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ORIGINAL RESEARCH ARTICLE

Different *EGFR* Gene Mutations in Exon 18, 19 and 21 as Prognostic and Predictive Markers in NSCLC: A Single Institution Analysis

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Abstract

Background Mutations of epidermal growth factor receptor (*EGFR*) in non-small cell lung cancer (NSCLC) predict longer overall survival (OS) and response to *EGFR* tyrosine kinase inhibitors (TKIs). The clinical relevance of different mutations in terms of response to TKIs and prognosis is still unclear.

Objectives The aims of the present study were to assess the relationship between mutations in exon 18, 19 and 21 in patients treated with TKIs and their clinical outcomes, and evaluate the role of specific point mutations.

Methods We included in this analysis 55 patients with metastatic NSCLC and mutations in exon 18, 19 and 21, treated in our center between 2004 and 2014. All patients received treatment with TKIs in first and/or subsequent lines. Endpoints analyzed were OS (primary) and time to progression (TTP) (secondary), according to exon mutations and specific point mutations.

Results A strong negative prognostic association for OS ($p = 0.02$) and TTP ($p = 0.03$) was found for exon 18 mutations compared with exon 19 deletions. A trend toward a longer median OS was observed in exon 19 deletions versus exon 21 point mutations (+6.6 months), although more exon 19-mutated patients had brain metastases at diagnosis. Comparing each mutation, *p.E746_A750del* and *p.E746_T751del* of exon 19 and *p.L858R* mutation of exon 21, a trend toward improved OS in *p.E746_A750del* was found.

Conclusion In this analysis, exon 19 deletions were associated with better outcomes, despite a higher percentage of brain metastases in this group. The prognostic relevance of *p.E746_A750del* requires further studies.

Key Points

Different *EGFR* mutations can determine different prognostic features.

Our series confirms worse survival for exon 18- versus exon 19-mutated patients and suggests possible differences between exon 19 deletions and exon 21 mutations.

The prevalence of brain metastases is higher in patients carrying exon 19 deletions compared with those with exon 21 mutations; despite these findings, exon 19-deleted patients had a longer overall survival.

Tyrosine kinase inhibitors seem to have different efficacy in specific exon 19 deletions.

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1 Introduction

Lung cancer is the leading cause of cancer-related death in both men and women worldwide, and about 75–80 % of lung cancers have a non-small cell histology [non-small cell lung cancer (NSCLC)] [1]. Historically, platinum-

based chemotherapy doublets represented the standard of care for metastatic NSCLC. However, in the last decade, a better understanding of molecular mechanisms in lung cancer pathogenesis and the discovery of new potential therapeutic targets have changed the current treatment for patients with advanced NSCLC. Epidermal growth factor receptor (*EGFR*) is a member of the *ERBB* receptor tyrosine kinase family that may influence angiogenesis, cellular proliferation, apoptosis and the epithelial-mesenchymal transition by activation of multiple downstream pathways (*RAS/RAF/MAPK*, *JAK-STAT* and *PIK3C/Akt*) [2–4]. Mutations of the *EGFR* gene in NSCLC predict both a better overall survival (OS) (>20 months) and response to *EGFR* tyrosine kinase inhibitors (TKIs) (gefitinib, erlotinib, afatinib) with a longer progression-free survival (PFS) (>9 months) [5–7].

Many retrospective and prospective studies confirmed that the objective response rate to TKIs in patients carrying *EGFR* mutations is 70–80 % [8–10], although no definitive data from randomized clinical trials have shown to date a clear survival benefit of TKIs compared with chemotherapy. Some clinical factors such as "never" smoking status, female gender, Asian ethnicity, adenocarcinoma histology with bronchioloalveolar carcinoma, and well or moderately differentiated tumor cells have been related to somatic mutations of the *EGFR* gene [11, 12]. *EGFR* mutations are present in the first four exons of the tyrosine kinase domain of the gene, and about 90 % of these are short in-frame deletions in exon 19 or point mutations in exon 21, with the substitution of arginine for leucine at the amino acid 858 (*p.L858R*) level [13]. The most frequent deletions in exon 19 of *EGFR* are *p.E746_A750del* (66.1 %), followed by *p.L747_P753>S* (56.8 %), *p.L747_A750>P* (4.0 %), and *p.L747_T751del* (3.7 %) [14]. *EGFR* mutations of exon 18 are rare and heterogeneous as they represent about 4 % of all *EGFR* mutations. Among these rare mutations, *p.G719S*, *p.G719A* and *p.E709X* of exon 18 are the most frequent [15]. Finally, exon 20 alterations represent 4 % of all *EGFR* gene mutations, but both insertions (with the exception of *p.A763_Y764insFQEA*) and *p.T790M* mutation confer resistance to *EGFR* TKIs [16].

