Lung Cancer 141 (2020) 56–63

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Cryobiopsy increases the EGFR detection rate in non-small cell lung cancer

Maik Haentschel[a,*], Michael Boeckeler[a], Ahmed Ehab[b], Robert Wagner[c], Werner Spengler[a], Volker Steger[d], Hans Boesmueller[e], Marius Horger[f], Richard A. Lewis[g], Falko Fend[e], Lothar Kanz[h], Irina Bonzheim[e], Juergen Hetzel[a]

[a] Department of Medical Oncology and Pneumology, Eberhard Karls University, Tuebingen, Germany
[b] Department of Pneumology, Klinik Loewenstein, Loewenstein, Germany
[c] Department of Diabetology, Endocrinology and Nephrology, Eberhard Karls University, Tuebingen, Germany
[d] Department of Thoracic and Cardiovascular Surgery, Eberhard Karls University, Tuebingen, Germany
[e] Institute of Pathology and Neuropathology, Eberhard Karls University, Tuebingen, Germany
[f] Department of Diagnostic and Interventional Radiology, Eberhard-Karls-University, Tuebingen, Germany
[g] NPARU, University of Worcester, Worcester, UK
[h] Department of Hematology, Oncology, Clinical Immunology and Rheumatology, Eberhard-Karls-University, Tuebingen, Germany

ARTICLE INFO

Keywords:
Cryobiopsy
Non-small cell lung cancer
Epidermal growth factor receptor mutation

ABSTRACT

Objectives: Detection of activating epidermal growth factor receptor (EGFR) mutation is crucial for individualized treatment of advanced non-small-cell lung cancer (NSCLC). However little is known about how biopsy technique affects the detection rate of EGFR mutations. This retrospective, single center study evaluated the detection rate of EGFR mutations in tissue obtained by bronchoscopic cryobiopsy and compared this to other standard tissue sampling techniques.

Materials and methods: We retrospectively analyzed 414 patients with histologically confirmed NSCLC and known EGFR mutation status between 3/2008-7/2014. Tumor specimens obtained by tissue preserving bronchoscopic cryobiopsy were compared to those obtained by other techniques.

Results and conclusion: Analysis of bronchoscopic cryobiopsy tissue detected 29 activating EGFR mutations in 27 (21.6 %) out of 125 patients, while analysis of tissue obtained by non-cryobiopsy techniques (bronchoscopic forceps biopsies, fine needle aspiration, imaging guided transthoracical and surgical procedures) detected 42 EGFR mutations in 40 (13.8 %) out of 298 patients ($p < 0.05$). Cryobiopsy increased detection rate of EGFR mutations in central tumors compared with forceps biopsy (19.6 % versus 6.5 %, $p < 0.05$), while an insignificant trend was detected also for peripheral tumors (33.3 % versus 26.9 %).

Bronchosopic cryobiopsy increases the detection rate of activating EGFR mutations in NSCLC in comparison to other tissue sampling techniques. This will help to optimize individualized treatment of patients with advanced tumors. Because of the retrospective nature of this analysis, a prospective trial is mandatory for final assessment.

1. Introduction

Lung cancer ranks among the most common cancers worldwide with approximately 1.8 million patients in 2012 [1,2]. Non small cell lung cancer (NSCLC) represents 75–85 % of all lung cancers. 2/3 of these patients are in non-curable stage III and IV at the time of diagnosis, and are usually treated systemically [3]. The current management of non-curable NSCLC has become more and more individualized and is based on immunohistochemical and molecular tumor characterization [4]. In this context, EGFR mutation is currently the most relevant molecular-genetic alteration [5] since target therapies directed to activating EGFR mutation not only have a much better side effect profile [6], but also lead to an improved clinical response [7], an improved progression free survival, and an improved overall survival in certain subgroups (e.g. with deletion in Exon 19 [8–14]).

As a consequence, precise and correct molecular characterization of advanced stage III and IV NSCLC is crucial for ensuring optimal treatment, and missing any targetable alteration may result in suboptimal therapy. Therefore, representative tumour tissue of adequate volume and quality forms the basis for optimal histological and molecular evaluation. EGFR testing lays the cornerstone for current NSCLC treatment in advanced stages [15]. Apart from surgical resection and

* Corresponding author at: Department of Medical Oncology and Pneumology, Eberhard Karls University, Otfried-Mueller-Str., 10, D-72076, Tuebingen, Germany.
E-mail address: maik.haentschel@med.uni-tuebingen.de (M. Haentschel).

https://doi.org/10.1016/j.lungcan.2019.12.008
Received 11 June 2019; Received in revised form 13 December 2019; Accepted 16 December 2019

radiologically guided biopsies, the standard techniques to obtain tissue samples in patients with suspected lung cancer are bronchoscopic forceps biopsy and fine needle aspiration [16]. However, there has been no detailed evaluation of the quality of biopsy specimen required for molecular testing, nor has there been an evaluation as to which biopsy technique provides the best material for molecular testing. As a result, guidelines on molecular testing of NSCLC do not discuss the quality of biopsy specimens required for EGFR testing, and limit their recommendations to statements like “a sufficient cancer cell content and appropriate DNA quantity and quality” [17,18] are required. This had led to the imprecise advice that each laboratory defines its own criteria [17]. Even in prospective large multicentre trials evaluating the effect of targeted therapies, criteria addressing the quality of biopsy specimens needed for molecular testing are usually not specified [19].

The recently developed technique of cryobiopsy for the diagnosis of patients with endobronchial tumor has revealed a significantly higher diagnostic yield for lung cancer than conventional forceps biopsy [20]. In addition, cryobiopsy samples are of sufficiently large size to routinely allow immunohistological staining [21]. Cryobiopsy provides better tissue preservation without biopsy artifacts. Nevertheless, it cannot be ruled out that physical alterations caused by the freezing and thawing cycle during cryobiopsy could influence the diagnostic value on a molecular level.

We have therefore evaluated the detection rate of EGFR mutations in cryobiopsies and compared these to other biopsy techniques in patients with pathologically proven non small cell lung cancer.

2. Patients and methods

2.1. Study design and population

This retrospective single center study was performed at the Department of Internal Medicine II in collaboration with the Institute of Pathology at the University of Tuebingen, Germany. All malignant tissue samples from the University Hospital which had been examined for EGFR mutations between March 2008 and July 2014 were included in this study. A total of 483 tissue samples were analyzed for mutations of the EGFR gene on exon 18, 19, 20, 21 using Sanger sequencing [22]. 60 tumor samples were excluded because of extrapulmonary tumor origin or unspecified diagnoses, and another 9 cases with cancer of unknown primary were excluded (Fig. 1). Altogether 414 patients with histologically confirmed NSCLC were included in the analysis. The study protocol and the retrospective evaluation of the patient specific data were approved by the ethics committee of the University of Tuebingen (Project number 466/2014BO1).

2.2. Sampling procedures and data source

In 125 cases (30.2 %) the tissue samples had been obtained by cryobiopsy in the manner as previously described [21] using cryoprobes of 1.9 or 2.4 mm diameter (Erbe Elektromedizin GmbH, Tuebingen, Germany) and a freezing time of up to 10 s depending on the freezing power and diameter of the cryoprobes. In the remaining 289 cases (69.8 %), the “non-cryobiopsy” group, other diagnostic procedures were performed (see Fig. 1). Of these, conventional bronchoscopic forceps biopsy was undertaken in 98 cases (23.7 %). M.B., W.S. and J.H. carried out all bronchoscopies, and type of biopsy (cryobiopsy, forceps biopsy or fine needle aspiration) was decided by each investigator. More than 80 % of radiologically guided transthoracic biopsies were performed by M.H., and by V.S. for surgical techniques. Diagnostic video assisted thoracic surgery was predominately performed for pleural resection (n = 12) showing pleural carcinosis and for resection of a peripheral nodule (n = 5). Only in a single case video assisted thoracoscopy was proceeded for lymph nodes extraction.

