Cancer Chemother Pharmacol (2012) 69:289–299
DOI 10.1007/s00280-011-1791-9

MINI REVIEW

Intratumor heterogeneity and chemotherapy-induced changes in EGFR status in non-small cell lung cancer

Jan Nyrop Jakobsen · Jens Benn Sørensen

Received: 13 September 2011 / Accepted: 16 November 2011 / Published online: 1 December 2011

Abstract
Introduction Biomarker expression is increasingly being used to customize treatment in non-small cell lung cancer (NSCLC). The choice of systemic treatment usually depends on biomarker expression in the initial diagnostic biopsy taken before initiation of first-line treatment. Chemotherapy induces DNA damages in the tumor cells, and thus, biomarker expression in the tumor after systemic treatment might not be identical to biomarker expression in the diagnostic biopsy. NSCLC is highly heterogeneous and biomarker expression may vary in different areas within the same tumor. This review explores the tumor heterogeneity and chemotherapy-induced changes in EGFR biomarker status in NSCLC.
Methods A literature search was performed in August 2011 using pubmed.
Results Fifteen trials explored EGFR status in primary tumor and subsequent resected primary tumor, lymph node metastases, or organ metastases. Four papers compared EGFR status in primary tumor or metastases before and after systemic treatment. All trials included relatively few patients and used different chemotherapy regimes, biopsy locations, or time intervals between biopsies.
Conclusions Tumor heterogeneity and probably also previous systemic treatment may be an obstacle for correct interpretation of EGFR status in NSCLC. Heterogeneity regarding EGFR mutations is probably rare and previously reported intra and intertumor heterogeneity may be due to methodological issues. In the current and future clinical scenario with many different options for systemic treatment both as 2nd line and beyond, it is increasingly important to further elucidate the role extent of chemotherapy-induced changes in biomarker expression for proper use of biomarkers in order to customize treatment and thus improve prognosis.

Keywords Non-small cell lung cancer · NSCLC · Biomarkers · Heterogeneity · Chemotherapy · EGFR

Abbreviations

AAH	Adenomatous hyperplasia
AD	Adenocarcinoma
AKT	A member of the non-specific serine/threonine-protein kinase family
ASC	Adenosquamous carcinoma
BAC	Broncoalveolar carcinoma
EGFR	Epidermal growth factor receptor
ERCC1	Excision repair cross-complementing
LCC	Large cell carcinoma
LEC	Lymphoepithelioma-like carcinoma
MAPK	Mitogen-activated protein kinase
NSCLC	Non-small cell lung cancer
SAC	Sarcomatoid carcinoma
SQ	Squamous carcinoma
TKI	Tyrosine kinase inhibitor

Introduction

Lung cancer accounts for 20–25% of all cancer deaths and is thus the most common cause of cancer death [1]. Non-small cell lung cancer (NSCLC) is the most frequent type of lung cancer and accounts for 75% of all lung cancer cases. The majority of patients are diagnosed with advanced disease where curable treatments are not possible.

J. N. Jakobsen (✉) · J. B. Sørensen
Department of Oncology, Finsencentre, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark
e-mail: jan.nyrop.jakobsen@rh.regionh.dk

Targeted agents have been extensively explored in NSCLC in order to improve treatment, either as single agents or in combination with chemotherapy both in first-line treatment and beyond. The choice of systemic treatment is decided by histological subtype and to some extend by the expression of molecular predictive markers in the tumor cells, e.g., such as epidermal growth factor receptor (EGFR) mutations [2]. Profiling of various biomarkers on cancer cells may further improve the prediction of clinical outcome and also toxicity in some cases, when treating with antineoplastic drugs [3].

The choice of subsequent systemic treatments, if first-line systemic treatment fails, usually remains dependent on the result from the initial diagnostic biopsy taken before the initiation of the first-line treatment. However, it is not well understood to which extent chemotherapy may influence the expression of different biomarkers in the tumor. One problem is that the initial biopsy might not necessarily be representative for the entire tumor and nor for the residual tumor cells after first-line treatment and accordingly not suitable as basis for the choice of subsequent systemic treatments. This may be due to alteration of surface protein expression on tumor cells or due to extermination of chemotherapy sensitive clones and thereby a selection of more resistant clones with alternative expression of biomarkers. Moreover, as malignant tumors acquire changes in metastatic phenotype through clonal evolution occurring during a multistep tumor progression [4], differences in biomarkers may vary between primary tumor and metastases or even within the primary tumor itself.

This review concerns intratumor heterogeneity of epidermal growth factor receptor (EGFR) status and possible chemotherapy-induced changes in EGFR status.

Materials and methods

A literature search was performed in August 2011 using pubmed. No time restraints were applied. Keywords used were combinations of "NSCLC, genetic changes, EGFR, epidermal growth factor, biomarkers, neoadjuvant treatment, comparison of biopsies, multiple biopsies, and chemotherapy induced changes in biomarkers." References in selected articles were scrutinized, and relevant articles were included in the review. Articles that explore heterogeneity in biomarkers within tumor or between primary tumor and metastases were included. Also, articles exploring treatment-induced changes in biomarkers were included if they contained data on patients included in the trial, together with systemic treatments used and time sequence between biopsies and treatment. Tables displaying included trials are presented. The terms positive and negative reflect the terms and definitions used by the authors of the studies included.

Tumor heterogeneity and EGFR biomarker

Lung cancers arise from precursor lesions [5], which can be situated extensively and multifocal in the respiratory mucosa. The development of lung cancer is not a static entity and involves a multistep cascade of molecular and genetic changes. Every succession of clonal expansion is driven by the acquisition of additional mutations, which contribute to the initiation, development, and maintenance of lung cancer [6]. This cascade leads to billions of malignant cells that have accumulated different genetic mutations [7], and more than 20 different mutations have been observed to be present in lung cancer tumor cells [8]. Various tumor clones can accumulate different mutations and can thus present different biomarker status and tumor heterogeneity. Heterogeneous tumors can lead to difficulties interpreting biomarker status of small biopsies acquired from a small part of the tumor, which may not be representative to the entire tumor.

