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ORIGINAL ARTICLE

Distribution of EGFR SNPs -191C/A and 181946G/A in patients with lung cancer depending on smoking status in the Republic of Srpska, Bosnia and Herzegovina

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Summary

Purpose: *To analyze the frequencies of two single nucleotide polymorphisms (SNPs) of EGFR gene, -191C/A and 181946G/A, among lung cancer patients from the Republic of Srpska, Bosnia and Hercegovina, as well as to assess the association of SNP genotypes with the cancer type and other demographic characteristics of patients, particularly with the smoking status.*

Methods: *This study enrolled 41 lung cancer patients from the territory of Republic Srpska, Bosnia and Herzegovina. Detection EGFR SNPs was performed using PCR-RFLP methodology. PCR was performed on 2720 Thermal Cycler (Applied Biosystems, United States). PCR, as well as RFLP products, were detected by gel electrophoresis. SPSS-17 software (SPSS, Inc.) was used for statistical analyses.*

Results: *There was significantly more male than female smokers in our cohort (p=0.006). In addition, the proportion of smokers was higher among patients with adenocarcinoma in comparison to patients with other lung cancer types (p=0.044). Adenocarcinoma was less common in patients older than 64 years (p=0.035). The wild type homozygous genotype of both SNPs was the most frequent genotype in all the tested demographic groups. Using dominant genetic model for -191C/A SNP, we observed statistically significant association of -191CC genotype and adenocarcinoma (p=0.043) in the subgroup of patients younger than 64 years. Namely, patients younger than 64 years and carriers of -191CC genotype had higher risk (odds ratio/OR=9.6; 95% confidence interval/CI= 0.8477 to 108.7214) for adenocarcinoma than the ones carrying -191CA or -191AA genotype.*

Conclusions: *Patients younger than 64 years and carriers of -191CC genotype have significantly higher risk for adenocarcinoma than carriers of -191CA or -191AA genotype. Further studies on larger cohorts are necessary to evaluate -191C/A SNP as a potential biomarker.*

Key words: *adenocarcinoma, EGFR, lung cancer, smoking status*

Introduction

Lung cancer is the most frequently diagnosed malignancy, and the leading cause of cancer-related mortality [1,2]. Since in the majority of cases conventional chemotherapy is ineffective with side effects, an extensive research is being conducted with the aim of introducing new therapeutics and targeted therapy based on molecular markers [3].

Pathways that regulate and control cell growth and proliferation are undoubtedly important for the etiology of cancer. Epidermal growth factor

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receptor (EGFR) is overexpressed in many cancers, leading to uncontrolled cell proliferation and carcinogenesis [4].

Due to specific mutations or polymorphisms in *EGFR* gene, there is a significant inter-patient heterogeneity of response to treatment with tyrosine kinase inhibitors (TKI). Hence, the accurate identification of patients who might benefit from *EGFR* TKI therapy has become an important step in the treatment decision-making [5,6]. The tyrosine kinase domain of EGFR is encoded by exons 18–25 of *EGFR* gene, and the majority of mutations associated with enhanced sensitivity to EGFR TKIs are located in exons 18–21 [7]. In addition, a study by Ma et al. revealed the association between the SNP 181946G/A (rs229334) in exon 25 and better response to TKI therapy [10].

Besides the variants in the coding exons, SNPs in the promoter region of *EGFR* have also been investigated for their role in modified promoter activity and response to EGFR-TKI therapy [8]. For example, SNP -191C/A has been associated with enhanced transcription of *EGFR* gene, thus increasing the production of EGFR protein [8,9].

In a previous study, Obradovic et al. reported the frequencies of polymorphisms -191C/A and -216G/T in the promoter region and 181946 G/A in the coding region of *EGFR* gene in patients with lung cancer in Serbia in comparison to healthy controls [11]. In this study we further investigated the frequencies of *EGFR* SNPs -191C/A and 181946G/A in different types of lung cancer and with focus to smoking status in patients from Republic Srpska, Bosnia and Herzegovina.

Methods

Subjects

The study included 41 DNA samples obtained from lung cancer patients admitted to the University Hospital Foca, Public Hospital Bjeljina and Public Hospital East Sarajevo, Republic of Srpska, Bosnia and Herzegovina, after confirmation of diagnosis at the Department of Pathology. The study was approved by the Ethics Committee of the University of East Sarajevo, Foca, Republic of Srpska, Bosnia and Herzegovina.

