Tumor Biol. (2016) 37:10479–10486
DOI 10.1007/s13277-016-4930-4

ORIGINAL ARTICLE

Frequencies of *EGFR* single nucleotide polymorphisms in non-small cell lung cancer patients and healthy individuals in the Republic of Serbia: a preliminary study

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Received: 10 November 2015 / Accepted: 28 January 2016 / Published online: 5 February 2016

Abstract The purpose of this study was to determine the frequencies of *EGFR* −216G>T, −191C>A, and 181946C>T in Serbian non-small cell lung cancer (NSCLC) patients, as well as to compare it with healthy individuals, in order to assess their potential importance for lung cancer in Serbia. The study involved 56 NSCLC patients and 53 unrelated healthy volunteers, and genotyping was performed on DNA samples obtained from formalin-fixed paraffin-embedded lung tumor tissue and blood, respectively. This was the first time to show genotype frequencies of those single nucleotide polymorphisms for this study group from the territory of the Republic of Serbia. There was very strong evidence of association between age and death due to lung cancer (Pearson chi-square = 43.439, $df = 2$, $p < 0{,}001$), as well as between ever smoking and death due to lung cancer (Pearson chi-square = 31.727, $df = 1$, $p < 0.001$). When dominant genetic model (GG vs. GT+TT) was used for −216G>T, we have found significant association ($p = 0.012$) between −216GG genotype and NSCLC patients within smokers' subgroup. So, carriers of −216GG genotype had higher risk (OR = 4.33, 95 % CI = 1.324–14.179) than noncarriers (GT and TT) for developing non-small cell lung cancer in our patients.

Keywords Epidermal growth factor receptor · Non-small cell lung cancer · Single nucleotide polymorphism

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Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane protein that participates in the regulation of cell growth and oncogene expression [1, 2]. Its intracellular tyrosine kinase (TK) domain, responsible for downstream cell signaling, is coded by *EGFR* exons 18 to 24 that are commonly affected by deletions, insertions, and point mutations [3]. As a single mutation in any of the key molecules of signal cascade can be a trigger for lung cancer development [4–6], EGFR has become a key molecule in oncologic research and tyrosine kinase inhibitors (TKIs) a promising treatment for lung cancer, including non-small cell lung cancer (NSCLC) form [7, 8]. Unfortunately, it has been noticed that not all of the treated patients are good responders to therapy [9–12]. In addition to other predictive biomarkers, *EGFR* polymorphisms have been associated with the outcome of the TKI therapy [13], suggesting that polymorphisms should be in the course of the future scientific concern.

Interethnic differences in the distribution of *EGFR* polymorphisms and mutations [14, 15], as well as in drug response [16, 17] implicate that ethnic groups differ in terms of lung cancer development, clinical course, and prognosis. Single nucleotide polymorphisms −216G>T (rs712829) and −191C>A (rs712830), located in promoter region, and

181946C>T (D994D) (rs2293347) in exon 25 have been observed to participate in the regulation of EGFR activity [18–21]. Previous reports also showed that all three of them display interethnic variability in terms of frequency distribution across different ethnic groups [14, 18–20, 22]. Yet, as to our best knowledge, studies reporting their frequency in Serbian population are currently lacking. Thus, the purpose of this study was to determine the distribution of *EGFR* −216G>T, −191C>A, and 181946C>T in NSCLC patients in Serbia, as well as to compare it with healthy individuals, in order to assess their potential importance for lung cancer in our population.

Materials and methods

Characteristics of SNPs presented in this study, their location in *EGFR* gene, functional significance, primers used for genotypization, melting temperature in PCR, and enzyme used for RFLP analysis were presented in Table 1.

Subjects

This retrospective study included 109 DNA samples obtained from 56 NSCLC patients treated at the Institute for Pulmonary Diseases of Vojvodina, Pulmonary Oncology Clinic, Sremska Kamenica, Serbia, as well as from 53 unrelated healthy volunteers recruited from the Institute of Molecular Genetics and Genetic Engineering, Belgrade, or from Clinical Center, Kragujevac. Healthy controls during the test are selected by those who did not have a fever, inflammation, and chronic disease and did not have any therapeutic protocols or drugs.

