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*Original scientific paper*

# DETECTION OF K-*RAS* GENE MUTATION IN BRONCHIAL ASPIRATE OF PATIENTS WITH LUNG CARCINOMA

Vesna ILIĆ [1], Ilija TOMIĆ [2], Gordana CVETKOVĆ [2], Radmila BOKUN [3], and Zvonko MAGIĆ [1]

Institute for Medical Research[1], Clinic of Pulmology[2], Institute of Pathology[3], Military Medical Academy, Belgrade, Serbia

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Lung cancer belongs to a group of tumors with bad prognosis resulting in limited therapeutic chances. It is the most common cause of cancer deaths and cancer-related deaths worldwide. Only 25-40% of lung cancers are considered resectable when a diagnosis is made and just 20% of patients have a confined disease at the time of surgery. That makes problem of early diagnosis of lung cancer one of the biggest challenges in clinical oncology. Our goal was to determine whether molecular genetic assays could augment conventional clinical and laboratory diagnostic procedures.

Bronchoalveolar aspirate of patients with different histological types and stages of NSCLC were analyzed for presence of K-*ras*

Corresponding author: Vesna Ilić, Institute for Medical Research, Military Medical Academy, Crnotravska 17, Belgrade, 2661-122/ 3608-857, vesnailic@hotmail.com

oncogene mutations (codons 12 and 13) and compared with cytological findings in the same samples.

Mutations in codons 12 and 13 of K- and H-*ras* genes in bronchoalveolar aspirate of 53 patients (pts) were examined by polymerase chain reaction and SSCP analysis.

Mutations in K-*ras* gene were identified in 18/53 (34%) specimens of bronchoalveolar aspirate, out of which 3/18 were adenocarcinomas, 11/18 squamous cell carcinoma, two were with anaplastic and two patients with chronical lung disease. The same samples were examined for presence of malignant cells by conventional cytological analysis. Normal cytological results were found in 6 samples of patients with malignant tumors out of which K-*ras* mutations were detected in 4 samples.

A presence of mutated K-*ras* gene may prove useful as an adjunct to cytological analysis and also could serve as additional criteria for early diagnosis in patients with bronchogenic carcinoma.

*Key words*: early detection, lung cancer, k-*ras* gene

## INTRODUCTION

Lung cancer is a tumor with limited therapeutic chances and bad prognosis. Only 25-40% of lung cancers can be surgically removed when a diagnosis is made and just 20% of people have a confined disease when they are surgically treated, so far, the most effective way of treating of the cancer (ANĐELIĆ *et al.* 2001).

Despite many efforts, there is no significant progress in treating lung cancer. Because of the increasing number of lung cancer patients, medical approach to lung cancer must be directed to prevention and early diagnosis of disease. During recent decades the development of molecular oncology strongly have influenced the understanding of molecular events including the initiation and progression of lung cancer, giving the hope that early detection of lung cancer cells will be possible in the near future.

Among the most common genetic changes in lung cancer are mutations of *ras* genes. *Ras* gene family contains three well characterized functional genes K-, H- and N-*ras* . Their products are similar proteins (molecular mass 21KD) having a crucial role in transduction of external signals which regulate cell growth and differentiation. Through many post translational modifications protein is located at internal side of cellular membrane. Thus both "*wild-type*" (WT) and mutated *ras* gene interact with proteins included in signal transduction. Point mutations in codons 12, 13 and 61 of *ras* genes activate them as oncogenes causing conformational changes that lead to permanent activation that stimulate autonomous and infinite cell growth. (BISHOP, 1991)

Changes in K-*ras* gene in codon 12 are the most common (80-90%) G-T transversion and predominantly present in 25-40% atypical adenomatous

hyperplasia's. (ZHANG, 2008.) Many molecular studies of the presence of K-*ras* mutations in lung cancer are based on analysis of body liquids, usually of sputum, bronchoalveolar fluid –lavage (BAL), aspirate or serum and plasma samples. Numerous PCR techniques, besides conventional PCR (like PCR-PIREMA, PCR-RFLP etc.) enable detection of mutated K- *ras* allele in a sample of BAL in patients (56%) with diagnosis of primary adenocarcinoma as well as in the patients (31%) with large cell lung carcinoma. (SOMERS, 1998). Significant percent of K-*ras* mutation was observed in lung tissue without neoplastic phenotype in 60% patients with NSCLC as well as in lung tissue of 12% patients with benign diagnosis. Furthermore, comparison of the results for the presence of mutations obtained by PCR method with negative cytological results in sputum, showed that tumor specific mutations were present in 7/8 samples (YAKUBOVSKAYA *et al*., 1995).

