UvA-DARE (Digital Academic Repository)

EGFR and KRAS quality assurance schemes in pathology: generating normative data for molecular predictive marker analysis in targeted therapy

Thunnissen, E.; Bovée, J.V.M.G.; Bruinsma, H.; van den Brule, A.J.C.; Dinjens, W.; Heideman, D.A.M.; Meulemans, E.; Nederlof, P.; van Noesel, C.; Prinsen, C.F.M.; Scheidel, K.; van de Ven, P.M.; de Weger, R.; Schuuring, E.; Ligtenberg, M.

[Link to publication](#)

Citation for published version (APA):

EGFR and KRAS quality assurance schemes in pathology: generating normative data for molecular predictive marker analysis in targeted therapy

Erik Thunnissen,[1] Judith V M G Bovée,[2] Hans Bruinsma,[3] Adriaan J C van den Brule,[4] Winand Dinjens,[5] Daniëlle A M Heideman,[1] Els Meulemans,[6] Petra Nederlof,[7] Carel van Noesel,[8] Clemens F M Prinsen,[9] Karen Scheidel,[10] Peter M van de Ven,[11] Roel de Weger,[12] Ed Schuuring,[13] Marjolijn Ligtenberg[14,15]

For numbered affiliations see end of article.

Correspondence to
Dr Erik Thunnissen, Department of Pathology, VU Medical Center, De Boelelaan 1117, 1081 HV Amsterdam 1081HV, The Netherlands; e.thunnissen@vumc.nl

ABSTRACT

Introduction The aim of this study was to compare the reproducibility of epidermal growth factor receptor (EGFR) immunohistochemistry (IHC), EGFR gene amplification analysis, and EGFR and KRAS mutation analysis among different laboratories performing routine diagnostic analyses in pathology in The Netherlands, and to generate normative data.

Methods In 2008, IHC, in-situ hybridisation (ISH) for EGFR, and mutation analysis for EGFR and KRAS were tested. Tissue microarray sections were distributed for IHC and ISH, and tissue sections and isolated DNA with known mutations were distributed for mutation analysis. In 2009, ISH and mutation analysis were evaluated. False-negative and false-positive results were defined as different from the consensus, and sensitivity and specificity were estimated.

Results In 2008, eight laboratories participated in the IHC ring study. In only 4/17 cases (23%) a consensus score of ≥75% was reached, indicating that this analysis was not sufficiently reliable to be applied in clinical practice. For EGFR ISH, and EGFR and KRAS mutation analysis, an interpretable result (success rate) was obtained in ≥97% of the cases, with mean sensitivity ≥96% and specificity ≥95%. For small sample proficiency testing, a norm was established defining outlier laboratories with unsatisfactory performance.

Conclusions The result of EGFR IHC is not a suitable criterion for reliably selecting patients for anti-EGFR treatment. In contrast, molecular diagnostic methods for EGFR and KRAS mutation detection and EGFR ISH may be reliably performed with high accuracy, allowing treatment decisions for lung cancer.

INTRODUCTION

Recently, an empirical treatment approach with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) showed spectacular responses in a subset of patients with advanced non-small cell lung cancer (NSCLC).[1–4] In those patients, EGFR mutations and DNA amplifications were detected, and several studies have since been performed on the adjuvant treatment of NSCLC with EGFR TKIs. For prediction of response to EGFR TKI treatment, EGFR gene copy number,[5] EGFR protein expression, EGFR mutation[4 6] and KRAS mutation are informative.[5 7–9] However, in those studies, biomarker analysis was performed in central laboratories.

Before the biomarkers can be used to select patients for this novel type of treatment, it is necessary to evaluate the reproducibility of testing by different laboratories. Recently, recommendations for the use of EGFR molecular assays have been reported, and these include guidelines for tissue storage, handling and processing.[10] In addition, recommendations for the standardisation of molecular assays have been discussed. For mutation analysis, sequencing has been the procedure of choice, although the development of more rapid and sensitive techniques is awaited. For gene copy number changes, fluorescence in-situ hybridisation (FISH) analysis was preferred, as true amplification of the EGFR gene in NSCLC is less frequent as polysomy. Since then, high correlation between EGFR FISH and chromogenic in-situ hybridisation (CISH) analysis has been shown.[11 12] For colorectal cancer, a norm for mutation analysis has been published recently,[13] and the required level of minimum performance was based on arbitrary grounds. For lung cancer, suggestions for a norm in mutation and amplification analysis have not been published.

Proficiency testing for molecular diagnostic pathology has been performed in The Netherlands for more than a decade.[14] In addition to intra-laboratory validation of any novel molecular assay for diagnostic purposes, the performance of molecular testing in different laboratories needs to be assessed before using new molecular markers in daily healthcare procedures. Therefore, in 2008 and 2009, EGFR and KRAS analysis was evaluated nationwide. In two ring studies, the reproducibility was evaluated and a performance level for mutation and amplification analysis obtained.

MATERIALS AND METHODS

Ring study design

In 2008 and 2009, a ring study for quality control of EGFR and KRAS was performed. Organisation of the ring study was according to a previously described procedure,[14] which was basically that a similar set of blinded samples was distributed to different laboratories, test results were returned to a central location, and after decoding they were summarised as a consensus result, thereby

delineating outliers. Subsequently, overall outcome was written in a concept report, distributed to participants for comments, and then finalised. Monitoring quality control of daily practice was performed with ethical approval according to 'code goed gebruik'. A letter of invitation was sent from a central office to all pathology laboratories in The Netherlands. According to the Dutch Pathology Society, participation in ring studies for proficiency testing has been part of the guidelines for molecular diagnostics in pathology since 2005, and therefore strongly recommended. Laboratories could sign up for one or more of the tests offered. In 2008, laboratories tested immunohistochemistry (IHC) for EGFR, in-situ hybridisation (ISH) for EGFR, and mutation analysis for EGFR and KRAS. Formalin-fixed paraffin-embedded NSCLC specimens and DNA isolated from cell lines were used. Based on the results obtained in 2008, IHC testing was omitted in 2009. An overview of the samples distributed is shown in table 1.

From 15 lung cancer resection specimens with known outcome of mutation analysis for EGFR and KRAS, as determined by cycle sequencing[15] alone or by high resolution melting followed by cycle sequencing,[16][17] 6 mm cores were used to prepare a tissue microarray (TMA). In two of the cases a second core was taken from a different paraffin block, resulting in 17 TMA samples for 2008. In 2009, one core was removed to avoid possible recognition based on orientation of the array. After cutting sections for 80 slides, only 13 cores remained with sufficient tumour in the cores, including one of the cases with a duplicate core. The starting point for numbering the cores within the TMA was different each year.

To test EGFR IHC variability between the different laboratories, two unstained TMA slides of 3 μm were sent to each participant. Participants were asked to use the following scoring system (adapted from Tsao et al[15]) for IHC data: 0, no staining visible; 1, weak membranous staining in ≤10% tumour cells; 2, moderate/continuous membranous staining in >10% tumour cells; 3, strong and continuous membranous staining in >10% of the tumour cells.

