Suitability of Small Bronchoscopic Tumour Specimens for Lung Cancer Genotyping

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Key Words

Bronchoscopy · Endobronchial ultrasound-guided transbronchial needle aspiration · EGFR mutation · Forceps biopsy · Non-small cell lung cancer

Abstract

Background: Biomarker-driven clinical trials in advanced non-small cell lung cancer (NSCLC) usually accept biopsy specimens only, as cytology specimens are supposed to be more challenging due to low neoplastic cell content and suboptimal DNA quantity. **Objectives:** We aimed to evaluate 2 aspects of bronchoscopic biopsy and cytology specimens: (1) the proportion of neoplastic cells and quantity of DNA extracted, and (2) the detection limit of the Scorpion amplification refractory mutation system on endoscopic samples obtained in daily clinical practice. **Methods:** We screened 679 patients with advanced-stage NSCLC for the presence of an activating EGFR mutation according to the guidelines of the European Society of Medical Oncology.

Their diagnostic tumour tissue samples were characterized. A dilution experiment was performed to determine the minimal proportion of neoplastic cells for a reliable test result. **Results:** Surgical biopsies, bronchoscopic forceps biopsy samples and needle aspiration cytology specimens exhibited a median tumour cell proportion of 70 versus 30 versus 20% and a DNA quantity of 2,500 versus 1,610 versus 1,440 ng, respectively. The overall EGFR mutation rate was 11%, with no differences between different sample types. Dilution experiments showed that the detection limit depends on the type of mutation. A neoplastic cell content of at least 10 and 25% for exon 19 deletions and exon 21 L858R point mutation, respectively, was required for a true negative result. **Conclusions:** Bronchoscopic forceps biopsy and needle aspiration cytology specimens are suitable for accurate EGFR mutation analysis using single-gene quantitative real-time polymerase chain reaction. Technologies with a better analytical sensitivity are evolving and should consider these endoscopic tumour specimens.

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Introduction

Approximately 80% of non-small cell lung cancer (NSCLC) patients present or relapse with advanced disease. The pathologic diagnosis is often based on small biopsy and/or cytology specimens obtained during flexible bronchoscopy. The lack of tissue architecture in these small tissue specimens limits NSCLC subtyping, but the advent of immunohistochemistry in modern pathology practice has led to a reduction of 'NSCLC not otherwise specified' (NSCLC-NOS) [1, 2]. The use of formalin-fixed paraffin-embedded (FFPE) small bronchoscopic tumour samples for clinical genetic mutation analysis poses several challenges, which are sample-related (limiting the amount and/or low neoplastic cellular content in tumour tissue samples with variable proportions of other cells such as inflammatory cells as well as suboptimal DNA quality due to formalin fixation) and methodology-related (e.g. the analytical sensitivity of the test). Recent guidelines and recommendations have focused on standardized and evidence-based material processing, analysis and reporting of results [2–4].

In samples with a tumour cell content of at least 50%, the performance of direct Sanger sequencing is comparable with quantitative real-time polymerase chain reaction (qPCR) methodology [5]. However, the performance of Sanger sequencing on small endoscopic samples might be lower because a tumour cell content of at least 50% is not often obtained [6]. Indeed, direct Sanger sequencing on small samples for clinical EGFR mutation testing has shown a suboptimal sensitivity of 67% compared to massively parallel sequencing [7]. Allele-specific qPCR methods are considered more sensitive than Sanger and therefore could be more suitable for mutation testing on small endoscopic tumour samples [8]. The Therascreen kit (Qiagen, Manchester, UK) is a well-established CE-labelled kit for diagnostic analysis of EGFR mutational status using real-time PCR. It combines allele-specific amplification with Scorpion detection technology for analysis of 29 mutations in exons 18–21. The reported in vitro detection limit is 1% mutant alleles against a background of wild-type genomic DNA from tumour tissue.