For both the cryobiopsy and non-cryobiopsy group specific patient data included age, gender, and smoking history, as well as tumor characteristics including localization, staging (according to the 7th lung cancer TNM classification and staging system) [23] and histopathological classification. Only those samples in which EGFR testing was possible were included in the analysis. For all tissue specimens histopathological diagnosis of NSCLC and EGFR mutation analysis were performed on the same tissue sample. All histological and EGFR mutation analyses were done at the Institute of Pathology at the University of Tuebingen.

2.3. DNA extraction and EGFR mutation detection (Sanger Sequencing)

Genomic DNA was isolated from 5 µm paraffin-embedded tissue sections, partially after macrodissection to enrich tumor cell content. Tissue was dewaxed and digested with proteinase K for 16 h and DNA was purified applying standard phenol/chloroform purification [24]. DNA was amplified for exon 18, 19, 20 and 21 of the EGFR gene using M13-tailed primers previously published (exon 18, 21 [25] and 19, 20 [26]).

PCR was performed using 100 ng DNA template in a final volume of 50 µl with 0.2 µM of each primer, 0,2 mM dNTPs, 1.5 mM MgCl2 and one Unit Phusion HotStart High Fidelity Polymerase (Finnzymes, Woburn, MA, USA). Cycling conditions entailed an initial denaturation at 98 °C for 30 s followed by 45 cycles of denaturation (98 °C for 10 s), annealing (60 °C for 75 s) and elongation (72 °C for 30 s), with a final elongation at 72 °C for 4 min.

PCR products were purified (AMPure, Beckman Coulter, Brea, CA, USA) and aliquots of 7 µl were used for the sequencing reaction with 1 µM of the universal M13 sequencing primer and 2 µl of GenomeLab DTCS-Quick Start Kit (Beckman Coulter, Brea, CA, USA) in a final volume of 10 µl according to the manufacturers protocol. Sequencing reactions were purified (CleanSEQ, Beckman Coulter, Brea, CA, USA) and analyzed in a GenomeLab GeXP Genetic Analysis System and evaluated by the GenomeLab GeXP software 10.2 (Beckman Coulter, Brea, CA, USA) to determine the mutation status.

2.4. Tumor cell proportion analysis

The proportion of tumor cells was evaluated independently by two pathologists at the Department of Pathology, University of Tuebingen, and defined as percentage (%) of tumor cells compared to non tumorous cells on fixed tissue specimens. In cases of reduced tumor cell percentage, microdissection was performed on unstained serial paraffin sections in order to increase tumor cell percentage and optimize further analysis.

2.5. Statistical analysis

For statistically analysis SAS jmp® 11.2 (SAS, Cary, United States) was used. Comparisons between groups were made using the following tests: age – *t*-test; localization of tumor and timeline of tumor biopsy in relation to cancer treatment –Fisher’s exact test; tumor staging, histopathological classification, subcharacterization and comparison of bronchoscopic techniques – chi-square test; EGFR mutation frequency – 2 × 2 contingency table; tumor cell proportion – *t*-test. Significance was defined as p- value < 0.05.

3. Results

3.1. Patient characteristics

Demographic data of the cryobiopsy group and the non-cryobiopsy group were comparable with regard to age and gender, as shown in Table 1. Almost all patients were Causasian (> 99 %). Due to the retrospective study design smoking status was unknown in a large proportion of patients, but when known, it did not differ between both groups.

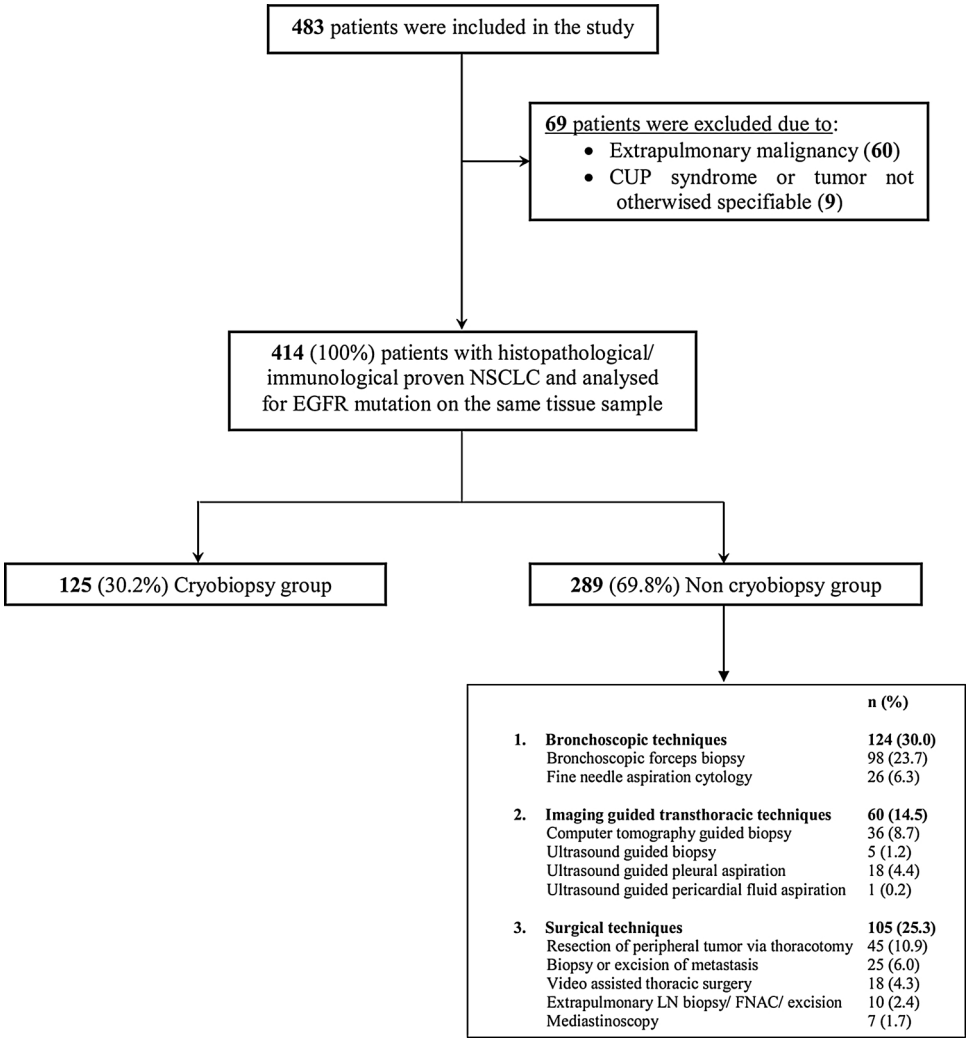

Fig. 1. Study population. n – absolute number; CUP – cancer of unknown primary; Extrapulmonary LN biopsy – extrapulmonary lymph node biopsy; FNAC – fine needle aspiration cytology.

Table 1
Patient characteristics[a].