The study included 31 males and 10 females, with a median age of 64 years (range 50-84). Non-small cell lung carcinoma (NSCLC) was diagnosed in 82.93% (34) patients, and small-cell lung carcinoma (SCLC) in 17.07% (7) patients. Eighteen (43.09%) patients had histologically confirmed adenocarcinoma, while 23 (56.15%) patients had other type of lung cancer. Demographic data of the study group are presented in Table 1.

DNA isolation

DNA was isolated from lung cancer patients` peripheral blood using Accuprep® Genomic DNA Extraction Kit (Bioneer, South Korea). Concentration of DNA was measured using NanoVue® 4282 Spectrophotometer (GE Healthcare, Milwaukee, WI, USA).

SNP genotyping

EGFR polymorphisms -191C/A and 181946G/A were genotyped using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, with a few modifications according to a previous report [11]. According to the final DNA concentrations, samples were divided into three groups. PCR reaction was carried out in a total volume of 25 µl of sample, with 5 µl genomic DNA from the sample group with concentration 5-12.4 ng/µl, 4 µl genomic DNA from the sample group with concentration 13.4-22 ng/µl and 3 µl genomic DNA from the sample group with concentration 23-77 ng/µl. PCR was performed in 2720 Thermal Cycler (Applied Biosystems, Foster City, United States).

The temperature profile of PCR reaction, using KAPA Taq DNA polymerase (KapaBiosystems, Boston, Massachusetts, USA), for -191C/A (rs712830) genotyping was as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 72°C for 1 min, and final extension at 72°C for 10 min. Detection of 197 bp PCR products was performed on 2% agarose gel stained with ethidium bromide and visualized by BioDoc Analyze (Analytik Jena, Germany).

The temperature profile of PCR reaction, using KAPA Taq DNA polymerase (KapaBiosystems, Boston, Massachusetts, USA), for 181946G/A (rs2293347) genotyping was as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, and final extension at 72°C for 10 min. Detection of 244 bp PCR products was performed on 2% agarose

Table 1. Demographic characteristics of the cohort enrolling 41 lung cancer patients from the Republic of Srpska, Bosnia and Herzegovina

Characteristics	*n (%)*
NSCLC	34 (82.93)
SCLC	7 (17.07)
Lung cancer patients	
Adenocarcinoma	18 (43.09)
Other type of lung cancer	23 (56.15)
Age, years	
<64	22 (53.66)
>64	19 (46.34)
Gender	
Male	31 (75.61)
Female	10 (24.39)
Smoking status	
Smoker	13 (31.71)
Ex smoker	17 (41.46)
Never smoker	11 (26.83)

gel stained with ethidium bromide and visualized by BioDoc Analyze (Analytik Jena, Germany).

To detect -191C/A polymorphism, PCR product was incubated at 37°C for 1 hr with restriction enzyme *SacII* (New England BioLabs, Ipswich, MA). Products of digestion (uncut 197 bp; cut 165 bp and 32 bp) were detected by 3% agarose gel electrophoresis and visualized by Vilber LourmatTransilluminator (Vilber, France).

To detect 181946G/A polymorphism, PCR product was incubated at 65°C for 1 hr with restriction enzyme *TfilI* (New England BioLabs, Ipswich, MA). Products of digestion (uncut 244 bp; cut 171 bp and 73 bp) were detected by 3% agarose gel electrophoresis and visualized by Vilber Lourmat Transilluminator (Vilber, France).

Statistics

The differences in genotype distribution for the two analyzed SNPs between NSCLC and SCLC, as well as between adenocarcinomas and other lung cancer types were analyzed using Chi square test and Fisher exact test, and contingency table analysis. The same tests were used to obtain the results for genotype distributions between different demographic groups (defined by age, gender and smoking status).

All statistical tests were carried out using SPSS-17 software (SPSS, Inc.). P values less than 0.05 were considered statistically significant.

Results

This study enrolled 41 lung cancer patients from the territory of Republic Srpska, Bosnia and Herzegovina. According to the smoking status, the patients were divided into 2 groups. In the first group, they were divided into 3 categories (never smokers, ex smokers and current smokers). The second group included 2 categories (never smokers *vs.* ex smokers merged with smokers) (Table 1).

