The frequency of EGFR and KRAS mutations in non-small-cell-lung cancer (NSCLC) – Routine screening data for Central Europe

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The frequency of EGFR and KRAS mutations in non-small-cell-lung cancer (NSCLC) – Routine screening data for Central Europe

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Article focus

- Frequency of clinically important mutations in non-small-cell lung carcinoma
- epidermal growth factor receptor (EGFR) and KRAS
- personalised medicine

Key messages

- The frequency of an activating EGFR mutation can not be expected in more than 4.9% of the "allcomers" with the diagnosis of NSCLC.

Strengths and limitations of this study' section

- Unselected and large cohort of lung cancer centre
- Analyses of three codons for EGFR
- ***Possible limitation***: Single centre study

Abstract:

Objectives: Due to novel therapy strategies in EGFR mutated patients, molecular analysis of the EGFR and KRAS genome has become crucial for routine diagnostics. Up to date these data derive mostly from clinical trials, and thus collected in pre-selected populations. We therefore screened "allcomers" with a newly diagnosed non-small-cell lung carcinoma (NSCLC) for the frequencies of these mutations.

Design: Cohort study

Setting: Lung cancer centre in a tertiary care hospital

Participants: Within 15 months a total of 552 cases with NSCLC were eligible for analysis.

Primary and secondary outcome measures: Frequency of scrutinizing exons 18, 19 and 21 for the presence of activating EGFR mutation and secondary codon 12 and 13 for activating KRAS mutations.

Results: Of the 552 patients, 27 (4.9%) showed a mutation of EGFR. 19 of these patients (70%) had deletion E746-A750 in codon 19 or deletion L858R in codon 21. Adenocarcinoma (ACA) was the most frequent histology among patients with EGFR mutations (ACA, 22/254 [8.7%] vs. non-ACA, 5/298 [1.7%]; p<0.001). Regarding only ACA, the percentage of EGFR mutations was higher in women (16/116 [14%] women vs. 6/138 [4.3%] men; p=0.008). Tumours with an activating EGFR mutation were more likely to be from non-smokers (18/27; 67%) rather than smoker (9/27; 33%).

KRAS mutation was present in 85 (15 %) of all cases. In 73 patients (86%), the mutation was found in exon 12 and in 12 cases (14%) in exon 13. Similarly, ACA had a higher frequency of KRAS mutations than non-ACA (67/254 [26%] vs. 18/298 [6.0%]; p<0.001).

Conclusions: We found a lower frequency for EGFR and KRAS mutations in an unselected Caucasian patient cohort as previously published. Taking our results into account, clinical

trials may overestimate the mutation frequency for EGFR and KRAS in NSCLC due to important selection biases.

Introduction

Lung cancer is still one of the most common cancers in Germany and worldwide, and the most common cause of death due to malignant tumours (1). Despite intensive research the prognosis of patients with non-small-cell lung carcinoma (NSCLC) continues to be restricted, which applies especially to patients with distant metastases. But in patients with NSCLC and an activating mutation of the epidermal growth factor receptor (EGFR), therapy has undergone a significant change. The application of first generation tyrosine-kinase inhibitors (TKI) Gefitinib an Erlotinib was able to achieve not only a better tolerability of the therapy, but also a significant improvement in progression-free survival (PFS) compared to platinum-based chemotherapy (2-6). Moreover, one study shows a significant increase in overall survival (OS) (7).

In the last years intensive research has been conducted to analyse the effect of TKIs in respect of the complex relationship of biomarkes which are associated with the EGFR pathway like v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). In addition to the superiority in EGFR mutated patients, TKIs seem to have an effect on the wild type NSCLC as well, while some studies have disputed this for KRAS mutated patients (5;6). However the significance of KRAS mutations for the diagnosis or treatment of NSCLC remains under debate. Since double mutations (EGFR and KRAS) have been described in only very few cases, KRAS may help to exclude EGFR mutation (8;9).

Therefore the question arises how many patients with NSCLC can benefit from the new treatment option and whether screening should be implemented in the routine diagnostics. In addition, numerous studies came from Southeast Asia, where patients with NSCLC have

higher EGFR mutation rates (10;11). Since the data available today are hampered either by biases due to study exclusion criteria or different geographical peculiarities, we established routine screening for consecutive patients in order to receive reliable data for the routine setting in Central Europe.

Patients and methods

Study population

From October 2009 until December 2010 we have screened prospectively all newly diagnosed patients with NSCLC (n = 753) for the ability to be analysed for EGFR and KRAS mutations. All subsequent biopsies have been obtained by surgery, routine bronchoscopy or CT guided biopsy. The histological diagnosis was performed according to WHO criteria (12). A total of 552/753 (75%) cases with NSCLC were eligible for analysis. In 183 patients (25%) none of the two analyses could be performed. In the majority of cases, this was explained by an insufficient amount of tissue sample. In addition, some patients refused their consent to carry out the mutation analysis. Informed consent was obtained from all patients prior to biopsy.

DNA extraction

After preparation of a 20 µm thick section from the formalin-fixed and paraffin-embedded tissue sample, the area of tumour cells was marked by comparison with the corresponding HE stained slide. This area with a size of 2 to 10 mm^2 was scraped with a pipette tip and transferred into 180 µl AL-buffer. The AL-buffer is included in the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Afterwards the DNA was extracted and dissolved in 150 µl AE-buffer according to the manufacturer's instructions using the QIAamp DNA Mini Kit.

Determination of EGFR mutations

For analysing the EGFR mutation status, the extracted DNA was subjected to conventional PCR for amplification of relevant portions of exons 18, 19 and 21 of the EGFR gene. Further, the PCR products were analyzed for the presence of a mutation by using the Pyrosequencing

PyroMark Instument (Qiagen, Hilden, Germany). The primers used for the PCR are listed in Table 1. The method of pyrosequencing has been performed according to the reagent manufacturer (Qiagen, Hilden, Germany) and the following primer names and sequences (3'-5') have been used for exons 18, 19 and 21:

- 18_F (forward) CAGCATGGTGAGGGCTGAG
- 18_R (reverse) GCCTGTGCCAGGGACCTTAC
- 19_F (forward) GTGGCACCATCTCACAATTGC
- 19_R (reverse) CACACAGCAAAGCAGAAACTCAC
- 21_F (forward) CCTCACAGCAGGGTCTTCTCTG
- 21_R (reverse) CTGGCTGACCTAAAGCCACC

Samples with a mutation in the pyrogram were additionally examined by dideoxy sequencing according to Sanger et al. for confirmation (Figure 1) (13).

Determination of KRAS mutations

The DNA was analyzed according to manufacturer's instructions using the K-RAS codon 12/13 kit from Molbiol TIB (Berlin, Germany) and the LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany).

This is a real-time PCR with FRET probes, which facilitate the differentiation of mutant and wild-type DNA in codon 12 and 13 on exon 1 of KRAS gene after successful amplification and analyses of the melting curve of the PCR products.

Statistical Analyses

Quantitative variables were summarized as means ± standard deviations (SD). For comparisons Student´s t-test was used and in cases with more than two groups, a oneway ANOVA with Bonferroni correction was calculated. Categorical variables are reported as

frequencies and for comparisons of categorical variables between groups chi-square tests were used. The level of significance for all tests was set to 5 percent and all tests were two-sided. Analyses were carried out with the Statistical Package for Social Sciences (SPSS, 13.0) on a Windows operating system.