DNA isolation

PureLink™ Genomic DNA Kits (Invitrogen/Life Technologies, Carlsbad, CA) were used for extraction of DNA from formalin-fixed paraffin-embedded lung tumor tissue, obtained from cancer patients. DNA samples from healthy volunteers were isolated from blood using QIAamp DNA Blood Mini Kit (Qiagen, Germany). Concentration of DNA was measured using Qubit® Fluorometer (Invitrogen/Life Technologies, Carlsbad, CA).

Polymorphism analyses

EGFR polymorphisms −216G>T and −191C>A were genotyped using the PCR-RFLP method according to Liu et al. [22], with optimisation strategy and final conditions already described in Obradovic et al. [23]. Briefly, the temperature profile of PCR using KAPA Taq HotStart PCR Kits (Kapabiosystems, Boston, MA, USA) was initial denaturation at 95 °C for 5 min, cycling steps (×45) of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 60 s, than final extension at 72 °C for 7 min. The total volume of PCR reaction was 25 μl, with 1 μl genomic DNA, 0.4 μl each primer, 0.2 mM each dNTPs, 5 % DMSO, and 1 U KAPA Taq DNA polymerase in 1× PCR buffer A (with 1.5 mM $MgCl_2$). Detection of 197 bp PCR products was performed by gel electrophoresis with ethidium bromide stained on 2 % agarose gel. 181946C>T (rs2293347) was genotyped according to Ma et al. [20], with modifications. Namely, temperature profile of PCR using KAPA Taq HotStart PCR Kits was initial denaturation at 95 °C, 5 min; 45 cycles of denaturation at 94 °C, for 30 s; annealing at 55 °C, for 30 s; extension at 72 °C, for 60 s; and final extension at 72 °C, for 7 min. PCR was performed in total volume of 25 μl, with 1 μl genomic DNA, 0.4 μM of each primer, 0.2 mM each dNTPs, magnesium concentration was adjusted for 1.7 mM MgCl2, and 1 U KAPA Taq DNA polymerase in 1× PCR buffer A. A detection of 244-bp PCR products was performed by gel electrophoresis with ethidium bromide stained on 2 % agarose gel.

Restriction enzymes BseRI (New England Biolabs, Ipswich, MA), Cfr42I, and Fast Digest TfiI (PfeI) restriction enzyme (Fermentas/Thermo Fisher Scientific, Vilnius, Lithuania) were used for RFLP digestion for −216G>T, −191C>A, and 181946C>T, respectively (Table 1). Products of restriction were detected by electrophoresis: for −191C>A and 181946C>T on 3 % agarose gel and for −216G>T on 8 % polyacrylamide gel.

Table 1 SNPs characteristics: location, functional significance, primers for genotypization, melting, temperature, enzyme for restriction. Modified according to Ma F. et al. [20]

No.	dbSNP-ID	Base change	Location	Position	Consequence type	PCR primers	Tm	Endonuclease
1.	rs712829	G>T	−216[a]	Promoter	Gene regulation	5′-CTCCTCCTCCTCTGCTCCTC-3′; 5′-GGGGCTAGCTCGGGACTC-3′	63 °C	BseRI
2.	rs712830	C>A	−191[a]	Promoter	Gene regulation	5′-CTCCTCCTCCTCTGCTCCTC-3′; 5′-GGGGCTAGCTCGGGACTC-3′	63 °C	Cfr42I (SacII)
3.	rs2293347	G>A	181946[a] (D994D)	Exon 25	Synonymous variant	5′-ATGAGGTACTCGTCGGCATC-3′; 5′-GAACCAAGGGGGATTTCATT-3′	55 °C	TfiI

[a] Nucleotide location counting from the ATG codon of the *EGFR* gene

Sequencing analysis

After purification of PCR products with QIAquickF PCR Purification Kit (Qiagen, Germany), using ABI PRISM® BigDyeTM Terminator v 3.1 Cycle Sequencing Kit, sequencing was conducted on ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA). Obtained sequence was compared and confirmed with the reference sequence of EGFR promoter region (http://www.ncbi.nlm.nih.gov; GenBank reference: M11234.1).

Statistical analyses

For statistical analyses, subjects were assigned to genotype groups based not only on each SNP, i.e., −191 C/C, C/A, or A/A, −216G/G, G/T, or T/T, and 181946G/G, G/A, or AA, but also according to dominant and recessive genetic model to wild-type homozygote or heterozygote + variant homozygote, and wild type homozygote + heterozygote or variant homozygote, respectively.