In our cohort, a significantly higher frequency of smokers *vs.* non smokers was detected among male patients in comparison to female patients (p=0.006) (Figure 1a). In addition, the predominance of adenocarcinoma over other lung cancer types was significantly higher in the group of smokers *vs.* the group of non smokers (p=0.044) (Figure 1b). Adenocarcinoma was also shown to be less common among patients older than 64 years in comparison to younger patients (p=0.035) (Figure 1c). Similar results for all these demographic variables were obtained when the patient cohort was divided into smokers, ex smokers and never smokers (data not shown).

Table 2. Genotype frequencies for -191 C/A

Demographic factors	*CC*		*CA*		*AA*		*Summary*		*p value*
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	
									0.303
NSCLC	26	63.41	6	14.63	2	4.88	34	82.93	
SCLC	4	9.76	3	7.32	0	0.00	7	17.07	
Total	30	73.17	9	21.95	2	4.88	41	100.00	
Lung cancer patients									0.297
Adenocarcinoma	15	36.59	3	7.32	0	0.00	18	43.90	
Other lung carcinomas	15	36.59	6	14.63	2	4.88	23	56.10	
Total	30	73.17	9	21.95	2	4.88	41	100.00	
Age, years									0.189
<64	17	41.46	3	7.32	2	4.88	22	53.66	
>64	13	31.71	6	14.63	0	0.00	19	46.34	
Total	30	73.17	9	21.95	2	4.88	41	100.00	
Gender									0.686
Male	22	53.66	7	17.07	2	4.88	31	75.61	
Female	8	19.51	2	4.88	0	0.00	10	24.39	
Total	30	73.17	9	21.95	2	4.88	41	100.00	
Smoking status									0.719
Smoker	9	21.95	3	7.32	1	2.44	13	31.71	
Ex smoker	14	34.15	3	7.32	0	0.00	17	41.46	
Never smoker	7	17.07	3	7.32	1	2.44	11	26.83	
Total	30	73.17	9	21.95	2	4.88	41	100.00	
									0.629
Smoker	23	56.10	6	14.63	1	2.44	30	73.17	
Non smoker	7	17.07	3	7.32	1	2.44	11	26.83	
Total	30	73.17	9	21.95	2	4.88	41	100.00	

For the purpose of statistical analysis, the patients were divided into groups which corresponded to SNPs' genotypes (three groups for each SNP): -191 CC, CA, AA and 181946 GG, GA, AA. In addition, we performed analyses under the dominant genetic model for each SNP (CC *vs.* CA+AA and GG *vs.* GA+AA).

The wild type -191CC and 181946GG genotypes were the most frequently detected, both in the group of NSCLC patients and SCLC patients. The differences in genotype distribution between NSCLC and SCLC patients for these SNPs did not reach statistical significance. The prevalence of the wild type homozygous genotype and the absence

Table 3. Genotype frequencies for 181946 G/A

Demographic factors and genotype frequencies	*GG*		*GA*		*AA*		*Summary*		*p value*
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	
Lung cancer patients									0.898
NSCLC	28	68.29	5	12.20	1	2.44	34	82.93	
SCLC	6	14.63	1	2.44	0	0.00	7	17.07	
Total	34	82.93	6	14.63	1	2.44	41	100.00	
Adenocarcinoma	15	36.59	2	4.88	1	2.44	18	43.90	
Other lung carcinoma	19	46.34	4	9.76	0	0.00	23	56.10	
Total	34	82.93	6	14.63	1	2.44	41	100.00	
Age, years									0.381
<64	19	46.34	2	4.88	1	2.44	22	53.66	
>64	15	36.59	4	9.76	0	0.00	19	46.34	
Total	34	82.93	6	14.63	1	2.44	41	100.00	
Gender									0.742
Male	26	63.41	4	9.76	1	2.44	31	75.51	
Female	8	19.51	2	4.88	0	0.00	10	24.39	
Total	34	82.93	6	14.63	1	2.44	41	100.00	
Smoking status									0.258
Smoker	12	29.27	0	0.00	1	2.44	13	31.71	
Ex smoker	13	31.71	4	9.76	0	0.00	17	41.46	
Non smoker	9	21.95	2	4.88	0	0.00	11	26.83	
Total	34	82.93	6	14.63	1	2.44	41	100.00	
Smoker	25	60.98	4	9.76	1	2.44	30	73.17	
Non smoker	9	21.95	2	4.88	0	0.00	11	26.83	
Total	34	82.93	6	14.63	1	2.44	41	100.00	