For EGFR ISH analysis two unstained TMA slides of 3 μm thickness in 2008 and 5 μm thickness in 2009 were distributed. For consistent data collection, participants were asked to use an EGFR copy number scoring system similar to the already routinely used HER2/neu testing in molecular diagnostic pathology laboratories in The Netherlands, according to the guidelines of the American Society of Clinical Oncology (ASCO)/College of Amercian Pathologists (CAP)[18]: 0, no amplification; 1—5, copies/nucleus in >50% tumour cells; 1, low amplification, small clusters or 6—10 copies/nucleus in >50% of tumour cells; 2, amplification, large clusters or >10 copies/nucleus in >50% of tumour cells. Since only the presence or absence of amplification is important in clinical practice, the categories between 0 and 1 were grouped together to generate one group for no amplification.

To estimate the concordance in EGFR and KRAS mutation analysis in 2008, DNA isolated from three cell lines with known mutations at a concentration of 10 ng/μl, and two 10 μm paraffin-embedded sections with a tissue area >10×10 mm^2 from two cases of pulmonary adenocarcinoma, were distributed. For each tumour specimen, one H&E-stained slide of 4 μm thickness was also included.

In 2009, four samples of a dilution series of DNA isolated from a cell line with a known mutation were mixed with DNA from another cell line with wild-type for that gene at a concentration of 30 ng/μl. In addition, one 4 μm section of the TMA along with the 13 samples were distributed for the mutation analysis of EGFR and KRAS, and tumour cells from each case should have been scraped off the TMA slide for DNA extraction. An additional section of the TMA was included for H&E staining to allow evaluation of tumour percentage by the participant. In 2008 and 2009, mutation analysis was performed according to Lynch et al[2] by participants CP, KS, RdW, EM and ML, and according to van Zandwijk et al[19] by participants PN, ES, AvdB and CvN. Mutation analysis was performed according to Janmaat et al[20] in 2008, and according to Kramer et al[17] and Heideman et al[17] in 2009, by participant DH.

Participating laboratories received coded cases and were unaware of the content with respect to presence or absence of mutations, EGFR amplification or EGFR expression status. Submission of results had to be within 6 weeks of receiving the tests.

Data analysis

For evaluation of the outcome of IHC and ISH, a consensus score using an arbitrarily chosen consensus threshold of 75% was used. Results denoting a 'not assessable' (NA) answer were not included in the calculation of the consensus score. The success rate (percentage) of the results of all laboratories combined was calculated as 1—fraction of NA results, this rate denotes the percentage of total requested tests resulting in an (incorrect or correct) answer.[13] Sensitivity and specificity were calculated compared with the consensus score, not taking the NA results into account. Results that differed from the consensus were considered to be either false-negative or false-positive results. For example, if most (>75%) laboratories detect a KRAS codon 12 mutation, but another laboratory identified the wrong mutation, this may have the same clinical relevance, but it was scored for technical error: false positive.

RESULTS
EGFR immunohistochemistry

In 2008, eight laboratories participated in the IHC ring study. The IHC results for the TMA and details of methods used are shown in table 2. In the eight laboratories, seven different antibodies were used. For six of the seven antibodies the epitope recognition site was on the extracellular domain of the EGFR protein and for the other it was on the intracellular domain of the EGFR protein.

The range of variation in high and low expression scores between laboratories was remarkable. For only four of 17 cases (23%, nos 4, 7, 9 and 14) the consensus threshold of 75% was reached, with a score range of one step difference maximum. For three cases, five of the eight laboratories (63%) showed the same result; one case showed a one step difference in score (range 2—3), and the other two showed a two step difference in score (range 0—2). In four samples the range of scores varied from absence of staining to strong membranous staining (scores 0—3).

Table 1 Overview of materials distributed in the ring studies of 2008 and 2009 according to test purpose

Study year	IHC	ISH	Mutation analysis
2008	TMA sections (n=17)	TMA sections (n=17)	Isolated DNA (n=3) from cell lines, paraffin sections (n=2)
2009	—	TMA sections (n=13)	Isolated DNA (n=4) from cell lines TMA (n=13)

IHC, immunohistochemistry; ISH, in-situ hybridisation; TMA, tissue microarray with 6 mm cores (for TMA sections, n is the number of cores on one slide).

Original article

Table 2 EGFR IHC scores and test details for tissue samples in each laboratory

Tissue sample	Laboratory							
	A	B	C	D	E	F	G	H
1	0	0	0	1	3	3	1	1
2	1	1	1	2	3	3	1	2
3	1	1	0	0	1	1	1	2
4	0	0	0	0	0	0	0	0
5	1	1	0	0	2	2	1	1
6	1	0	0	0	0	1	0	0
7	2	1	0	0	3	3	2	3
8	2	1	0	1	3	3	1	1
9	0	0	0	0	1	2	1	0
10	3	3	3	3	3	3	3	3
11	1	1	0	1	2	2	1	1
12	2	3	1	2	3	3	2	3
13	3	2	2	2	3	3	2	2
14	3	1	0	1	3	3	1	2
15	2	1	0	0	2	3	1	0
16	1	0	0	0	1	1	0	0
17	3	1	2	1	1	3	3	0
Antibody	EGFR.113*	Clone 31G7‡	Clone 111.6†	Clone 111.6†	Clone 31G7‡	Clone EGFR.25*	Clone 30§	Clone 2-18c9§
Epitope	Extracellular	Extracellular	Extracellular	Extracellular	Extracellular	Cytoplasm domain	Extracellular	Extracellular
Dilution	1:125, 45 min	1:40	1:50	1:400, 60 min RT	1:200, o/n 4°C	1:25	1:25	$
Epitope retrieval	10 min Tris-EDTA pH 9	Boiling DM809 buffer*** pH 6.1 10 min	Protease 2, 32 min‡‡	Protease type XIV, 0.05%, 5 min	Protease, 0.1 M, 15 min RT	Tris-EDTA pH 8.8, ER2**	Protease 8 min¶	Prot K 5 in RT
Staining	PowerVision, 30 min RT‡‡	Envision DM802§	††	PowerVision¶¶	PowerVision	PowerVision§§		$

The number and content of each tissue microarray sample is as described in the legend for Table 3. The laboratory codes do not correspond to those in Table 3.

Lab A uses 1) Poseidon Amsterdam, NL; Lab B uses 2) Vysis, Hoofddorp, NL; Lab C uses 2) Vysis, Hoofddorp, NL; Lab D uses 2) Vysis, Hoofddorp, NL; Lab E uses 3) Zytovision, Immunologic, Duiven NL; Lab F uses 3) Zytovision, Immunologic, Duiven NL; Lab G uses 3) Zytovision, Immunologic, Duiven NL; Lab H uses 4) DAKO, Glostrup, DK.