It is obvious that results of standard diagnostic procedures, such as cytology, radiography and bronchoscopy, are not quite satisfactory for early diagnosis of the disease and consecutively for better therapy results. Anamnestic information like history of smoking, professional exposure to carcinogens indicates the need for disease prevention and different approaches to a particular patient. Improving sensitivity of molecular testing will enable use of less invasive and more sensitive and specific diagnostic procedures, thus giving advantage in early diagnosis of disease.

This study was aimed to detect point mutations in K-*ras* oncogene in bronchial aspirate of patients with suspected lung cancer, and further to compare obtained results with cytological and patohistological results in the same samples.

## MATERIAL AND METHODS

*Patients*

Samples for analysis were obtained from patients aspirates during bronchoscopia at the functional diagnostics unit, Clinic for Pulmology (MMA). During the preliminary analysis we found out that bronchial aspirate samples appears to be better than BAL and pleural excretion, concerning that a particular material is obtained directly from the lesions without any additional procedures. After pathohistological examination from 53 collected samples, 8 were inflammatory changes, 3 patients had chronical obstructive pulmonal disease, 23 patients with planocelular carcinoma, 11 with adenocarcinoma, 3 adenosquamous and 3 with anaplastic lung carcinoma (Table 1.) The majority of patients with confirmed lung cancer (examined group consisted of 44 man and 9 women patients aging from 41 to 76 year), were classified as stage IIIb and IV, and all of them received presurgical irradiation.

| Diagnosis | | | | | | | Number |
|---|---|---|---|---|---|---|---|
| HOCD | | | | | | | 3 |
| Pleuritis | | | | | | | 8 |
| SCLC | | | | | | | 2 |
| | * | I | II | IIIa | IIIb | IV | |
| NSCLC | Ca.plano | 1 | 1 | 6 | 9 | 6 | 40 |
| | Ca.adskv. | | | | 3 | | |
| | Ca. anap. | | | 2 | 1 | | |
| | Ca. aden. | | | 1 | 8 | 2 | |
| Total | | 1 | 1 | 9 | 21 | 8 | 53 |

*Table 1. Diagnosis and tumor classification of examined group of patients*

HOCD-chronic opstructive lung disease;
Ca. plano.-Planocelular lung cancer;
Ca.adskv.-Adenosquamouslung cancer;
Ca. anap.-Anaplastic lung cancer;
Ca. aden.- Adenocarcinoma;
Pleuritis – inflamation of pleura;
SCLC – small cell lung cancer;
NSCLC – non-small cell lung cancer

## Methods

*DNA isolation*

DNA from bronchial aspirate was isolated by phenol-chlorophorm extraction. After precipitation of DNA at -20˚C, rinsing with absolute ethanol and drying, collected DNA was resuspended in the 100µl of redestilated water.

*Detection of K-ras mutation*

PCR was performed in 50µl of reaction mixture which finally include 400-600 ng templates DNA, 1XPCR buffer with 10mM KCl and 1, 5 mM $MgCl_2$, 0, 2 mM d NTP mix, 25 pmol of each primer and 1 U of *Taq* polymerase. The 50µl mixture was incubated in Eppendorf thermocycler for 35 cycles of denaturation, annealing and extension at 95ºC, 51ºC and 72ºC, respectively. First reaction was initiated with one 10-minute incubation cycle at 95ºC and ended with a 10 minute elongation at 72ºC. Primer sequences for the I exon of K-*ras* gene is:

5'- ATG ACT GAA TAT AAA CTT GT-3'

5'- CTC TAT TGT TGG ATC ATA TT -3'

PCR product was visualized by agarose and K-*ras* mutations were detected by acrilamide Single Strand Conformation Polymorphism (SSCP) electrophoresis.

Statistics

Statistical analysis of frequency distributions was evaluated by Student's *t* test, $\chi 2$ and/or Fisher′s exact test.

## RESULTS

We analyzed bronchial aspirates of patients from Clinic for Pulmology in order to detect presence of mutations into the first exon (codons 12 and 13) of K-*ras* gene, the most often symptoms were cough, pain, hoarseness, breath rhythm disturbance and a hart weakness. Among 53 patients, through further clinical examinations, 11 had inflammatory changes while 42 were positive for malignant lung neoplasia and further were classified as advanced stage of disease (III and IV stage).

K-*ras* mutations were present in 2/11 patients with inflammatory changes (18%) and in 16/42 with malignant neoplasm's (38%) (Figure 1.) There was no statistically significant difference between patients with inflammatory changes and malignant neoplasm′s in the frequency of K-*ras* mutations.