Diagnostic procedure	Cryobiopsy	Non cryobiopsy	p-value
Total number of patients, n	125	289	–
Age – year			
Median	65	66	n.s.
Sex - no (%)			
Male	77 (61.6)	183 (63.3)	
Female	48 (38.4)	106 (36.7)	n.s.
Smoking status			
Known, n (100 %)	69	165	
- Never smoker, n (%)	5 (7.2)	13 (7.9)	n.s.
- Ever smoker, n (%)	64 (92.8)	152 (92.1)	
Unknown	56	124	

[a] Patient characteristics (total number, median age, gender and smoking status) comparing the cryobiopsy group with the non cryobiopsy group.

3.2. Tumor characteristics

Tumor characteristics for both groups are shown in Table 2. Cryobiopsy was used more frequently for sampling of central tumors (85.6 %), while non-cryobiopsy techniques were more frequently used in peripheral tumors (66.1 %). The proportion of advanced cancers

Table 2
Tumor characteristics[a].

Diagnostic procedure	Cryobiopsy	Non cryobiopsy	p-value
Total number of patients, n (%)	125 (100)	289 (100)	
Localisation of tumor, n (%)			
• Central	107 (85.6)	98 (33.9)	< 0.05
• Peripheral	18 (14.4)	191(66.1)	
Tumor staging, n (%)			< 0.05
• I	3 (2.4)	27 (9.3)	
• II	3 (2.4)	19 (6.6)	
• III	14 (11.2)	39 (13.5)	
• IV	105 (84.0)	204 (70.6)	
Histopathological classification, n (%)			n.s.
• Adenocarcinoma/ Adenosquamous cell carcinoma	113 (90.4)	264 (91.3)	
• Large cell carcinoma	6 (4.8)	14 (4.8)	
• Squamous cell carcinoma	5 (4.0)	10 (3.5)	
• Carcinoma (not otherwise specified)	1 (0.8)	1 (0.4)	

[a] Tumor characteristics (total number, localization of tumor, tumor staging according to UICC and histopathological classification) comparing the cryobiopsy group with the non-cryobiopsy group.

Table 3
Frequency of patients with activating EGFR mutations[a].

Diagnostic procedure	Cryobiopsy	Non cryobiopsy	p-value
Total number of patients, n (%)	125 (100)	289 (100)	
Total number of patients*with* EGFR mutation(s)	27 (21.6)	40 (13.8)	< 0.05
Total number of Patients *without* EGFR mutation(s)	98 (78.4)	249 (86.2)	

[a] Frequency of patients with/ without activating EGFR mutation(s) comparing the cryobiopsy group with the non-cryobiopsy group.

according to the UICC classification [23] was significantly higher in the cryobiopsy group compared to the non cryobiopsy group. The distribution of histological tumor types was similar in both groups. Examples for preserved tumor tissue for bronchoscopic cryobiopsy and forceps biopsy are shown in Supplementary Fig. S1.

3.3. Frequency of patients with activating EGFR mutations

Among 125 tumor samples obtained by bronchoscopic cryobiopsy, 27 (21.6 %) tumors showed activating mutations in the EGFR gene, while in the non cryobiopsy group 40 out of 289 (13.8 %) EGFR mutated tumors were identified. This represented a significantly higher detection rate ($p < 0.05$) for activating EGFR mutation in tumor samples obtained using bronchoscopic cryobiopsy compared to non-cryobiopsy (Table 3). 29 activating EGFR mutations were detected in 27 patients in the cryobiopsy group with two patients harboring mutations in two EGFR Exons (18 and 20, respectively 19 and 20).

3.4. Subcharacterization/ specification of detected activating EGFR mutations

In both cryo- and non-cryobiopsy groups subcharacterization of activating EGFR mutations showed most changes in Exon 19 (cryobiopsy: 48.3 %; forceps biopsy 45.2 %) and 21 (cryobiopsy: 27.6 %; forceps biopsy 38.1 %) (Supplementary Table ST1). Detected alterations were consistent with the known predominant mutations e.g. deletion of exon 19 or mutation L858R in exon 21. There was no significant difference in the distribution of mutations between the two groups.

3.5. Frequency of activating EGFR mutations detected in the non cryobiopsy group

The frequency of activating EGFR mutations detected in the non-cryobiopsy group using different techniques is shown in Table 4.

3.6. Comparison of bronchoscopic tissue extraction: influence of tumor localization and biopsy technique

The overall comparison of bronchoscopic tissue extraction with conventional forceps biopsy (n = 98) and cryobiopsy (n = 125) did not reveal any significant difference in the detection rate of activating EGFR mutations (Supplementary Table ST2).

However, cryobiopsy detected significantly more EGFR activating mutations in central tumours than forceps biopsy (19.6 % versus 6.5 % $p < 0.05$), whereas in peripheral lung tumors the detection rate, although higher, was similar for both techniques (cryobiopsy (33.3 %) and forceps biopsy (26.9 %), Supplementary Table ST2).

In those patients diagnosed by bronchoscopic techniques a significantly higher proportion of EGFR mutations could be detected in peripheral tumors compared to central tumors (28.6 % versus 15.7 %, Supplementary Table ST3).

3.7. Proportion of tumor cells

Proportion of tumor cells of a tissue sample was defined as described above. Percentage of tumor cells was not evaluable in 3 patients in the cryobiopsy group and 35 patients in the non-cryobiopsy group due to technical problems (17 patients) or those with only cytology smear specimens (18 patients). Tumor tissue processing by microdissection was performed in 7 patients (5.6 %) after cryobiopsy and 30

Table 4
Frequency of activating EGFR mutation detected in NSCLC in the non-cryobiopsy group with regard to the applied technique[a].

	Absolute number of patients (n)	Specimen with mutation (n)	Muation detection rate (%)
Total	**289**	**40**	**13.8**
Bronchoscopy	**124**	**20**	**16.1**
Forceps biopsy	98	17	17.3
Fine needle aspiration cytology	26	3	11.5
Imaging guided transthoracic technique	**60**	**9**	**15.0**
Computer tomography guided biopsy	36	4	11.1
Ultrasound guided biopsy	5	0	0
Ultrasound guided pleural fluid aspiration	18	4	22.2
Ultrasound guided pericardial fluid aspiration	1	1	100.0
Surgical techniques	**105**	**11**	**10.5**
Resection of lung tissue via thoracotomy	45	3(5)°	6.7(11.1)[b]
Biopsy or excision of extrapulmonary metastasis	25	0	0
Diagnostic video assisted thoracic surgery	18	5	27.8
Extrapulmonary lymph node resection	10	3	30.0
Mediastioscopy	7	0	0

[a] Frequency of activating EGFR mutations comparing bronchoscopic, imaging guided and surgical techniques for tumor tissue sampling in the non-Cryobiopsy group. Table shows the absolute number of the different techniques and the corresponding absolute number and frequency of detected activating EGFR mutations, separately for each technique.

[b] In one patient with surgical resection of lung tissue three activating mutations in Exon 18, 19 and 21 could be detected simultaneously, leading to the increased number of 5 detectable mutations in 3 patients.

patients (10.4 %) in the non-cryobiopsy group to enhance tumor cell proportion, but this did not significantly differ between both groups (p = 0.09). Supplementary Table ST 4 shows the proportion of tumor cells in whole sample. Samples with microdissection were excluded for this analysis. There was a tendency towards higher tumor cell proportion in cryobiopsy samples (56.7 %) compared to non-cryobiopsy samples (51.1 %).

The percentage of tumor cells in cryobiopsy samples (56.7 %, SD ± 19.6) was significantly higher compared to bronchoscopic forceps biopsy samples (46.1 %, SD ± 20.3) ($p < 0.05$).