Some clinical studies suggested that patients with exon 19 deletions had longer PFS and OS than those with exon 21 point mutations [17–19]; other authors did not find any statistically significant difference between the two groups [20, 21]. Presently, the biological and clinical relevance of these mutations in terms of prognosis and clinical response to TKIs is still unclear. The aim of the present study was to assess the relationship between mutations in exon 18, 19 and 21 in patients treated with TKIs and their clinical outcome. In addition, we evaluated both the possibility of differences in terms of prognosis and the predictive role of specific deletions in exon 19.

2 Patients and Methods

2.1 Patient Selection

This study was designed as a retrospective analysis of 55 patients (aged ≥18 years) with histologically proven metastatic NSCLCs and mutations in exon 18, 19 or 21 treated in our center between 2004 and 2014. All mutational analyses were conducted in the laboratory of Diagnostic Molecular Pathology at the Catholic University of Sacred Heart (Rome, Italy). Additional criteria for selection were as follows: (a) first or subsequent lines of therapy with TKIs (gefitinib or erlotinib or afatinib), relying on clinical judgment; (b) imaging assessment [computed tomography (CT) or positron emission tomography-computed tomography (PET-CT)] performed at regular intervals (no longer than 3 months); and (c) complete information regarding previous or subsequent lines of chemotherapy. Patients were excluded in cases of concomitant mutation in two or more exons or if they harbored resistance mutations (*p.T790M* and exon 20 insertion). Patients whose diagnosis was performed after May 2014 were excluded to assure a minimum follow-up of at least 1 year. Finally, patients treated within clinical trials with any drug not previously approved were not included in the analysis. The study has been conducted in accordance with the rules of the local Ethics Committee and the Declaration of Helsinki. All patients provided written consent for use of their clinical data; a separate consent for molecular analyses was obtained.

2.2 Treatment

All patients received treatment with TKIs (gefitinib, erlotinib, afatinib) in first and/or subsequent lines. Only six patients (11 %) did not receive a TKI as first treatment, while five (9 %) received both gefitinib and erlotinib in different lines of therapy. Chemotherapy regimens included pemetrexed/taxanes (paclitaxel or docetaxel)/gemcitabine administered as mono-chemotherapy or in association with platinum salts (cisplatin or carboplatin). All treatments were continued until disease progression, unacceptable toxicity or patient's withdrawal. The clinical response to treatment was classified as complete response, partial response, stable disease or progressive disease according to the response evaluation criteria in solid tumors (RECIST) 1.1 criteria [22].

2.3 Immunohistochemistry and DNA Mutation Analysis

Formalin-fixed paraffin-embedded samples were obtained before starting any cancer therapy, as a set of ten 5-μm slides or as uncut tissue blocks. Mutational analysis was

performed by Sanger sequencing, or by Therascreen *EGFR* RGQ PCR Kit (Qiagen, Hilden, Germany). Genomic DNA was extracted from tumor lung tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen), according to the manufacturer's protocol. For Sanger sequencing, *EGFR* genes (exons 18, 19, 20 and 21) were amplified using the following primers: for exon 18, forward 5′-TCC AAA TGA GCT GGC AAG TG-3′ and reverse 5′-TCC CAA ACA CTC AGT GAA ACA AA-3′; for exon 19, forward 5′-GTG CAT CGC TGG TAA CAT CC-3′ and reverse 5′-TGT GGA GAT GAG CAG GGT CT-3′; for exon 20, forward 5′-ATC GCA TTC ATG CGT CTT CA-3′ and reverse 5′-ATC CCC ATG GCA AAC TCT TG-3′; and for exon 21, forward 5′-GCT CAG AGC CTG GCA TGA A-3′ and reverse 5′-CAT CCT CCC CTG CAT GTG T-3′. Polymerase chain reaction (PCR) conditions were as follows: initial denaturation at 95 °C for 10 min followed by 35 cycles at 95 °C for 40 s, 50 °C for 40 s and 72 °C for 40 s. After visualization onto agarose gel, PCR products were treated with ExoSAP-IT (USB Corp., Cleveland, OH, USA), following the manufacturer's protocol, amplified with BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems), using forward and reverse primers, and sequenced with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). For the Therascreen *EGFR* RGQ PCR Kit (Qiagen), 10 ng of DNA was amplified by real-time PCR in 25-μL reactions, according to the manufacturer's protocol. Real-time PCR was performed using the Rotor-Gene Q 5plex HRM (Qiagen). The PCR cycling conditions were as follows: 95 °C for 10 min and 40 cycles at 95 °C for 30 s and 60 °C for 60 s. The software program Rotor-Gene Q 2.0.2 was used to process the data. The sample Ct was compared with the cut-off point for the specific assay (cut-off ΔCt), according to instructions in the manual.