Results

Patients

The mean age of patients with wild type EGFR (n = 440; 66.7 ± 9.5 years) was comparable to the mean age of patients with EGFR mutation (n = 27; 70.3 ± 11.4 years) and that of patients with a KRAS mutation in the overall comparison (n = 85; 65.3 ± 9.8 years; p = 0.059). Table 1 summarises frequencies according to mutation, gender and histology.

A breakdown of patients according to the genealogy was not performed because all patients with a migration background except two came from other European countries. Patients from Asia were not diagnosed or treated in our clinic during the corresponding period.

EGFR mutation

Overall 27 (4.9%) of all 552 patients showed an activating EGFR mutation (3/27 mutations in exon 18 (11%), 15/27 mutations in exon19 (56%) and 8/27 mutations (30%) in exon 21; 1/27 tumour (3%) has shown a double mutation (exon 19 and 21)). The most common mutation in exon 19 was delE746-A750 with 10/15 (67%) cases. In exon 21 all detected 8 mutations were located on L848R (100%).

Considering the frequency of mutations according to histology, adenocarcinomas show mutations more often than any other histology (22/254 [8.7%] vs. 5/298 [1.7%]; p < 0.001).

When all histologies were analysed together, women also showed a significantly higher frequency of EGFR mutation compared to men (17/200 [8.5%] vs. 10/352 [2.8%]; p=0.003) (Table 2 upper and lower panel).

However, when the subgroup of adenocarcinomas was analyses among women the higher mutation rate compared with histologies remained (16/116 [14%] vs. 1/84 [1.2%], p = 0.002) (Table 2 upper panel). In contrast, among men with NSCLC no significant difference in the

rate of mutation could be detected with regard to histology (adenocarcinoma, 6/138 [4.3%] vs. Non- adenocarcinoma, 4/214 [1.9%]; p=0.172) (Table 2 lower panel).

Smoking status was closely associated with EGFR mutation rate. Among patients with EGFR mutations (n = 27) the rate of never-smokers was twice as high compared to smokers (18/27 [67%] vs. 9/27 [33%]) (Table 3).

KRAS mutation

A mutation in the KRAS gene was seen in a total of 85/552 (15%) of all analyzed tumours. While 73/85 (86%) of these mutations were found in exon 12, in exon 13 only 12/85 (14%) mutations were localized. With 29/85 (34%) cases the 12 Cys mutation was the most common. Similar to the EGFR the subgroups of adenocarcinomas had significantly (p <0.001) higher rate of KRAS mutations compared with other histologies (67/254 [26%] vs. 18/298 [6%]; Table 1). Within the subgroup of adenocarcinomas there was an even gender distribution regarding the frequency of KRAS mutations (women, 31/116 [27%] vs. men, 36/138 [26%]; p=0.909) (Table 2 upper and lower panel).

However, regardless of histology the rate of KRAS mutations was significantly (p = 0.007) more common in women than in men (42/200 [21%] vs. 43/352 [12%]). Besides that, the KRAS mutation in large cell carcinomas was significantly (p< 0.001) more frequent compared to squamous cell carcinomas (11/80 [14%] vs. 5/186 [2.7 %]) (Table 1). No patient had a double mutation (KRAS and EGFR).

Unlike in patients with an activating EGFR mutation the proportion of smokers is higher in patients with a KRAS mutation (75/85 [88%] vs. 10/85 [22%]) (Table 3).

Intention-to-treat analyses

Table 4 concludes the distribution according to histology and gender for all patients not included in this analysis (n = 180). The percentage of tumours that had to be left unclassified (NOS; non other specified) was substantially higher (38/180, 21%) compared to the patient group included (21/552; 3.8%; $p < 0.001$). This was most likely due to an insufficient probe size, which hampered both plain histology and mutation analysis. The percentage of patients with adencarcinomas excluded was slightly lower (39% vs. 46%), however, this difference was not statistically significant ($p = 0.112$) (Table 1 and 4).

Discussion

The present study delivers sound data on the frequency of EGFR and KRAS mutations in a large and unselected cohort of patients with newly diagnosed non-small cell lung cancer (NSCLC) over a well defined study period. The EGFR mutation rate reported here (4.9%) differs largely from frequencies reported in previous studies (8% -60%) (2;5;14). This suggests that previous study designs had import selection biases concerning this mutation analyses. In the most extensive study on the prevalence of activating EGFR mutations in NSCLC at 126 Spanish hospitals in the years 2005-2008 a total of 2105 patients were analyzed and a mutation rate of 16.6% was reported (15). One possible explanation for the higher mutation rate in this study lies in the unrepresentative distribution of the histological subgroups. While the expected proportion of adenocarcinomas in routine studies is between 32 to 54% depending on the literature, this study reported up to 78% adenocarcinomas. Since adenocarcinomas were also in our study more likely to exhibit an activating EGFR mutation, the frequency was overestimated in the Spanish study (15). The disproportion of adenocarcinomas is also found in other studies reporting EGFR mutation frequencies between 9% and 31% (5;16;17). In our analysis, however, the proportion of adenocarcinomas was well within the expected range (46%) with no selection bias induced by patient selection.

Another factor with great influence on the EGFR mutation rate is the geographical origin of the analysed patients. For quite some years it has been known that East Asian patients with NSCLC have a higher incidence of EGFR mutations while they show lower incidence of KRAS mutations compared to other ethnic groups at the same time (11;18;19). Studies with a high proportion of patients from Far Eastern countries or studies which have recruited their patients entirely in the East Asian region have consequently shown a corresponding effect

on the frequency of detected EGFR and KRAS mutations (3;20;21). None of our patients with a migration background came from an East Asian country and thus our figures are not prone to this bias.

A study from Basel seems to be the most comparable in terms of patient's cohort. Pathologists examined 307 NSCLC during a 4.5 year period for EGFR mutation and showed a mutation rate of 8.1%. However it remains unclear in this study, which preliminary selection bias may have been introduced by the clinicians, because mutation analyses were not requested for all samples. Moreover, it has not been reported how many patients with NSCLC were newly diagnosed in the participating hospitals (14).

As demonstrated in our study, patients with NSCLC and EGFR mutation are non-smokers in the majority of cases, which has been confirmed by numerous studies (2;22;23). In Europe and in Germany as well it is estimated that 85% of lung cancers related deaths are associated with cigarette smoking (24). Such a high proportion of smokers among patients with lung cancer make a high EGFR mutation rate questionable. On the other hand the question arises why this mutation occurs much more frequently in the Asian populations.

Independent of the rate of mutation in total, the frequency of EGFR mutations in exons 19 and 21 of our samples is comparable with those of numerous studies (20;25-27). In addition, the occurrence of the most common mutation in our evaluation delE746-A750 in exon 19 (10/27 [37%]) corresponds to the literature (28). Another interesting aspect is that the restriction on the search for the two most common mutations in exon 19 (delE746-A750) and exon 21 (L858R), as discussed in the literature, would have detected only 18/27 (69.2 %) of all EGFR mutations in our study.

Regarding the KRAS mutations our analysis confirms the known fact that KRAS mutations occur in smokers with NSCLC significantly more often than in non-smokers (29;30). This is similar to the distribution of mutations of KRAS at codon 12 (72/85 [85%]) and 13 (13/85 [15.3%]), which appears to be comparable with the frequency of the other studies (2;31). Therefore our results regarding the frequency of KRAS mutation are in keeping with the published reports. The missing gender bias and the higher frequency among smokers repeated in our sample support the validity of our data. Up to date, the clinical applicability of the KRAS mutation is limited, because there are no clinical associations strong enough for inclusion in clinical or therapeutic algorithms. However, KRAS mutation exclude or are only very rarely associated with other mutations (e.g. EGFR). KRAS may therefore help to build up a testing algorithm in order to get a reasonable genetic array for this type of cancer.

