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ORIGINAL ARTICLE

EGFR mutations and tumor metastases in patients with non-small cell lung cancer in the South of Russia

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Summary

Purpose: *To assess the frequencies of somatic EGFR mutations in the tumor tissues of patients with non-small cell lung cancer (NSCLC) residing in the South of Russia (SR), and to define the relationship between genetic subtypes of NSCLC and the emergence of different types of metastases.*

Methods: *DNA was extracted from formalin-fixed parrafin embedded (FFPE) samples of 721 patients. A total of 29 somatic EGFR mutations were detected using commercial Therascreen EGFR RGQ PCR Kit.*

Results: *EGFR mutations were significantly more frequent in females and non-smokers even when considering the combination of both factors. The frequency of activating EGFR mutations across three age groups (<51, 51-61, >61 years) of women with NSCLC was significantly different (x^2=10.94, p=0.004) and became higher with increasing age. Both activating and resistance mutations of EGFR were not associated with the frequency of regional or distant metastases. The frequencies of both regional and distant metastases were associated with higher disease stage (odds ratio/OR)=16.71; 95% confidence interval (CI): 9.5-29.38; p<0.0001, and OR=2.94; 95% CI: 2.22-3.88; p<0.0001, respectively) and adenocarcinona histology (OR=6.52; 95% CI: 2.03-20.92; p=0.002, and OR=1.99; 95% CI: 0.91-4.34; p=0.083, respectively) even when adjusted for age, gender, and smoking status. The risk for regional metastases development was associated with poor tumor differentiation (OR=2.91; 95% CI: 1.21-7.02; p=0.017).*

Conclusion: *EGFR mutations were not associated with the frequency of regional or distant metastases in SR patients with NSCLC.*

Key words: *adenocarcinoma, epidermal growth factor receptor, metastasis, mutations, non-small cell lung cancer*

Introduction

Treatment with *EGFR* tyrosine-kinase inhibitors (TKIs, e.g., erlotinib, gefitinib, and afatinib) in patients with NSCLC harboring *EGFR* mutations significantly improved the progression-free survival compared with chemotherapy [1]. In the context of determining the influence of genetic alterations in *EGFR* gene on the progression-free survival and metastases development in NSCLC, it is important to understand the role of various factors (age, gender, smoking status, histological subtype, tumor stage) in the metastatic process.

EGFR mutations are usually heterozygous, sensitizing or resistant (nonactivating). Sensitizing *EGFR* mutations increase the kinase activity of *EGFR*, leading to hyperactivation of downstream pro-survival signaling pathways [2]. Exon 19 deletion mutations and the single-point substitution mutation L858R in exon 21 are the most frequent in NSCLC and are termed "classical" sensitizing mutations [1]. These mutations cause structural alterations in the ATP-binding site of the intracellular domain of *EGFR*, thus increasing the affin-

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ity for TKIs and leading to clinical responses [3]. Therefore, analysis of the frequencies of mutations in the *EGFR* gene is essential for the prediction of treatment response. However, little is known about the relationship between *EGFR* mutations and the emergence of different types of metastases.

Targeted therapy may be used before the diagnosis of brain metastases, after diagnosis as initial therapy, and/or after diagnosis and cranial radiotherapy [4]. Thus, being able to detect metastatic lesions provides potential to greatly improve overall patient outcomes [5].

The present study aimed at determining the influence of genetic alterations in *EGFR* gene on metastases development in NSCLC and revealing the link between identified mutations and clinical characteristics, the stage of disease, histological type and type of metastasis.

Methods

This study was approved by the local research ethics board (Rostov Research Institute of Oncology). All of the study participants provided written informed consent. A total of 721 Caucasian (SR) patients diagnosed with NSCLC were included in the study.

We used FFPE samples fixed in 10% buffered formalin that contained at least 20% of the tumor tissue, which were obtained from 3 micron thick slices. DNA was extracted after double ortho-xylene/ethanol dewaxing using QIAamp® DNA FFPE Tissue Kit (Qiagene, Germany), according to the manufacturer's instructions. The concentration of DNA extracted from the samples was measured on fluorimeter Qubit2.0® using Quant-iT™ dsDNA Assay Kit, high sensitivity (Invitrogen, USA). A total of 29 somatic *EGFR* mutations were detected using commercial Therascreen *EGFR* RGQ PCR Kit (Qiagene, Germany) for the detection of mutations in real-time PCR using thermocycler Rotor-GeneQ (Qiagene, Germany) according to the manufacturer's instructions.

