EGFR-immunohistochemistry in colorectal cancer and non-small cell lung cancer : Comparison of 3 commercially available EGFR-antibodies

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Abstract

Background : **Epidermal Growth Factor Receptor (EGFR)-targeted therapies are currently used for the treatment of metastasized colorectal cancer (CRC) and non small cell lung cancer (NSCLC). Patient selection for this treatment is based on immunohistochemical (IHC) testing for EGFR. The rising amount of commercially available EGFR-antibodies makes standardisation of EGFR-IHC necessary. The goal of this study was to analyse possible discrepancies between 3 antibodies against EGFR.**

Patients and methods : **36 formalin-fixed samples of CRC (n = 26) and NSCLC (n = 10) were stained with 3 antibody-clones : 2-18C9 (Dako™) ; 31G7 (Ventana™) and 111.6 (Labvision Neomarkers™). Interpretation of stains includes assessment of % positive cells, evaluation of cut off values and staining intensity.**

Results : **With a 1% cut-off, the 2-18C9 clone stained 86% of the cases positive, the 31G7-clone 77% and the 111.6-clone 52%. With a 10% cut-off, percentages declined to 77%, 61% and 30% respectively. The 2-18C9-clone showed the highest staining intensity. The 2-18C9 clone and the 31G7-clone showed a concordance rate of 90%.**

Conclusions : **IHC staining with 3 different antibody clones directed against EGFR shows indeed differences in staining results : the percentage of positive cells and staining intensity are variable. A correct cut-off value for a positive result is important and can be different depending upon the antibody. Appropriate validation of an antibody is essential before it can be used for selection of patients.** (Acta gastroenterol. belg., **2008**, 71, **213-218**).

Introduction

EGFR is a 170 kD transmembrane receptor tyrosine kinase encoded by the erb proto- oncogene. Aberrant activation of EGFR in cells is associated with cell growth and proliferation, survival, invasion, metastasis and angiogenesis which all are key processes in tumorgenesis (1).

The past decade, EGFR has emerged as a rational target for anticancer therapy in a variety of solid tumours, especially colorectal cancer (CRC), non-small cell lung cancer (NSCLC) and head and neck cancer which are common tumours. In recent years, a monoclonal antibody (Cetuximab or Erbitux®) and a tyrosine kinase inhibitor (Erlotinib or Tarceva®) were commercialised as EGFR-targeted therapies and oncologists are using these therapies now in daily practice (1-3). Response prediction to EGFR-targeted therapies is however difficult and unresolved at present.

Current guidelines propose to reserve the treatment with these expensive targeted therapies for those patients who express the EGFR target in their tumour as membranous staining, demonstrated by an immunohistochemical test (4).

Questions arise about the use of immunohistochemistry (IHC) in this patient selection, since studies report a 25% response rate to Erbitux® in patients with EGFR-negative CRC (3,5,6). Moreover, no correlation has been found between clinical benefit from Erbitux® and intensity of EGFR-expression or percentage of EGFR-positive tumour cells on IHC (4).

Several explanations for this phenomenon were proposed in literature. One major reason may be that IHC is not the appropriate technique for patient selection for Erbitux® or Tarceva® because the antibodies used do not detect the relevant epitopes, or relevant receptor expression is only regional, such as receptor expression at the baso-lateral versus apical pole of the cell (3,5,7). Another possible reason may be the presence of additional molecular alterations in the tumour or metastases which may influence primary or secondary resistance to EGFR inhibitors. Furthermore, the composition of the tumour may be heterogeneous and so the lesion may not depend entirely on EGFR signalling or receptor activation may be more important than receptor expression.

IHC is the most commonly used method for determining EGFR-expression and it is applied in nearly all clinical studies concerning EGFR-targeted therapies. It is a relatively quick and simple technique that utilizes commonly available equipment and reagents. It preserves tissue morphology and gives additional topographic information about target molecule-expression in the tissue sample (6-8).

Despite its many advantages, IHC is however also a technique that harbours many pitfalls. These include the characteristics and number of the optimal samples suitable for the staining, the choice of primary antibody, especially when several primary antibodies are available, problems of antigen preservation, technical problems like antigen retrieval or detection systems used and interpretative aspects (scoring) (7).