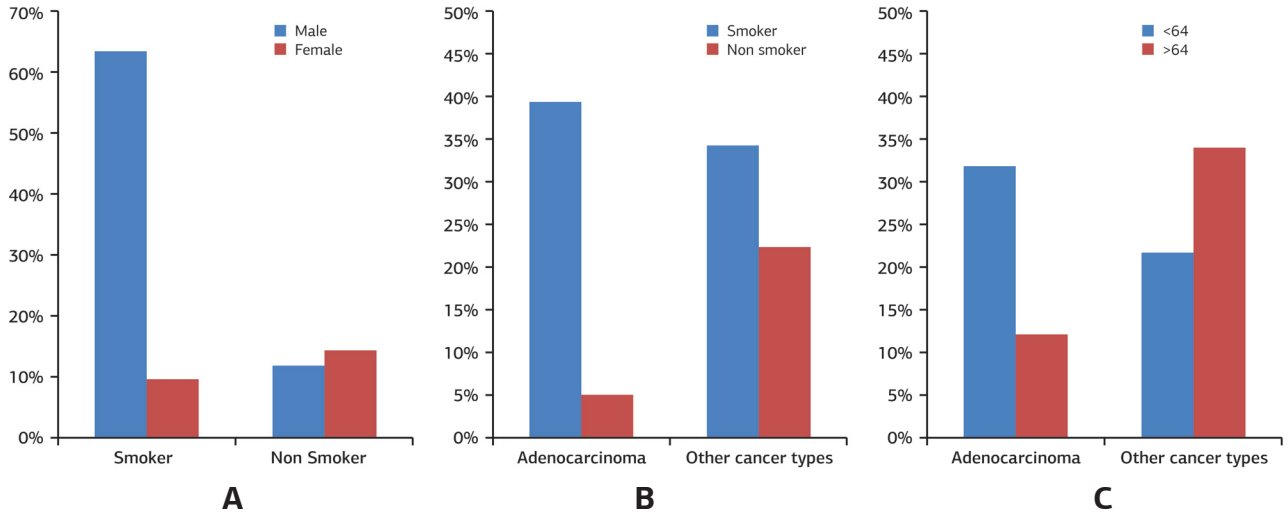

Figure 1. Demographic characteristics of lung cancer patients in Republic of Srpska, Bosnia and Herzegovina: **A)** frequency of smokers and non smokers according to gender, p=0.006; **B)** frequency of adenocarcinoma and other lung cancers according to smoking status, p=0.044; **C)** frequency of adenocarcinoma and other lung cancers according to age, p=0.035.

of different genotype distribution were also observed when we compared adenocarcinoma *vs.* other lung cancer types, as well as when the groups defined by other demographic variables (age, gender, smoking status) were compared (Tables 2 and 3).

In the subgroup of patients younger than 64 years, the difference of genotype frequency for -191 C/A SNP between adenocarcinoma and other lung cancer was tested under the dominant genetic model (CC *vs.* CA+AA). Based on the chi square analysis, statistically significant association of -191CC genotype and adenocarcinoma was detected (p=0.043). Furthermore, patients younger than 64 years and carriers of -191CC genotype were shown to have higher risk (OR=9.6; 95%CI= 0.8477 to 108.7214) for adenocarcinoma than the ones carrying -191CA or -191AA genotype (Figure 2).

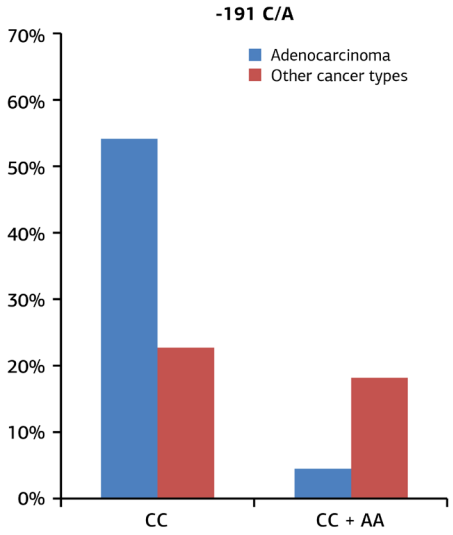

Figure 2. Genotype distribution for -191 C/A under the dominant genetic model between adenocarcinoma and other cancer types, p=0.043.