Landmark studies of molecularly targeted agents have focused on Sanger sequencing of single genes, but current methodologies used in a clinical setting perform multiplex PCR-based amplification for a whole range of somatic mutations of oncogenes and can detect and quantify mutation frequencies present in at least 10% of cells (e.g. mass array SNP Sequenom and SNaPshot mutation-

al analysis) [9–12]. More recently, cancer genome profiling based on massively parallel DNA sequencing which can detect mutation frequencies from at least 20% of mutation positive cells has been reported [13].

The goals of this study were to evaluate: (1) the percentage of malignant cells and the quantity of DNA extracted from bronchoscopic samples obtained by bronchial forceps biopsies and endobronchial ultrasound-guided transbronchial needle aspirations (EBUS-TBNA) in comparison with surgical biopsies, and (2) the in vivo analytical sensitivity of the Scorpion amplification refractory mutation system (ARMS) on endoscopic biopsies.

Patients and Methods

Patients and Tumour Samples

A total of 679 consecutive patients, diagnosed with advanced-stage NSCLC between 1 September 2010 and 30 October 2013 at the University Hospitals KU Leuven, were eligible (fig. 1). All adenocarcinoma, adenosquamous cell carcinoma, NSCLC-NOS or large-cell carcinoma patients underwent EGFR molecular testing irrespective of their smoking history. Patients with squamous-cell carcinoma with a negative or light (<10 pack-years) smoking history were also included. Clinical tumour samples from all the patients were analyzed for the presence of activating EGFR mutations. We included all diagnostic bronchoscopic tissue samples (i.e. from endobronchial and transbronchial lung biopsies and EBUS-TBNA), and samples from percutaneous core needle biopsy (PCNB), pleural biopsy (from medical thoracoscopy) and surgical diagnostic biopsy. In addition, we included archived specimens from patients resected for early-stage-disease NSCLC, which relapsed as stage IV disease. Bone biopsies, pleural fluid and bronchial lavage samples were excluded. A total of 679 samples were available from this single-institution database (fig. 1; table 1): 292 bronchial forceps biopsies, 155 EBUS-TBNA cell blocks, 37 pleural forceps biopsies, 45 PCNB and 135 surgical biopsies (97 pulmonary resections, 21 mediastinoscopies, 5 subcutaneous node resections, 5 brain metastasis resections, 3 abdominal node resections and 4 adrenal gland resections). Study approval was obtained from the Institutional Review Board of the University Hospitals KU Leuven.

Procedures

Biopsy Method

Endobronchial and EBUS-miniprobe-guided transbronchial lung biopsies were taken using a 2-mm forceps (single-use Radial Jaw 2-mm biopsy forceps, Boston Scientific, Boston, Mass., USA). EBUS-TBNA was performed with a 22-gauge needle (single-use Vizishot needle NA-201SX-4022, Olympus), PCNB was performed using an 18-gauge needle (single-use needle MC1810, BARD Biopsy Systems, Tempe, Ariz., USA), parietal pleural biopsies were performed using a rigid forceps during medical thoracoscopy (26170BD, KARL STORZ, Tuttlingen, Germany), and surgical biopsies were either multiple forceps biopsies (mediastinoscopy) or in toto resections of the target lesion (anatomical lung resection or resection of a metastasis).

Fig. 1. Tissue sample diagram. The asterisk indicates never-smokers, light smokers (<10 pack-years) and ex-smokers. AC = Adenocarcinoma; AdSqCCa = adenosquamous cell carcinoma, LCC = large-cell carcinoma; SqCCa = squamous cell carcinoma; NSCLC-NOS = non-small cell lung cancer not otherwise specified.

