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Preselection of EGFR mutations in non-small-cell lung cancer patients by immunohistochemistry: comparison with DNA-sequencing, EGFR wild-type expression, gene copy number gain and clinicopathological data

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Abstract

Targeting epidermal growth factor receptor (EGFR) in patients with non-small-cell lung cancer (NSCLC) having EGFR mutations is associated with an improved overall survival. The aim of this study is to verify, if EGFR mutations detected by immunohistochemistry (IHC) is a convincing way to preselect patients for DNA-sequencing and to figure out, the statistical association between EGFR mutation, wild-type EGFR overexpression, gene copy number gain, which are the main factors inducing EGFR tumorigenic activity and the clinicopathological data. Two hundred sixteen tumor tissue samples of primarily chemotherapeutic naïve NSCLC patients were analyzed for EGFR mutations E746-A750del and L858R and correlated with DNA-sequencing. Two hundred six of which were assessed by IHC, using 6B6 and 43B2 specific antibodies followed by DNA-sequencing of positive cases and 10 already genotyped tumor tissues were also included to investigate debugging accuracy of IHC. In addition, EGFR wild-type overexpression was IHC evaluated and EGFR gene copy number determination was performed by fluorescence *in situ* hybridization (FISH). Forty-one/206 (19.9%) cases were positive for mutated EGFR by IHC. Eight of them had EGFR mutations of exons 18–21 by DNA-sequencing. Hit rate of 10 already genotyped NSCLC mutated cases was 90% by IHC. Positive association was found between EGFR mutations determined by IHC and both EGFR overexpression and increased gene copy number (p=0.002 and p<0.001, respectively). Additionally, positive association was detected between EGFR mutations, high tumor grade and clinical stage (p<0.001). IHC staining with mutation specific antibodies was demonstrated as a possible useful screening test to preselect patients for DNA-sequencing.

Keywords: NSCLC, preselection of patients for DNA-sequencing, EGFR activating mutation, immunohistochemistry, fluorescence *in situ* hybridization, direct sequencing.

Introduction

Epidermal growth factor receptor (EGFR) is a receptor-tyrosine kinase targeted by several individualized therapies in a plethora of malignancies including non-small-cell lung cancer (NSCLC), with therapy regimens comprising different monoclonal antibodies and tyrosine kinase inhibitors (TKIs) [1]. Monoclonal antibodies inhibit the ligand binding to the extracellular domain of EGFR, while TKIs target the intracellular tyrosine kinase domain of EGFR [1].

For the treatment of adenocarcinomas displaying distinct activating mutations of EGFR, superiority of Erlotinib and Gefitinib compared to standard Platinum-based chemotherapy has been demonstrated [2–4]. Induction of the EGFR-pathway by activating mutations promotes tumor progression. Ligand binding to EGFR induces dimerization followed by activation of the intracellular protein kinase leading to auto-phosphorylation of the cytoplasmic domain and activation of downstream signaling pathways including the PI3K–AKT–mTOR (phosphatidylinositol 3-kinase–protein kinase B–mammalian target of rapamycin) and the RAS–RAF–MEK–MAPK (mitogen-activated protein kinase) pathways [5].

Activation of the EGFR-pathway can be induced by EGFR-overexpression, increased gene copy number and activating mutations [6]. Overexpression of EGFR is present in 40–80% of patients with NSCLC [7]. Activating EGFR kinase domain mutations are found in 10–20% of lung carcinomas [8]. They are located in the exons 18–21 and cause constitutive activation of the tyrosine kinase [9]. Ninety percent of these activating EGFR mutations are point mutations on exon 21 (L858R) or deletion/deletion-insertion mutations on exon 19 (*e.g.*, delE746-A750) [8, 10]. These activating mutations of the EGFR have been shown to be associated with an increased sensitivity to TKIs Erlotinib and Gefitinib in terms of longer progression-free survival (PFS), longer median overall survival (OS) and objective response rate (ORR) [11, 12]. In addition, affected patients had better prognosis compared to those treated with conventional chemotherapy [13, 14].

Activating EGFR mutations are associated with clinico-pathological features including female gender (42% *vs.*

14% in male gender), never-smoking status (51% *vs.* 10% in smokers or ever-smokers), Asian ethnicity (30% *vs.* 8% in non-Asian ethnicity) and adenocarcinoma (ADC) histology (40% *versus* 3% in other tumor histologies) [15].

Multitudes of methodical approaches for detecting these EGFR mutations have been tested. The standard testing method is direct sequencing of formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks. Application of sequencing may be constricted due to insufficient amounts of available DNA, especially in small biopsies. Furthermore, a less resource-consuming technique would be favorable [16].

Alternative screening methods have been developed including denaturing high-performance liquid chromatography (dHPLC), high-resolution melting analysis (HRMA) or next-generation sequencing, all differing in sensitivity and specificity [17–19].

Targeted detection methods for EGFR mutations consist among others of amplification refractory mutation system (ARMS), fragment length analysis and pyrosequencing.