*Novacastra, Newcastle, UK.
†NeoMarkers; Stratech Scientific, Newmarket, UK.
‡Zymed.
§Dako, Glostrup, Denmark
¶Ventana prepkit.
**Bond-maX.
††Ventana Benchmark XT.
‡‡nr pdvo-hrp 110.
§§Vision biosystems Bond-maX.
¶¶Immunologic via klinipath.
$Pharm Dx IHC Kit
EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; o/n, overnight.
RT = Room Temperature

Hardly any of the IHC EGFR expression patterns correlated with *EGFR* amplification or mutation status. The staining results for samples of the same tumour (nos 3 and 5) were the same for five laboratories, and there was a one score difference for the other three laboratories, indicating more consistency within the laboratories than among the laboratories. In 2009, IHC was not part of the proficiency testing.

EGFR in-situ hybridisation
In 2008, eight laboratories participated in ISH studies. Five laboratories used FISH and three used CISH. The ISH results are shown in table 3.

One laboratory had a success rate of 24%, while for the other laboratories the success rate was over 97%. Two laboratories using CISH had difficulties in obtaining reliable results: 15 of 51 cases (30%) could not be evaluated with CISH in contrast to only 1/102 (1%) with FISH. For the score range 0/1 and 2, a consensus score was obtained in 15/17 cases. For the distinction of 'amplification' (that is, score 2) versus 'no amplification' (that is, score 0 or 1) all cases reached the consensus score. One FISH laboratory missed the amplification in all three cases with amplification.

In 2009, eight laboratories delivered results for ISH. Five laboratories used FISH and three used CISH. An additional laboratory did not submit data because they wanted more slides for reliable estimation of amplification with CISH analysis. For the score range 0/1 versus 2, a 100% consensus score was obtained in all 13 cases using FISH, and in 11/13 using CISH. Two laboratories using CISH had difficulties in getting reliable information from the slide. In 2009, the overall consensus was 100% for FISH and CISH when non-evaluated slides were not considered as a false interpretation.

EGFR and *KRAS* mutation analysis
In 2008, 10 laboratories submitted results for the mutation analysis. The technique used in all laboratories was PCR followed by direct sequencing. One laboratory performed *KRAS* analysis only, since in case *KRAS* is wild-type, they send the case to another laboratory for *EGFR* mutation analysis. Exons 19 and 21 of *EGFR* were analysed by all nine laboratories. Exons 18 and 20 were tested by five and four laboratories, respectively. All the laboratories using sequence analysis indicated that the test might produce a false-negative result in cases with <50% tumour cells. For samples with <50% tumour cells, one

Table 3 ISH scores for tissue microarray samples for each laboratory participating in 2008 (n=17 samples) and/or 2009 (n=13 samples)

TMA	ISH	A		B	C		D		E	F*		G*		H*		I	J	N
		08	09	09	08	09	08	09	09	08	09	08	09	08	09	09	08	08
1		0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0
2		0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0
3		0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0
4		0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0
5		0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0
6		0	0/1	0	0	1	0	0	0	0	0	0	—	0	—	0	0	0
7		0	0	0	0	1	1	0	0	0	0	—	—	0	0	0	0	0
8		0	0	0	0	0	1	0	0	0	0	0	—	0	0	0	0	0
9		0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
10	AMP	1!	2	2	2	2	2	2	2	2	2	2	—	2	2	2	2	2
11		0	0	0	0	1	1	0	0	0	—	—	1	0	0	0	0	0
12		0	0	—	0	0	0	0	0	0	0	0	—	0	0	0	0	0
13	AMP	1!	2	2	2	2	2	1—2	2	2	2	2	—	2	2	1—2	2	2
14		1			1		1			0		1		0			0	1
15		0			1		1			0		2!		0			1	0
16		0			0		1			0		—		—			0	1
17	AMP	1!			2		2			2		2		2			2	2

Three cases had consensus score of 2 indicating amplification (AMP). Mono-coloured chromogenic in-situ hybridisation analysis was used in laboratories F—H (*), the other laboratories used dual-coloured fluorescence in-situ hybridisation. !, wrong answer (false positive or false negative); —, the test was received by the laboratory, but no test result was provided.
ISH, in-situ hybridisation; TMA, tissue microarray.

laboratory analysed exon 19 for fragment length alterations by using GeneScan (Applied Biosystems, Foster City, California, USA) and the exon 21 p.Leu858Arg mutation after digestion of the template DNA by the restriction enzyme Sau96I, which recognises only wild-type DNA at this position.

The results for isolated DNA in 2008 are shown in table 4. A consensus score was reached in all five samples. The results for the three DNA isolates were highly concordant. In DNA isolate 3, apart from the p.Leu858Arg mutation in exon 21, an additional p.Thr790Met mutation was detected by three out of the four laboratories examining exon 20 in this case: that is, one false-negative result. The percentage of tumour cells in the two paraffin samples was estimated to be at least 50% by all laboratories.

The p.Gly12Cys mutation in slide 1 and the p.Gly12Ala mutation in slide 2 were detected with a high rate of concordance. For slide 1, one laboratory reported a false-negative result and two laboratories could not give a reliable answer. Laboratory K performed inadequately in four out of five cases (that is, one false-positive results and three NA cases). The method of reporting mutations was variable due to the fact that almost no laboratory used the consensus nomenclature for mutations.

In 2009, 12 laboratories submitted results for *KRAS* mutation, and nine submitted results for *EGFR* mutation analysis. The techniques used were PCR combined with direct sequencing (n=11) and high-resolution melting followed by cycle sequencing for genotyping aberrantly melted PCR products (n=1). The mutation analysis results for DNA isolates are given in table 4 and for the TMA they are given in table 5. The analysis of the isolates revealed the same *KRAS* mutation in four of the samples, with a high rate of concordance. Only one laboratory obtained a false-negative result in a sample containing approximately 10% tumour cells. No false-positive results were found in the isolate containing wild-type DNA only.

The estimation of the percentage of tumour cells in the TMA samples ranged from 40% to 75% (see figure 1). For mutation analysis a consensus score (≥75%) was reached for 12 out of 13 TMA samples. In TMA sample no. 5, which was derived from the same tumour as sample no. 3, but from another block, seven out of 10 laboratories with a test result detected the same mutation as in TMA no. 3. One laboratory reported a negative result and an estimated tumour fraction below 50%, whereas the two other laboratories reported a negative result with tumour cell percentages of 60% and 65%. In TMA sample no. 7, two laboratories reported a false-positive result, and in TMA sample no. 8 two laboratories reported a false-negative result, although the estimated percentage of tumour cells was ≥50%. In TMA sample no. 13, one laboratory detected a *KRAS* p.Gly12Asp mutation instead of the p.Gly12Ala mutation detected by the other laboratories.