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Submission date : 11.05.2007
Revised version : 17.01.2008
Acceptance date : 06.03.2008

One of the most important technical issues concerning IHC, is the type of primary antibody used. Different antibodies may detect different epitopes. Sensitivity and specificity of the primary antibody are crucial factors in the immunohistochemical reaction (8). The rising amount of commercially available EGFR-antibodies makes standardisation of EGFR-IHC a difficult procedure, since different laboratories use different primary EGFR-antibodies (9-10). Both quality issues and financial considerations are important in the choice of primary antibody. The FDA-approved Dako EGFR pharmDx™ kit is considered as the "gold standard" for EGFR-IHC, but this primary antibody is also the most expensive EGFR-antibody in most countries.

Very few data are available about the performance of other EGFR-antibody clones compared to the Dako™-clone (9,10,17).

The goal of this study was to analyse possible discrepancies between 3 commercially available EGFR-antibodies for IHC in terms of percentage of positive cells, which may be relevant to the epitope detected, staining intensity and effect of cut-off values. In order to reach this goal, three different antibodies were used for the staining of serial sections of a variety of samples.

Methods

Tissue samples

In this study, 36 tissue samples were included. Of these 36 samples, 26 were samples of colorectal carcinoma (CRC) and 10 were samples of non-small cell lung carcinoma (NSCLC). Of each type of tumour, we included primary tumours and metastases, and both surgical resections and biopsy material (Table 1). Specimens were fixed in 6% (v/v) neutral buffered formalin for 24-48 hours.

All CRC-samples were adenocarcinomas, except 1 primary sigmoid CRC and 1 vaginal metastasis which were mucinous adenocarcinomas. Of the NSCLC-samples, 6 were squamous carcinomas, 2 were adenocarcinomas and 2 were undifferentiated large cell carcinomas.

Serial sections of the tissue samples were stained with the 2-18C9-clone (Dako™), the 31G7-clone (Ventana™) and the 111.6 clone (Klinipath™). These 3 antibodies are mouse monoclonal EGFR-antibody clones of the IgG1-isotype which react with an external domain of the 170 kD wild type EGFR and the 135kD EGFRvIII mutant.

EGFR immunohistochemistry

IHC with the 2-18C9-clone was performed manually, according to the manufacturer's instructions (11). Sections were deparaffinised in toluol and rehydrated. Subsequently, Proteinase K was applied for 5 min. followed by peroxidase block for 5 min. and incubation with the primary monoclonal antibody for 30 min.

Table 1. — **Included tissue samples**

Tissue samples	n
CRC	26
Primary tumours	17
– Resections	9
– Endoscopic biopsies	8
Metastases	9
– Liver	7
– Other	2
NSCLC	10
Primary tumours	7
– Resections	2
– Endoscopic biopsies	5
Metastases (biopsies)	3
TOTAL	36

Visualization was obtained with the ready-to-use visualization reagent based on dextran technology (Envision®). This reagent consists of both secondary goat anti-mouse antibody molecules and horseradish peroxidase molecules linked to a common dextran polymer backbone. The enzymatic conversion of the subsequently added chromogen results in formation of a visible reaction product to the antigen site. Specimens were then counterstained with haematoxylin and coverslipped. Control slides containing two formalin-fixed, paraffin-embedded human cell lines with staining intensity scores of 2+ and 0 were used for every procedure.

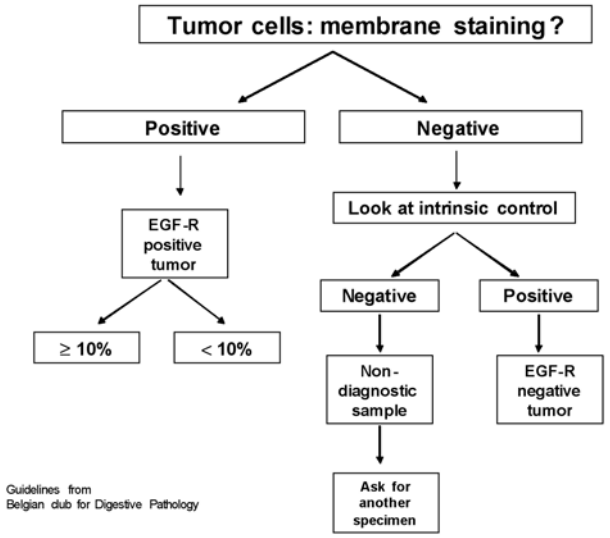
IHC with the 31G7-clone was performed automatically according to the manufacturer's instructions, using the Ventana™ Benchmark XT autostainer (XT ultraview DAB procedure). Briefly, sections were deparaffinised, pretreated with protease I for 4 min. and subsequently incubated with the primary antibody for 32 min. Sections were counterstained with haematoxylin for 4 min. and coverslipped. Skin biopsies were used as positive controls.