Another possibility is to detect predefined EGFR mutations by immunohistochemistry (IHC). Two mutation-specific antibodies directed against E746-A750del and L858R have been already employed for this purpose. Studies applying those antibodies reported high sensitivity and high specificity to identify patients who might benefit from EGFR-targeted therapies [20–22].

Amplification of EGFR gene copy number has already been shown to be predictive in patients treated with TKIs Gefitinib and Erlotinib. Patients harboring amplification or polysomy show improved response rates to Erlotinib or Gefitinib and also longer median time until progression of the disease and overall survival [23]. Furthermore, as shown in the FLEX (*First Line Treatment for Patients with EGFR-expressing Advanced NSCLC*) study, EGFR overexpression can be used as a predictor for efficacy in first-line treatment with Cetuximab plus chemotherapy in patients with advanced NSCLC [24].

The aim of this study was to figure out, whether the immunohistochemical detection of EGFR mutations E746-A750del and L858R can be used for a preselection of patients for direct DNA-sequencing. Moreover, we aimed to gain a more detailed insight into the biological connections of EGFR mutations, EGFR overexpression, amplification status and clinical data.

In light of this, we retrospectively analyzed the mutational status of EGFR concerning E746-A750del and L858R by IHC and compared the results with direct DNA-sequencing. Furthermore, wild-type EGFR protein expression was immunohistochemically evaluated and gene copy number was analyzed by fluorescence *in situ* hybridization (FISH). Finally, data of EGFR mutation specific IHC, direct sequencing, wild-type EGFR expression, EGFR gene copy number and the clinico-pathological status of the patients were analyzed for statistical association.

Patients, Materials and Methods

Selection of the patients

A total of 216 lung tumor specimens from patients with primary NSCLC were obtained from the Biomaterial Bank North after resection by the surgical department of the LungenClinic Grosshansdorf, Germany (Table 1), including 110 tumor tissues of ADCs, 86 squamous cell carcinoma (SSC) tumor tissues, 12 cases of large cell carcinomas (LCCs), and eight cases of other malignancies. This retrospective study was performed in compliance with the Ethical Committee of the University of Lübeck, Germany (Reference No. 12-220). All tumor samples were histologically classified according to the *International Association for the Study of Lung Cancer*/*American Thoracic Society*/*European Respiratory Society* International Multidisciplinary Classification of lung adenocarcinoma 2011 [25, 26]. All lung cancer tissue samples were fixed with formalin, dehydrated and paraffin-embedded according to standard procedures. Established clinical and histological factors of all tested patients were included in this study (age distribution, gender, histology, TNM classification, smoking status; Table 1).

Table 1 – ***Characteristics of 216 patients with non-small-cell lung cancer***

Category	Results, *N* (%)
Gender	
Males	136 (63)
Females	80 (37)
Age	
≥65 years old	134 (62)
<65 years old	82 (38)
Histological types	
Adenocarcinoma (ADC)	110 (51)
Acinar predominant	42 (39)
Papillary predominant	25 (24)
Micropapillary predominant	10 (9)
Solid predominant	28 (25)
Lepidic predominant	2 (1)
Invasive mucinous ADC	3 (2)
Squamous cell carcinoma	86 (40)
Large cell carcinoma	12 (6)
Others	8 (3)
**Tumor grade*	
Grade 1	5 (2)
Grade 2	82 (39)
Grade 3	122 (59)
**Pathological stage*	
I	68 (33)
II	60 (29)
III	73 (35)
IV	8 (3)
Available smoking behavior	
Current smokers	72
Former smokers	26
Never smokers	17

N: No. of cases; *Excluding seven endoscopic cases of genotyped mutated EGFR tumor tissues; EGFR: Epidermal growth factor receptor.

Tissue microarray (TMA) construction

Tissue microarrays (TMAs) of 206 tumor tissue specimens were constructed as previously described [27, 28]. Representative tumor punches (2 mm in diameter) of two core biopsies were taken after Hematoxylin and Eosin (HE) staining from two different viable parts of tumor tissue using a Beecher manual arrayer (Beecher instruments, AlphaMetrix Biotech Gmbh, Germany) to enhance representative analyzing of immunohistochemical

staining and FISH analysis. Single cores of the A549 cell line, which is known to express EGFR, was included on each array for means of positive on-slide control.

Additionally, 10 cases of already genotyped E746-A750del and L858R EGFR mutations previously confirmed by direct DNA-sequencing were also included in this study, seven cases of which were endoscopic biopsies.

The *International Association for the Study of Lung Cancer*/*American Thoracic Society*/*European Respiratory Society* (IASLC/ATS/ERS) International Multidisciplinary Classification of lung adenocarcinoma 2011 was used for the pathological classification of the cases [25, 26].

IHC staining and scoring

Two different mutation specific antibodies were used to detect the EGFR mutations: rabbit anti-EGF receptor (E746-A750del specific) mAb (D6B6), which detects endogenous levels of EGFR E746-A750del mutated protein and the rabbit anti-EGF receptor (L858R mutated specific) mAb (43B2), detecting endogenous levels of EGFR mutated L858R protein (Cell Signaling Technology, Danvers, MA, USA). Mouse monoclonal antibody 31G7 (Diagnostic BioSystems, Netherlands) was used for the detection of wild-type EGFR protein expression.