Table 4 *EGFR* and *KRAS* mutation analysis for isolated DNA and paraffin sections

Sample	EGFR	KRAS	A EGFR	A KRAS	B EGFR	B KRAS	C EGFR	C KRAS	D EGFR	D KRAS	E EGFR	E KRAS	F EGFR	F KRAS	G EGFR	G KRAS	H EGFR	H KRAS	I EGFR	I KRAS	J EGFR	J KRAS	K EGFR	K KRAS	L EGFR	L KRAS	M EGFR	M KRAS
2008																												
DNA08 1	WT	p.G12D																					■					
DNA08 2	-5 AA	WT																				■						
DNA08 3	M21	WT																										
Slide 1	WT	p.G12C						▨													■		▨					
Slide 2	WT	p.G12A																					▨					
2009																												
DNA09 1	WT	p.G12C																										
DNA09 2	WT	p.G12C																										
DNA09 3	WT	p.G12C	■																									
DNA09 4	WT	WT																										

EGFR and *KRAS* mutation analyses in 2008 for isolated DNA (DNA08 1—3) and paraffin sections (slides 1 and 2), and in 2009 for isolated DNA (DNA09 1—4), are shown for each laboratory (A—M). DNA samples 1, 2 and 3 contained 100%, 50% and 10% mutated DNA, respectively. Boxes: light grey, consensus score; black, wrong answer (false positive or false negative); grey, the test was received by the laboratory, but no test result was provided; white, no participation in the test.
WT, wild type; —5 AA, c.2235_2249del (p.Glu746_Ala750del or p.E746_A750del); M21, mutation exon 21: p.Leu858Arg cThr2573Gly, and in some laboratories pThr790Met also.

Original article

Table 5 Scores for tissue microarray samples for each laboratory participating in mutation analysis of *EGFR* and/or *KRAS* in 2009

| TMA	EGFR	KRAS	A EGFR	A KRAS	B EGFR	B KRAS	C EGFR	C KRAS	D EGFR	D KRAS	E EGFR	E KRAS	F EGFR	F KRAS	G EGFR	G KRAS	H EGFR	H KRAS	I EGFR	I KRAS	K KRAS	L KRAS	M KRAS
1		G12C																					
2	-5 AA																						
3		G12D																			■			
4																							
5		G12D				%															■	%	■
6																							
7											■		■										
8	+3 AA												■				■		■				
9	-5 AA																						
10																							
11																							
12	-5 AA																						
13	-4 AA																						

Scores for tissue microarray (TMA) samples (1—13) are shown for each laboratory (A—M) participating in mutation analysis of *EGFR* and/or *KRAS* in 2009. Boxes: light grey, consensus score; black, wrong answer (false positive or false negative); grey, the test was received by the laboratory, but no test result was provided. %, wrong answer with low percentage of tumour cells, implying that in a clinical setting the test would not be performed because the tumour cell fraction was too low. TMA samples: 1, *KRAS* mutation c.34G>T (p.Gly12Cys or p.G12C); 2, *EGFR* mutation c.2235_2249del (p.Glu746_Ala750del or p.E746_A750del); 3 and 5, *KRAS* mutation c.35G>A (p.Gly12Asp or p.G12D); 8, *EGFR* mutation c.2303_2311dup (p. Ser768_Asp770dup or p.S768_D770dup); 9, *EGFR* mutation c.2235_2249del (p.Glu746_Ala750del or p.E746_A750del); 12, *EGFR* mutation c.2240_2254del (p.Leu747_Thr751del or p.L747_T752del); 13, *EGFR* mutation c.2237_2253delinsTTGCT (p.Glu746_Thr751delinsValAla or p.E746_T751delinsVA). Samples 3 and 5 are duplicates. 4,6,7,10 and 11 are wild type = no mutation for *EGFR* or *KRAS*.

An overview of proficiency testing for 2008 and 2009 is shown in table 6. In 2008, the overall success rates for *EGFR* ISH, and *EGFR* and *KRAS* mutation analyses, were 89%, 98% and 88%, respectively. If two outlier laboratories with a low success rate for either *EGFR* ISH (24%) or *KRAS* mutation analysis (40%) were not taken into account, the success rate of all three tests was 98%. In 2009, the success rates for *EGFR* ISH, and *EGFR* and *KRAS* mutation analyses, were 97%, 99% and 99%, respectively.

The mean sensitivity of these analyses varied between 80% and 100% in 2008, and between 94% and 100% in 2009. The mean specificity of these analyses varied between 95% and 100% in 2008, and 98% and 100% in 2009. The mean accuracy was in the range of 93—100% when all laboratories were included. When the outlier laboratories were not included in the analyses, the range for mean accuracy for the four tests was 97—100%.
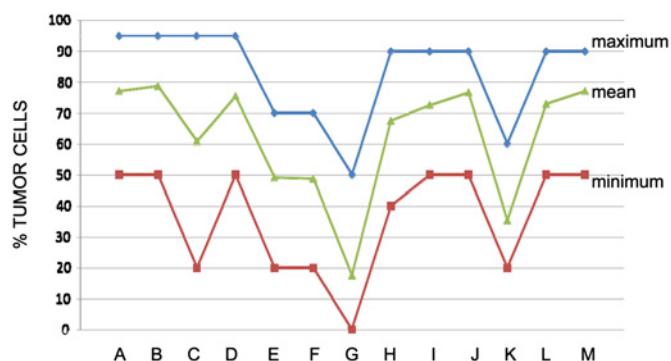
Figure 1 The minimum (red), mean (green) and maximum (blue) percentage of tumour cells scored for each tissue microarray sample in the 2009 ring study. For direct sequencing analysis, the laboratories reported that the test might be false negative due to sampling when a mutation was not detected in cases of estimated tumour percentage <50%.

DISCUSSION

This study describes the first nationwide proficiency testing for *EGFR* and *KRAS* mutation analysis, *EGFR* gene amplification analysis and EGFR expression. For EGFR expression analysis with IHC a consensus score of only 23% of the cases was reached, denoting a high variation in IHC results for most of the cases. Therefore, without further standardisation, EGFR expression analysis by IHC was considered not suitable for use in daily practice. In contrast, ISH and mutation analysis for *EGFR* and *KRAS* testing leads to a mean success rate of ≥97%, a sensitivity of ≥96% and a specificity of ≥95%.

To generate a norm for adequate molecular testing we propose that two cut-off levels should be chosen: one for the success rate (the percentage of correct answers is calculated as 100% minus the percentage of cases not leading to a test result and error cases combined), and one for the error level in the test outcome (the sum of false-positive and false-negative cases per test). In this study, two laboratories with success rate of 24—40% for ISH and *KRAS* mutation testing, respectively, clearly performed below a reasonable threshold. For the other laboratories taken together, the success rate was over 97%. To set a norm for success rate in a ring study with a limited number of test samples (<14) our study suggests that a maximum allowed error of one sample per test is reasonable. With this norm, two outlier laboratories were identified: one with two false-negative results and one with three false-negative results.

