Dominance of EGFR and Insignificant KRAS Mutations in Prediction of Tyrosine-kinase Therapy for NSCLC Patients Stratified by Tumor Subtype and Smoking Status

MILOS PESEK¹, LUCIE BENESOVA², BARBORA BELSANOVÁ², PETR Mukensnabl³, FRANTISEK BRUHA¹ and MAREK MINARIK²

Departments of ¹Pneumology and ³Pathology, Faculty Hospital Pilsen, Pilsen;
²Laboratory for Molecular Genetics and Oncology, Genomac International, Ltd., Prague, Czech Republic

Abstract. Background: Therapy by tyrosine kinase inhibitors (TKI) has become inevitable in treatment of advanced NSCLC. Mutations in EGFR and KRAS genes have been identified as the main potential predictive and prognostic factors. Here the clinical implications of EGFR/KRAS mutations in patients from two separate trials treated with gefitinib or erlotinib are analysed. Patients and Methods: A total of 360 patients (269 gefitinib and 91 erlotinib) were evaluated. Mutations in EGFR (exon 19 and 21) and KRAS (codons 12 and 13) and their impact on response and survival with respect to tumor subtype and smoking status were assessed. Results: Adenocarcinomas revealed 399 days to progression (TTP) and 548 days overall survival (OS) for EGFR mutated vs. 119 days to progression and 137 days survival for non-mutated, p<0.0001 (TTP) and p=0.0001 (OS). No EGFR effect was recorded for squamous cell tumors. For smoking status, both EGFR-mutated smokers and non-smokers profited from TKI therapy. Smokers: 243 vs. 122 days (mutated vs. non-mutated), p=0.0284 (TTP) and 244 vs. 126 days, p=0.0396 (OS); non-smokers: 390 vs. 71 days, p<0.0001, (TTP) and 548 vs. 135 days, p<0.0001 (OS). KRAS mutation in tumors did not result in a poorer prognosis in the subtype-selected groups, nor did it present as a negative factor in smokers. Conclusion: EGFR mutations possess statistical significance for a better therapy response and longer survival in all patients with adenocarcinomas (smokers as well as non-smokers). KRAS does not seem an “a priori” negative factor for TKI-based treatment of NSCLC.

Biologically targeted therapy of non-small cell lung cancer (NSCLC) based on tyrosine kinase inhibitors (TKI), directed at the epidermal growth factor receptor (EGFR), frequently delivers a long-term remission of advanced tumors as well as an increase of the time to progression and extended survival rates. A positive outcome can often be observed in patients after failure of the previous lines of chemotherapy (1-3). In 2004, Lynch and Paez reported a correlation between tumor gefitinib-induced remissions and an occurrence of somatic mutations in EGFR gene within the tumor tissue (2, 4). Over the subsequent years, TKI-sensitive EGFR mutations have been more often found in female non-smokers with adenocarcinomas or BAC type. Asians have typically exhibited EGFR mutation-positive tumors more frequently than Caucasians (3, 5-7). Patients with tumors bearing EGFR mutations typically show good response to TKI therapy and exhibit longer time to disease progression. On the other hand, there is still discussion about their effect on the overall survival rates. The results may be attributed to the different effect of various EGFR mutation types. Of the prevailing EGFR mutations, small deletions in exon 19 are perceived as more favorable than the point substitutions in exon 21 or in other exons (8, 9). The importance of mutation types as well as other EGFR-related indicators such as EGFR gene amplification or EGFR protein overexpression as prognostic factors or as predictors of therapy response have been scrutinized (7, 10-14). Somatic mutations in KRAS oncogene can be found in 15 to 25% of lung carcinomas (15). They are usually more frequent in men and are associated with smoking and adenocarcinoma type (16-18). Permanent activation of KRAS function, mostly by transversions in codon 12, has been widely recognized as a negative prognostic factor for biological therapy in colorectal cancer. Recent results of clinical trials indicate that tumors with mutated KRAS genes are unlikely to respond to a biological therapy by cetuximab.
and panitumumab (19, 20). Similarly in lung cancer, KRAS mutation is also widely perceived as a negative predictive and prognostic factor (21) with importance for adenocarcinomas undergoing surgical treatment in the very early stages. Since KRAS plays an important role in the EGFR tyrosine-kinase pathway, it is expected that its permanent activation will render TKI-based treatment by gefitinib and erlotinib ineffective (22, 23). Although such an assumption is reasonable, several papers have recently reported the lack of clinical evidence for negative prognostic value of mutated KRAS in gefitinib/erlotinib therapy (9, 11).