The registration of the samples, collection of the clinical data and *EGFR* mutation status were performed through a web-based resource (http://www.cancergenome.ru/page10). The following data were collected: patient characteristics (age, gender, smoking status), tumor characteristics (histological subtype, tumor stage, differentiation grade), and *EGFR* mutation status (presence/absence, and type of mutation). Patient and disease characteristics are summarized in Table 1.

Statistics

The frequencies were compared using non-parametric x^2 test. Multinomial logistic regression was used for the multivariate analysis to assess the association of different factors with the risk for metastasis development/frequency of mutations. P values of less than 0.05 were regarded as statistically significant. Statistical analysis was carried out using IBM SPSS Statistics 23 software.

Table 1. Patient and disease characteristics

Characteristics	*n*	*%*
Age, years		
<51	123	17.1
51-61	272	37.7
>61	326	45.2
Sex		
Female	304	42.2
Male	417	57.8
Smoking history		
Smokers	434	60.2
Former smokers	10	1.4
Never-smokers	277	38.4
Stage at diagnosis		
IA	152	21.1
IB	51	7.1
IIA	51	7.1
IIB	23	3.2
IIIA	159	22.7
IIIB	50	6.9
IV	235	32.6
Metastasis at the time of diagnosis		
Absent	237	32.8
Regional	231	32.1
Distant	253	35.1
Histological subtype		
Acinar adenocarcinoma	64	8.9
Papillary	13	1.8
Bronchoalveolar	2	0.3
Squamous	15	2.1
Mucinous	4	0.6
Acinar- papillary	5	0.7
Adenocarcinoma	618	85.7

Results

Most of the patients (62.2%) enrolled in the present study were diagnosed with stage IV disease, and 35.1% had distant metastases at the time of diagnosis. The median age was 60 years (range 28-85). Adenocarcinoma was the most frequent histological subtype found in 85.7% of the patients, followed by acinar adenocarcinoma, adenosquamous carcinoma and papillary carcinoma not otherwise specified (Table 1). *EGFR* mutations were detected in 25.8% of the patients that is consistent with the rate of 10-35% reported by My Cancer Genome resource (Table 2).

Results of statistical analyses

Ever-smoking patients with NSCLC had a higher risk for development of distant metastases (OR=1.94; 95%CI: 1.04-3.36; p=0.038) (Table 3).

As expected, an association was noted between higher stage of disease and frequencies of both regional (OR=11.33; 95%CI:7.24-17.73; p<0.0001) and distant metastases (OR=2.65; 95%CI:2.11-3.33; p<0.0001). After adjustment for age, gender, and smoking status of the patients, the association with stage became stronger: for regional metastases - OR=16.71; 95% CI: 9.5-29.38; p<0.0001, and for distant metastases - OR=2.94; 95%CI:2.22-3.88; p<0.0001.

Our results suggest that the risk for regional metastases development was associated with poor tumor differentiation (OR=2.91; 95%CI:1.21-7.02; p=0.017). However, this interaction disappeared after adjustment for age, gender, and smoking status (p=0.130). Furthermore, when compared with other pooled histological types, adenocarcinona was noted to be associated with the incidence of both regional and distant metastases development even after adjustment for clinical characteristics - OR=6.52; 95%CI:2.03-20.92; p=0.002, and OR=1.99; 95%CI:0.91-4.34; p=0.083, respectively.

EGFR mutations were not associated with the frequency of regional or distant metastases (Table 3).

Multinomial logistic regression of the putative association between smoking status of the patient and his/her *EGFR* mutation status demonstrated significantly increased incidence for sensitizing mutations in never-smokers (OR=4.31, 95%CI:2.75-6.75; p<0.0001) compared with ever-smokers group (Table 4).

Our results indicate that the rate of sensitizing *EGFR* mutations was 4.5-fold higher in women (95% CI: 3.06-6.47; p<0.0001); this association remained significant after correction for smoking status (OR=2.76, 95%CI:1.56-4.89; p<0.0001).