Expression analysis was assessed on 2 μm thick deparaffinized TMA sections. Negative controls omitting the primary antibody were always included.

The staining of each antibody, including antigen retrieval and antibody concentration was done following the manufacturer's protocol (Table 2).

The positive control for the mutation specific antibodies were a single sections of the FFPE block of a positive case with known EGFR mutational status by direct DNA-sequencing for exon 19 (in frame deletion E746-A750del) and exon 21 (L858R point mutation), respectively. Wild-type EGFR-staining specificity was ensured by using single sections of previously determined positive EGFR wild-type specimen. As previously shown, scoring system for wild-type EGFR expression of 10% positivity with 2+ and 3+ membranous staining intensities was used for evaluating staining with antibody clone 31G7 due to the best association with FISH analysis results.

Table 2 – ***Primary antibodies included in the study***

Clone	Manufacturer	Clonality	Host / Isotype	Reactivity	Antigen retrieval	Concentration
6B6	Cell Signaling Technology, USA	Monoclonal	Rabbit / IgG	Human	Tris/EDTA, pH 9 in steamer for 30 minutes	1:100
43B2	Cell Signaling Technology, USA	Monoclonal	Rabbit / IgG	Human	Tris/EDTA, pH 9 in steamer for 30 minutes	1:100
31G7	Diagnostic BioSystems, Netherlands	Monoclonal	Mouse / IgG	Human	Proteinase K for 2 minutes at room temperature	1:30

IgG: Immunoglobulin G; EDTA: Ethylenediaminetetraacetic acid.

The HE and IHC sections were examined by light microscopy, at 10× and 20× magnification. Scoring of EGFR expression was performed using two different scoring methods:

(*A*) *Staining intensity* was classified into four intensities: 0: negative staining, 1+ for weak staining intensity, 2+ for moderate staining intensity, and 3+ for strong staining intensity. Results were considered positive if ≥10% of tumor cells were positive with staining intensities from 1+ to 3+ (membranous/cytoplasmic) for the mutation specific antibodies and staining intensities 2+ and 3+ only (membranous) for the wild-type EGFR [21, 29].

(*B*) *H-score*: as applied in the retrospective FLEX study [24] is the product of the percentage of cancer cells positive for membranous/cytoplasmic EGFR protein expression multiplied by the overall intensity (ranging from 0 to 3+), producing scores ranging from 0 to 300, with score 100 as the positive threshold [30, 31].

FISH analysis

EGFR gene copy numbers were assessed by FISH using ZytoLight® SPEC EGFR/CEN 7 dual color probe (Zytomed Systems Gmbh, Berlin, Germany). The assay was done following the manufacturer's protocol. Before hybridization, sections were deparaffinized, dehydrated and immersed in citrate buffer followed by subsequent washing in distilled water. Afterwards, sections were air-dried and pretreated with pepsin, denatured and hybridized overnight. Next day, the slides were washed, counter-stained and mounted as previously described [28].

Analysis of FISH signals was performed with the Nikon Eclipse 80i H550L (Nikon, Japan) epifluorescence microscope, with the interference filters (AHF analysen-technik AG, Tübingen, Germany).

FISH was evaluated following the Colorado scoring system and classified into disomy (D), low trisomy (LT), high trisomy (HT), low polysomy (LP), high polysomy (HP), low and high amplifications (LA and HA, respectively). Gene to chromosome ratio 2.1 to 3 in LA and >3 in HA [32, 33].

DNA-sequencing for mutational analysis of exon 19 deletion and exon 21 L858R of the EGFR gene

Genomic DNA was extracted from tumor samples after IHC examination. Mutation positive tumor tissue samples were selected for DNA extraction and sequencing. HE-stained sections of FFPE blocks were first reviewed and sections composed of at least 50% of tumor cells were manually microdissected.

DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Germany). Scraped tumor tissues were transferred to a 1.5 mL microcentrifuge tube containing 180 μL ATL buffer and 20 μL proteinase K vortexed and incubated at 56°C, overnight. Afterwards, 200 μL of AL buffer was added to the sample, vortexed for 15 seconds and incubated at 70°C for 10 minutes. Two hundred μL of ethanol (100%) were added and vortexed for 15 seconds, followed by centrifugation at 6000×g for one minute. Samples were washed twice, once with AW1 buffer and centrifuged for one minute at 6000×g and once with AW2 buffer,

centrifuged for three minutes, at 20 000×g. Finally, elution was performed by adding 75 µL distilled water twice and centrifuged at 6000×g for one minute.

DNA-sequencing was carried out for the detection of EGFR mutations on exons 18–21 as described by Do *et al.* [34]. For these, 400 ng of genomic DNA was used in a final volume of 50 µL. Conditions were selected as follows: initial denaturation at 95°C for 15 minutes; 40 cycles of 94°C for one minute, 60°C for one minute, 72°C for 90 seconds, final elongation for 15 minutes, at 72°C [34].