The three SNPs were tested for Hardy-Weinberg equilibrium. The haplotypes and their frequencies were estimated by applying expectation-maximization (EM) algorithm. Gametic phase was evaluated using the Excoffier-Laval-Balding (ELB) algorithm, and afterwards, linkage disequilibrium (LD) coefficients between alleles at different loci were computed. For haplotype inference and LD estimation, Arlequin 3.5.1.3 software [24] was used.

Contingency table analysis and chi-square test (or Fisher's exact test, when necessary) were performed to assess the association of genotype frequencies between NSCLC patients and healthy controls for each polymorphism. Demographic data were compared across the genotype with the same test as well. According to age, subjects were divided into three groups: less than 41, 41–61, and more than 61. To test the effect of smoking, both ex and current smokers were considered smokers and compared (separately or in combination) with the non-smoker group.

Data were analyzed using SPSS-17 software (SPSS, Inc.). All p values less than 0.05 were considered statistically significant.

Results

Demographic data for all study subjects are presented in Table 2. There were more male (67.9 %) than female (32.1 %) smokers.

The median age for healthy subjects was 41 (range 25–66), which corresponds well to general Serbian population mean [25] and significantly differs ($p = 0.023$) from lung cancer patients (median 61, range 36–78). There was very strong evidence of association between age and death due to lung cancer (Pearson chi-square = 43.439, $df = 2$, $p < 0.001$), as well as between smoking and death due to lung cancer (Pearson chi-square = 31.727, $df = 1$, $p < 0.001$) (Table 2). The similar result was obtain when current and ex-smokers were separately compared with the non-smoking group (Pearson chi-square = 33.424, $df = 2$, $p < 0.001$). Frequency of genotypes for all tested SNPs for healthy controls and NSCLC patients is presented in Fig. 1.

Table 2 Demographic data for healthy controls and NSCLC patients from the territory of the Republic of Serbia

Demographic data	Healthy controls No. (%)	NSCLC patients No. (%)	p value[a]
EGFR samples	53	56	
Gender			0.100
Male	27 (51.2)	38 (67.9)	
Female	26 (49.1)	18 (32.1)	
Year			<0.01
<41	32 (60.4)	3 (5.4)	
41–61	18 (34.0)	28 (50.0)	
>61	3 (5.7)	25 (44.6)	
Smoking status			<0.01
Smokers	17 (37.0)	38 (67.9)	
Nonsmokers	27 (58.7)	4 (7.1)	
Ex-smokers	2 (4.3)	14 (25.0)	

[a] Chi-square test, two sided

There were no significant differences in genotype distribution between NSCLC patients and healthy controls, or in relation to any of the demographic characteristic ($p > 0.05$, Tables 3, 4, 5, and 6).

Results showed that the most frequent haplotypes for −191C/A and −216G/T in both NSCLC patients and healthy subjects were CG (56.23 %) and CT (26.94 %); AG was present at 15.55 % and AT was present at 1.28 %. Results of LD analysis ($D' = 0.792$, $r' = 0.05$) suggest that SNPs −191C>A and −216G>T are in linkage disequilibrium. The most frequent genotypes for both healthy controls and NSCLC patients were CG/CT and CG/CG (Fig. 2) without statistically significant differences between them ($p > 0.05$). Rare genotypes CT/AG and CT/AT were present within NSCLC patients and AG/AT that was present only in the control group (Fig. 2).

Next, we have analyzed association between −216G>T polymorphism and NSCLC disease within smokers' subgroup. By using dominant genetic model (GG vs. GT+TT), we have found significant association ($p = 0.012$) between −216GG genotype and NSCLC patients. Namely, carriers of −216GG genotype had higher risk (OR = 4.33, 95 % CI = 1.324–14.179) than noncarriers (GT and TT) for developing non-small cell lung cancer.