Currently, the most clinically relevant biomarker in NSCLC is EGFR, which is mutated in 30–40% of Asians and in 10–15% of Caucasians. The presence of EGFR mutations is also associated with female gender, non-smokers, and adenocarcinoma histology [9]. More than 80% of EGFR mutations are in-frame deletions in exon 19 or a single missense mutation in exon 21 (L858R) [7, 10].

Activating mutations in EGFR predicts sensitivity to treatment with EGFR tyrosine kinase inhibitors (EGFR-TKI) [11–16], with more than 70% objective response rate and improved progression-free survival compared to platinum-containing chemotherapy [16–18]). Due to the superior efficacy, the presence of EGFR mutations in the initial biopsy decides whether treatment with EGFR-TKI is initiated as first-line treatment [19].

It seems that EGFR mutations are found in the very early stage of adenocarcinoma development. Yoo et al. examined EGFR mutations in 20 atypical adenomatous hyperplasia (AAH), 43 bronchioloalveolar carcinoma (BAC) (which is also called adenocarcinoma in situ), and 47 small adenocarcinomas to elucidate the role of EGFR mutations through various stages in the early tumorigenesis. Thirty-five percentage of AAH, 35% of BAC, and 49% of adenocarcinomas were found harboring EGFR mutations, respectively, suggesting that EGFR mutations occur early in the development of adenocarcinoma, and those harboring mutations further progress to invasive and metastatic carcinomas [20]. Since EGFR mutations can occur early in the malignant transformation, the EGFR mutation also ought to occur in the subsequent invasive and metastatic tumor cells unless tumor cells can lose the EGFR mutations over time.

Another important aspect of EGFR is the number of gene copies. Increased EGFR gene copy number, e.g., amplification in NSCLC measured by FISH, is observed in

10–44% of NSCLC [21, 22] and most frequently results from chromosome 7 polysomy, whereas true EGFR amplification occurs less often [23–25]. Increased EGFR gene copy number also seems to have some correlation with response, PFS, and OS after treatment with EGFR-TKI [14, 15, 26], but the predictive value of EGFR amplification is significantly inferior to the predictive value of EGFR mutations [27]. Whereas EGFR may be mutated from the precancerous stage, EGFR amplification is detected mostly in the invasive stage and after, and EGFR amplification is correlated with high histological grade and/or invasive growth [28].

EGFR protein expression (or described as overexpression) evaluated by immunohistochemistry (IHC) is found in 40–90% of NSCLC patients [29], and there seem to be no correlation between EGFR expression and efficacy to EGFR-TKI treatment [27]. But still EGFR expression may be clinically relevant since a recent trial has shown that EGFR expression as a predictive marker for improved clinical outcome when combining standard first-line platinum-containing chemotherapy to cetuximab, an antibody-targeting EGFR, in patients with advanced NSCLC [30].

However, in clinical trials, some patients respond to the treatment with EGFR tyrosine kinase inhibitors irrespective of the expression and mutational status of EGFR [31].

Several trials have examined whether EGFR mutation status, EGFR gene copy number, and EGFR expression are correlated. It seems that there is somewhat correlation between EGFR gene copy number and EGFR mutations [22, 32, 33] When EGFR amplification occurs in EGFR-mutated cells, it interestingly seems that mainly the EGFR-mutated allele gets amplified [34]. Correlation between EGFR protein expression and EGFR gene copy number/EGFR mutation is unclear and varies between different studies [24, 25, 33, 35, 36].

Results

EGFR mutations

Selection of patients to EGFR-TKI treatment is most often based on the EGFR mutation status in the diagnostic biopsy, which has to be representative for the EGFR status throughout the entire tumor. Three trials have evaluated the occurrence intratumor heterogeneity regarding EGFR mutation. Nomonaga et al. [37] compared EGFR mutation status in different areas within resected mixed type adenocarcinomas from 38 patients. Heterogeneity between samples were observed in 9 patients, and heterogeneity was significantly correlated with smoking ($P = 0.043$). How such heterogeneity may impede efficacy of EGFR-TKI treatment, which potentially only affects EGFR-mutated cells, was evaluated by Taniguchi et al. [38]. Multiple areas within resected tumors were tested for EGFR mutations, and among the 21 patients, 6 patients had both EGFR-mutated and non-mutated NSCLC cells within the same primary tumor, and 15 patients had exclusively EGFR-mutated cells in the samples. The time to disease progression and overall survival after gefitinib treatment were significantly shorter in those patients with EGFR heterogeneity compared to patients harboring mutation-positive tumor cells only ($P = 0.009$ and $P = 0.003$, respectively) [38]. This suggests that intratumor variations in EGFR mutational status play a role for the efficacy of EGFR-TKI treatment (Table 1). These results are in contrast to a trial by Yatabe et al. [39], who examined the heterogeneity through transsectional analysis of EGFR mutation-positive primary tumors with adenocarcinoma histology. Three areas within the tumors were examined, and no heterogeneity between the sampled areas was discovered. To avoid missing minor heterogeneities in the tumor, 5 tumors were dissected into 100 parts and the subsequent EGFR mutation analysis did not reveal any intratumor heterogeneity.

EGFR mutation-positive patients who are to receive EGFR-TKI as first-line treatment may have organ or lymph node metastases, and since biomarker heterogeneity may be more likely to occur increasingly in metastases, it is equally important to elucidate potential discordance regarding EGFR mutations between primary tumor and metastasis.