IHC with the 111.6 clone was also performed manually according to the manufacturer's recommendations. Sections were first deparaffinised in toluol (2×5 min.) and rehydrated through ethanol (2×5 min.). Internal peroxidase was subsequently blocked in methanol with 0.3% H_20_2 (150 ml H_20_2 in 50 ml methanol) for 20 min. After washing with PBS, tissue sections were covered for 10 min. with protease XXV (ref AP-9006) in 1 mg/ml PBS at 37°C. Then the slides were incubated for 60 min at room temperature with the primary mouse monoclonal antibody (clone 111.6). After two rinses in the buffer, slides were incubated with Envision® and counterstained with DAB. Skin biopsies were used as controls.

EGFR staining scoring system

Slides stained with the 3 different antibody clones were independently scored by 2 pathologists and then compared. Assessment of EGFR-expression was realized according to the EGFR pharmDx scoring guidelines (11).

Table 2. — **Algorithm for scoring EGFR-stains**



Stains were considered positive when complete or incomplete membrane staining of any intensity occurred in tumour cells (+/- 1%) at magnification × 10, while positive controls were also stained and negative controls remained negative. Discrepancies were solved by examining the sections with a multi-headed microscope. Two-way kappa analysis was used as a measure of the agreement between the two pathologists. Values between, 0.81 and 1.0 represent an almost perfect agreement, 0.61 and 0.80 a substantial agreement, 0.41 and 0.60 moderate agreement, 0.21 and 0;40 fair agreement and 0 and 0.20 slight agreement (12). Positive samples were scored for a second time using a cut off level of 10% positive tumour cells because this cut off level is used in the official Belgian guidelines (Table 2).

According to their staining intensity, positive samples were classified as 1+, 2+ or 3+ (Fig. 1). The highest staining intensity was used as the final IHC result for that tumour.

Perineurium, normal colonic mucosa, normal bronchial mucosa and hepatocytes surrounding the liver metastases were considered as positive internal controls on tumour slides.

Results

Percentage positive tumours & effect of cut-off values

First, we scored all 108 stains and compared them in terms of EGFR-expression status and the possible effect of different cut-off levels in this scoring (Table 3a-3c).

On a total of 36 samples and using a cut-off 1%, the 2-18C9 clone stained 86% samples positive, the 31G7-clone 77% and the 111.6-clone 52%. Interobserver agreement was 0.74, 0.76 and 0.76. By using a cut-off 10%, the percentage of positive samples declined to 77%, 61% and 30% respectively (Table 4).

The effect of different cut-off levels was especially present in the CRC-samples.

Staining intensity

Secondly, all 78 positive stains were compared in terms of staining intensity.

Samples stained with the 2-18C9-clone had a nearly 25% higher amount of 2+ and 3+ positive tumours in comparison with the 31G7- or the 111.6-clone (Table 5, 6).

Concordance in EGFR-status

Thirdly, we wanted to know in which way, the different IHC-antibodies stained the same samples as positive or negative for EGFR, so that these antibodies can be considered as having comparable staining results.

In CRC, concordance between the 2-18C9 clone and the 31G7-clone, the 2-18C9 and the 111.6-clone and between the 31G7- and the 111.6-clone was 92%, 65% and 58% respectively. In NSCLC, concordance between the 2-18C9 clone and the 31G7-clone, the 2-18C9 and the 111.6-clone and between the 31G7- and the 111.6-clone was 90%, 50% and 60% respectively.

Discussion

With the ongoing use of EGFR-targeted therapies in clinical practice and the development of comparable drugs, the need for proper selection of patients and the development of tests which can help to predict the outcome increases. IHC for EGFR has been proposed as a possible technique for guiding EGFR-targeted therapies. However, the results of studies evaluating a positive staining result with the outcome are conflicting and do not support the use of IHC for the prediction of treatment response. Tests which could help to predict outcome of costly therapies would however be very interesting. In general, morphological tests could be suitable for cost benefit reasons. However, in order to be useful in clinical practice, these tests as well as tests based on other methodologies need to be reliable and reproducible. Several studies mentioned the IHC-technique as a possible reason for the lack of correlation between EGFR-status assessed by IHC and the clinical benefit from Erbitux®. The lack of correlation can be due to different factors. Technical shortcomings can explain why EGFR-negative patients do show therapy-response on Erbitux® (3, 5). It is indeed known that fixation (type of fixative and duration of fixation) and antigen retrieval can influence the results of IHC. In the present study all samples were fixed in the same fixative, the duration of fixation was also the same and antigen retrieval was based on the same principle in all three methodologies. There are however several other elements which can affect the results. The composition of cancers of the gastrointestinal tract is not usually homogeneous. Routine morphology shows variations in the grade of differentiation within the same tumour. Tumour cell kinetic studies have shown that intra-tumour variability is a confounding factor for the use of cell kinetics and potential doubling time as a