There was no difference in the tumor cell percentage in samples with activating EGFR mutations compared to those without activating EGFR muations in either cryo- or non-cryobiopsy samples.

3.8. Timeline of tumor biopsy and cancer treatment

As EGFR mutation detection may differ between treatment-naïve patients or pretreated patients with relapsed or progressive NSCLC we evaluated whether patients were treatment-naïve or have already had cancer treatment before biopsy (Supplementary Table ST 5). Most patients were untreated at time of biopsy (95.2 % in the cryobiopsy cohort, 90.3 % in the non-cryobiopsy cohort). There was no significant difference between both groups (p = 0.12).

4. Discussion

To our knowledge, this is the first analysis to study the influence of biopsy techniques on the rate of EGFR mutation detection in patients with NSCLC. Our restrospective analysis indicates that sampling by bronchoscopic cryobiopsy increased the detection rate of molecular genetic alterations in NSCLC in comparison to other biopsy techniques (21.6 % vs 13.8 % ($p < 0.05$). Thus cryobiopsy might be able to substantially increase the number of patients who go on to receive targeted therapy.

EGFR directed TKI therapy is based on acquired genetic alterations of the EGFR in Exon 18, 19, 20 and 21 as oncogenic drivers. Mutation detection serves as a predictor for treatment response. It plays a central role in the choice of treatment for each patient with advanced NSCLC due to the improvement of progression free survival and even overall survival under certain circumstances [9–14].

The prevalence of EGFR mutations varies substantially and is affected by ethnicity, age, gender and smoking history. In our study population from the surrounding area of Tuebingen in Southern Germany, with a Caucasian proportion of > 95 %, the EGFR mutation frequency with 13.8 % in the non cryobiopsy group is in accordance with the reported frequencies in large studies of predominantely Caucasian populations in both France and the United States (11–17%) [19,27]. However, the frequency of 21.6 % in the cryobiopsy group exceed the expected EGFR mutation level despite there being no observed difference between cryo- and non-cryo biopsy groups in terms of age, histopathological classification, smoking status or gender (Table 1). The medium age of 64 years in our study population is about 5 years lower than the expected median age at first diagnosis of 69–70 years in Germany.

The difference in tumor stage noted between the cryobiopsy and non-cryobiopsy group does not explain the observed differences in EGFR mutation rate and the higher perentage in the cryobiopsy group for the following reasons. Firstly, EGFR mutation develops in early stage malignancy and remains consistently present during further tumor development. To our knowledge, there are no data proving differences in EGFR- mutation rate as a reflection of tumor stage [28,29]. Secondly, driver mutations are usually consistent between primary tumor and metastasis [18,30]. Therefore, differences in tumor stage – whether primary tumor or metastasis – cannot serve as a definitive explanation for the differences in the observed frequencies of EGFR mutations.

In most cases diagnosis was based on the primary tumor tissue, only in few cases lymph node or tissue of metastasis was used. However, according to Yatabe et al. [30], a heterogeneous distribution of EGFR mutation is extremely rare in lung adenocarcinoma.

The proportion of tumors located in the central airways was higher in the cryobiopsy group compared to the non-cryobiopsy group. This may be explained by the utilization of different techniques in different situations, such as the use of transthoracic biopsy or surgical biopsy for peripherally located tumors and cryobiopsy for the more readily accessible central airway tumors.

In the subgroup of bronchoscopically diagnosed tumors EGFR mutation frequency was significantly higher in perpheral compared to central tumors, independent of the bronchoscopic technique, (forceps or cryobiopsy) (Supplementary Table ST3). However, the imbalance in tumor localization, with significantly more central tumors in the cryobiopsy groups compared to the non cryobiopsy group, should theoretically result in a higher increased EGFR mutation rate detected in the non-cryobiopsy group, which was not the case (Supplementary Table ST2). Thus the rate of EGFR mutations detected in the cryobiopsy group may be an underestimate of the detection rate if there had been an equal proportion of central to peripheral tumours in both groups.

The biopsy technique used is influenced by the accessibility of the neoplastic tissue. In the past there has been little attention paid to tissue quality in deciding which technique should be used. This is partly explained by the fact that there are no quality criteria for lung tumor tissue specimens. Adequacy of tissue has been decided on individual pathologists's assessments [31] and limitation of tissue is considered unavoidable with conventional biopsy techniques. Even in large multicenter trials molecular analysis of tumor tissue is not possible in a significant proportion of patients – sometimes this proportion is even not reported. Due to the lack of evidence, guidelines are forced to limit their requirements for tumor tissue to a recommendation for "sufficient cancer cell content" and an "adequate DNA quantity and quality" without precise definition. On the other hand, the importance of good quality tumor tissue for exact molecular diagnoses has been emphazied [17] and large specimens are generally recommended [32–37].

Bronchoscopic cryobiopsy directly addresses these requirements, since the specimens are of large size, of good quality and with fewer artifacts [15,20,38,39] compared to other biopsy or aspiration techniques. The size, representative nature and quality of tissue preservation found in cryo-biopsy specimens compared to forceps biopsy specimens probably explains the improved EGFR detection rate.

Undoubtedly, the continuous development and optimization of diagnoses in molecular tumor characterization, with its step from Sanger sequencing to next generation sequencing, has improved analytic sensitivity and specificity significantly, and allows tumor specimen characterization down to single cell level [32–37]. However, these highly sensitive methods are not infallible [40–43], and minimal tumor specimens may be declared to be representative of the entire tumor although they cannot cover the heterogeneity of tumors. Therefore these methods face limitations in sensitivity, too [44–47] and type of biopsy is still essential [48]. Reasons for false negative results may be due to an imbalance of tumor cells and normal tissue, including inflammatory and stromal cells [41], a low copy number DNA template [49] or DNA damage with consecutive sequence artifacts due to formalin fixation [50,51]. Tumor cell enrichment techniques such as laser capture microdissection may increase tumor cell content and compensate for some of the limitations of the sample size and quality, but diagnostic inaccuracy may still not be overcome completely [39,49,52,53]. Therefore by using highly sensitive detection techniques, harvesting of representative, and thus large, biopsies is still crucial. Cryobiopsy specimens provide the "greater amount of material and greater capacity to enrich the malignant content" as specified by Lindeman et al. in 2013 [17]. Thus increased tumour sample size, with consequentially more representative material, may be enough to explain the increase in detection of EGFR mutation in cryobiopsy specimens. Arimura et al.

showed that gene analysis and whole genome sequencing is possible on cryobiopsy samples and supposed an advantage compared to forceps biopsy [54,55]. Naito et al. measured a larger amount of DNA in cryobiopsies compared to forceps biopsies resulting in more successful whole-exome sequencing. [56].

Liquid biopsy for detection of cell-free tumor DNA and circulating tumor cells in the blood of advanced NSCLC patients has emerged as a non-invasive concept in EGFR mutation detection. This may overcome some of the risks and challenges of solide tissue sampling [57,58]. However, the sensitivity of these sequencing techniques has been reported to reach only 60–80 % as shedding of DNA into the bloodstream differs from tumor to tumor and from patient to patient [59,60]. This may explain the false negative rate of liquid biopsy. A clinically relevant example is the T790 M resistant mutation. In a case series liquid biopsy missed the detection of histologically proven mutation in 30 % of the cases [59,61]. Another limitation is the fact that PD-L1 tumor cell reactivity analysis can not be performed by using liquid biopsy. Thus liquid biopsy may serve as additive, sometimes substitutive techniques for molecular genetic NSCLC analysis, but has not demonstrated to replace solide tissue sampling reliably.