Two trials by Park et al. [40] and Chang et al. [41] included a total of 157 patients, who received curative intended resection of primary tumor and synchronous lymph node metastases. Subsequent EGFR mutation analysis by direct sequencing showed discordance in 29 and 12% of cases, respectively. Most often (22 and 11%) the discordance were due to a loss of EGFR mutation in the lymph node metastasis [40, 41]. Park et al. [41] retested the samples with a more sensitive heteroduplex analysis method, and the discordance between primary tumor and lymph node metastasis increased from 12 to 16.8% [42]. Mutations in primary tumor from 8 patients and in metastasis from 17 patients were undetectable by direct sequencing but were detected by heteroduplex analysis. One sample from primary tumor showed L858R mutation by direct sequencing but deletions in exon 19 by heteroduplex analysis [41]. These results indicate that direct sequencing may lack sensibility, which results in some false negative results.

Yatabe et al. [39] also examined the EGFR mutation status in lymph node metastases in 77 patients with known EGFR mutation-positive primary tumor. No discordant cases were observed. To examine this further, EGFR mutation-negative primary tumors from 50 patients with high probability of EFGR mutations (female, non-smoker, and lack of KRAS) were reanalyzed for EGFR mutations. This

Table 1 EGFR mutational status

Author	Histological subtype	No. of pts	Intratumor discordance (%)
Comparison of samples within resected primary tumor			
Tomonaga et al. [37]	Mixed type AD: 100%	38	24
Yatabe et al. [39]	AD: 100%	55	0

Author	Histological subtype (%)	No. of pts	Pos biomarker/ mutation in both PT and metastasis (%)	Neg biomarker in both PT and metastasis (%)	Pos in PT/neg in metastasis (%)	Neg in PT/pos in metastasis (%)	Discordance in biomarkers/ mutations between PT and metastasis (%)	Metastatic site (%)
Comparison of EGFR mutational status in primary tumor and metastasis in NSCLC								
Chang et al. [40]	AD: 61 SQ: 30 ASC: 7 SAC: 2	56	20	52	22	7	29	Lymph node: 100
Park et al. [41]	AD: 60 SQ: 38 LCC: 1 Other: 1	101	9 20[b]	79 64	11 10	1 7	12 17	Lymph node: 100
Kalikaki et al. [44]	AD: 72	25	4	68	16	8	28	Lung: 36 Thoracic wall: 20 Adrenal gland: 16 Brain: 12 Bone: 8 Liver: 4 Skin: 4
Gow et al. [55]	AD: 63 SQ: 31 LCC: 2 LEC: 4	67	13	74	13	25	38 27[a]	Brain: 38 Bone: 30 Other: 32
Matsumoto et al. [61]	AD: 100%	8	75	25	0	0	0	Brain
Cortot et al. [62]	AD: 76 SQ: 14 LCC: 5	21	0	0	0	0	0	Brain: 61.9 Bone: 9.5 Soft tissues:9.5 Lung: 19
Yatabe et al. [39]	AD	77	100	0	0	0	0	Lymph node: 100

SQ squamous carcinoma, *AD* adenocarcinoma, *ASC* adenosquamous carcinoma, *BAC* broncoalveolar carcinoma, *LCC* large cell carcinoma, *NOS* not otherwise specified, *SAC* sarcomatoid carcinoma, *LEC* lymphoepithelioma-like carcinoma, *PT* primary tumor

[a] By Hetoroduplex analysis

[b] By SARMS assay

subgroup of patients would be likely to be primarily EGFR mutation negative due to heterogeneity. But, no discordant cases were observed.

Two trials by Gow et al. [43], Kakilaki et al. [44] included 92 paired samples from primary tumor and corresponding metachronous organ metastases. Average time between primary resection of primary tumor and corresponding metastases was 9.3 and 30 months, respectively. Ten percent of patients included by Gow et al. [43] had previously received treatment with chemotherapy and none had received treatment with EGFR-TKI. EGFR mutation status was discordant in 38% of paired samples. Discordant samples were retested by the scorpion amplified refractory mutation system assay (SARMS), which is perceived as more sensitive than direct sequencing [43], and the proportion of discordant samples was reduced to 27%. Kakilaki et al. [44] included 3 patients who had previously been treated with gefitinib, and 15 out of 25 patients had received treatment with chemotherapy before the development of metachronous metastases. Discordance was seen in 28% of samples. In patients who had not received EGFR-TKI treatment, discordance was seen in 18% of samples. In 1 patient, EFGR mutation in the primary tumor was compared to 2 different metastases, and identical mutations were observed in the primary tumor (del19) and in 1 metastasis, but a different mutation (T790 M) in the other metastasis [44] (Table 2).

EGFR copy number

To elucidate the feasibility of fine needle aspiration samples when evaluating EGFR gene copy number status by

Table 2 EGFR expression

Author	Histological subtype	No. of pts	% biomarker pos on diagnostic biopsy	% biomarker pos on resected tumor	% biomarker pos in biopsy and neg in PT	% biomarker neg in biopsy and pos in PT	Discordance between diagnostic biopsy and resected tumor
Comparison of EGFR expression between initial biopsy and immediately subsequent resection of NSCLC primary tumor (%)							
Meert et al. [47]	SQ: 15 AD:12 LCC: 1	28	55	48	11	4	15