Table 1. Tissue specimen characteristics and performance

	Surgical biopsy (n = 135)	Bronchial biopsy (n = 292)	EBUS-TBNA (n = 155)	PCNB (n = 45)	Pleural biopsy (n = 37)
Histology, n (%)					
Adenocarcinoma	124 (92)	234 (81)	119 (77)	39 (86.6)	37 (100)
NSCLC-NOS	8 (6)	47 (15)	32 (20.6)	3 (6.6)	0 (0)
Adenosquamous cell carcinoma	2 (1.5)	3 (1)	0 (0)	0 (0)	0 (0)
Large-cell carcinoma	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Squamous cell carcinoma	0 (0)	8 (3)	4 (2.6)	3 (6.6)	0 (0)
Tumour content, %					
Mean ± SD	65±30	34±25	30±27	45±30	36±27
Median (range)	70 (5–100)	30 (5–100)	20 (1–100)	50 (5–100)	25 (5–100)
Total amount of DNA, ng					
Median (range)	2,500 (560–6,240)	1,610 (120–7,880)	1,440 (90–7,520)	1,020 (440–2,500)	1,690 (30–2,910)
DNA concentration, ng/μl					
Median (range)	50 (11–124)	32 (2–158)	29 (2–150)	20 (9–50)	34 (1–58)
DNA control curve, %					
Cp >35	1.5	0.7	1.9	0	0
EGFR mutation rate, %	11.9	10.6	10.3	13.3	13.5

Processing of Tumour Specimens

Endoscopic samples underwent fixation in 10 ml buffered formaldehyde (prefilled form 4%, Unifix, Klinipath, Duiven, The Netherlands). The fixation time was 6–12 h for bronchial biopsies, EBUS-TBNA samples, pleural biopsies and PCNB, and 8–18 h for surgical specimens. Routine EBUS-TBNA samples were rinsed in a liquid preservative collection medium (CytoRich) and processed to generate cell pellets; one ThinPrep Pap slide in a monolayer was used for cytopathology diagnosis and one FFPE cell block was used for immunohistochemistry and molecular testing. Immunohistochemistry for NSCLC phenotyping was performed on four con-

secutive 4-μm-thick paraffin sections with specific monoclonal antibodies: anti-CK7, anti-TTF-1, anti-K5.6 (all ready-to-use, Dako, Carpinteria, Calif., USA) and anti-p63 (1:30; Novocastra). Dako PT link was used for deparaffinization and heat-induced epitope retrieval (high, pH 9.0). Staining was performed using an automated immunostainer (Autostainer Link 48, Dako), followed by antibody detection using the EnVision FLEX visualization system (Dako). The antigen antibody immunoreaction was visualized using diaminobenzidine as a chromogen, and the slides were counterstained with Mayer's hematoxylin. For EGFR mutation analysis on small biopsies and cytology specimens (bronchoscopic, pleural

or PCNB samples), twelve consecutive 4- μ m-thick sections were prepared from the paraffin block. For surgical biopsies, seven consecutive 4- μ m-thick sections were prepared from the paraffin block. For both situations, the first and last section was stained with HE and evaluated for the presence and amount of tumour cells relative to the admixed non-neoplastic cells by an experienced pathologist (E.V.). The most representative area was marked on the HE slide and the proportion of tumour cells was estimated semi-quantitatively.

DNA Extraction

Serial unstained sections (4- μ m thick) from an FFPE block were used for genomic DNA extraction. Deparaffinization was performed with graded concentrations of xylene and alcohol. Using a mini-lambotte KA 480/04 (Devroe Instruments, Londerzeel, Belgium), the tumour foci in the marked areas were manually macro-dissected by a molecular biologist for tumour cell enrichment from 5 (for surgical biopsies) or 10 (for endoscopic biopsies) glass-mounted unstained sections and collected in microtubes. The tissue was digested overnight with proteinase K. DNA extraction was done using the Maxwell 16 FFPE tissue LEV DNA purification kit on the Maxwell 16 instrument (Promega Corporation, Madison, Wisc., USA) according to the manufacturer's instructions. The concentration of the extracted DNA was measured using Victor³ spectrophotometry (PerkinElmer).

EGFR Mutation Testing

Mutational analysis of EGFR was performed using the Therascreen EGFR RGQ PCR kit (Qiagen) on the LightCycler 480 Instrument II (Roche). The assay combines Scorpion and ARMS technologies to detect mutations in real-time PCRs, and allows the detection of 19 deletions between nucleotide positions 2,235 and 2,257 in exon 19, 3 insertions in exon 20 and point mutations leading to the following amino acid substitutions: T790M, L858R, L861Q, G719A/C/S and S768I. In keeping with the recommendations in the manual, samples in which the control PCR did not become positive until 35 cycles were considered technical failures due to poor DNA quality.

