

The Incidence of *EGFR*-Activating Mutations in Bone Metastases of Lung Adenocarcinoma

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Abstract Poor prognosis of lung adenocarcinoma is associated with early occurrence of distant metastases. This type of non-small-cell lung carcinoma more frequently involves *EGFR* gene abnormalities, which determine the efficacy of *EGFR* tyrosine kinase inhibitor therapies (*EGFR* TKIs). It is probable that genetic abnormalities present in primary tumor will also be present in metastases. Unfortunately little is known about the incidence of these mutations in the metastases and about the effectiveness of molecularly targeted therapy in such patients. Formalin-fixed, paraffin-embedded tumor tissue was prepared from 431 samples of primary adenocarcinoma, 61 of adenocarcinoma central nervous system (CNS) metastases and 8 of adenocarcinoma bone metastases. The presence of exon 19 deletions was examined using the PCR

technique and amplified PCR product fragment length analysis. The ASP-PCR technique was used to evaluate the L858R substitutions in exon 21, and the results were analyzed using ALF Express II sequencer. In the adenocarcinoma metastases to bone obtained from 8 patients, deletions in exon 19 of the *EGFR* gene were revealed in 3 smoking men and one non-smoking woman, while L858R substitution in exon 21 was found in one smoking woman and one man of unknown smoking status. The incidence of *EGFR* gene mutations in the bone metastases was 75 %, in the primary adenocarcinoma - 12.8 %, and in the adenocarcinoma metastases to CNS - 14.75 %. Five patients with *EGFR* gene mutation revealed in bone metastases were treated with *EGFR* TKIs; the majority of them had a satisfactory response to therapy.

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Abbreviations

AC	adenocarcinoma
ALK	anaplastic lymphoma kinase
ASP-PCR	allele-specific PCR
CNS	central nervous system
EGFR	epidermal growth factor receptor
EGFR TKI	EGFR tyrosine kinase inhibitor
FFPE	Formalin-fixed, paraffin-embedded
NSCLC	non-small-cell lung cancer
PCR	polymerase chain reaction
PFS	progression free survival

Introduction

Lung adenocarcinoma is currently the most common type of non-small-cell lung cancer (NSCLC) diagnosed in the populations of developed countries (more than 40 % of all NSCLC cases). Changes in habits related to smoking took place in these countries, which is especially apparent in female population. Due to the spread of smoking addiction among women (usually light cigarettes) and the ever-increasing number of ex-smokers, lung adenocarcinoma, which occurs more frequently in women (especially of Asian origin), and the etiology of which is moderately associated with the action of tobacco smoke carcinogens, has risen to first place in terms of incidence, surpassing squamous cell carcinoma (strongly associated with the action of tobacco smoke carcinogens). Lung adenocarcinoma not associated with smoking is characterized by different biology and molecular profile than smoking-related NSCLC. *EGFR* (epidermal growth factor receptor) gene mutations and *ALK* (anaplastic lymphoma kinase) gene rearrangements are often revealed in adenocarcinoma cells in non-smoking patients; this has been used for qualifying patients for EGFR tyrosine kinase inhibitor therapy (erlotinib, gefitinib) and ALK inhibitor therapy (crizotinib). Moreover, mutations in *KRAS*, *PIK3CA*, *BRAF*, *DDR2*, *MEK*, and other genes are more frequently revealed in adenocarcinoma patients than in patients with other types of NSCLC [1–4].

The molecular profile of lung adenocarcinoma and its moderate association with cigarette smoking influence the biology of this neoplasm. Adenocarcinoma (AC), more often than other types of NSCLC, originates from the epithelium of peripheral and small bronchi, or even pulmonary alveoli (e.g. adenocarcinoma of alveolar septa with lepidic growth pattern, previously bronchioloalveolar carcinoma associated with *EGFR* gene mutations) and often produces metastases to the lymph nodes and distant metastases to the central

nervous system, liver, adrenal glands, and bones. Because of this, less than 15 % of adenocarcinoma patients can be treated surgically. The remaining patients who have good performance status, but suffer from locally advanced and advanced adenocarcinoma, can receive systemic treatment in the form of chemoradiotherapy, chemotherapy or molecularly targeted therapy [1, 2, 4, 5].