			Pos biomarker/ mutation in both PT and metastasis (%)	Neg biomarker in both PT and metastasis (%)	Pos in PT/neg in metastasis (%)	Neg in PT/pos in metastasis (%)	Discordance in biomarkers/ mutations between PT and metastasis (%)	Metastatic site (%)
Comparison of EGFR expression in biopsies from primary tumor and metastasis in NSCLC								
Chang et al. [40]	AD: 61% SQ: 30% ASC: 7% SAC: 2%	56	39	27	27	7	34	Lymph node: 100
Rao et al. [48]	SQ: 35% AD: 53% BAC: 4% ASC: 8%	47	72	17	4	6	11	Lymph node: 100
Italiano et al. [24]	AD: 70% SQ: 23% LCC: 3% NOS: 3%	30 26	43	23	23	10	33	Adrenal gland: 3 Bone: 13 Brain: 67 Lung: 13 Soft tissue: 33
Gomez-Roca et al. [49]	AD: 47% SQ: 31% LCC: 12% BAC: 6% Other: 4%	49	51	16	27	6	33	Adrenal gland: 49 Brain: 18 Bone: 16 Soft tissue: 6 Liver: 8 Lymph node: 2
Kalikaki et al. [44]	AD: 72%	25	21 6 (p-EGFR)	68 44	5 13	5 38	11 50	Lung: 36 Thoracic wall: 20 Adrenal gland: 16 Brain: 12 Bone: 8 Liver: 4 Skin: 4

SQ squamous carcinoma, *AD* adenocarcinoma, *ASC* adenosquamous carcinoma, *BAC* broncoalveolar carcinoma, *LCC* large cell carcinoma, *NOS* not otherwise specified, *SAC* sarcomatoid carcinoma, *LEC* lymphoepithelioma-like carcinoma, *PT* primary tumor

FISH in primary tumor, Bozetti et al. [45] compared diagnostic fine needle aspiration samples to subsequent histological or resected sample. The EGFR gene copy number was discordant in 12% of paired samples, which may be due to heterogeneous distribution of EGFR amplification where tumor cells in the fine needle aspiration are not representative of the entire tumor. This was examined by Yatabe et al. [28] who examined 48 lung carcinomas and found EGFR amplification in 11 tumors. Out of the 11 EGFR amplified tumors, heterogeneous distribution was found in 9 tumors when comparing EGFR amplification by FISH in 3 independent parts of each tumor [28] (Table 3).

Three trials by Italiano et al. [24], Monaco et al. [46], and Bozzetti et al. including 101 patients compared EGFR amplification status between primary tumor and various organ metastasis of which 67, 17.5, and 45% was metachronous with a median time interval between samples of 8, 19, and 19.5 months, respectively. They found that EGFR amplification was discordant in 27, 32.5, and 32% of paired samples, respectively [24, 46].

EGFR expression

Meert et al. [47] compared biopsies from 49 NSCLC patients obtained from bronchoscopy of which 33% were cytological biopsies to histological material from subsequent surgical resections with regard to the expression of EGFR. The trial reported discordance between expression in biopsy and subsequently resected tumor of 15% [47].

Table 3 EGFR copy number

Author	Histological subtype	No. of pts	% biomarker pos on diagnostic biopsy	% biomarker pos on resected tumor	% biomarker pos in biopsy and neg in PT	% biomarker neg in biopsy and pos in PT	Discordance between diagnostic biopsy and resected tumor (%)
Comparison of EGFR copy number between initial biopsy and immediately subsequent resection of NSCLC primary tumor (%)							
Bozzetti et al. [45]	AD: 42.4 SQ: 42.4 ASC: 9 SAC: 3 NOS: 3	33	61	55	9	3	12

			Pos biomarker/ mutation in both PT and metastasis (%)	Neg biomarker in both PT and metastasis (%)	Pos in PT/neg in metastasis (%)	Neg in PT/pos in metastasis (%)	Discordance in biomarkers/ mutations between PT and metastasis (%)	Metastatic site (%)
Comparison of EGFR copy number in biopsies from primary tumor and metastasis in NSCLC (%)								
Italiano et al. [24]	AD: 70 SQ: 23 LCC: 3 NOS: 3	30 26	39	35	23	4	27	Adrenal gland: 3 Bone: 13% Brain: 67 Lung: 13 Soft tissue: 33
Bozzetti et al. [63]	SQ: 16 AD: 45 NOS: 36	28	32	36	4	29	32	Liver: 6 Pleura: 6 Abdomen: 3 Ribs: 6 Skin: 13 Lymph node: 65
Monaco et al. [46]	AD: 95 LCC: 3 SAC: 3	40	0	68	9	24	33	Lymph node: 60 Pleura: 17.5 Brain: 10 Other: 12.5

SQ squamous carcinoma, *AD* adenocarcinoma, *ASC* adenosquamous carcinoma, *BAC* broncoalveolar carcinoma, *LCC* large cell carcinoma, *NOS* not otherwise specified, *SAC* sarcomatoid carcinoma, *LEC* lymphoepithelioma-like carcinoma, *PT* primary tumor

Two trials by Rao et al. [48] and Chang et al. [40] compared EGFR expression in the resected primary tumor and corresponding resected lymph node metastasis in 103 patients. EGFR expression was discordant in 11 and 34%, respectively. These trials are not comparable since Chang et al. used a positive staining of more than 50% of cells to define EGFR positivity, while Rao et al. used a more common cut off value of 10%.

Three trials by Gomez-Roca et al. [49], Italiano et al. [24] and Kakilaki et al. [44] have explored the occurrence and concordance of EGFR expression in primary tumor and corresponding organ metastasis in 104 patients. Discordance was observed in 33, 33, and 11% of paired samples, respectively. In the trial by Gomez-Roca et al. [49], 61% of metastases were metachronous and 18% of the patients had received cisplatin-based neoadjuvant chemotherapy. Kakilaki et al. included 3 patients who had received treatment with gefitinib and 15 patients who had received treatment with chemotherapy before the development of metachronous metastases (Table 4).

EGFR status and chemotherapy

Another important factor when addressing changes in EGFR status is the influence of chemotherapy or targeted treatments, which potentially eradicates sensitive clones with a specific biomarker status or potentially changes the biomarker status in less sensitive tumor cells.