Discussion

To our knowledge, this is the first study analyzing the distribution of *EGFR*-191 C/A and 181946 G/A genotypes in lung cancer patients from Republic of Srpska, Bosnia and Herzegovina. The frequencies of EGFR polymorphisms were investigated in the patients' groups based on the classification NSCLC *vs.* SCLC type, as well as adenocarcinoma *vs.* other tumor types.

Lung cancer was originally described as NSCLC and SCLC, but the World Health Organization (WHO, Geneva, Switzerland) made improvements in 2015 in lung cancer classification, recognizing 4 major histological types: squamous cell carcinoma, adenocarcinoma, large cell carcinoma and small cell carcinoma [12,13]. Accumulating data in the field of molecular biology pointed to the need for further advances in lung cancer classification, particularly in recognizing differences between adenocarcinoma and other lung cancer types [12].

Increased EGFR protein production is common in lung cancer patients, although the genetic mechanism underlying its overexpression has not been elucidated yet. In addition, *EGFR* variants are frequently associated with lung adenocarcinoma. SNP -191C/A is located near the transcription initiation site of EGFR gene, which implies its effect on the regulation of *EGFR* expression [14-16]. Indeed, it has been demonstrated that this SNP increases *EGFR* promoter activity, leading to increased production of EGFR protein [8,9]. SNP 181946G/A, located in *EGFR* exon 25, has been shown previously to be associated with the treatment outcome, with G allele carriers responding better to TKI therapy [10].

It has been widely accepted that ethnic differences exist regarding cancer incidence and survival rates. Consequently, an extensive research has been aimed at studying mechanisms of cancer predispostition, and many polymorphisms with some functional significance have been recognized as candidates for that predisposition [17,18]. Ethnic difference in the distribution of *EGFR* variants has been observed in multiple studies [8,17,19]. Namely, -191C/A is present only in Caucasians; 181946G/A is also present in Caucasians, but is more frequent in Asian population [8,17]. However, it is still not clear whether these SNPs are associated with increased risk of developing lung cancer [20-22].

In previous studies, wild type homozygous was the most frequent -191C/A genotype, while heterozygous was the most frequent 181946 G/A genotype [20,21]. In our study, wild type homozygous was the most common genotype for both SNPs in the whole cohort, as well as within all the tested groups of patients. There was no evidence of association between a particular SNP genotype with any of the lung cancer types or demographic characteristics. The distributions of the tested SNPs' genotypes that we report here is in concordance with the findings for Caucasians from NCBI database [23-25].

Demographic characteristics of our cohort (predominance of males, smokers and older people) are comparable to those reported in other studies from Western European countries [26,27]. Generally, lung cancer affects mostly male patients from Central and Eastern Europe and from Eastern Asia [1]. Tobacco consumption, more common among men in comparison to women, is also

being reflected on the lung cancer incidence. Our data, with significantly more male than female smokers in Bosnia and Herzegovina, correlate with literature data [18,28-31]. In addition, in our cohort smokers were represented with higher frequency among patients with adenocarcinoma than among patients with other lung cancer types.

Cancer usually occurs in the elderly, as a result of the slow process of somatic mutations accumulation, and is generally being diagnosed in advanced stages [18,32-34]. In our study, adenocarcinomas were significantly more frequent in younger (<64 years) than in older patients (>64 years) when compared to other lung cancer types, which is in concordance with previous reports [34-36]. Furthermore, in the group of patients younger than 64 years we observed a significantly higher risk for developing adenocarcinoma among carriers of -191CC genotype than among carriers of -191CA or -191AA genotype. Interestingly, in a study by Obradovic et al. similar result was also obtained for -216G/T *EGFR* SNP [36].

Extensive scientific research of lung cancer has contributed to earlier diagnosis of the disease and to increase of the survivorship, but improvements of lifestyle, especially cessation of tobacco smoking are required [33,37]. Introduction of new molecular biomarkers, including *EGFR* SNPs, could improve worldwide the battle against lung cancer, but further research on larger cohorts is necessary for their evaluation and, ultimately, implementation in the routine clinical practice [38].

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Conflict of interests

The authors declare no conflict of interests.

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