Dilution Analysis to Determine the Minimum Tumour Cell Count Allowing Reliable EGFR Analysis with Therascreen ARMS/Scorpion Assay-Based PCR

Two histologic specimens consisting of approximately 100% nucleated tumour cells and harbouring either a known exon 19 deletion or exon 21 L858R point mutation were selected. The tumour-derived DNA was diluted with DNA from EGFR wild-type patients to obtain a final mutation concentration of 50, 25, 10, 5 and 1% mutant tumour cells. Amplification of a region of exon 2 (Control Reaction Mix), deletions in exon 19 (Deletions Reaction Mix) and L858R in exon 21 (L858R Reaction Mix) of the EGFR gene was performed using the Therascreen EGFR RGQ PCR mutation kit (Qiagen) on the LightCycler 480 Instrument II. qPCR reactions were carried out in a total volume of 25 μ l (19.5 μ l Reaction Mix + 0.5 μ l Taq DNA polymerase + 5 μ l sample) and the following cycle conditions: 95°C for 15 min, 45 cycles at 95°C for 30 s and 60°C for 1 min. A maximum amount of 250 ng of genomic DNA was used. The assay was carried out in duplicate. Sample Δ Cp (crossing point) values were calculated as the difference between the mutation assay Cp and control assay Cp from the same sample. Samples were classified mutation-positive if they gave a

Δ Cp less than the cutoff Δ Cp value for that assay. The cutoff for EGFR exon 19 deletions was a Δ Cp of 6.75; the cutoff for L858R was 6.4. Based on this experimental study, the test interpretation was applied to all NSCLC specimens referred to the Center for Human Genetics of the University Hospitals KU Leuven for molecular pathology analysis.

Results

The vast majority of the samples were adenocarcinoma cases (table 1). A mean ($\pm$ SD) of 7 ($\pm$ 2) bronchial biopsies were taken per patient using a 2-mm forceps, and a mean of 4.9 ($\pm$ 1.3) EBUS-TBNA were performed per patient using a 22-gauge needle. Only one PCNB was taken per patient within this cohort. The median proportion of malignant cells in the surgical samples was 70% (table 1). The median proportion of malignant cells in endoscopic samples (n = 447) was 25%, but the tumour content in the bronchoscopic forceps biopsy samples was significantly higher than in the EBUS-TBNA cell-block samples (median 30 vs. 20%; p = 0.026). Surgical biopsies generated a median of 2,500 ng of DNA (range 560–6,240) while FFPE small bronchoscopic forceps biopsies and EBUS-TBNA samples (10 unstained sections, 4- μ m thick) yielded a median of 1,610 and 1,440 ng DNA, respectively (table 1). While the median DNA yield obtained from surgical biopsies was significantly higher than from bronchoscopic samples, the median amount of DNA was not statistically different between bronchoscopic sample types (median 1,610 vs. 1440 ng; p = 0.16).

EGFR mutation testing was performed on all 664 samples. Overall, we observed a PCR failure rate of 1.2%, which was related to insufficient DNA quality (e.g. a high degree of DNA fragmentation). Activating EGFR mutations were found in 11.2% of our patients with advanced-stage NSCLC (74/662 patients). The frequency of activating EGFR mutations observed was similar in surgical biopsies (EGFR mutation rate 11.9%) and endoscopic biopsies (EGFR mutation rate 10.5%). A Cp of >36 for exon 19 deletions and >39 for exon 21 mutations carried a 5% risk for a false-positive finding, but these conditions were not encountered in our cohort.