Felip et al. [50] examined the effect of erlotinib with respect to treatment-related changes in EGFR expression and p-EGFR expression. Tumor biopsies were performed immediately before treatment and 6 weeks after disease progression, unacceptable toxicity, or consent withdrawal [50]. The trial included 14 patients who had progressed after at least one platinum-based chemotherapy regimen and who had suitable comparable pre and posttreatment biopsies. EGFR expression was observed in 79% and in 100% of pre and posttreatment samples, respectively. Five patients experienced clinical benefit defined as complete response, partial response, or stable disease more than 12 weeks. A reduction in p-EGFR expression ($P = 0.002$)

Table 4 Changes in biomarkers in primary tumor during antineoplastic drugs in NSCLC

Author	Histological subtype	No. of pts	Biomarker	% of biomarkers remaining pos	% of biomarkers remaining neg	% of biomarkers that changes from pos to neg	% of biomarkers that changes from neg to pos	% of changes in biomarker	Treatment	Timing of rebiopsy
De Pas [51]	N/A	47	EGFR expr	71%	6%	3%	11%	14%	N/A	N/A
			Mediastinal Node 85%		5%	2%	7%	9%		
			Node vs. PrTu							
Felip et al. [50]	LC: 57%	14	EGFR expr	79%	0%	0%	21%	21%	Erlotinib	Tumor biopsy from lung after 6 weeks
	AD: 21%		p-EGFR expr	7%	57%	36%	0%	35%		
	SQ: 21%			(p-EGFR)						
Badalian et al. [53]	AD: 82%	11	EGFR expr	Unchanged 46%	0%	Down regulated 18%	Up regulated 36%		Treatment (No pts) N/A (1)	Rebiopsy from bone metastases. Treatment - N/A
	SQ: 18%								Surgery (2)	
									Surgery + cyclophosphamide (1)	
									Surgery + doxorubicine (3)	
									Surgery + undefined chemo (1)	
									Surgery + cisplatin (2)	
Yatabe et al. [39]	AD: 100%	54	EGFR mutation	100%	0%	0%	0%	0%	N/A (EGFR-TKI)	From 1 to 212 days

SQ squamous carcinoma, *AD* adenocarcinoma, *ASC* adenosquamous carcinoma, *BAC* broncoalveolar carcinoma, *LCC* large cell carcinoma, *NOS* not otherwise specified, *SAC* sarcomatoid carcinoma, *LEC* lymphoepithelioma-like carcinoma, *PT* primary tumor

was observed when comparing pretreatment expression to posttreatment expression. No significant changes were observed regarding EGFR expression (0.0332). Regarding EGFR expression, 3 samples changed from negative to positive and no samples changed from positive to negative when using the defined H-score cut off value of 150.

De Pas et al. [51] examined the influence of neoadjuvant chemotherapy on the EGFR expression in 47 NSCLC patients with N2 disease. Preneoadjuvant mediastinal lymph node biopsies obtained by mediastinoscopy were compared to postneoadjuvant-resected primary tumor and resected mediastinal lymph nodes. Patients had received at least 3 cycles of chemotherapy. They observed that in 11% of cases, metastases in mediastinal lymph node changed from positive to negative and 3% from negative to positive EGFR expression when comparing pretreatment samples to posttreatment samples, respectively. This could indicate that a subsequent biopsy could be necessary after chemotherapy in the case of an EGFR-negative primary biopsy to evaluate EGFR expression.

Discussion

Determining EGFR expression by IHC to detect the presence of specific proteins in the cells is highly dependent on various factors such as techniques used for tissue collection and handling, antigen retrieval techniques, type of antibody, antibody detection protocol, and quality of the biopsy. In addition, there are currently no standard methodologies for handling or scoring EGFR expression which makes the frequencies of EGFR expression or overexpression difficult to compare between trials.

EGFR expression is usually considered positive when more than 10% of tumor cells are stained. This has proven to be the cut off value that provides the maximal differentiation in survival benefit between EGFR-positive and EGFR-negative patients treated with EGFR-TKIs [52]. Another method of scoring EGFR expression is by scoring the intensity (0–4) multiplied by the fraction of positive cells (0–100%) to get an H-score (0–400). An H-score above 200 is defined as EGFR expression positive. Most of the trials included in this paper have defined EGFR positivity as more than 10% stained cells [24, 44, 48, 49], except Chang et al. [40] who used a cut off value 50%. The trial by Meert et al. [47] defined samples as positive for EGFR when more than 1% of cells were stained, which is a similar cut off value as previously used in colon cancer. Felip et al. calculated the H-score, but used a cut off value of 150. Badalian et al. [53] also calculated an H-score but defined up or down regulation of EGFR as a change in H-score of 30% or above.

Direct sequencing has been widely used to detect EGFR mutations, but false negative results may occur when sam-

ples contain less than 30% tumor cells [54]. This is confirmed by the fact that the application of more sensitive techniques discovers additional EGFR mutations not discovered when the direct sequencing technique was applied [[41, 45, 55].]. Another influence on mutation status is the fact that formalin fixation may cause stand breaks and depurinate the samples. Mutation artifacts may be as frequent as every 500 base pairs [56]. This could lead to false negative or false positive testing of EGFR mutations, which could be interpreted as heterogeneity.

Another factor is EGFR amplification, which is distributed heterogeneously within the tumor with increased EGFR amplification-related invasive growth and metastasis. Since the mutant allele increases in number exclusively in EGFR amplification, it will lead to an increase in the mutant to wild-type ratio [34]. Thus, assuming that EGFR mutations are present in the entire tumor, the mutation signal would be stronger in the invasive areas with EGFR amplification. But, in other areas of the tumor where the EGFR mutation signal is low due to the lack of EGFR amplification and normal cells in the sample, the analysis of EGFR may not be sensible enough to detect the EGFR mutation as suggested by Yatabe et al. [39]. This is supported by the fact that Park et al. [41] discovered additional EGFR mutations after retesting samples with a more sensitive heteroduplex analysis.