2.4 Statistical Analyses

The primary endpoint was OS; time to progression (TTP) was considered as the secondary endpoint. OS was measured from diagnosis of metastatic disease until death or last follow-up contact. TTP was calculated from the beginning of first-line therapy until radiologically assessed disease progression. The outcome was censored if a patient had not progressed or was not dead at the time of last follow-up. The Kaplan–Meier method and the log-rank test were used to estimate OS and TTP. Multivariate Cox regression models were used to identify the prognostic and predictive effects of different mutations on survival. All reported p values are two-tailed, and a level of 0.05 or less was considered statistically significant.

3 Results

3.1 Patient Characteristics and Treatments

Fifty-five out of 79 patients with histologically proven diagnosis of NSCLC (adenocarcinoma or other non-small cell histology) and with an *EGFR* gene mutation treated in our center between February 2004 and May 2014 were considered eligible. Most patients were female (67 %) and never-smokers (71 %) and had adenocarcinoma histology (73 %). Median age at diagnosis was 68 years in both the exon 18 and 21 groups, and was 65 years in the exon 19 population. A total of 32 patients (58 %) had a deletion in exon 19, 18/55 (33 %) in exon 21, while only 5/55 (9 %) had a point mutation in exon 18. The most common in-frame deletions in the exon 19 group were *p.E746_A750del* ($n = 11$) and *p.E746_T751del* ($n = 4$). In the exon 21 group, 16 patients carried a *p.L858R* mutation, one patient three point deletions and another one had a *p.G779F* mutation. In the exon 18 population, the following point mutations were recognized: *p.S695I* ($n = 1$), *p.G719A* ($n = 1$), *p.G719D* ($n = 2$) and *p.E709_T710>D* ($n = 1$). All patients received TKIs: 49 in first line and six in second or subsequent lines. Most of them (50/55) received gefitinib, and five of these 50 received both erlotinib and gefitinib; two patients were treated only with erlotinib and three patients with afatinib. Eighteen patients received a second-line treatment after TKI, and in most of cases, it consisted in a platinum-based chemotherapy (61 %). The median duration of gefitinib administration was 12 months. Twenty-six of 46 patients (56 %) receiving first-line gefitinib experienced a partial response, whereas 4/46 had a stable disease and 16/46 a progressive disease as best response; no patients achieved a complete response. Patients' characteristics are summarized in Table 1.

3.2 Exon 18, 19 and 21 Mutations as Prognostic Markers

At the time of this analysis, 43 deaths (78 %) had occurred: five (100 %) in the exon 18 group, 23 (72 %) in the exon 19 group and 15 (83 %) in the exon 21 group. Brain/meningeal metastases—which are a recognized important prognostic factor—were identified in one patient in the exon 18 group (20 %), 16 patients with exon 19 deletion (50 %) and six patients with exon 21 mutation (33 %). Patients with exon 19 deletions had the longest median OS (22.73 months), in comparison with 16.16 and 11.26 months in those with exon 21 and exon 18 mutations, respectively ($p = 0.08$; Fig. 1). When we compared exon 18 and exon 19 groups separately, we