This work presents the analysis of patients that were treated by either gefitinib or erlotinib between 2002 and 2008 and their primary lung tumors tested for presence of EGFR and KRAS mutations. The importance of these two widely recognized molecular markers has been evaluated for therapy response and survival. The main emphasis was placed on stratification according to histological subtype and smoking status. The main clinical parameters observed were objective therapy response, time to progression and the overall survival time.

Patients and Methods

Patients enrolled in this study had received either gefitinib during the course of the Early Access Programme (EAP) or erlotinib according to regularly scheduled treatment schedule. The gefitinib cohort consisted of 269 patients. There were 200 males aged 36 to 85 (median age of 65) and 69 females aged 45 to 81 with a median age of 68. The erlotinib group included 91 patients, of which 57 were males aged 45 to 82 (median age of 63) and 34 females aged 41 to 80 with a median age of 62.5. The combined cohort of 360 patients included 298 smokers and 55 non-smokers (smoking status was not reported in 7 cases). There were 160 adenocarcinomas (including BAC), 175 squamous cell carcinomas and 25 non-differentiated non-small cell carcinomas. Most carcinomas were stage IV (48%); followed by stage IIIB (35%) and IIIA (12%), with stage I and II combined accounting for only 5%. In the therapy response prediction analyses, only patients that remained on the TKI therapy for at least 2 weeks were evaluated (338 out of 360, 94%).

Tumor tissue samples were collected during bronchoscopy examination and processed into either formalin-fixed paraffin-embedded histology sections or cytological slides. The genomic DNA was extracted by JetQuick Tissue DNA Isolation Kit (GENOMED GmbH, Loehne, Germany). Where necessary, tumor cells were carefully selected and removed from the samples by laser microdissection using P.A.L.M. microlaser instrument (Carl Zeiss MicroImaging GmbH, Germany). The microdissected cells were then corrected to account for the effect of chemical denaturant (24). For detection of EGFR exon 21 mutations, the primer sequences were \([\text{GC-clamp}]-\text{TCACAGCAGGTTCTTCTTC}\) for the forward primer and \(\text{TGCCTCCTTCTGCATGGTAT}\) for the reverse primer. The sequence of the GC-clamp was CGCCC GCGCGGGCCCGCGCGCGGTCGCCCGCCGCCGCCCACGC. The PCR was carried out with PPP Master Mix (Top-Bio). The program consisted of 34 cycles with an annealing temperature at 65°C. The PCR products were then denatured for 8 minutes at 95°C and then the heteroduplex were allowed to form for 30 minutes at 65°C. The heteroduplex PCR products for KRAS mutation detection were prepared with previously published primers (24) and PCR and heteroduplex programs identical to the above.

Theoretical melting temperature (Tm) for the EGFR exon 21 was calculated using WinMelt software (Medprobe, Oslo, Norway) and then corrected to account for the effect of chemical denaturant present in each respective CE and chip-CE instrument. Fragment analysis and sequencing was performed on a 96-capillary array sequencer (MegaBACE 1000; Amersham) equipped with Caddy 1000 robotic plate loader (Watrex Praha, Prague, Czech Republic) for unattended automated operation.

Kaplan-Meier survival curves as well as other statistical analyses were performed with the use of MedCalc software (MedCalc Software, Mariakerke, Belgium). Statistical significance was scrutinized by a two-tailed \(t\)-test at a 95% confidence level.