In summary we offer for the first time unbiased mutation rates for EGFR and KRAS in an unselected cohort of Caucasian patients. Previously described mutation rates for EGFR need to be corrected to approximately 5%, thus lowering the proportion of patients with NSCLC entitled to personalised medicine in Central Europe. However, in keeping with the literature non-smoking women with adenocarcinoma do have the highest frequency of tumours with an activating EGFR mutation.

Competing Interests

None

Contributorship

All authors contributed equally to the study and helped writing the manuscript.

C Boch wrote the manuscript and recruited patients

J Kollmeier wrote the manuscript and helped with the data analyses

A Roth wrote the manuscript and did the pathologic analyses

S Stephan-Falkenau wrote the manuscript and did the pathologic analyses

D Misch wrote the manuscript and analysed the data

W Grüning wrote the manuscript and did the sampling

TT Bauer wrote the manuscript, was involved in the study design and analysed the data

T Mairinger wrote the manuscript and did the pathologic analyses

Data sharing

No unpublished data exists. All data have been included in the intention to treat analyses.

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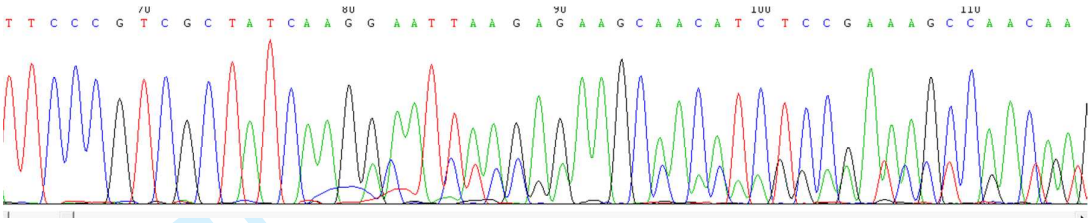
Figures

Figure 1: Representation of a mutation in exon 19 (del E746-A750) by using Sanger dideoxy (13).



Tables:

Table 1: Proportion of mutations found according to gender and histological type of non small cell lung carcinoma (NSCLC). No double mutations were found in this cohort.

		Gender		Histology					
	Total	Female	Male	Adeno	Squamos	Large cell	Sarcomat.	Adenosqua.	NOS
Total	552	200 (36.2%)	352 (63.8%)	254 (46.0%)	186 (33.7%)	79 (14.3%)	7 (1.3%)	5 (0.9%)	21 (3.8%)
EGFR pos.	27 **(4.9%)**	17 (8.5%)	10 (2.8%)	22 (8.7%)	2 (1.1%)	2 (2.5%)	0 (0%)	1 (20%)	0 (0%)
EGFR neg.	525	183 (91.5%)	342 (97.2%)	232 (91.3%)	184 (98.9%)	77 (97.5%)	7 (100%)	4 (80%)	21 (100%)
KRAS pos.	85 **(15.4%)**	42 (21.0%)	43 (12.2%)	67 (26.4%)	5 (2.7%)	11 (13.9%)	0 (0%)	0 (0%)	2 (9.5%)
KRAS neg.	467	158 (79.0%)	309 (87.8%)	187 (73.6%)	181 (97.3%)	68 (86.1%)	7 (100%)	5 (100%)	19 (90.5%)

NOS: Non other specified

Table 2: Proportion of EGFR and KRAS mutations found according to women (upper table) and men (lower table)

	Total	EGFR pos.		KRAS pos.	
Total number women	200	17	8.5%	42	21.0%
Adenocarcinoma	116	16	13.8%	31	26.7%
Squamous cell carzinoma	47	0	0.0%	2	4.3%
Large cell carcinoma	28	1	3.6%	7	25.0%
Sarcomatoid carcinoma	0	0	0.0%	0	0.0%
Adenosquamous carcinoma	0	0	0.0%	0	0.0%
NOS (non other specified)	9	0	0.0%	2	22.2%

	Total	EGFR pos.		KRAS pos.	
Total number of men	352	10	2.8%	43	12.2%
Adenocarcinoma	138	6	4.3%	36	26.1%
Squamous cell carzinoma	139	2	1.4%	3	2.2%
Large cell carcinoma	51	1	2.0%	4	7.8%
Sarcomatoides Karzinom	7	0	0.0%	0	0.0%
Adenosquamous carcinoma	5	1	20.0%	0	0.0%
NOS (non other specified)	12	0	0.0%	0	0.0%

Table 3: Smoker status and gender distribution of all 112 patients with either EGFR or KRAS mutation.

	Smokers		Never-smokers	
Total number of mutations	84	75.0%	28	25.0%
EGFR pos.	**9**	**33.3%**	**18**	**66.7%**
EGFR pos. Female	5	29.4%	12	70.6%
EGFR pos. Male	4	40.0%	6	60.0%
KRAS pos.	**75**	**88.2%**	**10**	**11.8%**
KRAS pos. Female	38	90.5%	4	9.5%
KRAS pos. Male	37	86.0%	6	14.0%

Table 4: Histology of the patients not included in the study, because of insufficient probe size or denial of informed consent.

	Total	%	Men	Women
Total	180		111	69
Adenocarcinoma	70	38.9%	38	32
Squamous cell carzinoma	47	26.1%	34	13
Large cell carcinoma	17	9.4%	10	7
Sarcomatoides Karzinom	8	4.4%	7	1
Adenosquamous carcinoma	0	0.0%	0	0
NOS (non other specified)	38	21.1%	22	16

The frequency of EGFR and KRAS mutations in non-small-cell-lung cancer (NSCLC) – Routine screening data for Central Europe

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The frequency of EGFR and KRAS mutations in non-small-cell-lung cancer (NSCLC) – Routine screening data for Central Europe

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Article focus

- Frequency of clinically important mutations in non-small-cell lung carcinoma
- epidermal growth factor receptor (EGFR) and KRAS
- personalised medicine

Key messages

- The frequency of an activating EGFR mutation can not be expected in more than 4.9% of the "allcomers" with the diagnosis of NSCLC.

Strengths and limitations of this study' section

- Unselected and large cohort of lung cancer centre
- Analyses of three codons for EGFR
- ***Possible limitation***: German Single centre study

Introduction

Lung cancer is still one of the most common cancers in Germany and worldwide, and the most common cause of death due to malignant tumours (1). Despite intensive research the prognosis of patients with non-small-cell lung carcinoma (NSCLC) continues to be restricted, which applies especially to patients with distant metastases. But in patients with NSCLC and an activating mutation of the epidermal growth factor receptor (EGFR), therapy has undergone a significant change. The application of first generation tyrosine-kinase inhibitors (TKI) Gefitinib an Erlotinib was able to achieve not only a better tolerability of the therapy and an improvement in progression-free survival (PFS) in observational studies (2-4), but also compared to platinum-based chemotherapy (5). However, the effect on overall survival (OS) is still controversial; one study could not detect a benefit (6), whereas Zhou and coworkers reported a significant increase in OS (7).

In the last years intensive research has been conducted to analyse the effect of TKIs in respect of the complex relationship of biomarkes which are associated with the EGFR pathway like v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). In addition to the superiority in EGFR mutated patients, TKIs seem to have an effect on the wild type NSCLC as well, while some studies have disputed this for KRAS mutated patients (2;8). However the significance of KRAS mutations for the diagnosis or treatment of NSCLC remains under debate. Since double mutations (EGFR and KRAS) have been described in only very few cases, KRAS may help to exclude EGFR mutation (9;10).