Biomarkers on biopsies from solid tumors are increasingly being used to determine the drug of choice as more targeted treatments are entering clinical use. If the choice of subsequent treatment modalities is based on biomarkers in the cells, then the biomarker expression and occurrence of mutations in biopsy have to be representative for the entire tumor. Currently, no consensus on whether the biopsy on which to decide therapy options should preferably be from primary tumor or from metastases or whether a histological material is mandatory or whether cytological material is sufficient, though the latter is likely to be true.

The degree of heterogeneous distribution of EGFR mutations in NSCLC is still unclear as Yatabe et al. [39] find no heterogeneous distribution after thorough examination of 55 adenocarcinomas, and yet Taniguchi et al. [38] finds that patients with heterogeneous tumors have relatively decreased survival when treated with gefitinib. If a biomarker is distributed heterogeneously within a tumor, then there most likely will be an even more profound heterogeneous distribution between primary tumor and metastasis. In this case, it is important to consider the location of the metastatic site and time between samples. Most likely different clones metastasize to different organs, and longer intervals between sampling lead to increased occurrence of new mutations and an increasingly variation in phenotype.

Some patient benefit from EGFR-TKI treatment irrespective of the EGFR status, and some patients do not benefit despite the presence of EGFR mutations. Whether this is due to actual intra and intertumor heterogeneity regarding EGFR mutation status or whether it is simply due to false negative or false positive tests caused by methodological errors are still unclear.

It is not well elucidated how systemic antineoplastic treatment affects tumor cells and their expression of biomarkers. Trials concerning chemotherapy-induced changes in the expression of biomarkers or occurrence of mutations are often demanding to execute due to the fact that tissue samples are required both before and after treatment. Ethics is another important aspect when performing a clinical trial, which might demand that patients undergo, repetitive biopsies during palliative chemotherapy treatment. Most feasible for such trials may be patients receiving neoadjuvant treatment followed by surgery. The trials included in this paper do seem that biomarkers have changed in some patients after antineoplastic treatment. [50, 51, 53]. These trials all include a very limited number of patients and different treatment modalities. It is difficult to estimate whether discordance between biomarker expression between the pre and post-treatment samples is due to a change in biomarker status or simply a reflection of the preexisting tumor heterogeneity. Tumors can acquire resistance to gefitinib during treatment through the induction of the mutation (T790 M) [57]. This mutation (T790 M) is very rare before treatment with gefitinib therapy, and most of these mutations are found after gefitinib treatment [58–60] indicating that antineoplastic treatment can influence tumor phenotype either through selection of resistant clones or may be by inducing DNA changes in the cells.

Conclusion

Treatment of patients with advanced NSCLC with curative intent requires that all tumor clones are eradicated irrespective of their potentially different biomarker status. Thus, as treatments based on new biomarkers get implemented, it gets increasingly important to elucidate the relevance and degree of heterogeneous distribution of the targeted biomarker. EGFR mutations are not found with the same frequency in various histological subtypes. Histological subtypes who harbor the same biomarker are possibly also different regarding the heterogeneity of the biomarker distribution throughout the tumor.

Another aspect is how chemotherapy affects the biomarker status in the tumor cells. Potentially, a biomarker that is predictive of response to chemotherapy will disappear during chemotherapy as the sensitive tumor clones that harbor the biomarker are eradicated or as the tumor cells get more resistant to the treatment. It is also possible that

the biomarker status stays unchanged in the tumor cells as the cell develops resistance mechanisms, which makes the predictive biomarker redundant.

Furthermore, various chemotherapy agents may affect the biomarker status in various histological subgroups of NSCLC differently, but this issue is also hitherto not firmly elucidated. In the current and future clinical scenario with many different options for systemic treatment both as 2nd and 3rd line and beyond, it is thus increasingly important to further elucidate these outstanding questions in relation to proper use of biomarkers such as EGFR in order to offer the patients an individually tailored treatment.

Conflict of interest None.