Table 1 Patients' characteristics

	Exon 18 ($n = 5$)	Exon 19 ($n = 32$)	Exon 21 ($n = 18$)	*p.E746_A750del* ($n = 11$)	*p.E746_T751del* ($n = 4$)	*p.L858R* ($n = 16$)
Sex (*n*)						
Male	3	8	7	3	0	5
Female	2	24	11	8	4	11
Median age at diagnosis (years)	68	65	68	64	64	67
Race						
European	5	30	18	11	4	16
Asian	0	1	0	0	0	0
African	0	1	0	0	0	0
TKI						
Gefitinib	5	30	15	11	3	14
Erlotinib	0	5	2	2	1	1
Afatinib	0	1	2	0	0	2
Metastases site						
CNS (brain, meninges)	1	16	6	8	1	5
Bone	5	17	9	6	3	8
Visceral	4	28	13	8	4	11
Lymphnodes	5	24	13	7	4	12
No. lines of therapies						
1	3	21	13	8	3	12
2	2	4	3	2	0	3
3	0	6	2	1	1	1
>3	0	1	0	0	0	0

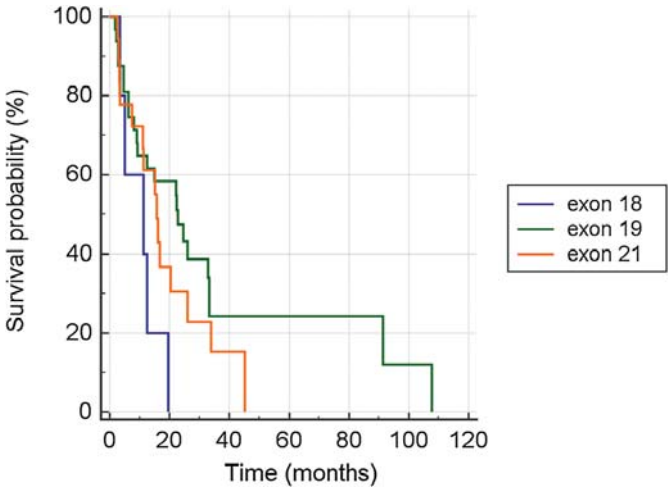

Fig. 1 Overall survival in exons 18, 19 and 21

found a significant difference suggesting a strong negative prognostic factor for exon 18 mutations ($p = 0.02$). No statistical significance for median OS values was found between exon 19 and 21 ($p = 0.3$), even with the important difference in favor of exon 19 deletions (+6.6 months).

3.3 Exon 18, 19 and 21 Mutations as Predictive Markers

At the time of the analysis, all patients with exon 18 mutations, 26 out of 32 patients in the exon 19 group and 15 out of 18 patients in the exon 21 population had experienced disease progression after TKI treatment. There were no differences in median TTP between exon 19 and 21 groups (13.4 vs. 11.3 months; $p = 0.89$). Conversely, patients carrying an exon 18 mutation had a significantly worse median TTP than those with exon 19 deletions (7.2 vs. 13.4 months; $p = 0.03$); no significant differences were observed in comparison with the exon 21 group (7.2 vs. 11.9 months; $p = 0.06$) (Fig. 2). No patients achieved a complete response. The response rates were calculated for exon 18, 19 and 21 and were 40, 71.8 and 66.6 %, respectively (Table 2). Median duration of treatment was 3, 12 and 9 months in the three groups, respectively.