Therefore the question arises how many patients with NSCLC can benefit from the new treatment option and whether screening should be implemented in the routine diagnostics. In addition, numerous studies came from Southeast Asia, where patients with NSCLC have higher EGFR mutation rates (11;12). Since the data available today are hampered either by

biases due to study exclusion criteria or different geographical peculiarities, we established routine screening for consecutive patients in order to receive reliable data for the routine setting in Central Europe.

Patients and methods

Study population

From October 2009 until December 2010 we have screened prospectively all newly diagnosed patients with NSCLC (n = 753) for the ability to be analysed for EGFR and KRAS mutations. All subsequent biopsies have been obtained by surgery, routine bronchoscopy or CT guided biopsy. Staging of the toumor war performed according to the Union for International Cancer Control UICC (Seventh Edition)(13). The complete staging was available for 735/753 patient (97.6%). The histological diagnosis was performed according to WHO criteria (14). A total of 552/753 (75%) cases with NSCLC were eligible for analysis. In 183 patients (25%) none of the two analyses could be performed. In the majority of cases, this was explained by an insufficient amount of tissue sample. In addition, some patients refused their consent to carry out the mutation analysis. Informed consent was obtained from all patients prior to biopsy.

DNA extraction

After preparation of a 20 µm thick section from the formalin-fixed and paraffin-embedded tissue sample, the area of tumour cells was marked by comparison with the corresponding HE stained slide. This area with a size of 2 to 10 mm^2 was scraped with a pipette tip and transferred into 180 µl AL-buffer. The AL-buffer is included in the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Afterwards the DNA was extracted and dissolved in 150 µl AE-buffer according to the manufacturer's instructions using the QIAamp DNA Mini Kit.

Determination of EGFR mutations

For analysing the EGFR mutation status, the extracted DNA was subjected to conventional PCR for amplification of relevant portions of exons 18, 19 and 21 of the EGFR gene. Further, the PCR products were analyzed for the presence of a mutation by using the Pyrosequencing PyroMark Instument (Qiagen, Hilden, Germany). The primers used for the PCR are listed in Table 1. The method of pyrosequencing has been performed according to the reagent manufacturer (Qiagen, Hilden, Germany) and the following primer names and sequences (3'-5') have been used for exons 18, 19 and 21:

- 18_F (forward) CAGCATGGTGAGGGCTGAG
- 18_R (reverse) GCCTGTGCCAGGGACCTTAC
- 19_F (forward) GTGGCACCATCTCACAATTGC
- 19_R (reverse) CACACAGCAAAGCAGAAACTCAC
- 21_F (forward) CCTCACAGCAGGGTCTTCTCTG
- 21_R (reverse) CTGGCTGACCTAAAGCCACC

Samples with a mutation in the pyrogram were additionally examined by dideoxy sequencing according to Sanger et al. for confirmation (Figure 1) (15).

Determination of KRAS mutations

The DNA was analyzed according to manufacturer's instructions using the K-RAS codon 12/13 kit from Molbiol TIB (Berlin, Germany) and the LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany).

This is a real-time PCR with FRET probes, which facilitate the differentiation of mutant and wild-type DNA in codon 12 and 13 on exon 1 of KRAS gene after successful amplification and analyses of the melting curve of the PCR products.

Statistical Analyses

Quantitative variables were summarized as means ± standard deviations (SD). For comparisons Student´s t-test was used and in cases with more than two groups, a oneway ANOVA with Bonferroni correction was calculated. Categorical variables are reported as frequencies and for comparisons of categorical variables between groups chi-square tests were used. The level of significance for all tests was set to 5 percent and all tests were two-sided. Analyses were carried out with the Statistical Package for Social Sciences (SPSS, 13.0) on a Windows operating system.

Results

Patients

The mean age of patients with wild type EGFR (n = 440; 66.7 ± 9.5 years) was comparable to the mean age of patients with EGFR mutation (n = 27; 70.3 ± 11.4 years) and that of patients with a KRAS mutation in the overall comparison (n = 85; 65.3 ± 9.8 years; p = 0.059). Table 1 summarises frequencies according to mutation, gender and histology.

A breakdown of patients according to the genealogy was not performed because all patients with a migration background except two came from other European countries. Patients from Asia were not diagnosed or treated in our clinic during the corresponding period.

EGFR mutation

Overall 27 (4.9%) of all 552 patients showed an activating EGFR mutation (3/27 mutations in exon 18 (11%), 15/27 mutations in exon19 (56%) and 8/27 mutations (30%) in exon 21; 1/27 tumour (3%) has shown a double mutation (exon 19 and 21)). The most common mutation in exon 19 was delE746-A750 with 10/15 (67%) cases. In exon 21 all detected 8 mutations were located on L848R (100%).

Considering the frequency of mutations according to histology, adenocarcinomas show mutations more often than any other histology (22/254 [8.7%] vs. 5/298 [1.7%]; p < 0.001).

When all histologies were analysed together, women also showed a significantly higher frequency of EGFR mutation compared to men (17/200 [8.5%] vs. 10/352 [2.8%]; p=0.003) (Table 2 upper and lower panel).

However, when the subgroup of adenocarcinomas was analyses among women the higher mutation rate compared with histologies remained (16/116 [14%] vs. 1/84 [1.2%], p = 0.002) (Table 2 upper panel). In contrast, among men with NSCLC no significant difference in the

rate of mutation could be detected with regard to histology (adenocarcinoma, 6/138 [4.3%] vs. Non- adenocarcinoma, 4/214 [1.9%]; p=0.172) (Table 2 lower panel).

Smoking status was closely associated with EGFR mutation rate. Among patients with EGFR mutations (n = 27) the rate of never-smokers was twice as high compared to smokers (18/27 [67%] vs. 9/27 [33%]) (Table 3).

KRAS mutation

A mutation in the KRAS gene was seen in a total of 85/552 (15%) of all analyzed tumours. While 73/85 (86%) of these mutations were found in exon 12, in exon 13 only 12/85 (14%) mutations were localized. With 29/85 (34%) cases the 12 Cys mutation was the most common. Similar to the EGFR the subgroups of adenocarcinomas had significantly (p <0.001) higher rate of KRAS mutations compared with other histologies (67/254 [26%] vs. 18/298 [6%]; Table 1). Within the subgroup of adenocarcinomas there was an even gender distribution regarding the frequency of KRAS mutations (women, 31/116 [27%] vs. men, 36/138 [26%]; p=0.909) (Table 2 upper and lower panel).

However, regardless of histology the rate of KRAS mutations was significantly (p = 0.007) more common in women than in men (42/200 [21%] vs. 43/352 [12%]). Besides that, the KRAS mutation in large cell carcinomas was significantly (p< 0.001) more frequent compared to squamous cell carcinomas (11/80 [14%] vs. 5/186 [2.7 %]) (Table 1). No patient had a double mutation (KRAS and EGFR).

Unlike in patients with an activating EGFR mutation the proportion of smokers is higher in patients with a KRAS mutation (75/85 [88%] vs. 10/85 [22%]) (Table 3).

Intention-to-treat analyses

Table 4 concludes the distribution according to histology and gender for all patients not included in this analysis (n = 180). The percentage of tumours that had to be left unclassified (NOS; non other specified) was substantially higher (38/180, 21%) compared to the patient group included (21/552; 3.8%; p < 0.001). This was most likely due to an insufficient probe size, which hampered both plain histology and mutation analysis. The percentage of patients with adencarcinomas excluded was slightly lower (39% vs. 46%), however, this difference was not statistically significant (p = 0.112) (Table 1 and 4).