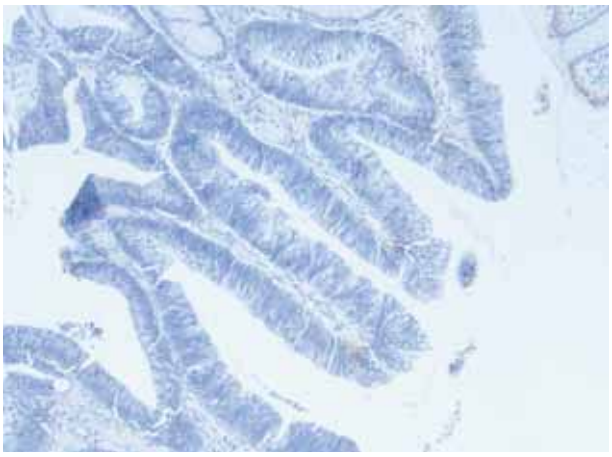


Fig. 1a. — Microphotograph of a colorectal cancer showing no EGFR staining (2-18C9-clone).

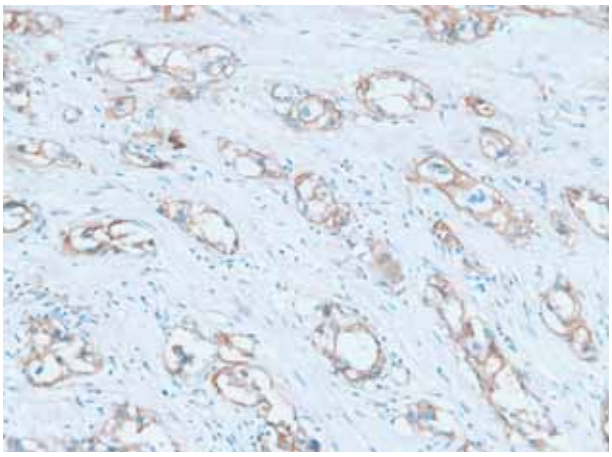


Fig. 1c. — Pleural metastasis of a non small cell lung cancer showing positive staining for EGFR scored as 2+ (31G7-clone).

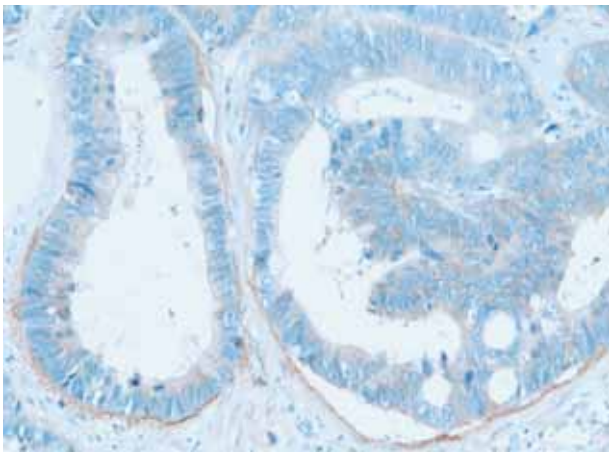


Fig. 1b. — Colorectal cancer showing positive staining for EGFR scored as 1+ (31G7-clone).

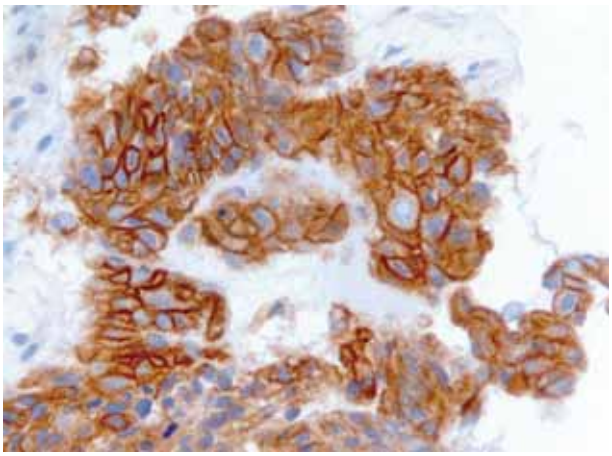


Fig. 1d. — Non small cell lung cancer showing positive staining for EGFR scored as 3+ (111.6-clone).