We performed dilution experiments to evaluate the minimal tumour cell content for the Therascreen ARMS/Scorpion assay-based PCR. On the one hand, this showed that the detection limit depends on the type of the mutation (table 2). In a dilution series of exon 19 deletions, we found that the critical Δ Cp value of <6.75 was only reached with an estimated tumour cell fraction of at least 10%, while lower tumour cell fractions typically yielded

Table 2. Dilution experiment for exon 19 deletion (del) and exon 21 L858R point mutation

Control reaction	Cp	Mutation mix	Cp	Δ Cp
<i>Deletions</i>				
100% del 19	29.06	100% del 19	31.72	2.66
50% del 19	27.68	50% del 19	32.09	4.41
25% del 19	27.23	25% del 19	32.76	5.53
10% del 19	27.05	10% del 19	33.79	6.74
5% del 19	26.96	5% del 19	34.91	7.95
1% del 19	26.81	1% del 19	36.83	10.02
<i>L858R</i>				0
100% L858R	27.63	100% L858R	32.69	5.06
50% L858R	27.21	50% L858R	33.19	5.98
25% L858R	27.03	25% L858R	33.71	6.68
10% L858R	26.96	10% L858R	34.99	8.03
5% L858R	26.96	5% L858R	35.85	8.89
1% L858R	26.83	1% L858R	37.23	10.40

Table 3. Percentage of biopsy or cytology specimens for relevant tumour content threshold

	Tumour content threshold	
	>25%	>10%
Surgical biopsy (n = 135)	90%	98%
Bronchial biopsy (n = 292)	57%	90%
EBUS-TBNA (n = 155)	44%	86%
PCNB (n = 45)	69%	93%
Pleural biopsy (n = 37)	51%	86%

higher Cp values. For exon 21 L858R point mutation, using the Δ Cp 6.4 cut-off threshold, an estimated tumour cell fraction of at least 25% was required for a positive result. On the other hand, the sensitivity of TheraScreen was considered sufficient to truly exclude mutations (based on the assumption that tumour cells are uniformly and not heterogeneously mutant, which is mostly the case) in samples with $\geq 25\%$ tumour cells. In our series, 57% of the diagnostic bronchoscopic forceps biopsy samples and 44% of the diagnostic EBUS-TBNA samples yielded a tumour content of $>25\%$; this was the case for 90% of the surgical biopsies (table 3). Considering a detection threshold of 10% tumour cells, we calculated that 90% of bronchoscopic forceps biopsy samples and 86% of EBUS-TBNA cell-block samples would fulfil this threshold.

Discussion

In patients presenting with advanced stage NSCLC, current routine diagnostic work-up is often based on small FFPE endoscopic samples. Biomarker-driven clinical trials in advanced-stage NSCLC usually accept biopsy specimens only, as TBNA samples are considered more challenging due to the lower neoplastic cell content and suboptimal DNA quantity. We found that the median proportion of neoplastic cells within bronchoscopic forceps biopsy samples was significantly higher than within EBUS-TBNA cell-block samples (30 vs. 20%; $p = 0.026$). DNA extracted from these bronchoscopic forceps biopsy and EBUS-TBNA cell-block samples yielded a median of 1,610 and 1,440 ng DNA, respectively. The overall EGFR mutation rate was 11.2%, without significant differences in EGFR mutation detection rate across different types of tumour tissue specimen.

For clinical mutation analysis, reliable and sensitive methods are needed [2]. This is related to the performance characteristics of the tests, which can be found in commercial package inserts but also depends heavily on sample characteristics. The tumour content of the obtained specimen is a key point. Pathologists should be conservative in their estimation [14–15]. Identifying areas with a maximal tumour content and applying manual macrodissection for tumour cell enrichment is fundamental as this should be above the detection limit of the assay used and should not rely upon the sensitivity of the test to prevent false-negative results or compensate for poor specimens [16]. The tumour content of bronchial forceps biopsy samples in our study was higher than in a study reporting a median at 20% in bronchial biopsies of squamous cell lung cancer or lung adenocarcinoma [17]. This could be related to the fact that a dedicated endoscopist managed to harvest 7 ± 2 forceps biopsies per patient. The DNA extracted from these bronchial forceps biopsy samples is identical to what has been reported by others. A study by Arcila et al. [18] showed an identical amount of DNA extracted from 10 unstained slides (4- μ m thick) from FFPE bronchial biopsies (1,690 ng, range 250–3,600 ng). Reported DNA extraction from TBNA cytology samples generated lower amounts of DNA, i.e. 230 ng (range 120–400 ng [18]) and 282 ng [19], compared to 1,440 ng DNA extracted from EBUS-TBNA samples in our cohort. This observation was most likely related to the number of needle passages taken by the endoscopist, i.e. 5 ± 1 TBNA per patient in our cohort. Therefore, we support a statement made by others that sufficient representative needle passes per sample per cell block are recommended to provide sufficient DNA [4, 20].