When we analysed the distribution according to clinical stage, we found a bias with regard to higher clinical stages in the non tested group (Table 5). However, when stages IIIB and IV were compared to the potentially operable stages (IA – IIIA) we found this difference not to be statistically significant (not tested: 102/180; 56.7% versus tested: 277/552; 50.2%, p = 0.077).

Discussion

The present study delivers sound data on the frequency of EGFR and KRAS mutations in a large and unselected cohort of patients with newly diagnosed non-small cell lung cancer (NSCLC) over a well defined study period. The EGFR mutation rate reported here (4.9%) differs largely from frequencies reported in previous studies (8% -60%) (2;5;16). This suggests that previous study designs had import selection biases concerning this mutation analyses. In the most extensive study on the prevalence of activating EGFR mutations in NSCLC at 126 Spanish hospitals in the years 2005-2008 a total of 2105 patients were analyzed and a mutation rate of 16.6% was reported (17). One possible explanation for the higher mutation rate in this study lies in the unrepresentative distribution of the histological subgroups. While the expected proportion of adenocarcinomas in routine studies is between 32 to 54% depending on the literature, this study reported up to 78% adenocarcinomas. Since adenocarcinomas were also in our study more likely to exhibit an activating EGFR mutation, the frequency was overestimated in the Spanish study (17). The disproportion of adenocarcinomas is also found in other studies reporting EGFR mutation frequencies between 9% and 31% (2;18;19). In our analysis, however, the proportion of adenocarcinomas was well within the expected range (46%) with no selection bias induced by patient selection.

Another factor with great influence on the EGFR mutation rate is the geographical origin of the analysed patients. For quite some years it has been known that East Asian patients with NSCLC have a higher incidence of EGFR mutations while they show lower incidence of KRAS mutations compared to other ethnic groups at the same time (12;20;21). Studies with a high proportion of patients from Far Eastern countries or studies which have recruited their patients entirely in the East Asian region have consequently shown a corresponding effect

on the frequency of detected EGFR and KRAS mutations (6;22;23). None of our patients with a migration background came from an East Asian country and thus our figures are not prone to this bias.

A study from Basel seems to be the most comparable in terms of patient's cohort. Pathologists examined 307 NSCLC during a 4.5 year period for EGFR mutation and showed a mutation rate of 8.1%. However it remains unclear in this study, which preliminary selection bias may have been introduced by the clinicians, because mutation analyses were not requested for all samples. Moreover, it has not been reported how many patients with NSCLC were newly diagnosed in the participating hospitals (16).

As demonstrated in our study, patients with NSCLC and EGFR mutation are non-smokers in the majority of cases, which has been confirmed by numerous studies (5;24;25). In Europe and in Germany as well it is estimated that 85% of lung cancers related deaths are associated with cigarette smoking (26). Such a high proportion of smokers among patients with lung cancer make a high EGFR mutation rate questionable. On the other hand the question arises why this mutation occurs much more frequently in the Asian populations.

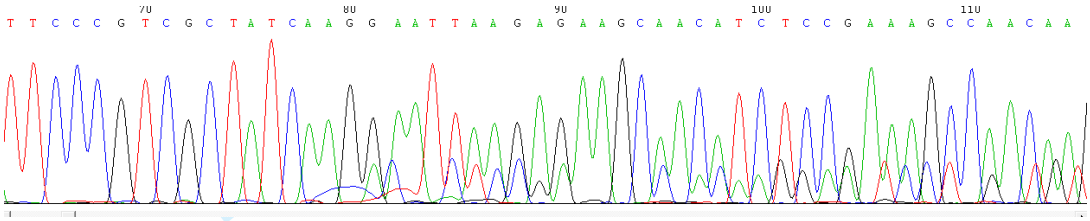
Independent of the rate of mutation in total, the frequency of EGFR mutations in exons 19 and 21 of our samples is comparable with those of numerous studies (22;27-29). In addition, the occurrence of the most common mutation in our evaluation delE746-A750 in exon 19 (10/27 [37%]) corresponds to the literature (30). Another interesting aspect is that the restriction on the search for the two most common mutations in exon 19 (delE746-A750) and exon 21 (L858R), as discussed in the literature, would have detected only 18/27 (69.2 %) of all EGFR mutations in our study.

Regarding the KRAS mutations our analysis confirms the known fact that KRAS mutations occur in smokers with NSCLC significantly more often than in non-smokers (31;32). This is similar to the distribution of mutations of KRAS at codon 12 (72/85 [85%]) and 13 (13/85 [15.3%]), which appears to be comparable with the frequency of the other studies (5;33). Therefore our results regarding the frequency of KRAS mutation are in keeping with the published reports. The missing gender bias and the higher frequency among smokers repeated in our sample support the validity of our data. Up to date, the clinical applicability of the KRAS mutation is limited, because there are no clinical associations strong enough for inclusion in clinical or therapeutic algorithms. However, KRAS mutation exclude or are only very rarely associated with other mutations (e.g. EGFR). KRAS may therefore help to build up a testing algorithm in order to get a reasonable genetic array for this type of cancer.

In summary we offer for the first time unbiased mutation rates for EGFR and KRAS in an unselected cohort of Caucasian patients. Previously described mutation rates for EGFR need to be corrected to approximately 5%, thus lowering the proportion of patients with NSCLC entitled to personalised medicine in Central Europe. However, in keeping with the literature non-smoking women with adenocarcinoma do have the highest frequency of tumours with an activating EGFR mutation.

Figures

Figure 1: Representation of a mutation in exon 19 (del E746-A750) by using Sanger dideoxy (15).



Tables:

Table 1: Proportion of mutations found according to gender and histological type of non small cell lung carcinoma (NSCLC). No double mutations were found in this cohort.

		Gender		Histology					
	Total	Female	Male	Adeno	Squamos	Large cell	Sarcomat.	Adenosqua.	NOS
Total	552	200 (36.2%)	352 (63.8%)	254 (46.0%)	186 (33.7%)	79 (14.3%)	7 (1.3%)	5 (0.9%)	21 (3.8%)
EGFR pos.	27 **(4.9%)**	17 (8.5%)	10 (2.8%)	22 (8.7%)	2 (1.1%)	2 (2.5%)	0 (0%)	1 (20%)	0 (0%)
EGFR neg.	525	183 (91.5%)	342 (97.2%)	232 (91.3%)	184 (98.9%)	77 (97.5%)	7 (100%)	4 (80%)	21 (100%)
KRAS pos.	85 **(15.4%)**	42 (21.0%)	43 (12.2%)	67 (26.4%)	5 (2.7%)	11 (13.9%)	0 (0%)	0 (0%)	2 (9.5%)
KRAS neg.	467	158 (79.0%)	309 (87.8%)	187 (73.6%)	181 (97.3%)	68 (86.1%)	7 (100%)	5 (100%)	19 (90.5%)

NOS: Non other specified

Table 2: Proportion of EGFR and KRAS mutations found according to women (upper table) and men (lower table)

	Total	EGFR pos.		KRAS pos.	
Total number women	200	17	8.5%	42	21.0%
Adenocarcinoma	116	16	13.8%	31	26.7%
Squamous cell carzinoma	47	0	0.0%	2	4.3%
Large cell carcinoma	28	1	3.6%	7	25.0%
Sarcomatoid carcinoma	0	0	0.0%	0	0.0%
Adenosquamous carcinoma	0	0	0.0%	0	0.0%
NOS (non other specified)	9	0	0.0%	2	22.2%

	Total	EGFR pos.		KRAS pos.	
Total number of men	352	10	2.8%	43	12.2%
Adenocarcinoma	138	6	4.3%	36	26.1%
Squamous cell carzinoma	139	2	1.4%	3	2.2%
Large cell carcinoma	51	1	2.0%	4	7.8%
Sarcomatoides Karzinom	7	0	0.0%	0	0.0%
Adenosquamous carcinoma	5	1	20.0%	0	0.0%
NOS (non other specified)	12	0	0.0%	0	0.0%

Table 3: Smoker status and gender distribution of all 112 patients with either EGFR or KRAS mutation.