Table 2. EGFR mutation status of patients with NSCLC

Characteristics	*n*	*%*
Type of mutation		
Exon 20 insertion	9	1.2
S768I	1	0.1
G719X	11	1.5
L861Q	3	0.4
L858R	64	8.9
Exon 19 deletion	91	12.6
T790M	7	1.0
Wild type	535	74.2
Classification of mutations		
Activating (sensitizing)*	169	23.4
Nonactivating (resistance)**	17	2.4
Wild type	535	74.2

* S768I, G719X, L861Q, L858R, exon 19 deletion;
** exon 20 insertion, T790M

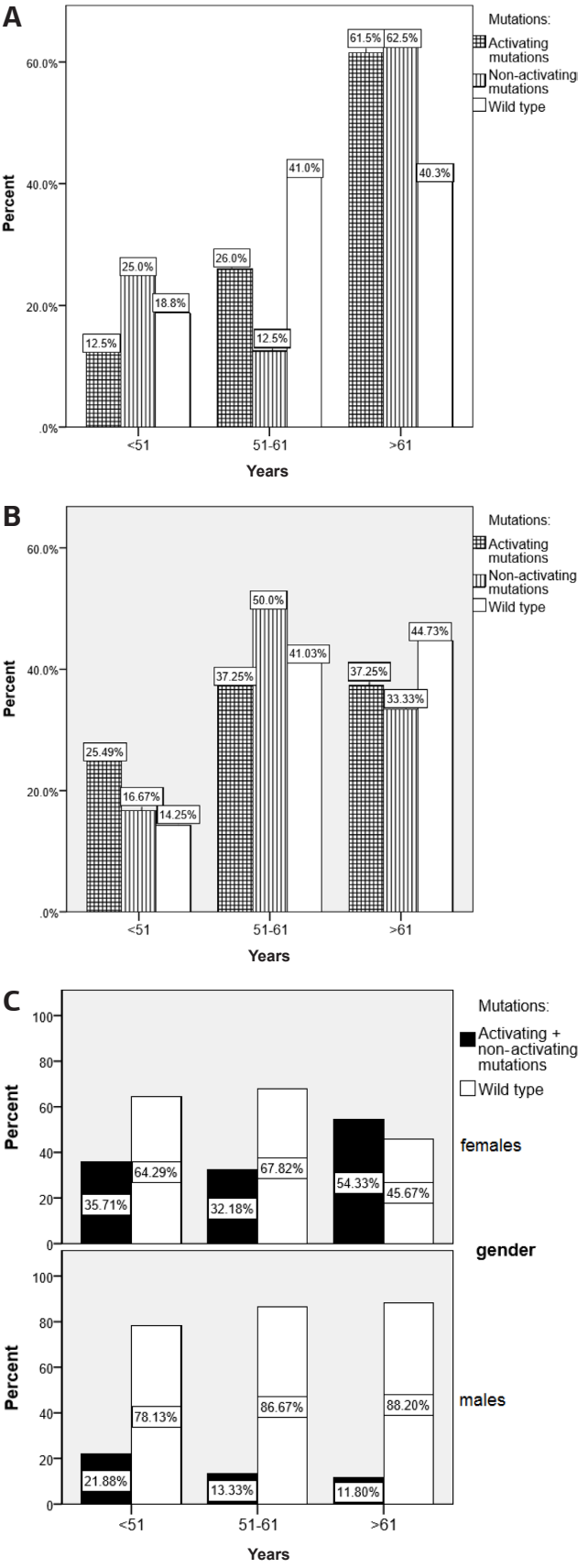

Figure 1. Distribution of the activating/non-activating mutations of EGFR across different age groups of the SR patients with NSCLC: **(A)** females; **(B)** males; **(C)** both. **(A)** and **(B)** mutations and wild type are shown as the percentage from all included patients; **(C)** mutations and wild type are shown as the percentage for each age group of patients.

Table 3. Multinomial logistic regression of the influence of clinical characteristics of the patients and tumor stage, differentiation grade, histological subtype, and EGFR mutations on the risk for regional/distant metastases development