Our study has several limitations. The retrospective approach does not allow a balance between both study groups, and this has led to an observed imbalance in tumor staging and localization in the groups. The type of bronchoscopic biopsy was not defined and the different techniques (cryobiopsy, forceps biopsy or fine needle aspiration) were used upon investigators choice. We did not differentiate between first diagnosis and relapse of disease in either group. However most patients were treatment-naïve when biopsy was taken (95.2 % in the cryobiopsy cohort, 90.3 % in the non-cryobiopsy cohort) (Suppementary Table ST5). As rebiopsy would rather be expected in a certain amount of known EGFR-mutated patients in order to evaluate for required T790 M mutation, the higher percentage of pretreated patients would rather favor a higher percentage of detectable EGFR muations in the non-cryobiopsy cohort.

We used Sanger sequencing as the former gold standard for EGFR mutation analysis in all cases, though nowadays it has been replaced stepwise by next generation sequencing or other techniques providing an increase in sensitivity. This could have resulted in missing some of the EGFR mutations. It has to be noted, that this less sensitive analyzing technique was used for both cohorts. However it cannot be concluded, that a more sensitive analyzing technique would have canceled out the differences.

The EGFR mutations rate in the non-cryobiopsy cohort was 13.8 % and thus even higher than previously reported 9.8 % and 10.3 % for a Caucasian European NSCLC cohort in Germany [62,63].

Interestingly, the largest metaanalysis on EGFR mutation prevalence, including 456 studies (only in 2 studies Sanger sequencing was used), showed an EGFR mutation prevalence of 14.1 % (95 % confidence interval 12.7 %–15.5 %) for the European population which is comparable to the rate in our study [64].

Due to the retrospective design of the study there was no strict protocol for indication when a cryobiopsy or another procedure was performed. Furthermore there were cases in which a cryobiopsy could not be applied e.g. mediastinal lymph node assessment.

We assume that the observed differences between biopsy techniques reflect the importance of adequate NSCLC tumor sampling for molecular characterization. Up till now this has only partially been taken into account or has been neglected completely when trying to optimise molecular diagnoses. However, correct molecular typing is crucial for prognosis, therapeutic choice, and quality of life in lung cancer patients. Any stepwise diagnostics are always limited by the step of least sensitivity. Therefore each step of the diagnostic pathway has to be optimized. The current study illustrates that some current biopsy techniques may be inadequate to reach this goal and even subsequent applied analytics of highest sensivitiy may not be able to overcome this problem. We suggest adhering to the current guidelines [17] but we are aware of the retrospective character of our study and therefore a prospective trial is required to prove our findings. Even a small increase in mutation detection in NSCLC would have significant implications for many patients' lives.

In conclusion, bronchosopic cryobiopsy is feasible for EGFR detection analysis in NSCLC and increases the detection rate of activating EGFR mutations in comparison to other tissue sampling techniques. Therefore it may optimize individualized treatment in a substantial number of patients and offer additional therapeutic options. However, due to the retrospective nature of our study, a prospective trial is mandatory for final assessment.

Authors' contributions

MH contributed to the concept, performed the bronchoscopic procedures, analyzed the data, performed statistical analysis and wrote the main part of the manuscript. MB contributed to the concept and performed the bronchoscopic procedures. AE analyzed the data and contributed to the manuscript. RW analyzed the data and performed statistical analysis. W.S. performed the bronchoscopic procedures. V.S. performed the surgical procedures. H.B. and F.F. did the histopathological evaluation. MHo performed the guided biopsies and aspirations. RAL and LK made revisions for the final manuscript.

I.B. performed the molecular genetic tissue analysis. JH conceived the research concept and strategies, performed the bronchoscopic procedures, analyzed the data, performed statistical analysis and wrote the manuscript. All the authors discussed the results and implications, commented on the manuscript at all stages, and reviewed the final version prior to submission. All authors read and approved the final manuscript.

Declaration of Competing Interest

JH has received personal fees from Erbe Elektromedizin GmbH for workshops and presentations. MH and MB have received personal fees from Erbe Elektromedizin GmbH for Workshops. AE, RW, WS, VS, HB, MHo, RAL, FF, LK, IB have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.12.008.

References

[1] R. Siegel, J. Ma, Z. Zou, A. Jemal, Cancer statistics, 2014, CA Cancer J. Clin. 64 (1) (2014) 9–29.
[2] T.Y. Cheng, S.M. Cramb, P.D. Baade, D.R. Youlden, C. Nwogu, M.E. Reid, The international epidemiology of lung cancer: latest trends, disparities, and tumor characteristics, J. Thorac. Oncol. 11 (10) (2016) 1653–1671.
[3] A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, M.J. Thun, Cancer statistics, 2009, CA Cancer J. Clin. 59 (4) (2009) 225–249.
[4] D.L. Aisner, C.B. Marshall, Molecular pathology of non-small cell lung cancer: a practical guide, Am. J. Clin. Pathol. 138 (3) (2012) 332–346.
[5] T.J. Lynch, D.W. Bell, R. Sordella, S. Gurubhagavatula, R.A. Okimoto, B.W. Brannigan, P.L. Harris, S.M. Haserlat, J.G. Supko, F.G. Haluska, D.N. Louis, D.C. Christiani, J. Settleman, D.A. Haber, Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib, N. Engl. J. Med. 350 (21) (2004) 2129–2139.
[6] R. Rosell, E. Carcereny, R. Gervais, A. Vergnenegre, B. Massuti, E. Felip, R. Palmero, R. Garcia-Gomez, C. Pallares, J.M. Sanchez, R. Porta, M. Cobo, P. Garrido, F. Longo, T. Moran, A. Insa, F. De Marinis, R. Corre, I. Bover, A. Illiano, E. Dansin, J. de Castro, M. Milella, N. Reguart, G. Altavilla, U. Jimenez, M. Provencio, M.A. Moreno, J. Terrasa, J. Munoz-Langa, J. Valdivia, D. Isla, M. Domine, O. Molinier, J. Mazieres, N. Baize, R. Garcia-Campelo, G. Robinet, D. Rodriguez-Abreu, G. Lopez-Vivanco, V. Gebbia, L. Ferrera-Delgado, P. Bombaron, R. Bernabe, A. Bearz, A. Artal, E. Cortesi, C. Rolfo, M. Sanchez-Ronco, A. Drozdowskyj, C. Queralt, I. de Aguirre, J.L. Ramirez, J.J. Sanchez, M.A. Molina, M. Taron, L. Paz-Ares, Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial, Lancet Oncol. 13 (3) (2012) 239–246.