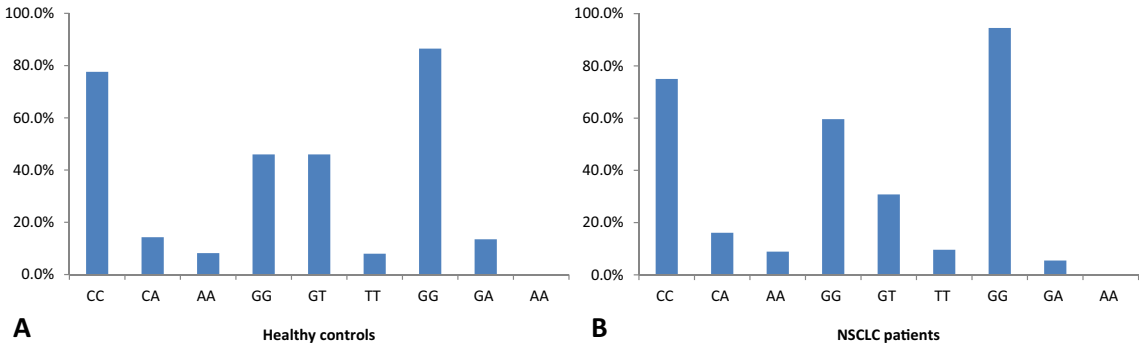

Fig. 1 Frequency of SNPs: CC, CA, AA refers to −191C/A; GG, GT, TT refers to −216G/T; and GG, GA refers to 181946G/A: for **a** healthy controls and **b** for NSCLC patients

Discussion

In the present study, we assessed the frequencies of *EGFR* −216G>T, −191C>A, and 181946C>T in NSCLC patients and healthy volunteers from the territory of the Republic of Serbia. Since SNPs are normally present in both healthy and tumor tissue of the same person, DNA for genotyping can be obtained from any tissue [18, 21, 26–28], and in our study, we used lung cancer tissue and blood. Genotype frequencies found in Serbs correspond well to NCBI database [29–31], with mostly homozygous wild type more frequent than any other genotype for all tested SNPs in NSCLC patients and healthy volunteers.

The most frequent haplotypes in the whole study group were CG and CT, which corresponds well to the literature data [22]. Lack of deviation from Hardy-Weinberg equilibrium for −216G>T and 181946G>A found in the whole study group implicates similar allele distributions in NSCLC patients and healthy subjects, while deviation observed for −191 C>A is probably due to small sample size.

Earlier reports in several different populations from East Asians [21, 27, 32] showed similar distribution frequency of polymorphisms −216G>T and −191C>A between healthy individuals and lung cancer patients. However, significant difference in 181946C>T genotype distributions between NSCLC patients and healthy controls was observed [18].

Ethnic differences in cancer development, mortality rate, and survival were already evidenced [33], as well as significant differences in SNP distributions among ethnic groups. SNP −216G>T was found to be more frequent in Caucasians and African-Americans than in Asian individuals, but −191C>A was present only in Caucasians. On the other hand, 181946C>T was most frequent in Asians [18, 22]. Our results correspond well to previous reports in Caucasians.

Position of two *EGFR* polymorphisms −216G>T and −191C>A indicate their significance in gene regulation, with

Table 3 Statistical analysis for SNPs of healthy controls and NSCLC patients

SNPs	Genotype/allele	Healthy controls *n*	NSCLC patients *n*	OR	95 % CI	$p^{a,c}$	HWE
−191C/A	CC	38	42	1.00		0.926	0.01
	CA	7	9	1.15	0.467–2.842		
	AA	4	5				
	C	83 (84.7 %)	93 (83.0 %)				
	A	15 (15.3 %)	19 (17.0 %)				
−216G/T	GG	23	31	1.92	0.863–4.250	0.168	0.610
	GT	23	16	1.00			
	TT	4	5				
	G	69 (69.0 %)	78 (69.6 %)				
	T	31 (31.0 %)	26 (23.2 %)				
181946G/A	GG	32	52	2.71		0.260[b]	0.663
	GA	5	3	1.00	0.606–12.109		
	AA	0	0				
	G	69 (93.2 %)	107 (97.3 %)				
	A	5 (6.8 %)	2 (2.7 %)				