Clinical studies have demonstrated the high efficacy of EGFR tyrosine kinase inhibitors (TKIs) in the 1st line of treatment for patients with NSCLC with activating mutations in the *EGFR* gene (most frequently with L858R substitution in exon 21 or deletion in exon 19), resulting in the extension of progression free survival (PFS) to 10 months in nearly 70 % of patients. Similar efficacy of EGFR TKIs was noted in patients with *EGFR* mutations after the failure of standard 1st line chemotherapy. Unfortunately, only 10 % of adenocarcinoma patients of Caucasian origin are carriers of *EGFR* gene mutations (this percentage amounts to over 40 % among Asian patients). What is more, the majority of studies concerned with *EGFR* gene mutations pertained to primary lung adenocarcinoma. Less information is available on the incidence of these mutations in metastatic adenocarcinoma tumors and on the efficacy of EGFR TKIs therapy in such cases. It is highly probable that genetic abnormalities present in the cells of the primary tumor will also be present in the metastases. This is why current molecular testing guidelines admit the possibility of labeling the *EGFR* mutations in the metastatic tumor if adequate material from the primary cancerous focus is not present. Nonetheless, taking into consideration the heterogeneity of primary NSCLC tumors, one cannot exclude the possibility of the occurrence of metastasis consisting of a different clone of neoplastic cells that have no *EGFR* gene mutations. It is also not known whether the presence of *EGFR* gene mutations favors the occurrence of metastases, and whether it is related to their organ location [3–8].

Bone environment is considered to be poorly differentiated, which could suggest resistance to foreign cell settlement. Nevertheless, metastases to bone are revealed in 30–40 % of NSCLC patients. As the NSCLC metastasizes to bone, numerous and complicated mechanisms must be activated. Their result is the movement of progenitor cancer cells from blood vessels and marrow to skeletal tissue (changed osteolytically by the activated osteoclasts), after which they achieve the capacity for proliferation and clonal growth [4, 5, 9–12].

The skeleton is one of the most common locations where the NSCLC metastases develop; their primary locations include the spine, pelvis, ribs, femur, and ilium. The extensive vascularization of these bone types is probably significant here, as it exposes the bones to more contact with cancer cells traveling through the vascular lumen. Significantly worse prognosis was noted in patients with appendicular bone metastases in comparison to patients with axial bone metastases [7, 8, 10]. However, the etiology of the development of

NSCLC metastases to bone has not been entirely explained, and the international literature almost completely lacks analysis of the status of the *EGFR* gene in NSCLC metastases to bone.

The research subject of the present study was the evaluation of the incidence of *EGFR* activating mutations in lung adenocarcinoma metastases to bone. We also analyzed the incidence of these mutations in the primary lung adenocarcinoma and in adenocarcinoma metastases to the central nervous system (CNS), as well as the efficacy of EGFR TKI therapy in 5 patients with *EGFR* gene mutations revealed in adenocarcinoma metastases to bone.

Material and Methods

Formalin-fixed, paraffin-embedded (FFPE) tumor tissue was prepared from 431 samples of locally advanced or advanced primary adenocarcinoma (AC), 61 samples of AC brain metastases and 8 samples of AC bone metastases. Patients characteristics of AC primary tumor and AC brain metastases are presented in Table 1. Detailed clinical data of the patients with *EGFR* gene mutations examined in AC bone metastases is shown in Table 2.

DNA was isolated from the FFPE tissue using a QIAmp DNA FFPE Tissue Kit (Qiagen, Canada) according to the manufacturer's instructions. Quantitative and qualitative analysis of the isolated DNA was performed on Biophotometer Plus (Eppendorf, Germany). The presence of exon 19 deletions was examined using the PCR technique and amplified PCR product fragment length analysis. In order to evaluate the L858R substitutions in exon 21, the ASP-PCR (allele-specific PCR) technique was used along with two pairs of PCR primers. PCR primers were labeled with Cy5 fluorochrome. The results were analyzed on an ALF Express II sequencer (Tables 2, 3 and 4).

To estimate the sensitivity of the applied techniques, the serial dilutions of DNA from lung cancer cell lines NCI-H1650 and NCI-H1975 with delE747-A750 and L858R mutations into DNA isolated from healthy donors' PBMCs were performed to reach 100 % wild type and 50 %, 25 %,

20 %, 10 %, 5 %, 2 % and 1 % of mutant DNA by volume. It was demonstrated that 10 % of mutated DNA diluted with the control DNA (wild-type) was the sensitivity cut-off point for PCR followed by fragment length analysis for delE746-A750 in exon 19 while for ASP-PCR detection for L858R mutation of *EGFR* it was 5 % of mutated-DNA diluted with 95 % wild-type DNA. Moreover the test was validated by European Molecular Quality Network. Therefore we did not apply direct sequencing as it is a low sensitivity method.