Type of metastasis	*Factor*	*OR*	*p*	*95% CI*	
				Lower	*Upper*
	Patient clinical characteristics				
Regional metastases	Age	1.226	0.163	0.921	1.633
	Females vs. males	0.707	0.245	0.394	1.269
	Ever-smokers vs. never-smokers	0.970	0.915	0.549	1.712
Distant metastases	Age	1.022	0.887	0.760	1.374
	Females vs. males	1.452	0.251	0.768	2.745
	Ever-smokers vs. never-smokers	**1.942**	**0.038**	**1.038**	**3.361**
	Before adjustment for age, gender, smoking status				
Regional metastases	Stage	**11.329**	**< 0.0001**	**7.238**	**17.734**
	Differentiation grade	**2.911**	**0.017**	**1.208**	**7.016**
	Adenocarcinoma histotype vs. other histotypes	**7.835**	**< 0.0001**	**2.620**	**23.437**
	Activating EGFR mutations vs. wild type	1.383	0.559	0.466	4.102
	Non-activating EGFR mutations vs. wild type	0.468	0.587	0.030	7.217
Distant metastases	Stage	**2.653**	**< 0.0001**	**2.112**	**3.332**
	Differentiation grade	1.173	0.647	0.593	2.319
	Adenocarcinoma histotype vs. other histotypes	**2.123**	**0.049**	**1.004**	**4.489**
	Activating EGFR mutations vs. wild type	1.623	0.243	0.720	3.656
	Non-activating EGFR mutations vs. wild type	0.164	0.216	0.009	2.871
	After adjustment for age, gender, smoking status				
Regional metastases	Stage	**16.709**	**< 0.0001**	**9.501**	**29.384**
	Differentiation grade	2.117	0.130	0.801	5.593
	Adenocarcinoma histotype vs. other histotypes	**6.522**	**0.002**	**2.034**	**20.919**
	Activating EGFR mutations vs. wild type	0.935	0.922	0.243	3.591
	Non-activating EGFR mutations vs. wild type	0.340	0.501	0.015	7.900
Distant metastases	Stage	**2.937**	**< 0.0001**	**2.224**	**3.879**
	Differentiation grade	1.007	0.986	0.494	2.053
	Adenocarcinoma histotype vs. other histotypes	1.991	0.083	0.914	4.336
	Activating EGFR mutations vs. wild type	1.182	0.732	0.454	3.074
	Non-activating EGFR mutations vs. wild type	0.120	0.163	0.006	2.371

* Bold numbers denote statistically significant results (p<0.05); OR: odds ratio, 95%CI: 95% confidence interval. Reference category for the dependent variables: absence of metastases

Table 4. Multinomial logistic regression of the influence of gender, smoking status, and age on the incidence of EGFR mutations

Type of mutations	*Factor*	*OR*	*p*	*95% CI*	
				Lower	*Upper*
	Before adjustment (influence of the single factor)				
Sensitizing	Never-smokers vs. ever-smokers	**4.307**	**< 0.0001**	**2.748**	**6.750**
(activating) mutations	Females vs. males	**4.448**	**< 0.0001**	**3.058**	**6.470**
	Age	0.830	0.150	0.645	1.069
Resistance	Never-smokers vs. ever-smokers	2.434	0.161	0.702	8.443
(non-activating) mutations	Females vs. males	**3.189**	**0.035**	**1.088**	**9.351**
	Age	0.989	0.977	0.478	2.048
	After adjustment (gender & smoking status)				
Sensitizing	Never-smokers vs. ever-smokers	**2.131**	**0.015**	**1.158**	**3.923**
(activating) mutations	Females vs. males	**2.763**	**< 0.0001**	**1.562**	**4.889**
Resistance	Never-smokers vs. ever-smokers	0.667	0.681	0.096	4.611
(non-activating) mutations	Females vs. males	5.982	0.069	0.867	41.256

* Bold numbers denote statistically significant results (p<0.05); OR: odds ratio, 95%CI: 95% confidence interval. Reference category for the dependent variables: EGFR wild type

Therefore, the presence of activating *EGFR* mutations was independently associated with the female gender and the non-smoking status.

The analysis revealed that the frequencies of *EGFR* mutations across three age groups (<51, 51-61, >61 y.o.) of women with NSCLC were different: x^2=12.7, p=0.013) – for activating mutations x^2=10.94, p=0.004; for non-activating mutations x^2=2.62, p=0.271. Therefore, the frequency of the sensitizing *EGFR* mutations in females became higher with increasing age (Figure 1A). In males, there were no significant differences in the distribution of *EGFR* mutations across age groups (x^2=4.6, p=0.331) - for activating mutations x^2=4.29, p=0.117; for non-activating mutations x^2=0.32, p=0.853 (Figure 1B and 1C).

Discussion

Previous reports have shown strong evidence of association between clinico-pathological features (e.g. smoking status, sex, age) and frequency of *EGFR* mutations in NSCLC for the Russian population [6]. There is a gradual age-related increase in the frequency of *EGFR* mutations in lung cancer patients. This increase is particularly evident for the L858R substitution in non-smokers. Women have higher frequency of *EGFR* mutation than men, even after adjustment for smoking status [6]. It was pointed out that there was strong evidence of association between age and death due to lung cancer, as well as between ever-smoking and death due to lung cancer [7].