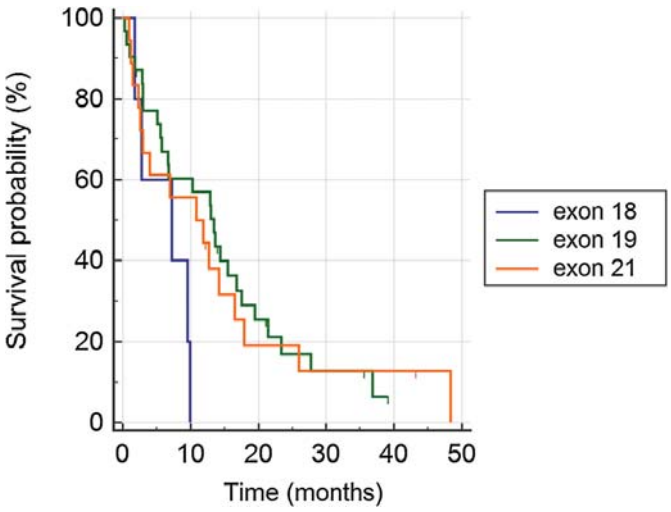

Fig. 2 Time to progression in exons 18, 19 and 21

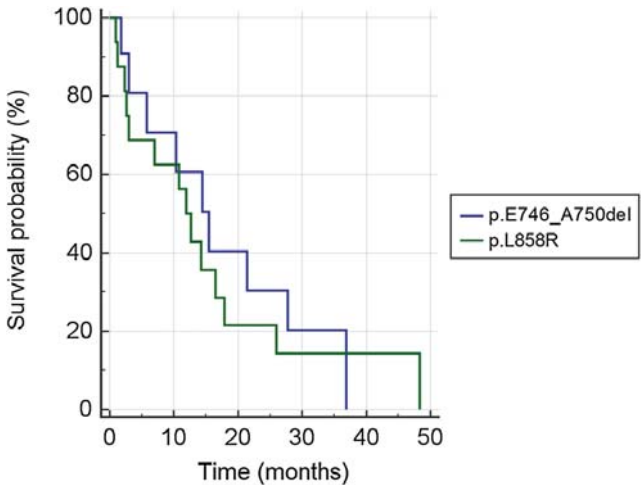

Fig. 3 Time to progression in *p.E746_A750del* and *p.L858R*

3.4 Comparison of Most Frequent Mutations in Exon 19 Versus Exon 21

In order to evaluate the role of single specific mutations, we compared the most common alterations in exon 19 and 21 groups. *p.E746_A750del* of exon 19 and *p.L858R* mutation in the exon 21 group were found in 11 and 16 patients, respectively. Radiologically assessed brain/meningeal metastases were found at diagnosis in eight out of 11 patients with *p.E746_A750del* (72 %) and in five out of 16 in the *p.L858R* group (31 %). All patients with *p.E746_A750del* received gefitinib as first-line treatment; 14 out of 16 patients with *p.L858R* received gefitinib, while in two cases, afatinib was administered as first-line therapy. No difference in median TTP between the two mutations was found: 15.5 versus 13.0 months, respectively ($p = 0.77$; Fig. 3). Nevertheless, an improved but not statistically significant OS was associated with *p.E746_A750del* (24.5 vs. 15.7 months; $p = 0.35$; Fig. 4). Response rate was 72.7 % in the *p.E746_A750del* group and 68.7 % in patients with the *p.L858R* point mutation (Table 2). Median duration of treatment with gefitinib was 12 and 11 months in *p.E746_A750del* and *p.L858R* patients, respectively.

3.5 Comparison of the Most Frequent Deletions in Exon 19

In addition to 11 patients carrying a *p.E746_A750del*, in the exon 19 population, four patients with *p.E746_T751del* were also found. Only one patient with *p.E746_T751del* had brain metastases. At the time of our analysis, ten deaths occurred (4/4 in the *p.E746_T751del* group and 6/11 in *p.E746_A750del*-mutated patients), and in 13 patients, disease progression was observed (4/4 in the *p.E746_T751del* group and 9/11 in *p.E746_A750del*-mutated patients). The median OS was 24.5 and 14.3 months in *p.E746_A750del* and *p.E746_T751del*, respectively; median TTP was 15.5 and 10.2, respectively. In spite of the apparently large difference, it was not statistically significant because of the small sample size (OS: $p = 0.52$; TTP: $p = 0.36$; Figs. 5, 6).

4 Discussion

Many studies have demonstrated that the presence of *EGFR* mutation is associated with longer survival independent of the treatment administered, suggesting that *EGFR* mutations are positive prognostic factors [13, 23,

Table 2 RR in exon 18, 19, 21 groups and in *p.E746_A750* and *p.L858R* groups

	Exon 18 ($n = 5$)	Exon 19 ($n = 32$)	Exon 21 ($n = 18$)	*p.E746_A750del* ($n = 11$)	*p.L858R* ($n = 16$)
PR	1	22	10	8	9
SD	1	1	2	0	2
PD	3	9	6	3	5
RR	40 %	71.8 %	66.6 %	72.7 %	68.7 %

PR partial respose, *SD* stable disease, *PD* progressive disease, *RR* response rate