Polymerase chain reaction (PCR) products were purified with the Rapid PCR Cleanup Enzyme Set (New England Biolabs, Ipswich, USA), followed by sequencing with BigDye® Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol and precipitated before running on the 3100 Genetic Analyzer (Applied Biosystems). Sequencing data were analyzed with the Sequencing Analysis software (version 5.3, Applied Biosystems).

Statistical analysis

Statistical analysis was performed using SPSS® (Statistical Package for the Social Sciences) Statistics 20 (SPSS Inc., Chicago, IL, USA). Association between different variables were performed using Pearson's χ^2 (*chi*)-square test in 2×2 table, if more than 20% of the cells have expected count less than 5 we used Fisher's exact test (FEP – Fisher's exact probability) and Monte Carlo significance test (MCP – Monte Carlo permutation) if more than 2×2 table. Cohen's kappa test was used for testing agreement between tests. All tests were two-sided and results were considered statistically significant for *p*-values ≤0.05.

Results

Patients' characteristics

A total of 216 tumor tissue samples of NSCLC patients were included in this study. Clinical parameters of the patients are shown in Table 1. The study included 136 (63%) males and 80 (37%) females, 82 (38%) patients <65 years old and 134 (62%) ≥65 years old. One hundred ten (51%) tumors were classified as ADC, 86 (40%) were grouped as SCC, 12 (6%) as LCC and eight (3%) as others. ADC subtypes according to IASLC classification 2011 were described as follows: 42 were classified as acinar, 25 as papillary, 28 were solid, 10 as micropapillary, two lepidic and three of invasive mucinous subtype.

Mutation specific IHC of EGFR exon 19 deletion E746-A750 and exon 21 L858R point mutation

IHC-based detection of EGFR mutations E746-A750del on exon 19 and point mutation L858R on exon 21 was performed in 206 cases of the TMAs. Forty-one (19.9%) cases were scored positive for EGFR mutation using scoring system A (results were considered positive if ≥10% of tumor cells had staining intensities from 1+ to 3+). Exon 19 del E746-A750 was detected in 12 (29%) of these tumor tissues with antibody clone D6B6. Twenty-five (61%) tumor samples were scored as positive for the point mutation L858R on exon 21 with antibody clone 43B2. Four (10%) of the tumor tissue samples were positive for both mutations.

Using modified *H*-score, 29 (14%) cases were positive for mutated EGFR, 10/29 (34%) cases of the tumor samples were positive with 6B6 antibody. Seventeen/29 (59%) cases were positive with 43B2 antibody and two/29 (7%) cases were positive using both antibodies. A strong agreement between both scoring systems (Table 3) was observed [κ=0.081 (95% CI – confidence interval, 0.717 to 0.902), p<0.001).

EGFR wild-type protein expression evaluated by IHC

The 206 tumor tissue samples were also immunohistochemically analyzed for the expression of wild-type EGFR. One hundred seventy-six (85%) tissue samples were scored EGFR positive, whereas 30 (15%) tumor tissues were classified as EGFR negative.

Table 3 – ***Association between IHC analysis of mutated EGFR (score A and B), wild-type EGFR and FISH analysis of gene copy number in 216 cases***

		31G7		FISH		EGFR mutation score B	
		Positive N (%)	*Negative N (%)*	*Positive N (%)*	*Negative N (%)*	*Positive N (%)*	*Negative N (%)*
EGFR mutation score A	Positive	12 (5.6)	38 (17.6)	4 (1.9)	46 (21.3)	0 (0)	50 (23.1)
	Negative	166 (76.9)	0 (0)	160 (74.9)	6 (2.8)	30 (13.9)	136 (63)
	P-value	0.002*		<0.001*		<0.001**	
EGFR mutation score B	Positive	38 (17.6)	0 (0)	37 (17.1)	1 (0.5)		
	Negative	148 (68.5)	30 (13.9)	15 (6.9)	163 (75.5)		
	P-value	0.003*		0.038*			

IHC: Immunohistochemistry; EGFR: Epidermal growth factor receptor; FISH: Fluorescence *in situ* hybridization; *N*: No. of cases; *Fisher's exact test, statistically significant p<0.05; **Cohen's kappa agreement test (κ=0.081, p<0.001).

IHC of EGFR genotyped mutated NSCLC tumor samples

In addition, 10 already-genotyped samples were IHC examined to enrich the population of EGFR-mutated tumor tissues. Both mutations – E746-A750del on exon 19 and point mutation L858R on exon 21 – have been analyzed and confirmed by sequencing.

Immunohistochemical analysis using a 10% cut-off yielded in nine (90%) positive scored tumor tissues and one (10%) negative case.

Six/seven (86%) of the exon 19 E746-A750del positive cases, by sequencing, were IHC positive with antibody 6B6. Three/three cases (100%), which had L858R point mutation on exon 21, by sequencing, were IHC positive with antibody 43B2. Using modified

H-score, nine (90%) cases were positive using both antibodies. All of the 10 tumor samples show wild-type EGFR protein overexpression.

Association between mutated EGFR evaluated by IHC and clinicopathological data

The relation between the IHC-positive mutated EGFR, for both mutations on exon 19 (deletion E746-A750) and the point mutation L858R on exon 21, using score A, 10% positivity as cut-off, was investigated in the whole cohort (including 206 TMAs and additional 10 genotyped mutated EGFR tumor tissues) (Table 4).