Results

Results for times to progression and overall survival for original datasets and for data stratified by tumor subtype and smoking status are summarized in Table I.

| Table I. Summary of results for time to progression (TTP) and overall survival (OS) (days) of patients receiving targeted biological therapy. |
|------------------|---------|----------|----------|----------|----------|
| Time to progression/overall survival | EGFR positive | EGFR negative | KRAS positive | KRAS negative |
| All | 307 | 127 | 103 | 137 |
| Adenocarcinomas | 227 | 95 | 79 | 105 |
| Squamous cell carcinomas | 139 | 27 | 107 | 33 |
| Smokers | 243 | 122 | 96 | 126 |
| Non-smokers | 247 | 111 | 177 | 126 |

Sequencing reaction was performed using Dynamic Terminator Sequencing kit (Amersham Biosciences, Piscataway, NJ, USA). Somatic point mutations in exon 21 of the EGFR gene and exon 1 of KRAS (codons 12 and 13) were detected using cycling-gradient capillary electrophoresis – a heteroduplex-based separation technique exhibiting high sensitivity in detecting somatic mutations in cancer tissue (24). For detection of EGFR exon 21 mutations, the primer sequences were \([\text{GC-clamp}]-\text{TCACAGCAGGTTCTTCTTC}\) for the forward primer and \(\text{TGCCTCCTTCTGCATGGTAT}\) for the reverse primer. The sequence of the GC-clamp was CGCCC GCGCGGGCCCGCGCGCGGTCGCCCGCCGCCGCCCACGC. The PCR was carried out with PPP Master Mix (Top-Bio). The program consisted of 34 cycles with an annealing temperature at 65°C. The PCR products were then denatured for 8 minutes at 95°C and then the heteroduplex were allowed to form for 30 minutes at 65°C. The heteroduplex PCR products for KRAS mutation detection were prepared with previously published primers (24) and PCR and heteroduplex programs identical to the above.

Theoretical melting temperature (Tm) for the EGFR exon 21 was calculated using WinMelt software (Medprobe, Oslo, Norway) and then corrected to account for the effect of chemical denaturant present in each respective CE and chip-CE instrument. Fragment analysis and sequencing was performed on a 96-capillary array sequencer (MegaBACE 1000; Amersham) equipped with Caddy 1000 robotic plate loader (Watrex Praha, Prague, Czech Republic) for unattended automated operation.

Kaplan-Meier survival curves as well as other statistical analyses were performed with the use of MedCalc software (MedCalc Software, Mariakerke, Belgium). Statistical significance was scrutinized by a two-tailed \(t\)-test at a 95% confidence level.
Of the original group of 360 patients, 199 were successfully tested for both EGFR and KRAS mutations (55%). Additionally, 25 patients (7%) were tested only for one of either EGFR or KRAS mutations due to the limited amount of genetic material extracted from their samples. For the remaining 136 patients, either no tumor sample was available or the quality of the acquired sample was too low for the analysis. A total of 33 out of 213 patients (15.5%) tested positive for mutation in EGFR, and 32 out of 208 patients (15.4%) for KRAS. In a total of 116 out of 199 patients (58.3%), none of the tested mutations was detected. The concurrent presence of both EGFR and KRAS mutation was discovered in two patients. These patients were subsequently removed from further statistical evaluations and a separate case report is now being prepared.

In the overall analysis of the mutation effect, patients with EGFR mutation-positive tumors (n=33) showed significantly longer times to progression compared to EGFR mutation-negative tumors (n=130) with 307 vs. 127 days (p=0.0002, two-tailed t-test, 95% CI). On the other hand, no difference in time to progression was observed for tumors bearing KRAS mutation (n=32) compared to those without (n=125) with 103 vs. 137 days (p=0.4597, two-tailed t-test, 95% CI). In terms of the overall survival, a similar trend was observed with a significant preference of EGFR-positive over EGFR-negative tumors (442 vs. 188 days, p<0.0001, two-tailed t-test, 95% CI) and insignificant difference between the KRAS-positive and KRAS-negative tumors (236 vs. 200 days, p=0.4965, two-tailed t-test, 95% CI).