In theory, a 90% Bayesian confidence interval (CI) may be calculated for a number of samples in proficiency testing, see table 7. For instance, in a laboratory with eight out of 10 correct answers in a proficiency testing study, the chance is 90% that between 53% and 92% of the routine analyses will be correct. The chance is 5% that in daily practice this laboratory will generate the correct answer for more than 92% of cases. As it seems reasonable to set a norm in proficiency testing at the smallest number of correct cases where the upper limit of the 90% CI is above the 95%, theoretically in a setting of 10 cases at least nine cases should be correct.

Table 6 Overview of proficiency testing for the different tests in 2008 and 2009

	EGFR IHC 2008	EGFR ISH		EGFR mutation		KRAS mutation	
		2008	2009	2008	2009	2008	2009
Consensus	4/17	17/17	13/13	5/5	17/17	5/5	17/17
No. of laboratories	8	8	9	9	9	10	12
NA cases		15/136	3/117	1/45	2/153	4/50	2/204
Success rate (%)	—	89±27*	97±4	98±7	99±4	92±19†	99±4
Positive cases	—	3	3	2	5	3	6
FN cases	—	3/22	0/27	0/18	1/44	1/26	4/71
Sensitivity (%)	—	88±35‡	100±0	100±0	98±7	96±11	94±11§
Negative cases	—	14	10	3	12	2	11
FP cases	—	1/99	0/87	0/26	0/107	1/20	2/131
Specificity (%)	—	96±12¶	100±0	100±0	100±0	95±16	98±4
Accuracy (%)	—	95±10**	100±0	100±0	99±2	93±16††	97±4

Consensus: number of cases for which a consensus was reached (cases with >75% agreement) between laboratories/total number of cases. Success rate: 1−fraction of cases with no answer (NA) that is reported as 'no reliable test result'. Positive/negative cases: number of consensus cases with/without amplification or mutation, respectively. False-negative (FN) and false-positive (FP) cases calculated over cases with reported reliable test result (number of FN or FP cases/total cases).
*Including one outlier laboratory of 24% NA in 13/17 samples, otherwise the result would be 98±4%.
†Including one outlier laboratory of 40%, otherwise the result would be 98±7%.
‡Including outlier of 0%, that is three FN cases in one laboratory, otherwise the result would be 100±0%.
§Including one laboratory with two FN cases, otherwise the result would be 97±7%.
¶Including the outlier laboratory mentioned in * with one FP case (2/3 remaining cases=67%), otherwise the result would be 100±0%.
**Including the outlier laboratory mentioned in *, otherwise the result would be 97±7%.
††Including the outlier laboratory mentioned in †, otherwise the result would be 98±7%
IHC, immunohistochemistry; ISH, in situ hybridisation; —, consensus too low for meaningful application.

For a specific set of samples in proficiency testing (n=10, 14, 20 or 30) the 90% CI (region between the 5th percentile and 95th percentile of the posterior probability distribution) is shown for the success rate (fraction of correct answers×100%). The 90% CI is constructed with Bayesian statistics,[21] assuming uniform prior probability for the success rate on the interval between 0 and 1.

Our data suggest that 97% sensitivity and 97% specificity for mutation detection is feasible. Unfortunately, to determine whether every individual laboratory reaches this level precisely, an unacceptably large amount of samples needs to be tested in a ring study. However, the main aim of proficiency testing, providing external feedback about test performance, was reached with a limited number of samples, as in this study laboratories performing within the proposed error norm and outlier laboratories for a certain test were clearly established. Moreover, the necessity of participating in quality control ring studies of molecular diagnostic pathology is demonstrated.

Recently, for KRAS mutation analysis in colorectal cancer, arbitrary thresholds for success rates of DNA extraction and correct KRAS mutation identification were set at 95% and 97%,

respectively.[13] For lung cancer, a norm has not been recommended in the guidelines for EGFR and KRAS mutation testing.[10] Importantly, our study supports that such mutation and gene amplification analyses can be performed with an average 95% success rate and 97% accuracy. Nevertheless, reporting the accuracy level, and also the sensitivity and specificity data, provides additional information.

In The Netherlands, participation in proficiency testing is part of the guideline, but a norm for proficiency testing is lacking. In the USA, in the 'Clinical Laboratory Improvement Act' of 1988, 'unsatisfactory' performance in proficiency testing is defined as failure to attain the minimum satisfactory score for an analyte for a single testing event. 'Unsuccessful' performance is defined as failure to attain the minimum satisfactory score for either two consecutive or two out of three consecutive events. Failure to return proficiency testing results to the proficiency testing programme within the time frame specified by the programme, or failure to participate, is also an unsatisfactory performance. In the USA (for clinical chemistry and microbiology) when testing a larger number of cases, failure to attain an overall testing event score of at least 80% is considered unsatisfactory. As to the

Table 7 90% posterior probability estimates based on performance in proficiency testing

No. of correct answers	No. of samples					
	10 No. of correct answers	10 90% CI	14	20	30 90% CI	30 No. of correct answers
n/n	10/10	76.2 to 99.5	81.9 to 99.7	86.7 to 99.8	90.8 to 99.8	30/30
n−1/n	9/10	63.6 to 96.7	72.1 to 97.6	79.3 to 98.3	85.6 to 98.8	29/30
n−2/n	8/10	53.0 to 92.1	63.7 to 94.3	72.9 to 96.0	81.1 to 97.3	28/30
n−3/n	7/10	43.6 to 86.5	56.0 to 90.3	67.1 to 93.2	76.8 to 95.5	27/30
n−4/n	6/10	35.0 to 80.0	48.9 to 85.8	61.6 to 90.1	72.9 to 93.4	26/30
n−5/n	5/10	27.1 to 72.9	42.3 to 80.9	56.3 to 86.8	69.0 to 91.2	25/30
n−6/n	4/10	20.0 to 65.0	36.0 to 75.6	51.3 to 83.2	65.3 to 88.9	24/30
n−7/n	3/10	13.5 to 56.4	30.0 to 70.0	46.4 to 79.4	61.7 to 86.5	23/30
n−8/n	2/10	7.9 to 47.0	24.4 to 64.0	41.7 to 75.5	58.2 to 83.9	22/30
n−9/n	1/10	3.3 to 36.4	19.1 to 57.7	37.2 to 71.4	54.8 to 81.3	21/30
n−10/n	0/10	0.5 to 23.8	14.2 to 51.1	32.8 to 67.2	51.5 to 78.7	20/30

For a specific set of samples in proficiency testing (n=10, 14, 20 and 30), the 90% confidence interval (CI), which is the region between the 5th percentile and 95th percentile of the posterior probability distribution) is shown for the success rate (fraction of correct answers×100%). The 90% CI is constructed with Bayesian statistics[21] assuming uniform prior probability for the success rate on the interval between 0 and 1.

consequences of an unsatisfactory performance the responsibility for improvement belongs to the laboratories performing the molecular test. Essential in this respect appears to be an internal quality control and validation programme with external samples. In Europe, the consequences for unsuccessful performance in molecular diagnostics have not yet been established. One option is that the laboratory itself directly withdraws the test from its diagnostic procedures. Alternatively, either the professional organisation or the government should establish consequences.