Fifty/216 (23.1%) patients were found to have EGFR mutations (deletion E746-A750 or L858R). Thirty-one/50 (62%) patients were classified as ADC, 17/50 (34%) were SCC patients and two/50 (4%) were classified as LCC. There was no statistically significant difference between the mutated EGFR and the histological subtypes (p=0.425). There was also no statistically significant difference found between mutated EGFR and the ADC predominant subtypes according to IASLC Classification of adenocarcinoma in 2011 [26] (p=0.281): 15/31 (49%) of patients with mutated EGFR were acinar predominant subtype, nine/31 (29%) cases were papillary predominant subtype, five/31 (16.1%) were solid predominant, one/31 cases (3%) was of the lepidic subtype and one/31 cases (3%) was of invasive mucinous subtype.

Table 4 – ***Association between clinicopathological data, EGFR wild-type expression, mutated EGFR score A (10%), mutated EGFR score B (H-score) and gene copy number by FISH***

		Total *N* (%)	EGFR wild-type	Mutated EGFR score A	Mutated EGFR score B	FISH
Age	≥65 years old	134 (62)	118 (63.4)	29 (58)	18 (62.1)	2 (61.5)
	<65 years old	82 (38)	68 (36.6)	21 (42)	11 (37.9)	20 (38.5)
	P-value		0.315	0.511	1.000	1.000
Gender	Males	136 (63)	120 (64.5)	27 (54)	16 (55.2)	28 (53.8)
	Females	80 (37)	66 (35.5)	23 (64)	13 (44.8)	24 (46.2)
	P-value		0.308	0.181	0.301	0.139
Histology	ADC	110 (51)	91 (48.9)	31 (62)	15 (51.7)	33 (63.5)
	SCC	86 (40)	83 (44.8)	17 (34)	12 (41.4)	17 (32.7)
	LCC	12 (6)	9 (4.8)	2 (4)	2 (6.9)	2 (3.8)
	Other	8 (3)	3 (1.6)	0 (0)	0 (0)	0 (0)
	P-value		<0.001*	0.425	0.985	0.430
ADC subtypes	Lepidic	2 (1)	2 (1)	1 (3)	1 (3.4)	2 (3.8)
	Acinar	42 (39)	36 (19.4)	15 (41)	8 (27.6)	15 (28.8)
	Papillary	25 (24)	24 (12.9)	9 (29)	4 (13.8)	10 (19.2)
	Solid	28 (25)	22 (11.8)	5 (16.1)	2 (6.9)	5 (9.6)
	Micropapillary	10 (9)	6 (3.2)	0 (0)	0 (0)	1 (1.9)
	Invasive mucinous	3 (2)	1 (0.5)	1 (3)	0 (0)	0 (0)
	P-value		0.014*	0.281	0.339	0.011*
**Grade*	Well differentiated	5 (2)	2 (1.1)	0 (0)	0 (0)	1 (1.9)
	Moderately differentiated	82 (39)	72 (38.7)	16 (32)	12 (41.4)	17 (32.7)
	Poorly differentiated	122 (59)	105 (56.5)	27 (54)	17 (58.6)	27 (51.9)
	P-value		0.053*	<0.001*	1.000	<0.001*
**Tumor size*	T1	39 (18.7)	31 (16.7)	4 (8)	3 (10.3)	5 (9.6)
	T2	118 (56.5)	102 (54.8)	27 (54)	16 (55.2)	27 (51.9)
	T3	32 (15.3)	29 (15.6)	7 (14)	5 (17.2)	7 (13.5)
	T4	20 (9.5)	17 (9.1)	5 (10)	5 (17.2)	6 (11.5)
	P-value		0.625	<0.001*	0.342	<0.001*
**Lymph node metastasis*	N0	100 (47.7)	83 (44.6)	13 (26)	8 (27.6)	15 (28.8)
	N1	50 (23.8)	44 (23.7)	14 (28)	9 (31)	14 (26.9)
	N2	44 (20.9)	38 (20.4)	13 (26)	8 (27.6)	13 (25)
	N3	16 (7.6)	14 (7.5)	3 (6)	4 (13.8)	3 (5.8)
	P-value		0.785	<0.001*	0.064	<0.001*
**Stage*	I	68 (33)	59 (31.7)	8 (16)	6 (20.7)	8 (15.4)
	II	60 (29)	47 (25.3)	16 (32)	8 (27.6)	16 (30.8)
	III	73 (35)	66 (35.5)	17 (34)	14 (48.3)	18 (34.6)
	IV	8 (3)	7 (3.8)	2 (4)	1 (3.4)	3 (5.8)
	P-value		0.296	<0.001*	0.348	<0.001*
Smoking behavior	Current smokers	72	64 (34.4)	16 (32)	12 (41.4)	15 (28.8)
	Former smokers	26	23 (12.4)	5 (10)	1 (3.4)	5 (9.6)
	Never smokers	17	15 (8.1)	8(16)	1 (3.4)	9 (17.3)
	P-value		0.286	0.200	0.567	0.080

EGFR: Epidermal growth factor receptor; FISH: Fluorescence *in situ* hybridization; *N*: No. of cases; ADC: Adenocarcinoma; SCC: Squamous cell carcinoma; LCC: Large cell carcinoma; *Excluding seven endoscopic cases of genotyped mutated EGFR tumor tissues.