For EGFR mutation testing, many laboratories still use either direct sequencing or the ARMS/Scorpion assay for mutation screening. Given the low (11%) prevalence of EGFR mutations in lung adenocarcinoma, every effort should be made to avoid the possibility of false-negative results. Direct Sanger sequencing is regarded as not sensitive enough for small bronchoscopic samples, as a tumour cell fraction of at least 30–50% is required for reliable EGFR mutation detection by this method. More sensitive PCR-based methods such as the Scorpion ARMS have a reported sensitivity of 1% of mutant alleles among wild-type alleles corresponding to a tumour cell content of 2%, and are therefore considered more reliable for EGFR mutation testing on small biopsy samples. An important caveat is that the package inserts of commercial tests usually refer to sample conditions, which cannot be achieved in routine clinical practice. We tested the performance characteristics of the Therascreen assay on a large series of endoscopic and surgical tumour samples obtained in routine clinical practice. On the one hand, our dilution experiment demonstrated that the detection limit of the Therascreen assay on FFPE small bronchoscopic specimens was substantially inferior to the advertised 1%. Based on the assumption that tumour cells are uniformly and not heterogeneously mutant, a tumour cell content of $\geq 25\%$ was required for a reliable wild-type result. In bronchoscopic samples with a tumour content of $< 25\%$, the possibility of potential false-negative findings should be considered. On the other hand, the frequency of activating EGFR mutations in our bronchoscopic samples was 10.5%, no different from what has been reported in multi-institutional lung cancer molecular testing platforms in Germany or France [21, 22]. The reason could be that the ability to detect mutations is limited by the mutant-allele frequency and not only by the histopathologic estimate of the tumour cell content [7]. In addition, we considered a false-positive result as unlikely, as we never encountered a Cp > 36 for exon 19 deletions or a Cp > 39 for exon 21 mutations in our bronchoscopic tumour samples.

For anaplastic lymphoma kinase (ALK) status testing, fluorescent in situ hybridization (FISH) is currently considered the gold standard. ALK-FISH can be performed on paraffin-embedded tissue material, but its interpretation may be difficult because of signal loss by section artifacts, target DNA integrity or incomplete penetration of probes. Several advantages (e.g. assessment of the entire cell nucleus) can be envisioned for performing ALK-FISH directly on ThinPrep slides compared to slides derived from FFPE cell blocks [23]. A large NSCLC cohort with liquid-based cytology material (mainly TBNA specimens) available for ALK status testing demonstrated that the routine use of ThinPrep-FISH is feasible and can reliably detect ALK gene rearrangements [23].

In conclusion, the integration of EBUS-TBNA and bronchoscopic forceps biopsy specimens can result in accurate analysis of the molecular alterations in patients with advanced-stage NSCLC, on the condition that the endoscopist takes enough tumour samples. Clinically applicable molecular testing platforms are evolving and should consider these small bronchoscopic tumour samples.

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None declared for all authors.

References

- 1 Rekhtman N, Brandt S, Sigel C, et al: Suitability of thoracic cytology for new therapeutic paradigms in non-small cell lung carcinoma: high accuracy of tumour subtyping and feasibility of EGFR and KRAS molecular testing. *J Thorac Oncol* 2011;6:451–458.
- 2 Thunnissen E, Kerr K, Herth F, et al: The challenge of NSCLC diagnosis and predictive analysis on small samples: practical approach of a working group. *Lung Cancer* 2012;76:1–18.
- 3 Lindeman N, Cagle P, Beasley M, et al: 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 Mol Diagn* 2013;15:415–453.