	Smokers		Never-smokers	
Total number of mutations	84	75.0%	28	25.0%
EGFR pos.	**9**	**33.3%**	**18**	**66.7%**
EGFR pos. Female	5	29.4%	12	70.6%
EGFR pos. Male	4	40.0%	6	60.0%
KRAS pos.	**75**	**88.2%**	**10**	**11.8%**
KRAS pos. Female	38	90.5%	4	9.5%
KRAS pos. Male	37	86.0%	6	14.0%

Table 4: Histology of the patients not included in the study, because of insufficient probe size or denial of informed consent.

	Total	%	Men	Women
Total	180		111	69
Adenocarcinoma	70	38.9%	38	32
Squamous cell carzinoma	47	26.1%	34	13
Large cell carcinoma	17	9.4%	10	7
Sarcomatoides Karzinom	8	4.4%	7	1
Adenosquamous carcinoma	0	0.0%	0	0
NOS (non other specified)	38	21.1%	22	16

Table 5: Lung cancer stages of the investigated cohort and the excluded patients (according to UICC 7th edition)(13).

	Intention to treat population (n = 732)	Percent	Study Population (n = 522)	Percent	Patients excluded from analyses (n = 180)	Percent
Clinical Stage (n;%)						
Staging not completed	13	1.8	9	1.6	4	2.2
IA	76	10.4	56	10.1	20	11.1
IB	50	6.8	36	6.5	14	7.8
IIA	44	6.0	35	6.3	9	5.0
IIB	54	7.4	44	8.0	10	5.6
IIIA	116	15.8	95	17.2	21	11.7
IIIB	82	11.2	70	12.7	12	6.7
IV	297	40.6	207	37.5	90	50.0

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The frequency of EGFR and KRAS mutations in non-small-cell-lung cancer (NSCLC) – Routine screening data for Central Europe

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ARTICLE SUMMARY

Article focus

- Frequency of clinically important mutations in non-small-cell lung carcinoma
- epidermal growth factor receptor (EGFR) and KRAS
- personalised medicine

Key messages

- The frequency of an activating EGFR mutation can not be expected in more than 4.9% of the "allcomers" with the diagnosis of NSCLC.

Strengths and limitations of this study' section

- Unselected and large cohort of lung cancer centre
- Analyses of three codons for EGFR
- ***Possible limitation***: German Single centre study

Abstract

Objectives: Due to novel therapy strategies in EGFR mutated patients, molecular analysis of the EGFR and KRAS genome has become crucial for routine diagnostics. Up to date these data derive mostly from clinical trials, and thus collected in pre-selected populations. We therefore screened "allcomers" with a newly diagnosed non-small-cell lung carcinoma (NSCLC) for the frequencies of these mutations.

Design: Cohort study

Setting: Lung cancer centre in a tertiary care hospital

Participants: Within 15 months a total of 552 cases with NSCLC were eligible for analysis.

Primary and secondary outcome measures: Frequency of scrutinizing exons 18, 19 and 21 for the presence of activating EGFR mutation and secondary codon 12 and 13 for activating KRAS mutations.

Results: Of the 552 patients, 27 (4.9%) showed a mutation of EGFR. 19 of these patients (70%) had deletion E746-A750 in codon 19 or deletion L858R in codon 21. Adenocarcinoma (ACA) was the most frequent histology among patients with EGFR mutations (ACA, 22/254 [8.7%] vs. non-ACA, 5/298 [1.7%]; p<0.001). Regarding only ACA, the percentage of EGFR mutations was higher in women (16/116 [14%] women vs. 6/138 [4.3%] men; p=0.008). Tumours with an activating EGFR mutation were more likely to be from non-smokers (18/27; 67%) rather than smoker (9/27; 33%).

KRAS mutation was present in 85 (15 %) of all cases. In 73 patients (86%), the mutation was found in exon 12 and in 12 cases (14%) in exon 13. Similarly, ACA had a higher frequency of KRAS mutations than non-ACA (67/254 [26%] vs. 18/298 [6.0%]; p<0.001).

Conclusions: We found a lower frequency for EGFR and KRAS mutations in an unselected Caucasian patient cohort as previously published. Taking our results into account, clinical

trials may overestimate the mutation frequency for EGFR and KRAS in NSCLC due to important selection biases.

Introduction

Lung cancer is still one of the most common cancers in Germany and worldwide, and the most common cause of death due to malignant tumours (1). Despite intensive research the prognosis of patients with non-small-cell lung carcinoma (NSCLC) continues to be restricted, which applies especially to patients with distant metastases. But in patients with NSCLC and an activating mutation of the epidermal growth factor receptor (EGFR), therapy has undergone a significant change. The application of first generation tyrosine-kinase inhibitors (TKI) Gefitinib an Erlotinib was able to achieve not only a better tolerability of the therapy and an improvement in progression-free survival (PFS) in observational studies (2-4), but also compared to platinum-based chemotherapy (5). However, the effect on overall survival (OS) is still controversial; one study could not detect a benefit (6), whereas Zhou and coworkers reported a significant increase in OS (7).

In the last years intensive research has been conducted to analyse the effect of TKIs in respect of the complex relationship of biomarkes which are associated with the EGFR pathway like v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). In addition to the superiority in EGFR mutated patients, TKIs seem to have an effect on the wild type NSCLC as well, while some studies have disputed this for KRAS mutated patients (2;8). However the significance of KRAS mutations for the diagnosis or treatment of NSCLC remains under debate. Since double mutations (EGFR and KRAS) have been described in only very few cases, KRAS may help to exclude EGFR mutation (9;10).

Therefore the question arises how many patients with NSCLC can benefit from the new treatment option and whether screening should be implemented in the routine diagnostics. In addition, numerous studies came from Southeast Asia, where patients with NSCLC have higher EGFR mutation rates (11;12). Since the data available today are hampered either by

biases due to study exclusion criteria or different geographical peculiarities, we established routine screening for consecutive patients in order to receive reliable data for the routine setting in Central Europe.

Patients and methods

Study population

From October 2009 until December 2010 we have screened prospectively all newly diagnosed patients with NSCLC (n = 753) for the ability to be analysed for EGFR and KRAS mutations. All subsequent biopsies have been obtained by surgery, routine bronchoscopy or CT guided biopsy. Staging of the toumor war performed according to the Union for International Cancer Control UICC (Seventh Edition)(13). The complete staging was available for 735/753 patient (97.6%). The histological diagnosis was performed according to WHO criteria (14). A total of 552/753 (75%) cases with NSCLC were eligible for analysis. In 183 patients (25%) none of the two analyses could be performed. In the majority of cases, this was explained by an insufficient amount of tissue sample. In addition, some patients refused their consent to carry out the mutation analysis. Informed consent was obtained from all patients prior to biopsy.

DNA extraction

After preparation of a 20 µm thick section from the formalin-fixed and paraffin-embedded tissue sample, the area of tumour cells was marked by comparison with the corresponding HE stained slide. This area with a size of 2 to 10 mm^2 was scraped with a pipette tip and transferred into 180 µl AL-buffer. The AL-buffer is included in the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Afterwards the DNA was extracted and dissolved in 150 µl AE-buffer according to the manufacturer's instructions using the QIAamp DNA Mini Kit.