A statistically significant difference was found between mutated EGFR cases and the high tumor grade and pathological stage (p<0.001 each). Additional, statistically significant difference was also found between the positive mutated EGFR and the size of the tumor and the lymph node stage (p=0.001 each).

There was no statistical significance found between EGFR mutation and the smoking behavior (p=0.200), 47.1% of the never-smokers harbored positive EGFR mutation compared to 22.2% of the current smokers carried mutated EGFR.

No statistical difference was found between IHC-positive mutated EGFR and the gender (p=0.181) or the patient age (p=0.511).

Relationship between the mutational status of EGFR and wild-type EGFR expression

All of the IHC determined EGFR-mutated positive tumor samples (including the 206 TMA specimens and the additional 10 genotyped mutated EGFR tumor tissues) were also positive for the overexpression of wild-type EGFR using a 10% cut-off point. The statistically significant difference accounts (p=0.002) (Table 3).

Figure 1 illustrates one case with point mutation L858R on exon 21, confirmed by sequencing, showing IHC positivity for mutated EGFR clone 43B2, and wild-type EGFR clone 31G7.

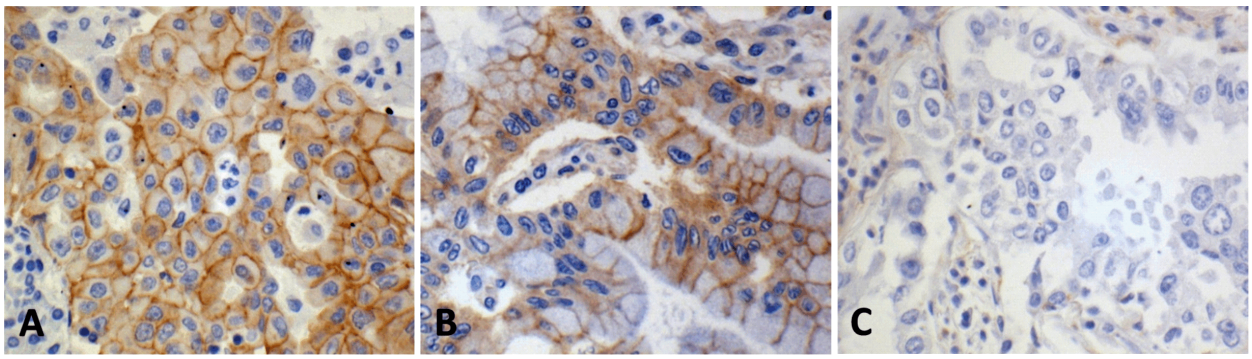

Figure 1 – ***Immunohistochemical staining of ADC showing different EGFR expressions (original magnification, ×400): (A) EGFR wild-type overexpression, score 2+ (clone 31G7, Diagnostic BioSystems, Netherlands); (B) EGFR mutated, positive, score 2+, point mutation L858R (clone 43B2, Cell Signaling Technology, USA); (C) EGFR negative for exon 19 deletion (E746-A750) (clone 6B6, Cell Signaling Technology, USA). ADC: Adenocarcinoma; EGFR: Epidermal growth factor receptor.***

EGFR gene copy number evaluated by FISH analysis, association with IHC

Of 206 TMAs tumor samples, 164 (80%) were negative for EGFR gene amplification, while 42 (20%) showed positive FISH results. Of these positive cases, 28/42 (67%) showed high polysomy and 14/42 (33%) showed amplification.

The FISH analysis of the 10 genotyped mutated EGFR tumor tissues was positive in 100%.

A significant association between gene copy number evaluated by FISH and mutated EGFR-positive cases in the whole cohort (including 206 TMAs and additional 10 genotyped mutated EGFR tumor tissues) was determined using both scoring methods [10% cut-off point and modified *H*-score (p-value <0.001 and 0.038, respectively)] (Table 3).

Using 10% cut-off point, 46/50 (90%) cases of EGFR mutant positive tumor samples evaluated by IHC showed FISH positivity, while four/50 (8%) cases of these EGFR-mutated positive tumor tissues were FISH negative.

Using the modified *H*-score, 37/38 (97%) cases of IHC EGFR-mutated samples were FISH positive; one/38 (3%) cases of EGFR-mutated positive cases was FISH negative.

The relation between EGFR-mutated positive cases analyzed by IHC and FISH positivity is represented in Figure 2.