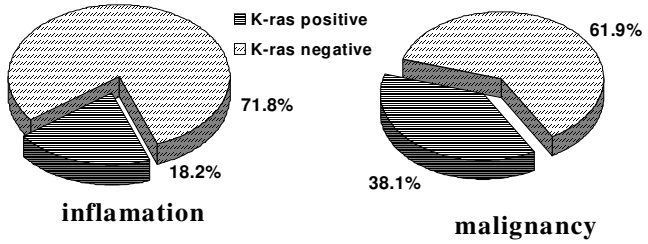


Figure 1. Distribution of K-*ras* mutation in a group of patients

K-*ras* mutations were distributed within different histological types as following: in squamous cell carcinoma (11/23), adenocarcinoma (3/12), as well as in two patients with diagnosis of anaplastic carcinoma and two with chronic obstructive lung disease (Figure 2.)

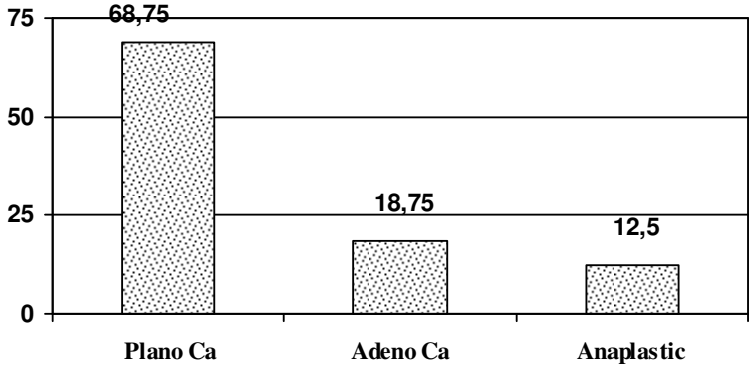


Figure 2. Distribution of K-*ras* mutation in examined group of patients according to histological classification of NSCLC

9/11patients with metastatic disease (metastasis in pleura, chilus and supraclavicular region) had mutated K-*ras* gene (Figure 3.)

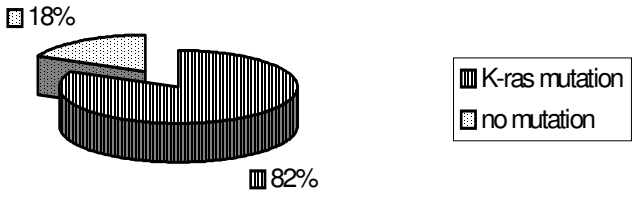


Figure 3.Incidence of K-*ras* gene mutation in lung cancer patients with metastatic disease

Comparison of personal anamnestic data with the incidence of K-*ras* mutation in the bronchial aspirate samples of patients showed that a considerable number of them was in the group of active or former smokers (30.8 ± 12.6 year) (Figure 4.)

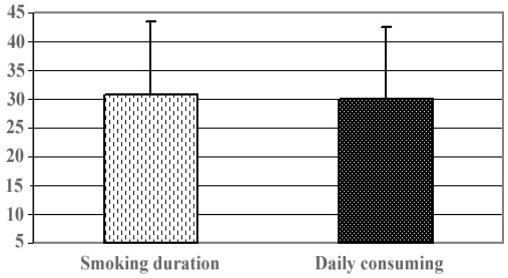


Figure 4.Median value with standard deviation of smoking duration and daily cigarete consuming in examined group

Concerning total number of patients with K-*ras* mutations (n=18), 17/18 were active smokers (Figure 5.)

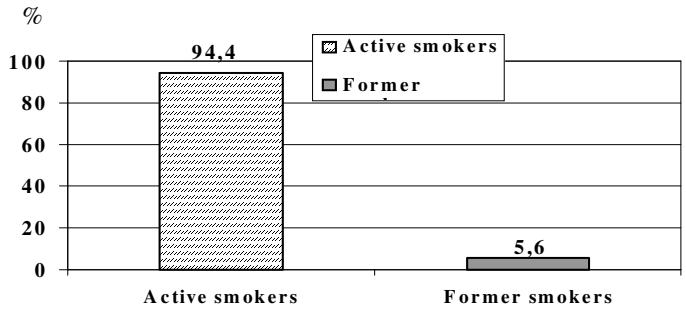


Figure 5. Distribution of active and former smokers in a group of patients with detected K-*ras* mutation

PCR and cytological analysis were performed from the same samples of bronchial aspirate. In the 6 samples with confirmed diagnosis of cancer (patohistological examination) cytological findings were normal. Furthermore, the presenc of K-*ras* mutations in the 4 of these samples was detected. ( Figure 6.)