Table 3a. — **% positive cells in CRC**

	2-18C9	**31G7**	**111.6**
Cut off 1%	21 (81%)	19 (73%)	14 (54%)
Cut off 10%	18 (69%)	13 (50%)	6 (23%)

Table 3b. — **% positive cells in NSCLC**

	2-18C9	**31G7**	**111.6**
Cut off 1%	10 (100%)	9 (90%)	5 (50%)
Cut off 10%	10 (100%)	9 (90%)	5 (50%)

Table 3c. — **% positive cells (all samples)**

	2-18C9	**31G7**	**111.6**
Cut off 1%	31 (86%)	28 (77%)	19 (52%)
Cut off 10%	28 (77%)	22 (61%)	11 (30%)

predictor for treatment outcome (13). The expression of proteins in a tumour may also be highly variable. There are differences between different tumour types and there is also intra-tumour variability. We found a higher expression of EGFR in NSCLC. In colorectal cancer a higher expression of EGFR is constantly observed at the edge of the tumours compared to the surface. Loss of membranous E-cadherin has also been described at the edge of tumours in areas reported as epithelial to mesenchymal transition (14). This change in expression correlates with gain of nuclear ß-catenin. The tumour environment apparently may influence the expression of proteins. The characteristics and the optimal number of samples that should be examined has not been determined for EGFR although it is known that especially colorectal cancer may be heterogeneous in composition. We tested this heterogeneity of EGFR expression in a small series of 22 patients by examining 3 blocks for each patient. For 8 patients (36%), positivity was seen in only one block.

Table 4. — **Percentage positive tumours depending on cut-off levels**

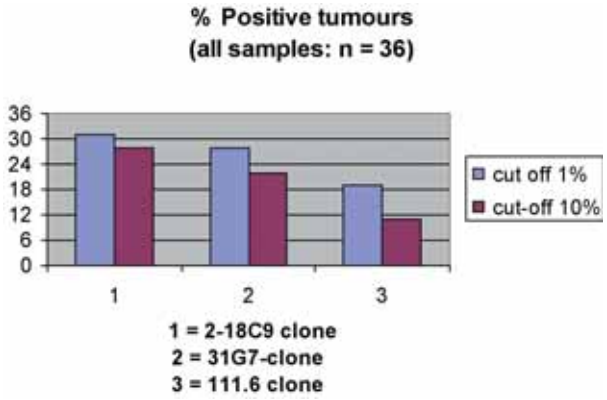


Table 5. — **Staining intensity**

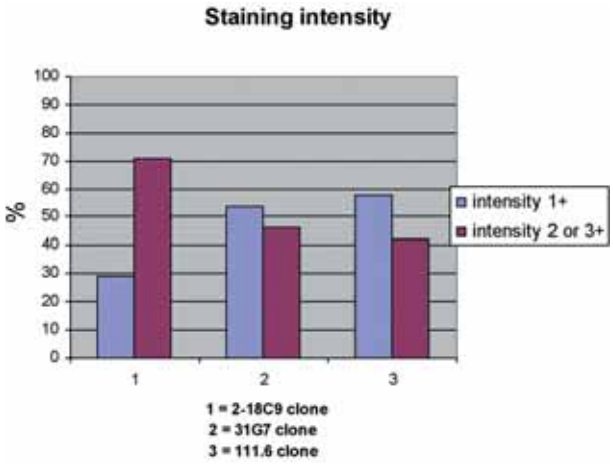
	2-18C9	31G7	111.6
1+	9 (29%)	15 (54%)	11 (58%)
2-3+	22 (71%)	13 (46%)	8 (42%)
Total	31 (86%)	28 (77%)	19 (52%)

Furthermore, the expression may be different in primary lesions and metastases. This constitutes a problem in clinical practice because it implies that stainings should be performed on several specimens, preferentially surgical specimens which are not always available, and for a metastasis on samples from the primary lesion as well as from the metastasis. Furthermore, the heterogeneous expression may imply that more than one tissue sample of a lesion needs to be examined. This heterogeneity may also affect the results obtained with other techniques than IHC. Analysis of EGFR and HER2 gene copy number by fluorescence in situ hybridization is also not showing a significant association between copy numbers and objective response (15). However, before these issues can be solved, it is essential to have a test which reliably detects the antigen. The objective of this study therefore was to compare the performance of three EGFR-antibodies used for immunohistochemical testing and to see if the choice of primary EGFR-antibody has an influence in determining EGFR-expression status. Such a study can help to improve the quality of EGFR-IHC testing and IHC testing in general.