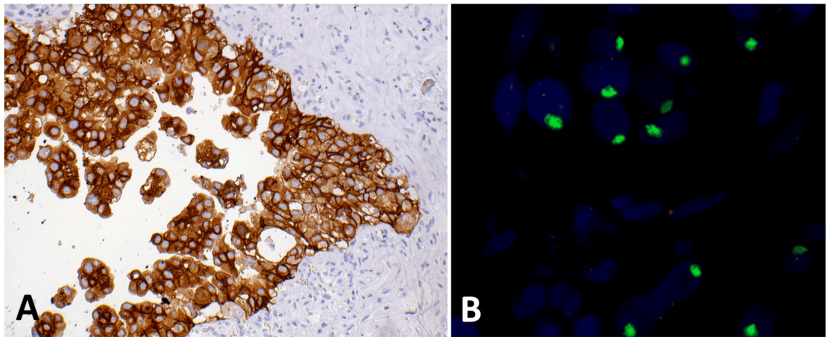

Figure 2 – ***(A) IHC of ADC with EGFR mutation for exon 19 deletion (E746-A750) using mutated specific antibody clone 6B6, scored 3+ (original magnification, ×400); (B) FISH analysis of the same case showing high amplification (HA) (original magnification, ×400). IHC: Immunohistochemistry; ADC: Adenocarcinoma; EGFR: Epidermal growth factor receptor.***

EGFR mutation detection by direct DNA-sequencing

Of the 206 TMAs samples, DNA-sequencing was performed on the 41 cases, which were immunohistochemically determined as EGFR positive. Genotyping of exons 18–21 of these IHC EGFR-mutated positive cases led to the identification of two/41 (5%) cases with A746-A750del on exon 19 and four/41(10%) cases with point mutation L858R on exon 21 by sequencing.

In addition, two/41 (5%) tumor samples showed exon 18 mutation G719C – one (2.5%) of them showed also S768I mutation on exon 20.

None of the tumor tissues classified as EGFR-mutated by IHC had mutations in both exons 19 and 21 together when sequenced.

The sequencing of the four different types of EGFR mutations detected on exons 18–21 is shown in Figure 3.

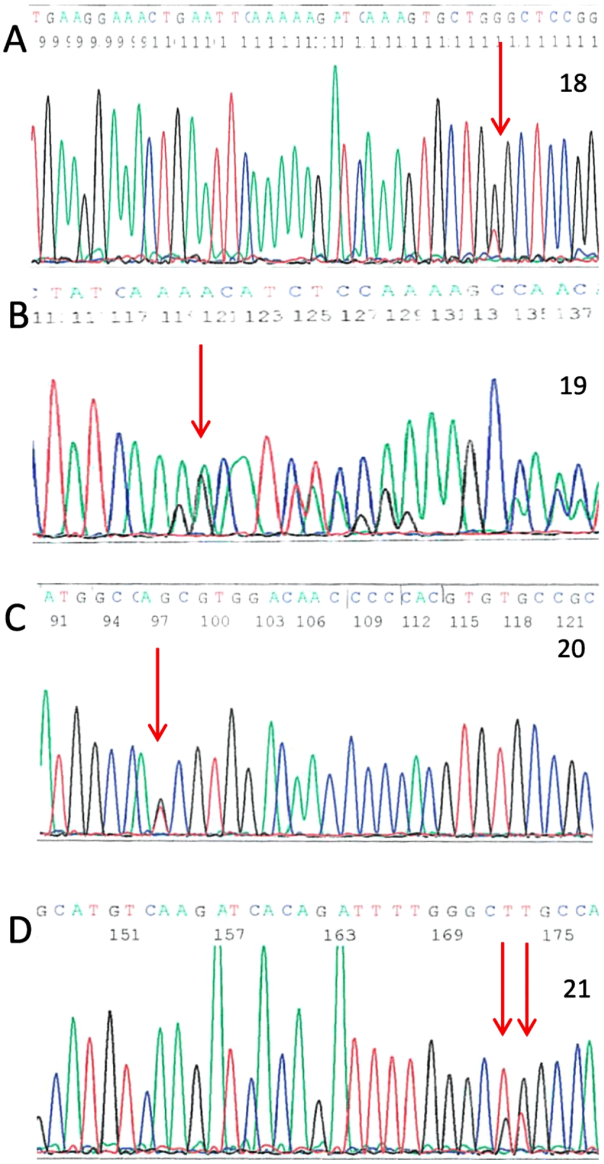

Figure 3 – ***DNA-sequencing: (A) Exon 18 mutation G719C; (B) Exon 19 del E746-A750; (C) Exon 20 S768I mutation; (D) Exon 21 L858R point mutation. del: Deletion.***

Discussion

Immunohistochemical detection of mutated EGFR on protein level constitutes a fast and routinely applicable procedure to identify patients who can profit of TKI therapy compared with the state of the art method of molecular testing by sequencing.

In our current study, we immunohistochemically analyzed the frequency of exon 19 deletion E746-A750 and the point mutation L858R on exon 21. These EGFR mutations were identified in 41/206 (20%) patients with NSCLC using scoring method A and in 29/206 (14%) patients using the modified *H*-score (scoring method B) demonstrating the dependence of interpreting the IHC-staining results. The determined percentage of these EGFR mutations is in accordance with other studies [21, 22]: Yu *et al.* [21] already reported a frequency of 15.3% positive for both EGFR mutations in NSCLC patients, whereas Brevet *et al.* [22] observed EGFR mutations in 28% of lung ADC samples.