Statistical analysis employed a chi-square test to compare the number of patients with and without *EGFR* mutation in different subgroups.

The study was approved by the Ethical Committee of the Medical University in Lublin (decision no. KE-0254/131/2011).

Results

Activating mutations in the *EGFR* gene occurred significantly more frequently in adenocarcinoma bone metastases (75 %) than in the primary lung adenocarcinoma (12.8 %, $\chi^2=25.43$, $p<0.00001$) or in the adenocarcinoma metastasis to CNS (14.75 %, $\chi^2=15.09$, $p<0.0001$). Mutations in the primary lung adenocarcinoma and in its metastases to CNS were significantly more common among women than among men (respectively: $\chi^2=9.17$, $p=0.0025$ and $\chi^2=4.03$, $p<0.05$). Activating mutations in the *EGFR* gene were revealed in both women with adenocarcinoma metastases to bone. These mutations were also revealed in the AC bone metastases in 4 men; however, the presence of *EGFR* mutations was excluded in two men in this material. It is noteworthy that the vast majority of patients with *EGFR* gene mutations revealed in bone metastases were smokers. The median age of the patients with *EGFR* gene mutations found in the primary tumor (63 years) was insignificantly higher than the median age of patients with the presence of this mutation revealed in the metastatic tumor to CNS (50 years) or bones (59.5 years).

In the material obtained from primary lung adenocarcinoma tumors and the material from AC bone metastases, deletions in

Table 1 Patients characteristic

Feature		Primary adenocarcinoma	Brain metastases		
Age	Median (mean±standard deviation)	63 (63±9 years)	59 (60±9 years)		
Gender	Female	121	17		
	Male	310	44		
Type of material	Surgical	Resection	191	Brain	53
		Mediastinoscopy	32	Cerebellum	6
	Intrabronchial biopsy			Spinal cord	2
		EBUS-TBNA	91		
	Transthoracic FNA	65			
		52			

Table 2 Clinical data of patients with lung adenocarcinoma metastases to the skeleton

Patient	Gender	Age	Tobacco smoking status	<i>EGFR</i> gene status	Site of metastasis
J.A	Woman	65	Non-smoker	Deletion in exon 19	Femur
Z.Z	Man	55	Smoker (45 pack-years)	Deletion in exon 19	Spine, humerus
F.J	Man	82	Smoker (180 pack-years)	Wild-type	Radial bone
G.Z	Man	44	Smoker (15 pack-years)	Deletion in exon 19	Ilium
J.R	Man	60	Former smoker (15 pack-years)	Deletion in exon 19	Numerous bone metastases
K.K	Woman	74	Former smoker (pack-years - no data)	Substitution in exon 21	Ribs and skull bones
D.T	Man	53	No data	Wild-type	Skull bones
B.M	Man	59	No data	Substitution in exon 21	Rib bones

exon 19 were slightly more common than L858R substitutions in exon 21 of the *EGFR* gene. In the adenocarcinoma metastases to CNS, mutation in exon 21 was more common than deletions in exon 19.

The presence of *EGFR* mutations in both bone metastases and the primary lung tumor was confirmed only in patient J.A. In the remaining patients, mutation was only revealed in bone metastases. In these patients, the material from primary tumors was not available for the analysis of mutations in the *EGFR* gene.

Four patients with activating mutations in the *EGFR* gene, which were revealed in adenocarcinoma bone metastases, were treated with EGFR TKIs. Patient J.A. did not receive this kind of treatment due to the presence of symptomatic metastases in the CNS, while patient B.M., after receiving the results concerning *EGFR* gene mutations, had poor performance status (PS=3) and died shortly after.

