological subtypes of NSCLC since it gives an early metastasis. Although in the group we studied there were more patients with SCC than with AC, we can conclude that mutations in K-*ras* gene appear early in genesis of AC because 50% of analyzed AC stage I harbor mutation in this gene.

In most studies published so far, SCC harbored *ras* mutations only exceptionally, in less than 5% of cases (23). It has been thought that even when K-*ras* mutations occur as an early event during the development of the SCC, it probably does not confer a growth advantage to cells, at least in a subset of mutated tumors. Graziano found K-*ras* mutations in 1.6% of analyzed SCC (10), Nelson in 2.8% (20) and Rodenhuis (24) did not find any mutation in 43 analyzed SCC. Our results disagree with these findings showing the incidence of K-*ras* mutations in SCC even higher than in AC - 36.8%. Possible explanations for the inconsistent results include the specificities of geographic regions and populations, nutrition habits, life style, environmental factors and genetic predisposition of particular populations. Influence of demographic characteristics of populations was confirmed by the results of some other non-US studies. Rosell detected mutations in K-*ras* gene in 18% of analyzed SCC (25) and the similar findings reported Cho in a South Korean study (26).

Our results are consistent with the most recent data showing that over 80% of all mutations in *ras* genes are found in K-*ras*. Beside that, over 80% of mutations in this gene are detected in codon 12, which seems to be more affected by cancerogenic agents from cigarette smoke (27). All epidemiological studies confirmed that tobacco smoke, a complex mixture containing many carcinogens, is a major etiological agent of lung cancer, but we cannot discuss that because all patients included in our study were smokers or former smokers.

The development of NSCLC is a multistep process involving accumulation of genetic and epigenetic alterations of malignant cells. A number of literature data has showed that an oncogene activation of K-*ras* appears early in the process of lung carcinogenesis, but the exact time of its activation has not yet been estimated. We detected K-*ras* mutations in 45% of NSCLC stage I and the found incidence was significantly lower in analyzed carcinomas G3 grade than in G2 grade. Based on these findings, we could make conclusion that the oncogene activation of K-*ras* is an early but not an initial event in the genesis of NSCLC. Since the genetic instability is the characteristic of the malignant cell, every tumor represent genetic heterogeneous population of the cells. Because of high sensitivity PCR method used for amplification of K-*ras* gene, it is possible to detect subpopulation of cells that do not have to evolve into superinvasive clones of high-grade carcinomas. That is the reason why very often mutations in *ras* genes detected in primary carcinomas, could not be found in lymph nodes or metastatic lesions. Thus, there is still an unanswered question whether mutations of this gene provide a selective growth advantage to malignant cells. If a selective advantage exists, it may manifest itself clinically in the form of aggressive tumor growth and/or resistance to various medical interventions. It has been reported that *ras* oncogene confers resistance to ionizing radiation, but there are also conflicting results, therefore demanding further investigations (9).

The prognostic significance of K-*ras* mutations in NSCLC has also been studied in a number of retrospective series. In some of them, the presence of K-*ras* mutations has been found to be a negative prognostic factor, associated with early relapse and shortened survival (8). However, this finding needs to be confirmed in studies that will provide a clearer understanding of the clinical relevance of this gene and suggest avenues for future research.

CONCLUSION

Our results indicate that H-*ras* mutations apparently play an inferior role in lung carcinogenesis. However, mutations in K-*ras* gene probably present an early event in genesis of NSCLC, and not only in adenocarcinomas, as the majority of previous reports indicate, but also in squamous cell carcinomas as well.

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