Our previous results demonstrate that the smoking status and gender are important factors influencing the rate of somatic mutations in the *EGFR* gene. The frequency of *EGFR* mutations in the group of non-smoking men was increased 19-fold compared with the group of male smokers; in the group of non-smoking women – 2-fold higher than in the group of smoking women; in the group of non-smoking men - 2.2-fold lower than in the group of non-smoking women. The frequency of *EGFR* mutations in male smokers was decreased almost 23-fold compared with the group of female smokers [8]. Herein we demonstrated increased incidence of sensitizing mutations of *EGFR* in non-smokers when compared with smokers, and in women when compared with men, without and after adjustment for the smoking status. We detected *EGFR* mutations in 25.8% of SR patients with NSCLC, and adenocarcinoma was the predominant histological subtype.

These results are consistent with the following studies. The global map of *EGFR* mutations by ethnicity (n = 33,162 patients with NSCLC of adenocarcinoma histology, of which 9,749 patients had *EGFR* mutation-positive NSCLC/adenocarcinoma) has shown that in all regional (geographic) subgroups where data were available, *EGFR* mutation frequency in NSCLC/adenocarcinoma was higher in women compared with men without adjustment for the smoking status, and in never-smokers compared with ever-smokers [9]. The pooled meta-analysis of 3,688 patients with NSCLC showed that the incidence of *EGFR* mutations differs according to cigarette-smoking history: OR for the *EGFR* mutation in non-smokers relative to smokers was 4.829 (95% CI:3.598-6.482; p<0.001) [10]. This prevalence was confirmed by the study of an unselected Caucasian population of Denmark [11]. Vallee et al. [12] have detected *EGFR* mutations in 13.5% of 1,403 genotyped tumor samples, with female and adenocarcinoma histology predominance. Mutated patients were significantly older than non-mutated ones, consistently with our present findings.

Epidemiologic studies suggest that East Asians are more susceptible to smoking-unrelated lung cancer but less susceptible to smoking-related cancer compared with Caucasians. Mutations in the *EGFR* gene are more common in Asian females and never-smokers; low frequency of *EGFR* mutations in smokers may be the result of dilution by smoking-related cancer [13]. However, a study of 1,018 NSCLC cases of Indian ethnicity has reported higher incidence of exon 21 mutations in male smokers, while exon 19 mutations were predominant among non-smokers [14].

Recent investigations have focused on the association between the frequency of *EGFR* mutations and the risk for metastases to the different sites of the body. For instance, the strong association between *EGFR* mutations and brain metastasis remained significant in the current research [2].

Our results show that *EGFR* mutations were not associated with the frequency of regional or distant metastases in genotyped population, both before and after adjustment for clinical characteristics of the patients (p>0.05, Table 3).

Similarly, Li et al. and Stanic et al. have previously found no difference between the incidence of brain metastases in patients with lung adenocarcinoma of wild-type *EGFR* and those with *EGFR* mutations [15,16]. However, among patients with *EGFR* mutations, the incidence of brain metastases was significantly higher in patients with mutation at exon 19 than in patients with mutation at other sites [15]. With respect to these results, our analysis has detected no association between *EGFR* mutation at exon 19 and incidence of distant metastases in patients with NSCLC when

compared with other pooled types of *EGFR* mutations ($p<0.05$, data not shown).

The logistic regression analysis revealed that the female gender, adenocarcinoma, distant metastasis and chemotherapy are factors associated with *EGFR* gene mutation in NSCLC patients: females resulted in an increased incidence (2.438 times higher compared with males) of *EGFR* mutation; adenocarcinoma, distant metastasis and chemotherapy exhibited an increase in *EGFR* mutation risk (by 2.571, 2.810 and 0.367 times, respectively) [17]. *EGFR*-positive patients with brain metastasis at diagnosis had a longer survival [15]. Therefore, further studies are needed to assess the influence of *EGFR* mutations and other biomarkers on lung cancer metastases, which will provide an opportunity to detect tumor metastases, to reveal groups of NSCLC patients at risk, and to provide therapies to treat metastatic cancer – an invariably fatal process.

Conflict of interests

The authors declare no conflict of interests.

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