[7] J.G. Paez, P.A. Janne, J.C. Lee, S. Tracy, H. Greulich, S. Gabriel, P. Herman, F.J. Kaye, N. Lindeman, T.J. Boggon, K. Naoki, H. Sasaki, Y. Fujii, M.J. Eck, W.R. Sellers, B.E. Johnson, M. Meyerson, EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy, Science 304 (5676) (2004) 1497–1500.
[8] R. Rosell, T. Moran, C. Queralt, R. Porta, F. Cardenal, C. Camps, M. Majem, G. Lopez-Vivanco, D. Isla, M. Provencio, A. Insa, B. Massuti, J.L. Gonzalez-Larriba, L. Paz-Ares, I. Bover, R. Garcia-Campelo, M.A. Moreno, S. Catot, C. Rolfo, N. Reguart, R. Palmero, J.M. Sanchez, R. Bastus, C. Mayo, J. Bertran-Alamillo, M.A. Molina, J.J. Sanchez, M. Taron, Screening for epidermal growth factor receptor mutations in lung cancer, N. Engl. J. Med. 361 (10) (2009) 958–967.
[9] J.C. Yang, Y.L. Wu, M. Schuler, M. Sebastian, S. Popat, N. Yamamoto, C. Zhou, C.P. Hu, K. O'Byrne, J. Feng, S. Lu, Y. Huang, S.L. Geater, K.Y. Lee, C.M. Tsai, V. Gorbunova, V. Hirsh, J. Bennouna, S. Orlov, T. Mok, M. Boyer, W.C. Su, K.H. Lee, T. Kato, D. Massey, M. Shahidi, V. Zazulina, L.V. Sequist, Afatinib versus cisplatin-based chemotherapy for EGFR mutation-positive lung adenocarcinoma (LUX-Lung 3 and LUX-Lung 6): analysis of overall survival data from two randomised, phase 3 trials, Lancet Oncol. 16 (2) (2015) 141–151.
[10] Y.L. Wu, C. Zhou, C.K. Liam, G. Wu, X. Liu, Z. Zhong, S. Lu, Y. Cheng, B. Han, L. Chen, C. Huang, S. Qin, Y. Zhu, H. Pan, H. Liang, E. Li, G. Jiang, S.H. How, M.C. Fernando, Y. Zhang, F. Xia, Y. Zuo, First-line erlotinib versus gemcitabine/cisplatin in patients with advanced EGFR mutation-positive non-small-cell lung cancer: analyses from the phase III, randomized, open-label, ENSURE study, Ann. Oncol. 26 (9) (2015) 1883–1889.
[11] C. Zhou, Y.L. Wu, G. Chen, J. Feng, X.Q. Liu, C. Wang, S. Zhang, J. Wang, S. Zhou, S. Ren, S. Lu, L. Zhang, C. Hu, C. Hu, Y. Luo, L. Chen, M. Ye, J. Huang, X. Zhi, Y. Zhang, Q. Xiu, J. Ma, L. Zhang, C. You, Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study, Lancet Oncol. 12 (8) (2011) 735–742.
[12] M. Fukuoka, Y.L. Wu, S. Thongprasert, P. Sunpaweravong, S.S. Leong, V. Sriuranpong, T.Y. Chao, K. Nakagawa, D.T. Chu, N. Saijo, E.L. Duffield, Y. Rukazenkov, G. Speake, H. Jiang, A.A. Armour, K.F. To, J.C. Yang, T.S. Mok, Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS), J. Clin. Oncol. 29 (21) (2011) 2866–2874.
[13] L.V. Sequist, J.C. Yang, N. Yamamoto, K. O'Byrne, V. Hirsh, T. Mok, S.L. Geater, S. Orlov, C.M. Tsai, M. Boyer, W.C. Su, J. Bennouna, T. Kato, V. Gorbunova, K.H. Lee, R. Shah, D. Massey, V. Zazulina, M. Shahidi, M. Schuler, Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations, J. Clin. Oncol. 31 (27) (2013) 3327–3334.
[14] J.C. Yang, V. Hirsh, M. Schuler, N. Yamamoto, K.J. O'Byrne, T.S. Mok, V. Zazulina, M. Shahidi, J. Lungershausen, D. Massey, M. Palmer, L.V. Sequist, Symptom control and quality of life in LUX-Lung 3: a phase III study of afatinib or cisplatin/pemetrexed in patients with advanced lung adenocarcinoma with EGFR mutations, J. Clin. Oncol. 31 (27) (2013) 3342–3350.
[15] L.M. Ofiara, A. Navasakulpong, S. Beaudoin, A.V. Gonzalez, Optimizing tissue sampling for the diagnosis, subtyping, and molecular analysis of lung cancer, Front. Oncol. 4 (2014) 253.
[16] J. Dionisio, Diagnostic flexible bronchoscopy and accessory techniques, Rev. Port. Pneumol. 18 (2) (2012) 99–106.
[17] N.I. Lindeman, P.T. Cagle, M.B. Beasley, D.A. Chitale, S. Dacic, G. Giaccone, R.B. Jenkins, D.J. Kwiatkowski, J.S. Saldivar, J. Squire, E. Thunnissen, M. Ladanyi, Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology, J. Thorac. Oncol. 8 (7) (2013) 823–859.
[18] N.B. Leighl, N. Rekhtman, W.A. Biermann, J. Huang, M. Mino-Kenudson, S.S. Ramalingam, H. West, S. Whitlock, M.R. Somerfield, Molecular testing for selection of patients with lung cancer for epidermal growth factor receptor and anaplastic lymphoma kinase tyrosine kinase inhibitors: American Society of Clinical Oncology endorsement of the College of American Pathologists/International Association for the study of lung cancer/association for molecular pathology guideline, J. Clin. Oncol. 32 (32) (2014) 3673–3679.
[19] M.G. Kris, B.E. Johnson, L.D. Berry, D.J. Kwiatkowski, A.J. Iafrate, I.I. Wistuba, M. Varella-Garcia, W.A. Franklin, S.L. Aronson, P.F. Su, Y. Shyr, D.R. Camidge, L.V. Sequist, B.S. Glisson, F.R. Khuri, E.B. Garon, W. Pao, C. Rudin, J. Schiller, E.B. Haura, M. Socinski, K. Shirai, H. Chen, G. Giaccone, M. Ladanyi, K. Kugler, J.D. Minna, P.A. Bunn, Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs, Jama 311 (19) (2014) 1998–2006.
[20] J. Hetzel, R. Eberhardt, F.J. Herth, C. Petermann, G. Reichle, L. Freitag, I. Dobbertin, K.J. Franke, F. Stanzel, T. Beyer, P. Moller, P. Fritz, G. Ott, P.A. Schnabel, H. Kastendieck, W. Lang, A.T. Morresi-Hauf, M.N. Szyrach, R. Muche, P.L. Shah, A. Babiak, M. Hetzel, Cryobiopsy increases the diagnostic yield of endobronchial biopsy: a multicentre trial, Eur. Respir. J. 39 (3) (2012) 685–690.
[21] J. Hetzel, M. Hetzel, C. Hasel, P. Moeller, A. Babiak, Old meets modern: the use of traditional cryoprobes in the age of molecular biology, Respiration 76 (2) (2008) 193–197.
[22] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. U. S. A. 74 (12) (1977) 5463–5467.
[23] S. Mirsadraee, D. Oswal, Y. Alizadeh, A. Caulo, E. van Beek Jr., The 7th lung cancer TNM classification and staging system: review of the changes and implications, World J. Radiol. 4 (4) (2012) 128–134.
[24] K. Sotlar, L. Escribano, O. Landt, S. Mohrle, S. Herrero, A. Torrelo, U. Lass, H.P. Horny, B. Bultmann, One-step detection of c-kit point mutations using peptide nucleic acid-mediated polymerase chain reaction clamping and hybridization probes, Am. J. Pathol. 162 (3) (2003) 737–746.