Patient G.Z. received erlotinib as the third line of treatment, after the failure of first line chemotherapy with cisplatin and vinorelbine, and second line monotherapy with the use of docetaxel. The patient was qualified for erlotinib treatment based on the high amplification of the *EGFR* gene in 80 % of the cancer cells; mutation in exon 19 of the *EGFR* gene was, in this case, revealed retrospectively. The result of the erlotinib treatment was the complete remission of metastatic lesions in the CNS, partial remission of diffuse lesions in the lungs and liver metastases, and stabilization of bone lesions. The disease was under control for 9 months, after which rapid progression took place and the patient died after

10 months from the commencement of erlotinib therapy [13]. Patient K.K., after the failure of first line chemotherapy with cisplatin and vinorelbine, received second line therapy with erlotinib. This enabled the achievement of partial remission of lesions in the lungs and stabilization of bone metastases, which has been sustained to this day (for 13 months). Patient Z.Z. received first line gefitinib treatment and achieved stabilization of bone and lung lesions, which has been sustained to this day (4 months). Patient J.R. was qualified for first line gefitinib treatment; however, after 2 months, further progression of the disease was revealed in his case.

Discussion

Mutations in the tyrosine kinase domain of the *EGFR* gene constitute independent predictive factors in EGFR TKI therapy, determining the occurrence of therapy response in over 70 % of patients [1, 4, 6]. They are most often found in Asians (30–50 %), women and non-smokers. Its incidence in the Caucasian population of NSCLC patients does not exceed 10 % [14].

Y. Togashii et al. found that, in half of the patients with *EGFR* gene mutations, distant metastases were also diagnosed (11 out of 22 patients with *EGFR* gene mutations). Moreover, metastasis was diagnosed much less frequently in the cases of lung adenocarcinoma with wild-type *EGFR* gene (4 out of 33 patients with wild-type *EGFR* gene). What is more, the majority of patients with diagnosed *EGFR* gene

Table 3 The incidence of activating mutations of the *EGFR* gene in primary tumors of lung adenocarcinoma

Primary lung adenocarcinoma, <i>n</i> =431			
<i>EGFR</i> gene mutations in primary lung adenocarcinoma tumors, <i>n</i> =55 (12.8 %)			
Deletions in exon 19	<i>n</i> =32 (58.2 %)	L858R substitution in exon 21	<i>n</i> =23 (41.8 %)
Women	<i>n</i> =16	Women	<i>n</i> =14
Men	<i>n</i> =16	Men	<i>n</i> =9
Age (median, mean±SD, years)	63, 62±10	Age (median, mean±SD, years)	63, 63±9

Table 4 The incidence of activating mutations of the *EGFR* gene in the material from lung adenocarcinoma metastases to CNS

Lung adenocarcinoma CNS metastases, <i>n</i> =61			
<i>EGFR</i> gene mutations in lung adenocarcinoma brain metastases, <i>n</i> =9 (14.8 %)			
Deletions in exon 19	<i>n</i> =3 (33.3 %)	L858R substitution in exon 21	<i>n</i> =6 (66.7 %)
Women	<i>n</i> =1	Women	<i>n</i> =4
Men	<i>n</i> =2	Men	<i>n</i> =2
Age (median, mean±SD, years)	50, 56±14	Age (median, mean±SD, years)	59, 62±9

mutation in distant NSCLC metastases smoked cigarettes [15]. This research may suggest that the presence of the mutation may be a factor conducive to the occurrence of distant metastases in NSCLC patients.

Sun et al. assessed the status of the *EGFR* and *KRAS* genes in a population of 80 NSCLC in whom material from both the primary tumors and the lymph node metastases was available. Mutations of the *KRAS* gene, which may cause a more aggressive course of the disease and resistance to EGFR TKIs treatment, were found in 7 samples of lymph node metastases; only in one case was this mutation found in the primary tumor as well. On the other hand, *EGFR* gene mutations were found in 21 primary tumors and 26 lymph node metastases; in all patients with mutations in the primary tumors, their presence was also confirmed in the metastases. This research does not confirm the relation between the presence of *EGFR* mutations and the higher likelihood of NSCLC metastases to the lymph nodes [7].

Few studies assessing the presence of *EGFR* mutations in lung cancer metastases to CNS have been conducted in Asian patients. Matsumoto et al. examined 21 tissue samples from metastatic brain tumors found in 19 NSCLC patients (68 % of whom were smokers). *EGFR* mutations were identified in 12 out of these 19 cases (63 %), including 10 short in-frame deletions in exon 19 and two L858R substitutions in exon 21. In all cases in which *EGFR* mutations were found in the primary tumors, they were also present in the corresponding brain metastases. It is postulated that, in Asian patients, the frequency of *EGFR* mutations in metastatic brain tumors of lung adenocarcinoma is higher than in primary tumors (c. 40 %) [16]. In the studies Caucasian population, the frequency of *EGFR* mutations in brain metastases of AC is only slightly higher than in primary tumors (12,8 % vs. 14,8 %).