For the detection of *EGFR* amplification/polysomy in 2008, one laboratory reported three false-negative results, whereas in 2009 results were optimal. Although a high correlation between *EGFR* FISH and CISH analyses has been shown,[11][12] the success rate of FISH was 100% in both years (in total 150 results), but for CISH the success rate was 67% (34 /51) in 2008 and 95% (37/39) in 2009 (see table 6). The low success rate for ISH in 2008 was largely due to a single laboratory (with a success rate of 24%) that had just begun to use *EGFR* ISH. It can be argued that a laboratory should not participate in a ring test as long as the test is not internally validated successfully. The scoring system to detect *EGFR* copy number changes in NSCLC used in this study differed from the more recently published guidelines using the Colorado criteria.[22][23] Interestingly, that scoring system is significantly different from the *HER2* scoring system, mainly because of the more frequent occurrence of polysomy in lung cancer compared with breast cancer. Therefore, the presence of cases with polysomy could affect the consensus rate in proficiency testing when using scoring systems other than the Colorado criteria.

A relation between *EGFR* mutation and gene amplification has been described: *EGFR* gene amplification often accompanies *EGFR* mutation with preferential amplification of the mutant allele.[27] In our study, one out of two amplification-positive cases tested for *EGFR* mutations showed a mutation. For clinical relevance, the molecular diagnostic methods appear to be more important than IHC for the identification of lung adenocarcinoma patients who may benefit from EGFR inhibitor treatments.[28]

In the ring studies of 2008 and 2009 most laboratories did not use standard nomenclature. The *EGFR* mutations were interpreted as correct as long as the correct numbers of nucleotides that were deleted or inserted were mentioned. However, for future reference the use of standard nomenclature is strongly advised. Guidelines for standard nomenclature of mutations can be found on the website of the Human Genome Variation Society (http://www.hgvs.org/rec.html).

Most discordant sequencing data were found in samples containing less than 50% tumour cells; therefore, it is essential to mention the percentage of tumour cells of the material used for DNA isolation and the sensitivity of the assay and/or reliability of the specific analysis in the pathology reports. Remarkably, the variation in estimation of tumour cell percentage varies tremendously between laboratories for tissue cores that, from the first to the last section (4 μm each), were cut within a 400 μm paraffin block thickness (figure 1). This may be due to variation in interpretation between pathologists; for example, confusion between the percentage of nuclei that are in the tumour (the correct measurement) and the percentage area of the sample that is tumour, which is not the correct estimate but is the percentage that pathologists often measure; this will be subject of further research. Of utmost importance is the relation between the fraction of tumour cells in a clinical specimen and the analytical sensitivity of the method used. The latter denotes the threshold of the technique for mutation detection, which can be expressed as number of copies necessary

to detect a signal or the minimum number of mutated cells required to detect a signal in a background of wild-type cells. For PCR and sequencing, a minimum amount of 20−30% tumour cells is required,[29−32] while for other techniques, such as PCR heteroduplex analysis with denaturing high-performance liquid chromatography, this is 1%,[33] for scorpion ARMS methodology it is around or below 1%, for PCR high-resolution melting sequencing it is around 2.5%,[16][34] for peptide nucleic acid clamped PCR it is 1−10%,[35] and for mutation-specific PCR it is 2.5%.[35] Theoretically not only the fraction of tumour cells is relevant, but also the amount of mutated heterozygous or homozygous gene copies per tumour cell, possibly increased by either polysomy[24][36−38] or (mutant allele specific) amplification.[39][40] If this biological phenomenon occurs, the fraction of tumour cells may in theory be slightly lower, but still lead to mutation detection. Therefore, clinical samples harbouring a tumour cell fraction at the detection limit of the technique may be used for mutation detection, but in this situation only a mutation-positive test result will be regarded as informative.

Generally, a deletion mutation is easier to detect than a point mutation. The present series contained only one point mutation sample (DNA08). Indeed, the Thr790Met mutation was detected by only three of the four laboratories that examined exon 20. It is possible that with mutation sequencing analysis a difference in analytical sensitivity may be present between deletion and point mutations, especially in cases where the tumour cell fraction is close to the detection limit.

For proficiency testing in molecular diagnostics (DNA-based ISH/mutation testing) in The Netherlands, a 75% consensus has been reached since 1998 for >95% of the samples (Schuuring E, Thunnissen E unpublished data). In order to achieve a high consensus (for example, US standard 80%), high-quality test samples are required. An interlaboratory survey of *HER2* IHC in breast cancer revealed that 20% of the 'challenges' (samples) showed too much variation in staining to 'be considered valid proficiency testing challenges'. A similar sample number of 25% with low consensus (<80%) was reported in another study for *HER2*,[41][42] and a study for CD117.[43] In fact these data underscore the higher variation present in IHC compared with ISH and mutation analyses.

IHC detection of EGFR expression did not lead to a consensus score in 13 of the 17 cases. This may be explained by: (a) the use of six different antibodies (see table 2), (b) different epitope retrieval approaches,[44] (c) variations in signal enhancement, (d) use of automatic or manual staining, (e) a tissue handling procedure not exactly similar to the one used in house, or (f) variation in interpretation of stained slides. The first three explanations probably have the largest impact. For different antibodies the range of variation is large, with maximum difference of around 30−50% in the number of percentage positive cases.[45−47] Although the epitope-binding site may differ between antibodies, the affinity of binding may be more important, as laboratories E and F had the highest values for IHC scores, while they were targeting different domains, and also using different antigen retrieval methods. Basically, IHC analysis is not quantitative, but can at best be considered a semiquantitative method with certain limits. In a model system on quantitative IHC it was shown that a difference in epitope concentration by a factor of only four was the range between minimum staining and maximum staining intensity.[48] The linear part of the dynamic range of IHC staining is close to a factor of two to three. Moreover after binding of the primary antibody, signal enhancement systems lead to an amplification of four to nine times, which is the difference between

Take-home messages

▶ External quality assurance ring studies are useful to monitor molecular diagnostic tests performed in different pathology laboratories.

▶ Epidermal growth factor receptor (EGFR) production measured by immunohistochemistry shows a high variation between laboratories, and, under the current circumstances, this prohibits its use as biomarker for treatment selection.

▶ *EGFR* mutation and in-situ hybridisation analysis may reliably be performed on a routine basis, allowing wider use of *EGFR* as a biomarker in lung cancer.

a maximum and a negative IHC outcome. Alternative explanations are less likely, such as: (a) the scoring system—scoring was standardised and performed according to a method described previously,[15] which is similar to the *HER2/neu* IHC scoring for breast cancer in the Netherlands[49]; (b) the tissue sample—it was fixed for all laboratories in the same manner; (c) the paraffin TMA block—it was cut at the same time, avoiding possible influences over time[50]; (d) depth levels—these vary only minimally: <400 μm for both ring studies, leaving minimal space for heterogeneity; (e) difference between biopsy and resection—this was not an issue in this study.[47] The lack of consensus for scoring EGFR IHC has also been reported in head and neck squamous cell carcinoma and colorectal carcinoma,[51] and consequently not used for the selection of patients that might benefit from EGFR-targeted therapy. In this type of ring study, it is not necessary to standardise methodology beforehand, as it may well be that the techniques used are different, but that they still lead to the same outcome, as was previously shown for a ring study on tissue identification[14] and also for the outcome of amplification and mutation analysis of this study. Nevertheless, this study clearly shows that further standardisation is required for EGFR IHC and that it should not be used to select lung cancer patients for EGFR TKI therapy at present. It is not excluded that future IHC studies with EGFR mutation-specific antibodies may yield more reproducible results.[52][53] Currently, EGFR IHC plays no role in selection for clinical treatment.