[a] Pearson chi-square

[b] Fisher's exact test

[c] Calculated values under dominant genetic model

Table 4 Genotype frequencies for −191C>A

Factors	−191C>A						Summary		p^a
	CC		CA		AA				
	No.	%	No.	%	No.	%	No.	%	
Status									0.954
Healthy controls	38	36.2	7	6.7	4	3.8	49	46.7	
NSCLC patients	42	40.0	9	8.6	5	4.8	56	53.3	
Summary	80	76.2	16	15.2	9	8.6	105	100.0	
Age									0.466
<41	27	25.7	4	3.8	2	1.9	33	31.4	
41–61	32	30.5	6	5.7	6	5.7	44	41.9	
>61	21	20.0	6	5.7	1	1.0	28	26.7	
Summary	80	76.2	16	15.2	9	8.6	105	100.0	
Gender									0.112
Male	52	49.5	6	5.7	6	5.7	64	61.0	
Female	28	26.7	10	9.5	3	2.9	41	39.0	
Summary	80	76.2	16	15.2	9	8.6	105	100.0	
Smoking status									0.326
Smoker	42	42.4	6	6.1	6	6.1	54	54.5	
Non smoker	23	23.2	6	6.1	0	0.0	29	29.3	
Ex-smoker	12	12.1	3	3.0	1	1.0	16	16.2	
Summary	77	77.8	15	15.2	7	7.1	99	100.0	

[a] Chi-square test for contingency table analysis

former located in binding site of a transcription factor Sp1 and latter near the initiation site of transcription [34, 35]. Previous studies confirmed that these two SNPs are associated with increased promoter activity and gene and protein expression, with CT haplotype compared to CG having greater influence on mRNA expression [19, 22]. On the other hand, 181946C>T is placed in the coding region (exon 25), conveys worse response to Gefitinib treatment [20], have a protective

Table 5 Genotype frequencies for −216G>T

Factors	−216G>T						Summary		p^a
	GG		GT		TT				
	No.	%	No.	%	No.	%	No.	%	
Status									0.284
Healthy controls	23	22.5	23	22.5	4	3.9	50	49.0	
NSCLC patients	31	30.4	16	15.7	5	4.9	52	51.0	
Summary	54	52.9	39	38.2	9	8.8	102	100.0	
Age									0.730
<41	18	17.6	13	12.7	2	2.0	33	32.4	
41–61	21	20.6	19	18.6	4	3.9	44	43.1	
>61	15	14.7	7	6.9	3	2.9	25	24.5	
Summary	54	52.9	39	38.2	9	8.8	102	100.0	
Gender									0.838
Male	31	30.4	24	23.5	6	5.9	61	59.8	
Female	23	22.5	15	4.7	3	2.9	41	40.2	
Summary	54	52.9	39	38.2	9	8.8	102	100.0	
Smoking status									0.697
Smoker	25	26.3	19	20.0	6	6.3	50	52.6	
Non smoker	13	13.7	14	14.7	2	2.1	29	30.5	
Ex-smoker	10	10.5	5	5.3	1	1.1	16	16.8	
Summary	48	50.5	38	40	9	9.5	95	100.0	

[a] Chi-square test for contingency table analysis

Table 6 Genotype frequencies for 181946G>A (D994D)

Factors	181946G>A (D994D)						Summary		p^a
	GG		GA		AA				
	No.	%	No.	%	No.	%	No.	%	
Status									0.260[b]
Healthy controls	32	34.8	5	5.4	0	0.0	37	40.2	
NSCLC patients	52	56.5	3	3.3	0	0.0	55	59.8	
Summary	84	91.3	8	8.7	0	0.0	92	100.0	
Age									0.067
<41	17	18.5	4	4.3	0	0.0	21	22.8	
41–61	40	43.5	4	4.3	0	0.0	44	47.8	
>61	27	29.3	0	0.0	0	0.0	27	29.3	
Summary	84	91.3	8	8.7	0	0.0	92	100.0	
Gender									0.474[b]
Male	53	57.6	4	4.3	0	0.0	57	62.0	
Female	31	33.7	4	4.3	0	0.0	35	38.0	
Summary	84	91.3	8	8.7	0	0.0	92	100.0	
Smoking status									0.626
Smoker	47	53.4	4	4.5	0	0.0	51	58.0	
Nonsmoker	18	20.5	3	3.4	0	0.0	21	23.9	
Ex-smoker	15	17.0	1	1.1	0	0.0	16	18.2	
Summary	80	90.9	8	9.1	0	0.0	88	100.0	

[a] Chi-square test for contingency table analysis

[b] Fisher's exact test

effect in ever-smokers, and displays interethnic variability in frequency distribution [18].