[25] D.H. Lee, G.K. Lee, S.Y. Kong, M.C. Kook, S.K. Yang, S.Y. Park, S.H. Park, B. Keam, D.J. Park, B.Y. Cho, S.W. Kim, K.W. Chung, E.S. Lee, S.W. Kim, Epidermal growth factor receptor status in anaplastic thyroid carcinoma, J. Clin. Pathol. 60 (8) (2007) 881–884.
[26] M. Yang, B. Shan, Q. Li, X. Song, J. Cai, J. Deng, L. Zhang, Z. Du, J. Lu, T. Chen, J.P. Wery, Y. Chen, Q. Li, Overcoming erlotinib resistance with tailored treatment regimen in patient-derived xenografts from naive Asian NSCLC patients, Int. J. Cancer 132 (2) (2013) E74–84.
[27] F. Barlesi, J. Mazieres, J.P. Merlio, D. Debieuvre, J. Mosser, H. Lena, L. Ouafik, B. Besse, I. Rouquette, V. Westeel, F. Escande, I. Monnet, A. Lemoine, R. Veillon, H. Blons, C. Audigier-Valette, P.P. Bringuier, R. Lamy, M. Beau-Faller, J.L. Pujol, J.C. Sabourin, F. Penault-Llorca, M.G. Denis, S. Lantuejoul, F. Morin, Q. Tran, P. Missy, A. Langlais, B. Milleron, J. Cadranel, J.C. Soria, G. Zalcman, Routine molecular profiling of patients with advanced non-small-cell lung cancer: results of a 1-year nationwide programme of the French Cooperative Thoracic Intergroup (IFCT), Lancet (London, England) 387 (10026) (2016) 1415–1426.
[28] E.C. de Bruin, C. Cowell, P.H. Warne, M. Jiang, R.E. Saunders, M.A. Melnick, S. Gettinger, Z. Walther, A. Wurtz, G.J. Heynen, D.A. Heideman, J. Gomez-Roman, A. Garcia-Castano, Y. Gong, M. Ladanyi, H. Varmus, R. Bernards, E.F. Smit, K. Politi, J. Downward, Reduced NF1 expression confers resistance to EGFR inhibition in lung cancer, Cancer Discov. 4 (5) (2014) 606–619.
[29] Z. Zhang, A.L. Stiegler, T.J. Boggon, S. Kobayashi, B. Halmos, EGFR-mutated lung cancer: a paradigm of molecular oncology, Oncotarget 1 (7) (2010) 497–514.
[30] Y. Yatabe, K. Matsuo, T. Mitsudomi, Heterogeneous distribution of EGFR mutations is extremely rare in lung adenocarcinoma, J. Clin. Oncol. 29 (22) (2011) 2972–2977.
[31] M.S. Tsao, A. Sakurada, J.C. Cutz, C.Q. Zhu, S. Kamel-Reid, J. Squire, I. Lorimer, T. Zhang, N. Liu, M. Daneshmand, P. Marrano, G. da Cunha Santos, A. Lagarde, F. Richardson, L. Seymour, M. Whitehead, K. Ding, J. Pater, F.A. Shepherd, Erlotinib in lung cancer - molecular and clinical predictors of outcome, N. Engl. J. Med. 353 (2) (2005) 133–144.
[32] S. Billah, J. Stewart, G. Staerkel, S. Chen, Y. Gong, M. Guo, EGFR and KRAS mutations in lung carcinoma: molecular testing by using cytology specimens, Cancer Cytopathol. 119 (2) (2011) 111–117.
[33] C.M. Chen, J.W. Chang, Y.C. Cheung, G. Lin, J.J. Hsieh, T. Hsu, S.F. Huang, Computed tomography-guided core-needle biopsy specimens demonstrate epidermal growth factor receptor mutations in patients with non-small-cell lung cancer, Acta Radiol. (Stockholm, Sweden: 1987) 49 (9) (2008) 991–994.
[34] L. Boldrini, S. Gisfredi, S. Ursino, T. Camacci, E. Baldini, F. Melfi, G. Fontanini, Mutational analysis in cytological specimens of advanced lung adenocarcinoma: a sensitive method for molecular diagnosis, J. Thorac. Oncol. 2 (12) (2007) 1086–1090.
[35] A. Fassina, A. Gazziero, D. Zardo, M. Corradin, E. Aldighieri, G.P. Rossi, Detection of EGFR and KRAS mutations on trans-thoracic needle aspiration of lung nodules by high resolution melting analysis, J. Clin. Pathol. 62 (12) (2009) 1096–1102.
[36] J.H. Smouse, E.S. Cibas, P.A. Janne, V.A. Joshi, K.H. Zou, N.I. Lindeman, EGFR mutations are detected comparably in cytologic and surgical pathology specimens of nonsmall cell lung cancer, Cancer 117 (1) (2009) 67–72.
[37] M.D. Lozano, J.J. Zulueta, J.I. Echeveste, A. Gurpide, L.M. Seijo, S. Martin-Algarra, A. Del Barrio, R. Pio, M.A. Idoate, T. Labiano, J.L. Perez-Gracia, Assessment of epidermal growth factor receptor and K-ras mutation status in cytological stained smears of non-small cell lung cancer patients: correlation with clinical outcomes, Oncologist 16 (6) (2011) 877–885.
[38] S. Griff, W. Ammenwerth, N. Schonfeld, T.T. Bauer, T. Mairinger, T.G. Blum, J. Kollmeier, W. Gruning, Morphometrical analysis of transbronchial cryobiopsies, Diagn. Pathol. 6 (2011) 53.
[39] S. Querings, J. Altmuller, S. Ansen, T. Zander, D. Seidel, F. Gabler, M. Peifer, E. Markert, K. Stemshorn, B. Timmermann, B. Saal, S. Klose, K. Ernestus, M. Scheffler, W. Engel-Riedel, E. Stoelben, E. Brambilla, J. Wolf, P. Nurnberg, R.K. Thomas, Benchmarking of mutation diagnostics in clinical lung cancer specimens, PLoS One 6 (5) (2011) e19601.
[40] A. Marchetti, C. Martella, L. Felicioni, F. Barassi, S. Salvatore, A. Chella, P.P. Camplese, T. Iarussi, F. Mucilli, A. Mezzetti, F. Cuccurullo, R. Sacco, F. Buttitta, EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment, J. Clin. Oncol. 23 (4) (2005) 857–865.
[41] P.A. Janne, A.M. Borras, Y. Kuang, A.M. Rogers, V.A. Joshi, H. Liyanage, N. Lindeman, J.C. Lee, B. Halmos, E.A. Maher, R.J. Distel, M. Meyerson, B.E. Johnson, A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening, Clin. Cancer Res. 12 (3 Pt 1) (2006) 751–758.
[42] Q. Pan, W. Pao, M. Ladanyi, Rapid polymerase chain reaction-based detection of epidermal growth factor receptor gene mutations in lung adenocarcinomas, J. Mol. Diagn. 7 (3) (2005) 396–403.
[43] H. Sasaki, K. Endo, A. Konishi, M. Takada, M. Kawahara, K. Iuchi, A. Matsumura, M. Okumura, H. Tanaka, T. Kawaguchi, T. Shimizu, H. Takeuchi, M. Yano, I. Fukai, Y. Fujii, EGFR Mutation status in Japanese lung cancer patients: genotyping analysis using LightCycler, Clin. Cancer Res. 11 (8) (2005) 2924–2929.
[44] K. Endo, A. Konishi, H. Sasaki, M. Takada, H. Tanaka, M. Okumura, M. Kawahara, H. Sugiura, Y. Kuwabara, I. Fukai, A. Matsumura, M. Yano, Y. Kobayashi, K. Mizuno, H. Haneda, E. Suzuki, K. Iuchi, Y. Fujii, Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay, Lung Cancer (Amsterdam, Netherlands) 50 (3) (2005) 375–384.
[45] K. Hoshi, H. Takakura, Y. Mitani, K. Tatsumi, N. Momiyama, Y. Ichikawa, S. Togo, T. Miyagi, Y. Kawai, Y. Kogo, T. Kikuchi, C. Kato, T. Arakawa, S. Uno, P.E. Cizdziel,