Conclusion

This study describes nationwide proficiency testing for *EGFR* and *KRAS* mutation analysis, EGFR expression, and *EGFR* gene amplification analysis. EGFR expression detected by IHC is not a suitable test for reliably selecting patients for anti-EGFR treatment. In contrast, molecular diagnostic methods for *EGFR* and *KRAS* mutation detection and *EGFR* ISH can be performed reliably in clinical pathology laboratories in The Netherlands.

Author affiliations
[1]Department of Pathology, Vrije Universteit Medical Centre, Amsterdam, The Netherlands
[2]Department of Pathology, Leiden University Medical Centre, Leiden, The Netherlands
[3]Department of Pathology, Sint Elisabeth Ziekenhuis, Tilburg, The Netherlands
[4]Department of Pathology, PAMM Laboratory, Eindhoven, The Netherlands
[5]Department of Pathology, Erasmus University Medical Centre, Rotterdam, The Netherlands
[6]Department of Pathology, Academic Medical Centre, Maastricht, The Netherlands
[7]Department of Pathology, Nederlands Kanker Instituut, Amsterdam, The Netherlands
[8]University of Amsterdam, Department of Pathology, Academic Medical Centre, Amsterdam, The Netherlands
[9]Department of Pathology, Canisius Wilhelmina Ziekenhuis, Nijmegen, The Netherlands
[10]Department of Pathology, Antonius Ziekenhuis, Nieuwegein, The Netherlands
[11]Department of Epidemiology and Biostatistics, Vrije Universiteit Medical Centre, Amsterdam, The Netherlands
[12]Department of Pathology, University Medical Centre, Utrecht, The Netherlands
[13]Department of Pathology, University Medical Centre Groningen, Groningen, The Netherlands
[14]Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[15]Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

REFERENCES

1. **Shepherd FA,** Rodrigues PJ, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005;**353**:123—32.
2. **Lynch TJ,** Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;**350**:2129—39.
3. **Fukuoka M,** Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol* 2003;**21**:2237—46.
4. **Mok TS,** Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;**361**:947—57.
5. **Carlson JJ,** Garrison LP, Ramsey SD, et al. Epidermal growth factor receptor genomic variation in NSCLC patients receiving tyrosine kinase inhibitor therapy: a systematic review and meta-analysis. *J Cancer Res Clin Oncol* 2009;**135**:1483—93.
6. **Rosell R,** Moran T, Queralt C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009;**361**:958—67.
7. **Dahabreh IJ,** Linardou H, Siannis F, et al. Somatic EGFR mutation and gene copy gain as predictive biomarkers for response to tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res* 2010;**16**:291—303.
8. **Linardou H,** Dahabreh IJ, Kanaloupiti D, et al. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet Oncol* 2008;**9**:962—72.
9. **Gupta R,** Dastane AM, McKenna RJ, et al. The predictive value of epidermal growth factor receptor tests in patients with pulmonary adenocarcinoma: review of current "best evidence" with meta-analysis. *Hum Pathol* 2009;**40**:356—65.
10. **Eberhard DA,** Giaccone G, Johnson BE. 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* 2008;**26**:983—94.
11. **Sholl LM,** John IA, Chou YP, et al. Validation of chromogenic in situ hybridization for detection of EGFR copy number amplification in nonsmall cell lung carcinoma. *Mod Pathol* 2007;**20**:1028—35.
12. **Yoo S,** Lee H, Park J, et al. Reliability of chromogenic in situ hybridization for epidermal growth factor receptor gene copy number detection in non-small-cell lung carcinomas: A comparison with fluorescence in situ hybridization study. *Lung Cancer* 2010;**67**:301—5.
13. **van Krieken JHJM,** Jung A, Kirchner T, et al. KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. *Virchows Arch* 2008;**453**:417—31.
14. **Thunnissen FB,** Tilanus MG, Ligtenberg MJ, et al. Quality control in diagnostic molecular pathology in the Netherlands; proficiency testing for patient identification in tissue samples. *J Clin Pathol* 2004;**57**:717—20.
15. **Tsao MS,** Sakurada A, Cutz JC, et al. Erlotinib in lung cancer - molecular and clinical predictors of outcome. *N Engl J Med* 2005;**353**:133—44.
16. **Kramer D,** Thunnissen FB, Gallegos-Ruiz MI, et al. A fast, sensitive and accurate high resolution melting (HRM) technology-based assay to screen for common K-ras mutations. *Cell Oncol* 2009;**31**:161—7.
17. **Heideman DA,** Thunnissen FB, Doeleman M, et al. 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* 2009;**31**:329—33.
18. **Wolff AC,** Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007;**25**:118—45.

19. **van Zandwijk N,** Mathy A, Boerrigter L, *et al*. EGFR and KRAS mutations as criteria for treatment with tyrosine kinase inhibitors: retro- and prospective observations in non-small-cell lung cancer. *Ann Oncol* 2007;**18**:99—103.

20. **Janmaat ML,** Rodriguez JA, Gallegos-Ruiz M, *et al*. Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or phosphatidyl inositol-3 kinase pathways in non-small cell lung cancer cells. *Int J Cancer* 2006;**118**:209—14.

21. *Bayesian data analysis*. Boca Raton: Chapman & Hall/CRC, 2003.

22. **Varella-Garcia M.** Stratification of non-small cell lung cancer patients for therapy with epidermal growth factor receptor inhibitors: the EGFR fluorescence in situ hybridization assay. *Diagn Pathol* 2006;**1**:19.

23. **Varella-Garcia M,** Diebold J, Eberhard DA, *et al*. EGFR fluorescence in situ hybridisation assay: guidelines for application to non-small-cell lung cancer. *J Clin Pathol* 2009;**62**:970—7.

24. **Cappuzzo F,** Hirsch FR, Rossi E, *et al*. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* 2005;**97**:643—55.

25. **Hirsch FR,** Varella-Garcia M, McCoy J, *et al*. 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* 2005;**23**:6838—45.

26. **Hirsch FR,** Varella-Garcia M, Bunn PA Jr, *et al*. Molecular predictors of outcome with gefitinib in a phase III placebo-controlled study in advanced non-small-cell lung cancer. *J Clin Oncol* 2006;**24**:5034—42.