The inter-observer agreement between the pathologists in our series is substantial for positivity. Staining intensity and percent positivity are less reliable features. Kappa values of 0.54 and 0.48 have been obtained for percent scores for respectively cytoplasmic and membranous EGFR positivity in a tissue microarray study of 1,197 colorectal cancers (16).

Overall, the current study shows : (1) a clearly higher percentage EGFR-positive tumours in samples stained with the EGFR-pharmDx-kit compared to both other EGFR-antibody clones, both in CRC and NSCLC. In NSCLC, we detected a consistently higher percentage

Table 6. — **Staining intensity**



EGFR-positive tumours in comparison with CRC, independently of which primary antibody-clone used. (2) When using a cut-off level of 10% instead of 1%, the amount of EGFR-positive CRC declined with 12% (2-18C9-clone), 23% (31G7-clone) and 29% (111.6-clone). This effect was not seen in the NSCLC-samples. (3) The EGFR-pharmDx-kit has a consistently higher staining intensity compared to the 2 other antibody-clones. (4) In 33 of 36 stains, the 2-18C9-clone and the 31G7-clone have the same EGFR-expression status. This is a concordance of 92%. When comparing our data with results published in literature, we find that Bhargava et al. published a 94% concordance rate between the 2-18C9-clone and the 31G7-clone (9). Also Chung *et al.* compared retrospectively these 2 antibody-clones in a small amount of CRC- tumour samples and found comparable results (5). Our results are in contrast with the data published by Penault-Llorca *et al.* This study mentions a higher number of EGFR-positive tumours with the 31G7-clone in comparison with the 2-18C9-clone. Penault-Llorca reported 75% EGFR-positive CRC with the 2-18C9-clone and 93% EGFR-positive CRC with the 31G7-clone (10). A comparable result was obtained in a series of 65 advanced colorectal cancer. A positive result was obtained with Clone 31G7 in 41 (63%) tumours while the Dako pharmDx kit showed a positivity in 35 samples (53%) (17). In comparison, we obtained 81% and 73% respectively. In the present literature, only CRC-tumour samples were used to compare IHC-staining results of primary antibodies (5,9,10).

These data show that the 2-18C9-clone and the 31G7-clone probably do not recognize exactly the same domain of EGFR. As a consequence, it might be indicated to use a second antibody when staining with a first antibody gives a negative result. We want to emphasize further the difference between staining results in the CRC-samples on one hand, and the NSCLC-samples on the other hand. In the latter group of samples, a consistently higher percentage of EGFR-expression was found

with the 3 antibody clones and we could not find any effect of cut-off percentages in the scoring of stains. This can be a proof of the different role or of differences in expression of EGFR in different tumour-types.

We should emphasize however the potential sources for bias in this study. First, we are aware of the small amount of EGFR-stains included. Studies with more tumour-samples, of different tumour-types have to be conducted for a better understanding of EGFR-IHC.

Second, only 3 available EGFR-antibodies are used but several other EGFR-antibodies are commercialised. Performance of these other EGFR-antibodies also has to be studied. Finally, other factors than the primary antibody play a role in the final result of EGFR-IHC. Atkins *et al.* emphasized the importance of standardized tissue processing and handling in the EGFR-IHC process (18,19). Although we have tried to handle our material in a standardised way, we can not exclude technical variations because we followed the staining procedures proposed by the manufacturers.

In conclusion, IHC as a technique to select patients for EGFR-targeted therapy needs to be further analysed for its validity. A better identification of patients which may respond to the EGFR-targeted therapy is important, given the costs of this treatment.

Aspects as the primary antibody used, but also other aspects of the IHC-process need to be standardised. These include the choice of type (surgical material) and number (one or more) of samples. Furthermore, other mechanisms besides EGFR signalling such as angiogenesis are most probably involved in tumour growth and development. Therefore it seems unlikely that one test would predict the patient's outcome. Most probably a panel of properly validated tests which may include tests based on IHC will be needed.

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