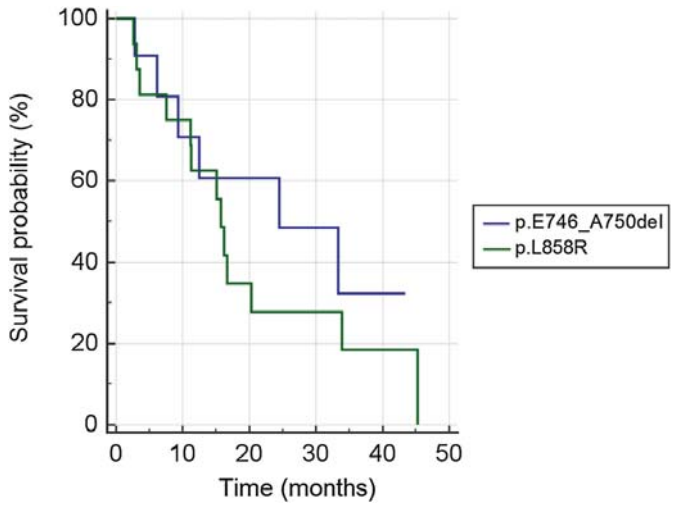

Fig. 4 Overall survival in *p.E746_A750del* and *p.L858R*

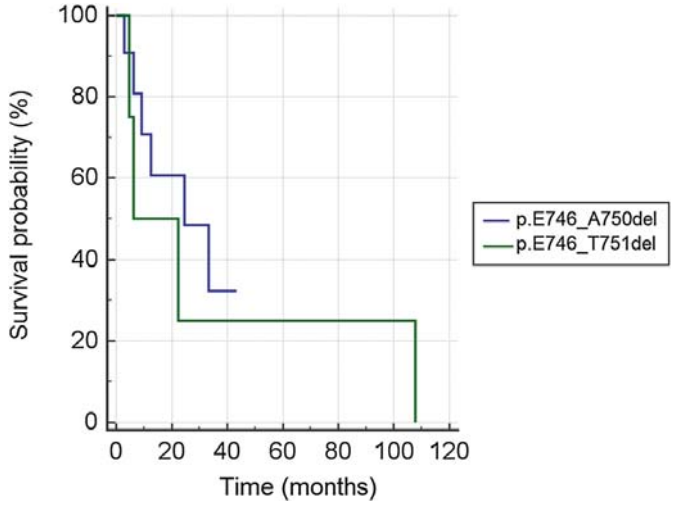

Fig. 5 Overall survival in *p.E746_A750del* and *p.E746_T751del*

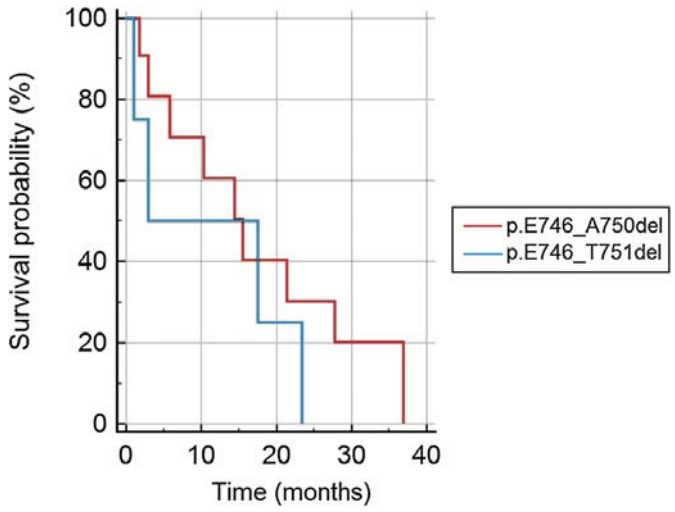

Fig. 6 Time to progression in *p.E746_A750del* and *p.E746_T751del*

24]. The aim of this retrospective analysis was to evaluate if different *EGFR* mutation genotypes may predict different survival or different response to treatment with TKIs in patients with NSCLC.

According to other studies [15, 25], the worst variant in terms both of prognosis and response to a first-line therapy was exon 18 mutation. The percentage of patients carrying this mutation is fortunately small (only 9 % in our population), but differences in OS ($p = 0.02$) and TTP ($p = 0.03$) are significant when exon 18 mutations are compared with exon 19 deletions.

When comparing patients with deletion of exon 19 and patients with mutation in exon 21, no statistically significant differences were detected in terms of OS (22.7 vs. 16.1 months) and response rate (71.8 vs. 66.6 %), although a trend toward a better prognosis was recorded. Moreover, OS was much longer in *p.E746_A750del* patients than in *p.L858R*-mutated patients (24.5 vs. 15.7 months), while response to TKI therapy was similar, with a median duration of treatment of 12 and 11 months, respectively. These data are in contrast with those reported by Riely et al. [19] and by Jackman et al. [17], who found a significant survival advantage for patients carrying an exon 19 deletion in comparison with those carrying a *p.L858R* mutation (34 vs. 8 months, respectively, in the study from Riely et al.). However, in the first one of these studies, lung cancers at any stage (I–IV) were considered, and most of stage IV patients were not treated with TKI inhibitors. Won et al., instead, found a significantly longer PFS following a TKI treatment in exon 19 deletions compared with *p.L858R* mutation (9.3 vs. 6.9 months; $p = 0.02$), without differences in response rate and OS [26].