27. **Li AR,** Chitale D, Riely GJ, *et al*. EGFR mutations in lung adenocarcinomas: clinical testing experience and relationship to EGFR gene copy number and immunohistochemical expression. *J Mol Diagn* 2008;**10**:242—8.

28. **Pinter F,** Papay J, Almasi A, *et al*. Epidermal growth factor receptor (EGFR) high gene copy number and activating mutations in lung adenocarcinomas are not consistently accompanied by positivity for EGFR protein by standard immunohistochemistry. *J Mol Diagn* 2008;**10**:160—8.

29. **Engelman JA,** Mukohara T, Zejnullahu K, *et al*. Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. *J Clin Invest* 2006;**116**:2695—706.

30. **Fan X,** Furnari FB, Cavenee WK, *et al*. 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* 2001;**18**:1023—6.

31. **Bosari S,** Marchetti A, Buttitta F, *et al*. Detection of p53 mutations by single-strand conformation polymorphisms (SSCP) gel electrophoresis. A comparative study of radioactive and nonradioactive silver-stained SSCP analysis. *Diagn Mol Pathol* 1995;**4**:249—55.

32. **Park S,** Holmes-Tisch AJ, Cho EY, *et al*. 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* 2009;**4**:809—15.

33. **Janne PA,** Borras AM, Kuang Y, *et al*. A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. *Clin Cancer Res* 2006;**12**:751—8.

34. **Molina-Vila MA,** Bertran-Alamillo J, Reguart N, *et al*. A sensitive method for detecting EGFR mutations in non-small cell lung cancer samples with few tumor cells. *J Thorac Oncol* 2008;**3**:1224—35.

35. **Ohnishi H,** Ohtsuka K, Ooide A, *et al*. A simple and sensitive method for detecting major mutations within the tyrosine kinase domain of the epidermal growth factor receptor gene in non-small-cell lung carcinoma. *Diagn Mol Pathol* 2006;**15**:101—18.

36. **Daniele L,** Cassoni P, Bacillo E, *et al*. Epidermal growth factor receptor gene in primary tumor and metastatic sites from non-small cell lung cancer. *J Thorac Oncol* 2009;**4**:684—8.

37. **Italiano A,** Vandenbos FB, Otto J, *et al*. 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* 2006;**17**:981—5.

38. **Sequist LV,** Engelman JA, Lynch TJ. Toward noninvasive genomic screening of lung cancer patients. *J Clin Oncol* 2009;**27**:2589—91.

39. **Soh J,** Okumura N, Lockwood WW, *et al*. Oncogene mutations, copy number gains and mutant allele specific imbalance (MASI) frequently occur together in tumor cells. *PLoS ONE* 2009;**4**:e7464.

40. **Sholl LM,** Yeap BY, Iafrate AJ, *et al*. Lung adenocarcinoma with EGFR amplification has distinct clinicopathologic and molecular features in never-smokers. *Cancer Res* 2009;**69**:8341—8.

41. **Dowsett M,** Hanna WM, Kockx M, *et al*. Standardization of HER2 testing: results of an international proficiency-testing ring study. *Mod Pathol* 2007;**20**:584—91.

42. **Fitzgibbons PL,** Murphy DA, Dorfman DM, *et al*. Interlaboratory comparison of immunohistochemical testing for HER2: results of the 2004 and 2005 College of American Pathologists HER2 Immunohistochemistry Tissue Microarray Survey. *Arch Pathol Lab Med* 2006;**130**:1440—5.

43. **Dorfman DM,** Bui MM, Tubbs RR, *et al*. The CD117 immunohistochemistry tissue microarray survey for quality assurance and interlaboratory comparison: a College of American Pathologists Cell Markers Committee Study. *Arch Pathol Lab Med* 2006;**130**:779—82.

44. **Derecskei K,** Moldvay J, Bogos K, *et al*. Protocol modifications influence the result of EGF receptor immunodetection by EGFR pharmDx in paraffin-embedded cancer tissues. *Pathol Oncol Res* 2006;**12**:243—6.

45. **Lee H,** Xu X, Choe G, *et al*. Protein overexpression and gene amplification of epidermal growth factor receptor in nonsmall cell lung carcinomas: Comparison of four commercially available antibodies by immunohistochemistry and fluorescence in situ hybridization study. *Lung Cancer* 2010;**68**:375—82.

46. **Buffet W,** Geboes KP, Dehertogh G, *et al*. EGFR-immunohistochemistry in colorectal cancer and non-small cell lung cancer: comparison of 3 commercially available EGFR-antibodies. *Acta Gastroenterol Belg* 2008;**71**:213—18.

47. **Mathieu A,** Weynand B, Verbeken E, *et al*. Comparison of four antibodies for immunohistochemical evaluation of epidermal growth factor receptor expression in non-small cell lung cancer. *Lung Cancer* 2010;**69**:46—50.

48. **Prinsen CF,** Klaassen CH, Thunnissen FB. Microarray as a model for quantitative visualization chemistry. *Appl Immunohistochem Mol Morphol* 2003;**11**:168—73.

49. **Giaccone G,** Iacona RB, Fandi A, *et al*. Epidermal growth factor receptor expression analysis in chemotherapy-naive patients with advanced non-small-cell lung cancer treated with gefitinib or placebo in combination with platinum-based chemotherapy. *J Cancer Res Clin Oncol* 2009;**135**:467—76.

50. **Atkins D,** Reiffen KA, Tegtmeier CL, *et al*. Immunohistochemical detection of EGFR in paraffin-embedded tumor tissues: variation in staining intensity due to choice of fixative and storage time of tissue sections. *J Histochem Cytochem* 2004;**52**:893—901.

51. **Valentini AM,** Pirrelli M, Caruso ML. EGFR-targeted therapy in colorectal cancer: does immunohistochemistry deserve a role in predicting the response to cetuximab? *Curr Opin Mol Ther* 2008;**10**:124—31.

52. **Brevet M,** Arcila M, Ladanyi M. Assessment of EGFR mutation status in lung adenocarcinoma by immunohistochemistry using antibodies specific to the two major forms of mutant EGFR. *J Mol Diagn* 2010;**12**:169—76.

53. **Yu J,** Kane S, Wu J, *et al*. Mutation-specific antibodies for the detection of EGFR mutations in non-small-cell lung cancer. *Clin Cancer Res* 2009;**15**:3023—8.

EGFR and KRAS quality assurance schemes in pathology: generating normative data for molecular predictive marker analysis in targeted therapy

Erik Thunnissen, Judith V M G Bovée, Hans Bruinsma, et al.

Updated information and services can be found at:

http://jcp.bmj.com/content/64/10/884.full.html

These include:

References	This article cites 52 articles, 16 of which can be accessed free at: http://jcp.bmj.com/content/64/10/884.full.html#ref-list-1
Email alerting service	Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections	Articles on similar topics can be found in the following collections Lung cancer (oncology) (98 articles) Lung cancer (respiratory medicine) (98 articles) Molecular genetics (285 articles)

Notes