Determination of EGFR mutations

For analysing the EGFR mutation status, the extracted DNA was subjected to conventional PCR for amplification of relevant portions of exons 18, 19 and 21 of the EGFR gene. Further, the PCR products were analyzed for the presence of a mutation by using the Pyrosequencing PyroMark Instument (Qiagen, Hilden, Germany). The primers used for the PCR are listed in Table 1. The method of pyrosequencing has been performed according to the reagent manufacturer (Qiagen, Hilden, Germany) and the following primer names and sequences (3'-5') have been used for exons 18, 19 and 21:

- 18_F (forward) CAGCATGGTGAGGGCTGAG
- 18_R (reverse) GCCTGTGCCAGGGACCTTAC
- 19_F (forward) GTGGCACCATCTCACAATTGC
- 19_R (reverse) CACACAGCAAAGCAGAAACTCAC
- 21_F (forward) CCTCACAGCAGGGTCTTCTCTG
- 21_R (reverse) CTGGCTGACCTAAAGCCACC

Samples with a mutation in the pyrogram were additionally examined by dideoxy sequencing according to Sanger et al. for confirmation (Figure 1) (15).

Determination of KRAS mutations

The DNA was analyzed according to manufacturer's instructions using the K-RAS codon 12/13 kit from Molbiol TIB (Berlin, Germany) and the LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany).

This is a real-time PCR with FRET probes, which facilitate the differentiation of mutant and wild-type DNA in codon 12 and 13 on exon 1 of KRAS gene after successful amplification and analyses of the melting curve of the PCR products.

Statistical Analyses

Quantitative variables were summarized as means ± standard deviations (SD). For comparisons Student´s t-test was used and in cases with more than two groups, a oneway ANOVA with Bonferroni correction was calculated. Categorical variables are reported as frequencies and for comparisons of categorical variables between groups chi-square tests were used. The level of significance for all tests was set to 5 percent and all tests were two-sided. Analyses were carried out with the Statistical Package for Social Sciences (SPSS, 13.0) on a Windows operating system.

Results

Patients

The mean age of patients with wild type EGFR (n = 440; 66.7 ± 9.5 years) was comparable to the mean age of patients with EGFR mutation (n = 27; 70.3 ± 11.4 years) and that of patients with a KRAS mutation in the overall comparison (n = 85; 65.3 ± 9.8 years; p = 0.059). Table 1 summarises frequencies according to mutation, gender and histology.

A breakdown of patients according to the genealogy was not performed because all patients with a migration background except two came from other European countries. Patients from Asia were not diagnosed or treated in our clinic during the corresponding period.

EGFR mutation

Overall 27 (4.9%) of all 552 patients showed an activating EGFR mutation (3/27 mutations in exon 18 (11%), 15/27 mutations in exon19 (56%) and 8/27 mutations (30%) in exon 21; 1/27 tumour (3%) has shown a double mutation (exon 19 and 21)). The most common mutation in exon 19 was delE746-A750 with 10/15 (67%) cases. In exon 21 all detected 8 mutations were located on L848R (100%).

Considering the frequency of mutations according to histology, adenocarcinomas show mutations more often than any other histology (22/254 [8.7%] vs. 5/298 [1.7%]; p < 0.001).

When all histologies were analysed together, women also showed a significantly higher frequency of EGFR mutation compared to men (17/200 [8.5%] vs. 10/352 [2.8%]; p=0.003) (Table 2 upper and lower panel).

However, when the subgroup of adenocarcinomas was analyses among women the higher mutation rate compared with histologies remained (16/116 [14%] vs. 1/84 [1.2%], p = 0.002) (Table 2 upper panel). In contrast, among men with NSCLC no significant difference in the

rate of mutation could be detected with regard to histology (adenocarcinoma, 6/138 [4.3%] vs. Non- adenocarcinoma, 4/214 [1.9%]; p=0.172) (Table 2 lower panel).

Smoking status was closely associated with EGFR mutation rate. Among patients with EGFR mutations (n = 27) the rate of never-smokers was twice as high compared to smokers (18/27 [67%] vs. 9/27 [33%]) (Table 3).

KRAS mutation

A mutation in the KRAS gene was seen in a total of 85/552 (15%) of all analyzed tumours. While 73/85 (86%) of these mutations were found in exon 12, in exon 13 only 12/85 (14%) mutations were localized. With 29/85 (34%) cases the 12 Cys mutation was the most common. Similar to the EGFR the subgroups of adenocarcinomas had significantly (p <0.001) higher rate of KRAS mutations compared with other histologies (67/254 [26%] vs. 18/298 [6%]; Table 1). Within the subgroup of adenocarcinomas there was an even gender distribution regarding the frequency of KRAS mutations (women, 31/116 [27%] vs. men, 36/138 [26%]; p=0.909) (Table 2 upper and lower panel).

However, regardless of histology the rate of KRAS mutations was significantly (p = 0.007) more common in women than in men (42/200 [21%] vs. 43/352 [12%]). Besides that, the KRAS mutation in large cell carcinomas was significantly (p< 0.001) more frequent compared to squamous cell carcinomas (11/80 [14%] vs. 5/186 [2.7 %]) (Table 1). No patient had a double mutation (KRAS and EGFR).

Unlike in patients with an activating EGFR mutation the proportion of smokers is higher in patients with a KRAS mutation (75/85 [88%] vs. 10/85 [22%]) (Table 3).

Intention-to-treat analyses

Table 4 concludes the distribution according to histology and gender for all patients not included in this analysis (n = 180). The percentage of tumours that had to be left unclassified (NOS; non other specified) was substantially higher (38/180, 21%) compared to the patient group included (21/552; 3.8%; $p < 0.001$). This was most likely due to an insufficient probe size, which hampered both plain histology and mutation analysis. The percentage of patients with adencarcinomas excluded was slightly lower (39% vs. 46%), however, this difference was not statistically significant (p = 0.112) (Table 1 and 4).

When we analysed the distribution according to clinical stage, we found a bias with regard to higher clinical stages in the non tested group (Table 5). However, when stages IIIB and IV were compared to the potentially operable stages (IA – IIIA) we found this difference not to be statistically significant (not tested: 102/180; 56.7% versus tested: 277/552; 50.2%, p = 0.077).

Discussion

The present study delivers sound data on the frequency of EGFR and KRAS mutations in a large and unselected cohort of patients with newly diagnosed non-small cell lung cancer (NSCLC) over a well defined study period. The EGFR mutation rate reported here (4.9%) differs largely from frequencies reported in previous studies (8% -60%) (2;5;16). This suggests that previous study designs had import selection biases concerning this mutation analyses. In the most extensive study on the prevalence of activating EGFR mutations in NSCLC at 126 Spanish hospitals in the years 2005-2008 a total of 2105 patients were analyzed and a mutation rate of 16.6% was reported (17). One possible explanation for the higher mutation rate in this study lies in the unrepresentative distribution of the histological subgroups. While the expected proportion of adenocarcinomas in routine studies is between 32 to 54% depending on the literature, this study reported up to 78% adenocarcinomas. Since adenocarcinomas were also in our study more likely to exhibit an activating EGFR mutation, the frequency was overestimated in the Spanish study (17). The disproportion of adenocarcinomas is also found in other studies reporting EGFR mutation frequencies between 9% and 31% (2;18;19). In our analysis, however, the proportion of adenocarcinomas was well within the expected range (46%) with no selection bias induced by patient selection.