Regarding this, one of the main findings of our study is that the prevalence of brain metastases at diagnosis was much higher in patients carrying an exon 19 deletion compared with patients with an exon 21 mutation. Brain metastases were diagnosed in 50 % of exon 19-deleted patients versus 33 % of exon 21-mutated patients. Moreover, if the analysis is restricted to the most common exon 19 deletion (*p.E746_A750del*), the incidence of brain metastases becomes much higher (72 %). This interesting data might provide a reason for the discrepancies between our study and other reports in terms of OS, even considering the small sample size. Nevertheless, other hypotheses cannot be excluded, such as an increased risk of brain involvement for specific molecular subtypes. In addition, the activity of different TKI inhibitors might play a role. In a pooled analysis of LUX-Lung 3 and LUX-Lung 6 trials [27] and in a review by Joshi et al. [28], the authors suggested that patients with deletions in exon 19 could benefit from first-line treatment with afatinib much more than patients with *p.L858R* mutations. As well, Mitsudomi et al. [10] noted a prolonged OS in *p.E746_A750del* compared

with *p.L858R* mutations and a better response to gefitinib in patients harboring an exon 19 deletion.

Although most patients in our sample were treated with gefitinib, first-line therapy with a TKI other than gefitinib or the use of a TKI in second-line therapy in patients treated with conventional chemotherapy as first-line therapy could have affected our results to a certain extent. In this regard, some evidence suggests that even chemotherapy efficacy could be affected by specific *EGFR* mutations. Cappuzzo et al. [29] reported a response to chemotherapy of 46.6 % in patients with exon 19 deletion and 0 % in the case of other mutations ($p = 0.02$). Unfortunately, this evaluation was not possible in our population, since our sample size was quite small and very few patients underwent conventional chemotherapy.

The scenario described above is further complicated by the observation that different mutations in the same exon could also indicate a different prognostic or predictive role. Several deletions in exon 19 are known, and most of them involve the amino acids from codon L747 to E749 (LRE fragment). Chung et al. [30] observed that therapeutic response associated with *EGFR* TKI treatment was different in different exon 19 deletions and, in particular, patients with non-LRE deletions had a worse response to TKIs than those with LRE deletions. In addition, a more recent study by Ma et al. [31] elegantly postulated a different response driven by the free binding energy of specific *EGFR* mutants with different TKIs. In our patients, the difference in terms of OS (24.5 vs. 14.3 months) and TTP (15.5 vs. 10.2 months) between *p.E746_A750del* and *p.E746_T751del* groups did not reach statistical significance. Nevertheless, considering most *p.E746_A750del* patients had brain metastases compared with one *p.E746_T751del* patient, the hypothesis of a superior TKI efficacy in this subgroup appears to be plausible.

Our findings suggest a correlation between *EGFR* genotype and survival; specific mutations may have additional implications in predicting survival benefit, but there are no remarkable outcome differences after treatment with TKIs, except for exon 18. The prognostic and predictive value of different *EGFR* mutations in NSCLC remains still uncertain, and the question of a therapy targeted on different *EGFR* mutations requires further molecular and clinical studies.

4.1 Limitations of the Study

The results of this study are affected by its retrospective nature. The main bias is the small sample size of exon 18, 19 and 21 groups and their subgroups (*p.E746_A750del*, *p.E746_T751del* and *p.L858R*). Moreover, not all patients received an up-front therapy with TKIs, and many of them did not undergo a second-line treatment; these facts could affect mainly the OS data.

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Compliance with Ethical Standards

Conflicts of interest Sabrina Rossi, Ettore D'Argento, Michele Basso, Antonia Strippoli, Vincenzo Dadduzio, Eleonora Cerchiaro, Maurizio Martini, Alessandra Cassano and Carlo Barone have no conflicts of interest that are directly relevant to the content of this article.

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Ethical approval and informed consent The study has been conducted in accordance with the rules of the local Ethics Committee and the Declaration of Helsinki. All patients provided a written consent for use of their clinical data; a separate consent for molecular analyses was obtained.

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