References

1. United States Cancer Statistics 2002–2004 (2007) Incidence and Mortality Web-based Report Version. Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute. J Nat Cancer Inst
2. Taron M, Ichinose Y, Rosell R, Mok T, Massuti B, Zamora L et al (2005) Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. Clin Cancer Res 11(16):5878–5885
3. Vilmar AC, Sorensen JB (2011) Customising chemotherapy in advanced nonsmall cell lung cancer: daily practice and perspectives. Eur Respir Rev: An Off J Eur Respir Soc 20(119):45–52
4. Coghlin C, Murray GI (2010) Current and emerging concepts in tumour metastasis. J Pathol 222(1):1–15
5. Wistuba II, Gazdar AF (2006) Lung cancer preneoplasia. Annu Rev Pathol 1:331–348
6. Fong KM, Sekido Y, Gazdar AF, Minna JD (2003) Lung cancer. 9: molecular biology of lung cancer: clinical implications. Thorax 58(10):892–900
7. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II et al (2005) Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 97(5):339–346
8. Sekido Y, Fong KM, Minna JD (2003) Molecular genetics of lung cancer. Annu Rev Med 54:73–87
9. Prudkin L, Wistuba II (2006) Epidermal growth factor receptor abnormalities in lung cancer. Pathogenetic and clinical implications. Ann Diagn Pathol 10(5):306–315
10. Tokumo M, Toyooka S, Kiura K, Shigematsu H, Tomii K, Aoe M et al (2005) The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. Clin Cancer Res 11(3):1167–1173
11. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I et al (2004) EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 101(36):13306–13311
12. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW et al (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350(21):2129–2139
13. Milas I, Komaki R, Hachiya T, Bubb RS, Ro JY, Langford L et al (2003) Epidermal growth factor receptor, cyclooxygenase-2, and BAX expression in the primary non-small cell lung cancer and brain metastases. Clin Cancer Res 9(3):1070–1076
14. Hirsch FR, Varella-Garcia M, McCoy J, West H, Xavier AC, Gumerlock P et al (2005) Increased epidermal growth factor receptor gene copy number detected by fluorescence in situ hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a southwest oncology group study. J Clin Oncol 23(28):6838–6845
15. Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L et al (2005) Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Nat Cancer Inst 97(9):643–655
16. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S et al (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304(5676):1497–1500
17. van Zandwijk N, Mathy A, Boerrigter L, Ruijter H, Tielen I, de Jong D et al (2007) EGFR and KRAS mutations as criteria for treatment with tyrosine kinase inhibitors: retro- and prospective observations in non-small-cell lung cancer. Ann Oncol 18(1):99–103
18. Yoshida K, Yatabe Y, Park JY, Shimizu J, Horio Y, Matsuo K et al (2007) Prospective validation for prediction of gefitinib sensitivity by epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer. J Thorac Oncol 2(1):22–28
19. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N et al (2009) Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 361(10):947–957
20. Yoo SB, Chung JH, Lee HJ, Lee CT, Jheon S, Sung SW et al (2010) Epidermal growth factor receptor mutation and p53 overexpression during the multistage progression of small adenocarcinoma of the lung. J Thorac Oncol 5(7):964–969
21. Dacic S, Flanagan M, Cieply K, Ramalingam S, Luketich J, Belani C et al (2006) Significance of EGFR protein expression and gene amplification in non-small cell lung carcinoma. Am J Clin Pathol 125(6):860–865
22. Takano T, Ohe Y, Sakamoto H, Tsuta K, Matsuno Y, Tateishi U et al (2005) Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J Clin Oncol 23(28):6829–6837
23. Cappuzzo F (2008) EGFR FISH versus mutation: different tests, different end-points. Lung Cancer 60(2):160–165
24. Italiano A, Vandenbos FB, Otto J, Mouroux J, Fontaine D, Marcy PY et al (2006) Comparison of the epidermal growth factor receptor gene and protein in primary non-small-cell-lung cancer and metastatic sites: implications for treatment with EGFR-inhibitors. Ann Oncol 17(6):981–985
25. Hirsch FR, Varella-Garcia M, Bunn PA, Di Maria MV, Veve R, Bremmes RM et al (2003) Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. J Clin Oncol 21(20):3798–3807
26. Tsao MS, Sakurada A, Cutz JC, Zhu CQ, Kamel-Reid S, Squire J et al (2005) Erlotinib in lung cancer—molecular and clinical predictors of outcome. N Engl J Med 353(2):133–144
27. Miller VA, Riely GJ, Zakowski MF, Li AR, Patel JD, Heelan RT et al (2008) Molecular characteristics of bronchioloalveolar carcinoma and adenocarcinoma, bronchioloalveolar carcinoma subtype, predict response to erlotinib. J Clin Oncol 26(9):1472–1478
28. Yatabe Y, Takahashi T, Mitsudomi T (2008) Epidermal growth factor receptor gene amplification is acquired in association with tumor progression of EGFR-mutated lung cancer. Cancer Res 68(7):2106–2111
29. Hirsch FR, Scagliotti GV, Langer CJ, Varella-Garcia M, Franklin WA (2003) Epidermal growth factor family of receptors in