It was already indicated that genetic factors may affect cancerogenesis, creating susceptible phenotypes, especially for tobacco consumers [36]. Also, it was shown that carriers of −216GG genotype show lower response rates for EGFR-TKI therapy and shorter progression-free survival than patients with the GT genotype [13]. In addition, it was observed that presence of at least one T allele of −216G>T improves progression-free survival [9]. In our study, *EGFR* −216GG genotype was a risk factor for smokers to develop lung cancer, confirming T allele as a possible protective element.

There are thousands of chemicals in tobacco smoke; some of them confirmed to be carcinogens [37]. The influence of smoking on mutational spectrum has already been shown [38], with nitrosamines and benzopyrenes as particularly important triggers of mutagenesis and cancerogenesis [39–41]. Our results conform well to the previous reports, with ever smoking being associated with death due to lung cancer in Serbs. Furthermore, we found tumor-related death to be associated with older age [25]. Our results showed that there are more lung cancer patients among individuals older than 61, which correlate with literature data [33].

Better responders on TKI therapy were patients positive for somatic mutations in tyrosine kinase (TK) domain of EGFR in NSCLC [13]. Still, it is not completely an elucidated mechanism of developing somatic mutations in tumors, but it is proposed that is higher influence of ethnic than environmental factors. This conclusion is based on results on Asian patients that change their host countries without change in mutation

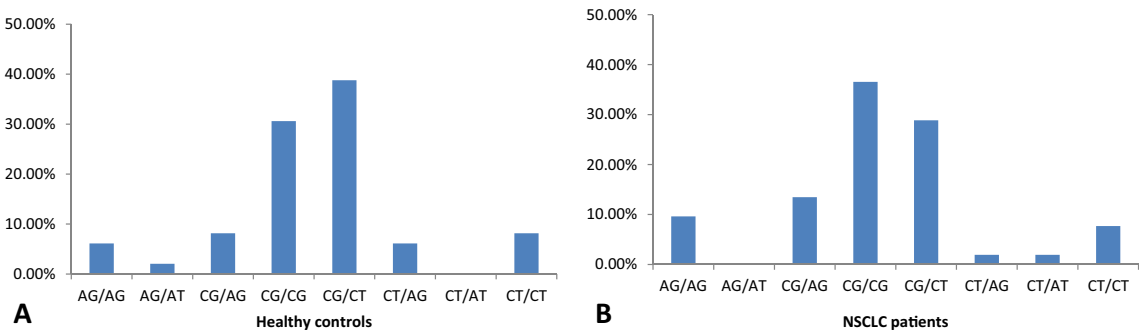

Fig. 2 The most frequent haplotype combinations for −191C>A and −216G>T: **a** for healthy controls and **b** for NSCLC patients

incidence [42, 43]. Although interethnic differences in environmental, demographic, genetic factors, response to therapy, survival, and prognosis for polymorphisms was noted [44], it is not still elucidated that they could be a potential cause of somatic mutations in tumors. Even though in Asian patients polymorphisms contributed development of *EGFR* mutations [42], still confirmation of this finding is necessary for other ethnicities. But due to clear interethnic differences in allele frequencies for many functional EGFR polymorphisms, potential influence of polymorphisms for appearance of somatic mutations in NSCLC indicates different ethnic susceptibility for lung cancer. Investigation for differences among ethnic groups is not important for racist connotation, but to search for different combination of factors that might be the most effective in personalized treatment approach.

There are some studies that propose SNPs to be predictors for effectiveness of EGFR-TKI treatment [13]; others are more suspicious [45], suggesting extensive analyses to confirm independent influence of polymorphisms as a predictors. When reliable and independent predictor for TKI effectiveness and safety would be found, NSCLC will probably be cured. We propose that it is hardly one single factor to be responsible for lung cancerogenesis, but it is probably a combination of variables contributing to the occurrence and development of NSCLC, so seeking for that combination that probably has ethnic background is a remaining challenge for further studies.

In conclusion, our study revealed *EGFR* polymorphism and smoking as risk factors for NSCLC development and mortality in Serbs. Due to small sample size, further larger studies are warranted to confirm our findings.

Acknowledgments The study was financially supported by the Ministry of Science, Republic of Serbia, 175056.

Compliance with ethical standards

Conflict of interests None

Ethical standards The study was approved by local ethics committees.

Informed consent Written informed consent was obtained from all study subjects.

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