Sequencing of the 41 IHC EGFR-mutation positive-tumor tissues confirmed exon 19 E746-A750 mutation in 5% (two/41) of the mutated samples and L858R in 10% (four/41) of the NSCLC lung tissues. Therefore, IHC-based preselection of EGFR mutational status is a cost-efficient possibility to bypass sequencing of a large patient collective for the treatment with EGFR-specific inhibitors. Forecasting the frequency up to the 206 tumor tissues, 3.8% of the patients had an activating mutation within the EGFR. This determined frequency is nearly in agreement with the studies of Boch *et al.* [35] the frequency of mutated EGFR of different histological NSCLC tumors was 4.9%, ascertained by pyrosequencing [35]. The higher frequency in our study is due to the fact of preselecting the EGFR-mutated tumor tissues before sequencing; strengthen the meaning of IHC pre-analysis.

In addition, direct DNA-sequencing is a reliable tool to detect all mutations but shows low sensitivity [36]. Other molecular tests show better results regarding the sensitivity and specificity compared to direct DNA-sequencing in the detection of mutated EGFR as the ARMS, the fragment length analysis, pyrosequencing and cationic conjugated polymer (CCP)-based fluorescence resonance energy transfer (FRET) [37]. For all these approaches, upstream preselection by IHC could be performed to enable cost effective and reasonable detection of EGFR mutations in patients with NSCLC. As mentioned by Ellison *et al.* [37], the method for the determination of EGFR mutational status could be predicated also on the properties of the tumor tissues including facts like for example the tumor cell content [37].

Many studies evaluated the role of EGFR protein overexpression and an increased gene copy number by FISH analysis to select patients who might benefit of EGFR-specific therapies with EGFR-specific antibodies and TKIs [30, 38–40]. Results of the phase 3 FLEX study have shown that high expression of EGFR in the tumors of patients was associated with survival benefit for EGFR-targeted therapy added to first-line chemotherapy [24, 30]. In our study, there is statistically significant association between wild-type EGFR overexpression and mutated EGFR expression evaluated by IHC in the 216 analyzed tumor tissue samples (p=0.002). These results are in accordance with the study of Liang *et al.* [41].

Likewise, EGFR gene copy number has already been shown to be a predictive biomarker for the effect of Gefitinib in patients with advanced NSCLC [38] and also to select patients for the treatment with Cetuximab [40]. As shown in our previous study, there is an association between EGFR gene copy numbers and the overexpression of wild-type EGFR evaluated by IHC [28].

In the current study, association between EGFR gene

copy number evaluated by FISH analysis and the mutated EGFR status IHC evaluated is shown (p<0.001 for scoring systems using 10% cut-off point and p=0.038 scoring with the modified *H*-score).

Different studies have already demonstrated the presence of significant statistical association between gene amplification status, expression level of EGFR and the presence of EGFR mutations [41, 42].

The frequency of 47.1% mutated EGFR among never smokers in our study compared to 22.2% positivity for mutated EGFR of the current smokers (without significant statistical association) is comparable to previously published data [35, 43, 44].

In the current study, 62% of the EGFR-mutated cases were ADCs, which is in accordance as already shown in the literature [10, 35, 44]. The distribution of the IHC-positive EGFR-mutated cases within the ADC histological predominant subtypes according to IASLC/ATS/ERS Classification in 2011 reveals that mutated EGFR is more frequent in cases of acinar predominant subtype (41%) followed by the papillary predominant (29%), solid predominant (16%), lepidic (3%) and invasive mucinous subtype (3%).

Regarding positive EGFR mutation by sequencing, the predominant subtypes were acinar and lepidic. This result is in concordance with the result of Russell *et al.*, which demonstrated the presence of mutated EGFR by Sequenom in the acinar predominant subtype (44%) [45] and different from Song *et al.* study, in which the EGFR mutation was more common in the micropapillary and lepidic subtype [46]. The relation between EGFR mutation and the histological predominant subtypes according to IASLC/ATS/ERS Classification in 2011 is warranted for further assessment due to their importance and relevance in clinical practice [47, 48].

Our study underlines the meaningful combination of several EGFR testing methods to allow the best possible selection for NSCLC patients who should have the chance of profiting of EGFR targeted therapies. To predict efficacy of TKI treatment, it would also make sense to look for the activated EGFR immunohistochemically with phosphorylation-specific antibodies.

Conclusions

IHC staining using EGFR-mutation specific antibodies was demonstrated as a useful sensitive prescreening method upstream DNA-sequencing. Altogether, wild-type EGFR overexpression, mutational status of EGFR and an increased gene copy number of EGFR can be consulted to select patients for EGFR specific therapies. This study accentuated the complexity of parameters for selecting patients for EGFR-targeting therapies and emphasize the importance of analyzing not just one parameter like for example the expression level or gene amplification status but also of the interplay of expression level, gene amplification status, mutational status and at least the potential analysis of the activated status of EGFR investigated with phosphorylation-specific antibodies.

Conflict of interests

The authors declare that they have no conflict of interests.

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