Tumor subtype. After stratification by tumor subtype, a group of adenocarcinomas revealed 21 positives out of 101 tested for EGFR (21%) and 23 positives out of 97 tested for KRAS (24%) mutation. The time to progression for patients on therapy was significantly longer for EGFR-mutated compared to EGFR-negative adenocarcinomas, with 399 days (n=21) vs. 119 days (n=57), p=0.0001. No significant difference was observed in time to progression between mutated and non-mutated KRAS adenocarcinomas, with 113 days (n=23) vs. 139 days (n=53), p=0.6093. The impact of either mutation on the overall survival of patients suffering with adenocarcinomas is illustrated in Figure 1 with the most prospective prognosis being for EGFR-positive patients (n=21) with 548 days vs. 137 days for non-mutated adenocarcinomas (n=48) (p=0.0001). KRAS positivity does not seem to be a negative factor in prognosis with 156 days for KRAS mutated (n=23) compared to the 137 days of non-mutated adenocarcinomas (n=48) (p=0.2863).

In a group of squamous cell carcinomas (SCC), the rate of EGFR mutations was 11 in 81 tested (14%) and the rate of KRAS mutations was 8 in 78 tested (10%). The corresponding times to progression for SCC patients on therapy were not significantly affected by the presence of EGFR mutation, with EGFR-mutated (n=11) at 161 days vs. 144 days for EGFR-negative (n=62), p=0.8544. There was a difference in the mean value of the time to progression between mutated and non-mutated KRAS SCC tumors with 84 days (n=8) vs. 151 days (n=59), but the difference was not statistically significant (p=0.5365). Figure 2 shows Kaplan-Meier curves for overall survival of the various mutation states of SCC. Neither of the mutation types seems to affect its prognosis, with rates of 207 days for EGFR- mutated (n=11; p=0.5967), 128 days for non-mutated (n=56) and 133.5 days for KRAS-mutated (n=8; p=0.9277) SCC tumors.

Due to the low numbers of samples, the impact of mutations could not be evaluated in non-differentiated lung cancer.

Smoking status. The smoking status was obtained in a combined group of 353 patients. The frequency of EGFR mutations was 18 out of 168 tested smokers (11%) while KRAS mutations were found in 33 out of 163 tested smokers (20%). In a group of non-smokers, the EGFR mutations were found in 15 out of 40 tested (38%) and KRAS mutations in 4 out of 39 tested.

From the 383 patients with known smoking status, 332 (98%) that had received biological therapy for more than 2 weeks were taken for further analysis. In a subgroup of 285 smokers, the mean time to progression for EGFR-mutated (n=18) was 243 days vs. 122 days for non-mutated (n=112) showing a statistical significance (p=0.0284, two-tailed t-test, 95% CI). The mean time to progression for KRAS-mutated smokers (n=29) was 96 days vs. 126 days for non-mutated without statistical significance (p=0.4791, two-tailed t-test, 95% CI). Figure 3 shows Kaplan-Meier curves for overall survival of the smokers with respect to the mutations found. There was a statistically significant better prognosis for patients with EGFR-mutated tumors in smokers (n=18) showing a median survival of 244 days vs. 126 days of non-mutated smokers (n=102) with p=0.0396 (two-tailed t-test, 95% CI). The survival of KRAS mutated smokers (n=29) was 90 days vs. 126 days in non-mutated smokers (n=102). The difference is not statistically significant (p=0.5187).

In 47 non-smokers, the mean time to progression for those with mutated EGFR (n=14) was 390 days vs. 71 days for non-mutated (n=15) showing a strong statistical significance (p=0.0001, two-tailed t-test, 95% CI). Time to progression for KRAS mutated non-smokers (n=3) was 177 days vs. 125 days for non-mutated (n=14) without statistical significance (p=0.6362, two-tailed t-test, 95% CI).

Overall survival of non-smokers with the three mutation states is depicted in Figure 4. There the median survival for EGFR-mutated non-smokers (n=14) was 548 days (p=0.0414), while only 135 days for non-smokers with non-mutated EGFR (n=10). The impact of KRAS mutation in non-smokers could not be reliably evaluated due to insufficient data as there were only 3 KRAS mutation-positive patients who had received biological therapy for more than 2 weeks.
Discussion