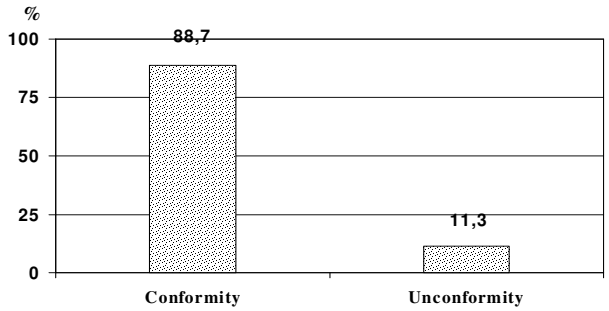


Figure 6. Distribution of the K-*ras* presence and citological findings conformity in bronchial aspirate samples

## DISCUSSION

Lung cancer may develop without any symptoms. It appears in the form of little knots at the lung edges, and patohistological and cytological analyses are crucial for a final diagnosis. When lesions are not available for bronchobiopsy or when biopted sample is not easy to be analyzed hystopathologically, a diagnosis relies on cytological analysis of aspirate and bronchoalveolar lavage. This analysis are not always reliable enough especially when lesions are smaller than 2 cm (OSHITA *et al*., 1999).

The purpose of our study was to analyze the presence of activating point mutations in the K-*ras* gene in the bronchial aspirate of patients which came to pulmologist for the first time. Aspirate was taken during routine bronchoscopic diagnostic procedure. Using PCR-SSCP method, mutations in exon I (codons 12 and 13) of K-*ras* gene were found in 18 out of 53 tested patients (34%), what is in accordance with incidence of mutations, which is set by use of the same technique in previous studies, and it varied from 25 to 48% (MINAMOTO *et al*., 2000). Although, mutations in *Ras* gene family are usually found in adenocarcinoma we have detected in 18% of tested patients what is less than in other studies (30-56%),

while we found more mutations in K-*ras* gene in advanced stage of planocellular lung cancer (48%) (SOMERS *et al.,* 1998).

In the study of Graziano and Gamble (GRAZIANO *et al*., 1999), one out of 61 patients with squamous cell carcinoma had K-*ras* mutation. Other studies found no K-*ras* mutations in the 43 samples of squamous cell carcinomas (RODENHUIS and SLEBOS, 1992). Also, one mutation was found in 37 squamous cell carcinoma samples (3%) (KEOHAVONG *et al.,* 1996). Contrary to these results, some non-US studies reported significant percentage of squamous cell carcinoma with K-*ras* mutation (up to 59% ) (ROSELL, 1996; TOMIDA, 2004).

The contradictory findings of different groups indicate the possibility that detection of mutations also depends on the sensitivity and specificity of used method, as well as on the quality of analyzed samples. Previous detection of K-*ras* mutation in human cancers is changed by the use of sensitive techniques such as PCR-pirema, Point-exact method (which using coupled capture technique and exonucleases, enables detection of one mutated cells out of 15000 normal cells), PCR-RFLP method, Southern blot, etc.

Since genetic basis of malignant changes is relatively well examined sufficient importance is attached to an early detection (molecular-genetic level) of cancer cells. It is a widespread opinion that *ras* mutations occurs relatively early in lung cancer (WRIGHT and GRUIDL, 2000). In human cancers K-*ras* mutations are detected in adenomas of colon, pancreatic hyperplasia where they were defined as an early event of cancer development (MINAMOTO *et al*., 2000). *Ras* mutations are detected in the case of former smokers who have not smoked for more than 15 years before the occurrence of malignancy, as well as in a sputum samples taken a year before clinical diagnosis of lung cancer (KIRSI and WILIAM, 2001).

Numerous studies (CLAYTON *et al.,* 2000) confirm the possibility of difference in PCR and cytological results in detection of malignant cells in BAL. Percentage of detection of K-*ras* mutation in samples which are not cytological diagnosed as malignant, sometimes is more than 20% (KEOHAVONG and DE MICHELLE, 2000). Comparing cytological findings with detection of K-*ras* mutations in the samples of patients with lung cancer we have detected that out of 43 lungs cancer patients 6 were with normal cytology, and in the 4 of these samples K-*ras* mutation was found. According to accessible information (SOMERS *et al.,* 1998) time between K-*ras* point mutation detection in sputum, aspirate cytology and clinical diagnosis of lung cancer varied from 1 month to almost 4 years. Molecular screening of genes involved in lung carcinogenesis thus would be valuable addition to standard cytological analysis as we have found in our study.