Another factor with great influence on the EGFR mutation rate is the geographical origin of the analysed patients. For quite some years it has been known that East Asian patients with NSCLC have a higher incidence of EGFR mutations while they show lower incidence of KRAS mutations compared to other ethnic groups at the same time (12;20;21). Studies with a high proportion of patients from Far Eastern countries or studies which have recruited their patients entirely in the East Asian region have consequently shown a corresponding effect

on the frequency of detected EGFR and KRAS mutations (6;22;23). None of our patients with a migration background came from an East Asian country and thus our figures are not prone to this bias.

A study from Basel seems to be the most comparable in terms of patient's cohort. Pathologists examined 307 NSCLC during a 4.5 year period for EGFR mutation and showed a mutation rate of 8.1%. However it remains unclear in this study, which preliminary selection bias may have been introduced by the clinicians, because mutation analyses were not requested for all samples. Moreover, it has not been reported how many patients with NSCLC were newly diagnosed in the participating hospitals (16).

As demonstrated in our study, patients with NSCLC and EGFR mutation are non-smokers in the majority of cases, which has been confirmed by numerous studies (5;24;25). In Europe and in Germany as well it is estimated that 85% of lung cancers related deaths are associated with cigarette smoking (26). Such a high proportion of smokers among patients with lung cancer make a high EGFR mutation rate questionable. On the other hand the question arises why this mutation occurs much more frequently in the Asian populations.

Independent of the rate of mutation in total, the frequency of EGFR mutations in exons 19 and 21 of our samples is comparable with those of numerous studies (22;27-29). In addition, the occurrence of the most common mutation in our evaluation delE746-A750 in exon 19 (10/27 [37%]) corresponds to the literature (30). Another interesting aspect is that the restriction on the search for the two most common mutations in exon 19 (delE746-A750) and exon 21 (L858R), as discussed in the literature, would have detected only 18/27 (69.2 %) of all EGFR mutations in our study.

Regarding the KRAS mutations our analysis confirms the known fact that KRAS mutations occur in smokers with NSCLC significantly more often than in non-smokers (31;32). This is similar to the distribution of mutations of KRAS at codon 12 (72/85 [85%]) and 13 (13/85 [15.3%]), which appears to be comparable with the frequency of the other studies (5;33). Therefore our results regarding the frequency of KRAS mutation are in keeping with the published reports. The missing gender bias and the higher frequency among smokers repeated in our sample support the validity of our data. Up to date, the clinical applicability of the KRAS mutation is limited, because there are no clinical associations strong enough for inclusion in clinical or therapeutic algorithms. However, KRAS mutation exclude or are only very rarely associated with other mutations (e.g. EGFR). KRAS may therefore help to build up a testing algorithm in order to get a reasonable genetic array for this type of cancer.

In summary we offer for the first time unbiased mutation rates for EGFR and KRAS in an unselected cohort of Caucasian patients. Previously described mutation rates for EGFR need to be corrected to approximately 5%, thus lowering the proportion of patients with NSCLC entitled to personalised medicine in Central Europe. However, in keeping with the literature non-smoking women with adenocarcinoma do have the highest frequency of tumours with an activating EGFR mutation.

Tables:

Table 1: Proportion of mutations found according to gender and histological type of non small cell lung carcinoma (NSCLC). No double mutations were found in this cohort.

		Gender		Histology					
	Total	Female	Male	Adeno	Squamos	Large cell	Sarcomat.	Adenosqua.	NOS
Total	552	200 (36.2%)	352 (63.8%)	254 (46.0%)	186 (33.7%)	79 (14.3%)	7 (1.3%)	5 (0.9%)	21 (3.8%)
EGFR pos.	27 **(4.9%)**	17 (8.5%)	10 (2.8%)	22 (8.7%)	2 (1.1%)	2 (2.5%)	0 (0%)	1 (20%)	0 (0%)
EGFR neg.	525	183 (91.5%)	342 (97.2%)	232 (91.3%)	184 (98.9%)	77 (97.5%)	7 (100%)	4 (80%)	21 (100%)
KRAS pos.	85 **(15.4%)**	42 (21.0%)	43 (12.2%)	67 (26.4%)	5 (2.7%)	11 (13.9%)	0 (0%)	0 (0%)	2 (9.5%)
KRAS neg.	467	158 (79.0%)	309 (87.8%)	187 (73.6%)	181 (97.3%)	68 (86.1%)	7 (100%)	5 (100%)	19 (90.5%)

NOS: Non other specified

Table 2: Proportion of EGFR and KRAS mutations found according to women (upper table) and men (lower table)

	Total	EGFR pos.		KRAS pos.	
Total number women	200	17	8.5%	42	21.0%
Adenocarcinoma	116	16	13.8%	31	26.7%
Squamous cell carzinoma	47	0	0.0%	2	4.3%
Large cell carcinoma	28	1	3.6%	7	25.0%
Sarcomatoid carcinoma	0	0	0.0%	0	0.0%
Adenosquamous carcinoma	0	0	0.0%	0	0.0%
NOS (non other specified)	9	0	0.0%	2	22.2%

	Total	EGFR pos.		KRAS pos.	
Total number of men	352	10	2.8%	43	12.2%
Adenocarcinoma	138	6	4.3%	36	26.1%
Squamous cell carzinoma	139	2	1.4%	3	2.2%
Large cell carcinoma	51	1	2.0%	4	7.8%
Sarcomatoides Karzinom	7	0	0.0%	0	0.0%
Adenosquamous carcinoma	5	1	20.0%	0	0.0%
NOS (non other specified)	12	0	0.0%	0	0.0%

Table 3: Smoker status and gender distribution of all 112 patients with either EGFR or KRAS mutation.

	Smokers		Never-smokers	
Total number of mutations	84	75.0%	28	25.0%
EGFR pos.	**9**	**33.3%**	**18**	**66.7%**
EGFR pos. Female	5	29.4%	12	70.6%
EGFR pos. Male	4	40.0%	6	60.0%
KRAS pos.	**75**	**88.2%**	**10**	**11.8%**
KRAS pos. Female	38	90.5%	4	9.5%
KRAS pos. Male	37	86.0%	6	14.0%

Table 4: Histology of the patients not included in the study, because of insufficient probe size or denial of informed consent.

	Total	%	Men	Women
Total	180		111	69
Adenocarcinoma	70	38.9%	38	32
Squamous cell carzinoma	47	26.1%	34	13
Large cell carcinoma	17	9.4%	10	7
Sarcomatoides Karzinom	8	4.4%	7	1
Adenosquamous carcinoma	0	0.0%	0	0
NOS (non other specified)	38	21.1%	22	16

Table 5: Lung cancer stages of the investigated cohort and the excluded patients (according to UICC 7th edition)(13).

	Intention to treat population (n = 732)	Percent	Study Population (n = 522)	Percent	Patients excluded from analyses (n = 180)	Percent
Clinical Stage (n;%)						
Staging not completed	13	1.8	9	1.6	4	2.2
IA	76	10.4	56	10.1	20	11.1
IB	50	6.8	36	6.5	14	7.8
IIA	44	6.0	35	6.3	9	5.0
IIB	54	7.4	44	8.0	10	5.6
IIIA	116	15.8	95	17.2	21	11.7
IIIB	82	11.2	70	12.7	12	6.7
IV	297	40.6	207	37.5	90	50.0

Figure Legend

Figure 1: Representation of a mutation in exon 19 (del E746-A750) by using Sanger dideoxy (15).

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