A. Lezhava, N. Ogawa, Y. Hayashizaki, H. Shimada, Rapid detection of epidermal growth factor receptor mutations in lung cancer by the SMart-amplification process, Clin. Cancer Res. 13 (17) (2007) 4974–4983.
[46] D.A. Heideman, F.B. Thunnissen, M. Doeleman, D. Kramer, H.M. Verheul, E.F. Smit, P.E. Postmus, C.J. Meijer, G.A. Meijer, P.J. Snijders, A panel of high resolution melting (HRM) technology-based assays with direct sequencing possibility for effective mutation screening of EGFR and K-ras genes, Cell. Oncol. 31 (5) (2009) 329–333.
[47] H. Miyazawa, T. Tanaka, Y. Nagai, M. Matsuoka, A. Sutani, K. Udagawa, J. Zhang, T. Hirama, Y. Murayama, N. Koyama, K. Ikebuchi, M. Nagata, M. Kanazawa, T. Nukiwa, S. Takenoshita, K. Kobayashi, K. Hagiwara, Peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based detection test for gefitinib-refractory T790M epidermal growth factor receptor mutation, Cancer Sci. 99 (3) (2008) 595–600.
[48] I.S. Hagemann, S. Devarakonda, C.M. Lockwood, D.H. Spencer, K. Guebert, A.J. Bredemeyer, H. Al-Kateb, T.T. Nguyen, E.J. Duncavage, C.E. Cottrell, S. Kulkarni, R. Nagarajan, K. Seibert, M. Baggstrom, S.N. Waqar, J.D. Pfeifer, D. Morgensztern, R. Govindan, Clinical next-generation sequencing in patients with non-small cell lung cancer, Cancer 121 (4) (2015) 631–639.
[49] M. Akbari, M.D. Hansen, J. Halgunset, F. Skorpen, H.E. Krokan, Low copy number DNA template can render polymerase chain reaction error prone in a sequence-dependent manner, J. Mol. Diagn. 7 (1) (2005) 36–39.
[50] C. Wong, R.A. DiCioccio, H.J. Allen, B.A. Werness, M.S. Piver, Mutations in BRCA1 from fixed, paraffin-embedded tissue can be artifacts of preservation, Cancer Genet. Cytogenet. 107 (1) (1998) 21–27.
[51] C. Williams, F. Ponten, C. Moberg, P. Soderkvist, M. Uhlen, J. Ponten, G. Sitbon, J. Lundeberg, A high frequency of sequence alterations is due to formalin fixation of archival specimens, Am. J. Pathol. 155 (5) (1999) 1467–1471.
[52] D.A. Eberhard, G. Giaccone, B.E. Johnson, 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) (2008) 983–994.
[53] N.L. Sieben, N.T. ter Haar, C.J. Cornelisse, G.J. Fleuren, A.M. Cleton-Jansen, PCR artifacts in LOH and MSI analysis of microdissected tumor cells, Hum. Pathol. 31 (11) (2000) 1414–1419.
[54] K. Arimura, M. Kondo, Y. Nagashima, M. Kanzaki, F. Kobayashi, K. Takeyama, J. Tamaoki, E. Tagaya, Comparison of tumor cell numbers and 22C3 PD-L1 expression between cryobiopsy and transbronchial biopsy with endobronchial ultrasonography-guide sheath for lung cancer, Respir. Res. 20 (1) (2019) 185.
[55] K. Arimura, E. Tagaya, H. Akagawa, Y. Nagashima, S. Shimizu, Y. Atsumi, A. Sato, M. Kanzaki, M. Kondo, K. Takeyama, P.P. Massion, J. Tamaoki, Cryobiopsy with endobronchial ultrasonography using a guide sheath for peripheral pulmonary lesions and DNA analysis by next generation sequencing and rapid on-site evaluation, Respir. Investig. 57 (2) (2019) 150–156.
[56] T. Naito, H. Udagawa, K. Kirita, T. Ikeda, Y. Zenke, S. Matsumoto, K. Yoh, S. Niho, G. Ishii, K. Goto, D.A. Haber, V.E. Velculescu, Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA, Cancer Discov. 4 (6) (2014) 650–661.
[57] D.A. Haber, V.E. Velculescu, Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA, Cancer Discov. 4 (6) (2014) 650–661.
[58] Y.L. Wu, L.V. Sequist, C.P. Hu, J. Feng, S. Lu, Y. Huang, W. Li, M. Hou, M. Schuler, T. Mok, N. Yamamoto, K. O'Byrne, V. Hirsh, N. Gibson, D. Massey, M. Kim, J.C. Yang, EGFR mutation detection in circulating cell-free DNA of lung adenocarcinoma patients: analysis of LUX-Lung 3 and 6, Br. J. Cancer 116 (2) (2017) 175–185.
[59] G.R. Oxnard, K.S. Thress, R.S. Alden, R. Lawrance, C.P. Paweletz, M. Cantarini, J.C. Yang, J.C. Barrett, P.A. Janne, Association between plasma genotyping and outcomes of treatment with osimertinib (AZD9291) in advanced non-small-cell lung cancer, J. Clin. Oncol. 34 (28) (2016) 3375–3382.
[60] A.G. Sacher, C. Paweletz, S.E. Dahlberg, R.S. Alden, A. O'Connell, N. Feeney, S.L. Mach, P.A. Janne, G.R. Oxnard, Prospective validation of rapid plasma genotyping for the detection of EGFR and KRAS mutations in advanced lung cancer, JAMA Oncol. 2 (8) (2016) 1014–1022.
[61] J.D. Merker, G.R. Oxnard, C. Compton, M. Diehn, P. Hurley, A.J. Lazar, N. Lindeman, C.M. Lockwood, A.J. Rai, R.L. Schilsky, A.M. Tsimberidou, P. Vasalos, B.L. Billman, T.K. Oliver, S.S. Bruinooge, D.F. Hayes, N.C. Turner, Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review, J. Clin. Oncol. 36 (16) (2018) 1631–1641.
[62] S. Gahr, R. Stoehr, E. Geissinger, J.H. Ficker, W.M. Brueckl, A. Gschwendtner, S. Gattenloehner, F.S. Fuchs, C. Schulz, R.J. Rieker, A. Hartmann, P. Ruemmele, W. Dietmaier, EGFR mutational status in a large series of Caucasian European NSCLC patients: data from daily practice, Br. J. Cancer 109 (7) (2013) 1821–1828.
[63] W. Schuette, P. Schirmacher, W.E. Eberhardt, J.R. Fischer, J.M. von der Schulenburg, J. Mezger, C. Schumann, M. Serke, S. Zaun, M. Dietel, M. Thomas, EGFR mutation status and first-line treatment in patients with stage III/IV non-small cell lung cancer in Germany: an observational study, Cancer Epidemiol. Biomarkers Prev. 24 (8) (2015) 1254–1261.
[64] Y.L. Zhang, J.Q. Yuan, K.F. Wang, X.H. Fu, X.R. Han, D. Threapleton, Z.Y. Yang, C. Mao, J.L. Tang, The prevalence of EGFR mutation in patients with non-small cell lung cancer: a systematic review and meta-analysis, Oncotarget 7 (48) (2016) 78985–78993.