preneoplasia and lung cancer: perspectives for targeted therapies. Lung Cancer 41(suppl 1):S29–S42
30. Pirker E, Paz Ares L, Eberhardt W, Krzakowski M, Störkel S, Heeger S et al. (2011) Epidermal growth factor receptor (EGFR) expression as a predictor of survival for first-line chemotherapy plus cetuximab in FLEX study patients with advanced non-small cell lung cancer (NSCLC). Presented at WCLC in Amsterdam. Abstract 01.06.2011
31. Sharma SV, Bell DW, Settleman J, Haber DA (2007) Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer 7(3):169–181
32. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S et al (2005) Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 353(2):123–132
33. Liang Z, Zhang J, Zeng X, Gao J, Wu S, Liu T et al. (2010) Relationship between EGFR expression, copy number and mutation in lung adenocarcinomas. BMC Cancer 10:376
34. Soh J, Okumura N, Lockwood WW, Yamamoto H, Shigematsu H, Zhang W et al (2009) Oncogene mutations, copy number gains and mutant allele specific imbalance (MASI) frequently occur together in tumor cells. PloS one 4(10):e7464
35. Dacic S (2008) EGFR assays in lung cancer. Adv Anat Pathol 15(4):241–247
36. Eberhard DA, Giaccone G, Johnson BE, Non-small-cell lung cancer working group (2008) Biomarkers of response to epidermal growth factor receptor inhibitors in non-small-cell lung cancer working group: standardization for use in the clinical trial setting. J Clin Oncol 26(6):983–994
37. Tomonaga N, Nakamura Y, Yamaguchi H, Ikeda T, Mizoguchi K, Motoshima K et al. (2011) Analysis of intra-tumor heterogeneity of EGFR mutations in mixed-type lung adenocarcinoma. Poster presented at IASCL 14th world conference on lung cancer, Amsterdam
38. Taniguchi K, Okami J, Kodama K, Higashiyama M, Kato K (2008) Intratumor heterogeneity of epidermal growth factor receptor mutations in lung cancer and its correlation to the response to gefitinib. Cancer Sci 99(5):929–935
39. Yatabe Y, Matsuo K, Mitsudomi T (2011) Heterogeneous distribution of EGFR mutations is extremely rare in lung adenocarcinoma. J Clin Oncol 29(22):2972–2977
40. Chang YL, Wu CT, Shih JY, Lee YC (2011) Comparison of p53 and epidermal growth factor receptor gene status between primary tumors and lymph node metastases in non-small cell lung cancers. Ann Surg Oncol 18(2):543–550
41. Park S, Holmes-Tisch AJ, Cho EY, Shim YM, Kim J, Kim HS et al (2009) Discordance of molecular biomarkers associated with epidermal growth factor receptor pathway between primary tumors and lymph node metastasis in non-small cell lung cancer. J Thorac Oncol 4(7):809–815
42. Jänne PA, Borras AM, Kuang Y, Rogers AM, Joshi VA, Liyanage H et al (2006) A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. Clin Cancer Res 12(3 Pt 1):751–758
43. Morinaga R, Okamoto I, Fujita Y, Arao T, Sekijima M, Nishio K et al (2008) Association of epidermal growth factor receptor (EGFR) gene mutations with EGFR amplification in advanced non-small cell lung cancer. Cancer Sci 99(12):2455–2460
44. Kalikaki A, Koutsopoulos A, Trypaki M, Souglakos J, Stathopoulos E, Georgoulias V et al (2008) Comparison of EGFR and K-RAS gene status between primary tumours and corresponding metastases in NSCLC. Br J Cancer 99(6):923–929
45. Bozzetti C, Tiseo M, Lagrasta C, Nizzoli R, Guazzi A, Graiani G et al (2010) Is cytology reliable for epidermal growth factor receptor gene evaluation in non-small cell lung cancer? J Thorac Oncol 5(4):551–553
46. Monaco SE, Nikiforova MN, Cieply K, Teot LA, Khalbuss WE, Dacic S et al (2010) A comparison of EGFR and KRAS status in primary lung carcinoma and matched metastases. Hum Pathol 41(1):94–102
47. Meert AP, Martin B, Verdebout JM, Paesmans M, Berghmans T, Ninane V et al (2004) Correlation of different markers (p53, EGF-R, c-erbB-2, Ki-67) expression in the diagnostic biopsies and the corresponding resected tumors in non-small cell lung cancer. Lung Cancer 44(3):295–301
48. Rao C, Hu Q, Ma J, Li J, Zhang C, Shen L et al. (2010) Comparison of the epidermal growth factor receptor protein expression between primary non-small cell lung cancer and paired lymph node metastases: implications for targeted nuclide radiotherapy. J Exp Clin Cancer Res 29:7
49. Gomez-Roca C, Raynaud CM, Penault-Llorca F, Mercier O, Commo F, Morat L et al (2009) Differential expression of biomarkers in primary non-small cell lung cancer and metastatic sites. J Thorac Oncol 4(10):1212–1220
50. Felip E, Rojo F, Reck M, Heller A, Klughammer B, Sala G et al (2008) A phase II pharmacodynamic study of erlotinib in patients with advanced non-small cell lung cancer previously treated with platinum-based chemotherapy. Clin Cancer Res 14(12):3867–3874
51. De Pas T, Pelosi G, de Braud F, Veronesi G, Curigliano G, Leon ME et al (2004) Modulation of epidermal growth factor receptor status by chemotherapy in patients with locally advanced non-small-cell lung cancer is rare. J Clin Oncol 22(24):4966–4970
52. Clark GM, Zborowski DM, Culbertson JL, Whitehead M, Savoie M, Seymour L et al (2006) Clinical utility of epidermal growth factor receptor expression for selecting patients with advanced non-small cell lung cancer for treatment with erlotinib. J Thorac Oncol 1(8):837–846
53. Badalian G, Barbai T, Rásó E, Derecskei K, Szendrôi M, Tímár J et al (2007) Phenotype of bone metastases of non-small cell lung cancer: epidermal growth factor receptor expression and K-RAS mutational status. Pathol Oncol Res 13(2):99–104
54. Fan X, Furnari FB, Cavenee WK, Castresana JS (2001) Non-isotopic silver-stained SSCP is more sensitive than automated direct sequencing for the detection of PTEN mutations in a mixture of DNA extracted from normal and tumor cells. Int J Oncol 18(5):1023–1026
55. Gow CH, Chang YL, Hsu YC, Tsai MF, Wu CT, Yu CJ et al (2009) Comparison of epidermal growth factor receptor mutations between primary and corresponding metastatic tumors in tyrosine kinase inhibitor-naive non-small-cell lung cancer. Ann Oncol 20(4):696–702
56. Williams C, Pontén F, Moberg C, Söderkvist P, Uhlén M, Pontén J et al (1999) A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol 155(5):1467–1471
57. Nguyen KSH, Kobayashi S, Costa DB (2009) Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancers dependent on the epidermal growth factor receptor pathway. Clin Lung Cancer 10(4):281–289
58. Kosaka T, Yatabe Y, Endoh H, Yoshida K, Hida T, Tsuboi M et al (2006) Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. Clin Cancer Res 12(19):5764–5769
59. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF et al (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2(3):e73
60. Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M et al (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 352(8):786–792

61. Matsumoto S, Takahashi K, Iwakawa R, Matsuno Y, Nakanishi Y, Kohno T et al (2006) Frequent EGFR mutations in brain metastases of lung adenocarcinoma. Int J Cancer 119(6):1491–1494
62. Cortot AB, Italiano A, Burel-Vandenbos F, Martel-Planche G, Hainaut P (2010) KRAS mutation status in primary nonsmall cell lung cancer and matched metastases. Cancer 116(11):2682–2687
63. Bozzetti C, Tiseo M, Lagrasta C, Nizzoli R, Guazzi A, Leonardi F et al (2008) Comparison between epidermal growth factor receptor (EGFR) gene expression in primary non-small cell lung cancer (NSCLC) and in fine-needle aspirates from distant metastatic sites. J Thorac Oncol 3(1):18–22