Research results (HECHT, 1999) emphasize fact that carcinogens from cigarettes and smoke induce *ras* oncogene mutations, and in connection (HEATHER *et al*., 1999) also showed the increasing incidence of K-*ras* mutations in lung cancer in women. In our study all female patients were smokers for a long time and K-*ras* mutation was detected in two patients, one with HOBP and the other with lung adenocarcinoma. According to recent information one of the mechanisms of selecting K-*ras* mutated clone is considered to be hormonally mediated by

estrogens (PATEL and PETER, 2004). Actually it is known that precursor cells of adenocarcinoma increase expression of estrogen receptors and thus cause the promotion of growth factors caused by this hormone unlike the other cellular lines of non-small cell lung cancer.

Patients with III stadium of tumor are usually treated with additional radiotherapy. The decision about selection of patients which will be treated by radiotherapy is made, first of all, according to histological and clinical parameters (CHOI *et al*., 2001). It is noticed that patients with tumors of similar histological characteristics react differently to radiotherapy (ADJEI, 2001). Having in mind that K-*ras* mutation increase resistance to radiotherapy, detection of K-*ras* mutation may be an additional information which could be included in prediction of tumor sensitivity to radiotherapy (BERNHARD *et al.,* 2000) enabling in that way appropriate treatment and better prognosis. Determination of K-*ras* gene status in advanced-stage tumor´s can be of a great importance in prognostic and guiding treatment decisions. (ELAINE *et al*., 2009). Also, presence of K-*ras* mutations in the tumor tissue of patients with colon cancer is a strong predictive factor to the therapy with monoclonal antibody against epidermal growth factor receptor (TOL *et al.,* 2009).

## CONCLUSION

Results obtained in this study indicate that usage of molecular markers can be valuable additional diagnostic procedure to standard cytological analyses for early detection of malignant cells in patients with lung cancer. Furthermore, these results can be used for improvement of patent treatment.

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# DETEKCIJA MUTACIJA U K-*RAS* ONKOGENU IZ ASPIRATA BRONHA PACIJENATA SA NESITNOĆELIJSKIM KARCINOMOM PLUĆA

Vesna ILIĆ [1], Ilija TOMIĆ [2], Gordana CVETKOVIĆ [2], Radmila BOKUN [3], i Zvonko MAGIĆ [1]

Institut za medicinska istraživanja[1]
Klinika za pulmologiju[2]
Institut za patologiju[3]
Vojno medicinska Akademija, Beograd, Srbija

I z v o d

Karcinom pluća je maligni tumor sa lošom prognozom koji pruža male terapijske mogucnosti. Svega 25-40% karcinoma pluća je operabilno u vreme kada se postavi dijagnoza, a samo 20% njih ima ograničenu bolest u vreme kada se podvrgavaju hirurškom lecenju - do sada najefikasnijem vidu lečenja ovog karcinoma. Pri tome je petogodišnje preživljavanje samo 13% a veliki broj pacijenata umire već u toku dve godine po postavljanju dijagnoze. Kako je efikasnost lečenja ovog karcinoma jos uvek niska, neophodni su novi pristupi bolesti koji se tiču pre svega rane dijagnostike primenom molekularnih markera. Pojava *ras* mutacija u ranim stadijumima karcinoma pluća predstavljala bi koristan marker koji bi omogućio raniji početak lečenja i time poboljšao njegov ishod. U radu su analizirane mogućnosti detekcije tačkastih mutacija u K-*ras* onkogenu u aspiratu bronha dobijenim tokom rutinske bronhoskopske dijagnostike u cilju blagovremenog otkrivanja bolesti kao i korelacija prisustva mutacija sa dobijenim citološkim i patohistološkim rezultatima. Mutacije u K-*ras* genu su nađene u 18/53 (34%) uzorka bronhijalnog aspirata od kojih je 3/18 histološkog tipa adenokarcinoma, 11/18 planocelularnog, kod dva pacijenta sa anaplastičnim karcinomom i dva sa dijagnozom hronične opstruktivne bolesti pluća. Nepodudarnost rezultata citološkog pregleda i histopatološkog nalaza je nađena u 6 uzoraka, kod kojih je patohistoloskom analizom nadjena maligna bolest a citoloski nalaz je bio normalan. Kod 4 od 6 uzoraka nadjene su K-*ras* mutacije. Sledstveno tome, izmena u K-*ras* genu detektovana *in vitro* testom u kliničkom uzorku može biti dodatni dijagnosticki i prediktivni marker karcinoma pluca.

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