- 4 Kossakowski C, Morresi-Hauf A, Schnabel P, et al: Preparation of cell blocks for lung cancer diagnosis and prediction: protocol and experience of a high-volume center. *Respiration* 2014;87:432–438.
- 5 Goto K, Satouchi M, Ishii G, et al: An evaluation study of EGFR mutation tests utilized for non-small-cell lung cancer in the diagnostic setting. *Ann Oncol* 2012;23:2914–2919.
- 6 Pirker R, Herth F, Kerr K, et al: Consensus for EGFR mutation testing in non-small cell lung cancer: results from a European workshop. *J Thorac Oncol* 2010;5:1706–1713.
- 7 Querings S, Altmüller J, Ansén S, et al: Benchmarking of mutation diagnostics in clinical lung cancer specimens. *PLoS One* 2011;6:e19601.
- 8 Van Krieken J, Jung A, Kirchner T, et al: KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for a European quality assurance program. *Virchows Arch* 2008;453:417–431.
- 9 Li T, Kung H, Mack P, Gandara D: Genotyping and genomic profiling of non-small cell lung cancer: implications for current and future therapies. *J Clin Oncol* 2013;31:1039–1049.
- 10 Paik P, Hasanovic A, Wang L, et al: Multiplex testing for driver mutations in squamous cell carcinomas of the lung (abstract 7505). *J Clin Oncol* 2012;30(suppl).
- 11 Sequist LV, Heist RS, Shaw AT, et al: Implementing multiplexed genotyping of non-small-cell lung cancers into routine clinical practice. *Ann Oncol* 2011;22:2616–2624.
- 12 Kris M, Johnson B, Kwiatkowski D, et al: Identification of driver mutations in tumour specimens of 1,000 patients with lung adenocarcinoma: the NCI's Lung Cancer Mutation Consortium (LCMC). *J Clin Oncol* 2011;29:477.
- 13 Frampton G, Fichtenholtz A, Otto G, et al: Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. *Nat Biotechnol* 2013;31:1023–1031.
- 14 Viray H, Li K, Long TA, et al: A prospective, multi-institutional diagnostic trial to determine pathologist accuracy in estimation of percentage of malignant cells. *Arch Pathol Lab Med* 2013;137:1545–1549.
- 15 Smits A, Kummer J, de Bruin P, et al: The estimation of tumor cell percentage for molecular testing by pathologists is not accurate. *Mod Pathol* 2014;27:168–174.
- 16 Kerr K: Personalized medicine for lung cancer: new challenges for pathology. *Histopathology* 2012;60:531–546.
- 17 Coghlin C, Smith L, Bakar S, et al: Quantitative analysis of tumor in bronchial biopsy specimens. *J Thorac Oncol* 2010;5:448–452.
- 18 Arcila M, Oxnard G, Nafa K, et al: Rebiopsy of lung cancer patients with acquired resistance to EGFR inhibitors and enhanced detection of the T790M mutation using a locked nucleic acid-based assay. *Clin Cancer Res* 2011;17:1169–1180.
- 19 Van Eijk R, Licht J, Schruppf M, et al: Rapid KRAS, EGFR, BRAF and PIK3CA mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. *PLoS One* 2011;6:e17791.
- 20 Gately K, O'Flaherty J, Cappuzzo F, et al: The role of the molecular footprint of EGFR in tailoring treatment decisions in NSCLC. *J Clin Pathol* 2012;65:1–7.
- 21 Zander T, Heukamp LC, Bos MCA, et al: Regional screening network for characterization of the molecular epidemiology of non-small cell lung cancer (NSCLC) and implementation of personalized treatment. *J Clin Oncol* 2012;30(suppl):663.
- 22 Barlesi F, Blons M, Beau-Faller M, et al: Biomarkers (BM) France: results of routine EGFR, HER2, KRAS, BRAF, PI3KCA mutations detection and EML4-ALK gene fusion assessment on the first 10,000 non-small cell lung cancer (NSCLC) patients (pts abstract 8000). *J Clin Oncol* 2013;31(suppl).
- 23 Minca E, Lanigan C, Reynolds J, et al: ALK status testing in non-small cell lung carcinoma by FISH on ThinPrep slides with cytology material. *J Thorac Oncol* 2014;9:464–468.