To date there has been no direct comparison of the efficacy of gefitinib and erlotinib. While the ISEL study reported a better survival only for non-smokers and with a preference for Asians (25), the BR.21 study resulted in significant improvement in survival for all patients, including all subtypes (26, 27). These results suggest a better performance of erlotinib, especially in patients with negative clinical predictors (tumor subtype, smoking status). An initial analysis of the presented data (gefitinib vs. erlotinib) revealed no differences in either the time to progression or overall survival for the two treatment types (28). Therefore, all subsequent analyses were carried out using a combined group of gefitinib and erlotinib patients.
Although somatic mutations in both EGFR and KRAS genes are found at the early stages of carcinogenesis (lung adenocarcinomas in particular), tumors with either of the two types exhibit predominantly different signaling pathways. The incidence of EGFR mutations often relates to the tumor subtype and non-smoking status (18). The above data have confirmed the highest frequency of EGFR mutation in adenocarcinomas and low frequency in SCC. The detected EGFR mutations decreased with cigarette exposure. KRAS mutations are not specific to the tumor subtype, but more related to the smoking status. The notion of a large proportion of lung tumors from heavy smokers (17) harboring KRAS mutations was confirmed.

There is no consensus on the role of EGFR-related molecular markers in the estimation of expected TKI therapy outcome. Some authors recommend evaluation of EGFR mutations in combination with EGFR amplifications, while others oppose there being an effect of either marker for therapy outcome prediction (12, 14, 29-31). These somewhat contradicting reports may be related to a variety of methodologies applied to genetic testing, variability of tumor profiles (genetic makeup), or sample types (paraffin fixed histology sections, cytology smears). In addition, the variability is further increased as the molecular profile (genetic makeup) of a tumor may be affected by any preceding chemotherapy.

The data show significantly longer times to progression as well as overall better survival for patients with EGFR-mutated adenocarcinomas for both smokers and non-smokers. The strongest effect was observed in adenocarcinomas and non-smokers, with close to 5-fold increase in the mean time to progression and 3-fold longer survivals for each group. A statistically significant 2-fold improvement in time to progression was obtained also for a group of smokers. The only group not affected by the EGFR mutation status was SCC tumor type. This is an important finding since many literature reports evaluating TKI therapy performance do not distinguish tumor subtypes with respect to EGFR mutation status (9-11, 29-32). In addition to EGFR mutation positivity, the presence of a BAC component within the tumor tissue may also play role in TKI effectiveness (33). In an overall conclusion, the data suggest a strong positive role of EGFR mutations for administration of TKI-based therapy for NSCLC patients. A higher frequency of positive therapy response and longer survival times have been observed for EGFR-mutated tumors, especially for EGFR-mutated adenocarcinomas compared to non-mutated ones. This clearly suggests an opportunity for effective treatment in those groups. In particular, patients with low performance status who exhibit EGFR mutations may benefit from TKI therapy in the first-line of treatment as also suggested by the recent IPASS study (34).

A presence of KRAS oncogene mutation is overwhelmingly perceived as a negative prognostic factor and also as a negative predictor of the effect of TKI therapy (35-37). Yet some recent papers challenge such a concept (9, 10). From the presented data, it is possible to see a relatively lower frequency of response for tumors with KRAS mutations; however, the survival analysis also resulted in a certain benefit for these patients. Overall, a statistically significant decline in survival rates was not observed when compared to non-mutated tumors in any of the statistically evaluated subgroups (adenocarcinomas, squamous cell carcinomas, smokers). Accordingly, KRAS mutations may not present such a strong negative predictive and prognostic factor. The slightly lower frequency of responses for patients with KRAS-mutated tumors did not show a statistical significance. Overall survival of patients with KRAS-mutated adenocarcinoma was fully comparable to that of those with non-mutated ones. Based on these findings, KRAS mutation should not exclude a patient from receiving TKI therapy without evaluation of other clinical predictors (38).

Acknowledgements

The authors would like to thank all patients at the Department of Pneumology, Faculty Hospital in Pilsen for voluntarily taking part in this study. Help and assistance from our colleagues in Pilsen and Prague Hana Krosnarova, Gabriela Krakorova, Radka Bittenglova, Patrik Sekerka and Andrea Krajecova is also acknowledged. This work was completed with support from the Czech Ministry of Health grant No. NR/9087-3.

References


34 Mok T: Phase III, randomised, open-label, first-line study of gefitinib vs carboplatin/paclitaxel (C/P) in clinically selected patients With advanced non-small-cell lung cancer (NSCLC), presented at the european society of medical oncology meeting, Stockholm. Abs LA2. 2008.