When it comes to the incidence of *EGFR* mutations in bone metastases of adenocarcinoma, we are not aware of any available reports on this subject. The incidence of this mutation in our material is surprisingly high. On the one hand, this may result from the pre-selection of patients for molecular examination with regard to their qualification for EGFR TKIs therapy, as well as from the very low number of studied patients. On the other hand, it could be hypothesized that the presence of *EGFR* mutations is conducive to the occurrence

of distant metastases to bone and, on the basis of the studies mentioned above, also to other locations, including the brain. What is more, this phenomenon may be to some degree independent from the influence of tobacco smoke carcinogens. It is not, therefore, possible to exclude the theory that the neoplastic progenitor cells settling in distant organs are carriers of various genetic abnormalities, which may not be found in the cells of the primary tumor [7, 8, 10, 12].

In the only study concerning the EGFR status in bone metastases of NSCLC, Badalian et al. studied the expression of the EGF receptor in 11 metastatic bone tumors and primary tumors coming from the same patients. The authors demonstrated that in 45.5 % of patients, high EGFR expression occurred in both tumor types. In turn, EGFR expression was higher in bone metastases than in the primary focus of the neoplasm in 36.4 % of patients; in 18.2 % of patients, the opposite relation was observed. Moreover, the status of the *KRAS* gene was assessed in the same tissue material. It was established that the incidence of this mutation exceeded 27 % in both the primary tumors and metastases. However, not in all patients were these mutations found in both the studied samples at the same time [11].

The assessment of the genetic profiles of primary and metastatic tumors serves the purpose of choosing the best therapy, which could extend the progression-free survival and improve the quality of life of the patients in advanced stages of the disease. Even though it is assumed that molecular diagnostics should be performed for primary tumors, it is also acceptable to examine the *EGFR* gene status in metastatic tumors. The meta-analysis performed by Petrelli et al. indicates that EGFR TKIs are indisputably effective in NSCLC patients with confirmed activating mutation in the *EGFR* gene, regardless of the stage of the disease and the location of distant metastases (in numerous clinical studies, patients with stage IV NSCLC were qualified for EGFR TKI therapy) [4].

Sugiura et al. retrospectively selected 83 Asian patients with bone metastases of lung adenocarcinoma, 52 of whom had good performance status despite the advancement of the disease; 14 patients were qualified for gefitinib therapy and the remaining 38 for systemic treatment. It turned out that even without previous diagnosis of *EGFR* mutation, the total survival time was longer by 7 months on average in the group of patients receiving

EGFR TKIs (17.6 months) in comparison to the group not treated with gefitinib (10.8 months). Nevertheless, the authors emphasize that the previous establishment of the *EGFR* gene status would have enabled the precise selection of those patients for whom the therapy would be most beneficial [8].

Furugaki et al. conducted an animal model analysis of the mechanism behind the action of erlotinib in the treatment of NSCLC bone metastases with the use of the NCI-H292 lung cancer cell line (which exhibits high potential for bone metastasis). They demonstrated that the use of molecularly targeted therapy led to the suppression of EGF receptor-dependent proliferation, a decrease in the production of osteolytic factors and, the inhibition of osteolysis influencing the RANKL/RANK signaling pathway in osteoclasts, despite the wild status of the *EGFR* gene [17].

Conclusions

All these reports suggest that detailed assessment of the *EGFR* gene status in the available AC bone metastasis material is highly useful in qualifying patients for EGFR TKIs therapy. The high percentage of patients in whom the mutation was found in AC bone metastases suggests that the presence of deletion in exon 19 or, less frequently, L858R substitution in exon 21 of the *EGFR* gene may be conducive to the development of metastasis. On the other hand, the study group was very small and the status of the mutation was assessed in the patients qualified for EGFR TKI treatment (preliminary selection of patients). The patients with *EGFR* gene mutations found in bone metastases of AC benefited from EGFR TKIs therapy, which further confirms the usefulness of such material in molecular diagnostics.

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