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Original article

EGFR immunohistochemistry as biomarker for antibody-based therapy of squamous NSCLC – Experience from the first ring trial of the German Quality Assurance Initiative for Pathology (QuIP®)

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ABSTRACT

Background: EGFR and its downstream signaling pathway are important targets for cancer therapy. Recently, the monoclonal anti-EGFR antibody Necitumumab in combination with gemcitabine and cisplatin was approved (EMA/14106/2016) for first-line treatment of squamous non-small cell carcinoma (SqNSCLC). Eligibility was restricted to cases with positive EGFR expression. In this context, a ring trial of the Quality Assurance Initiative for Pathology (QuIP®) was launched to prepare the German pathology community for a reliable and reproducible, immunohistochemically based biomarker test.

Materials and methods: The trial was set up by a three-step approach. Two lead institutes were nominated to organize the trial process and to select appropriate cancer samples. These were first tested by the H-score (range 0–300) to identify positive and negative cases. Seven additional pathology institutes with experience in EGFR immunohistochemistry each tested the selected panel of identical cases (internal ring trial) to confirm the suitability of samples and scoring criteria. Then the open ring trial for all institutes of pathology in German speaking countries was announced.

Results: For the internal trial 8 EGFR-positive and 2 negative lung sqNSCLC samples were selected. A cut-off value of cell membranous staining in ≥ 1% of tumor cells was introduced to define a case as EGFR negative or positive. Two points were attainable per correctly assessed sample leading to a maximum of 20 points, ≥ 18 points were required for a successful participation. All 7 panel institute passed this barrier, 5 with the maximum of 20 points and two with one error (18 points) being related to one case with incorrect interpretation of cytoplasmic versus membranous staining and one case with an H-score of 2 as being considered EGFR positive. A second cut-off value (H-score ≥ 3) was therefore introduced. In the open ring trial, 34 institutions participated of which 28 were successful according to the above criteria. The trial revealed a high reproducibility despite the use of different EGFR antibodies and detection systems. There was no association between technical parameters and trial failure. Again, one participant misinterpreted the subcellular EGFR localization.

Conclusions: The first nationwide ring test for determination of EGFR IHC expression in sqNSCLC could be successfully performed in a very tight time frame. By this, the national pathology community was prepared to

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incorporate this marker in the panel of predictive cancer tests in a quality assessed manner and to initiate and accompany future studies on EGFR pathway pathology.

1. Introduction

The epidermal growth factor receptor and its associated signaling pathways are well established targets in cancer therapy. In non-small cell lung cancer (NSCLC), this is particularly true for the application of tyrosine kinase inhibitors in lung adenocarcinomas with activating EGFR mutations [5]. Thus EGFR mutation analysis became a standard biomarker test in lung cancer. In addition, there were already hints by the FLEX study that the amount of EGFR protein-expression in NSCLC with wild-type EGFR may be predictive for combinatory therapy with the anti-EGFR antibody Cetuximab [20–22]. However, Cetuximab was not approved for NSCLC as it showed limited clinical benefit. So far, EGFR immunohistochemistry and its quantification did not enter the repertoire of standard biomarker analysis of NSCLC in Germany.

This situation has changed with the recent approval of the anti-EGFR antibody Necitumumab in combination with a gemcitabine and cisplatin chemotherapy for the treatment of progressed sqNSCLC [24,28,29]. A prerequisite for the use of the drug is the positive detection of EGFR expression (EMA/14106/2016). In the corresponding approval study (SQUIRE), there was an influence of the extent of EGFR expression on overall and progression-free survival, carcinomas with high expression (H-score ≥ 200, the H-score spans from 0 to 300) had a better hazard ratio than those with low expression [29]. Positivity of a tumor was assumed when at least one tumor cell showed clear EGFR expression [19].

In this context, a first German-wide ring trial for the quality control of EGFR IHC test algorithms in lung sqCC was set up by the German Quality Assurance Initiative for Pathology (QuIP® GmbH) to prepare the national pathology community for an immunohistochemically based EGFR test in sqNSCLC in a quality controlled manner. Pathological institutes wishing to carry out this biomarker analysis were given the opportunity to evaluate their test procedure and to demonstrate their testing performance within the framework of the QuIP ring trial system. In this study we report on the set-up of the interlaboratory test as well as results and experiences of the open ring trial on EGFR immunohistochemistry.

2. Materials and methods

The set-up of the interlaboratory trial followed the formal procedures that have been established within the QuIP initiative. The time course was as follows. In summer 2016 the manufacturer of Necitumumab (Eli Lilly and Company) contacted the steering-committee (QuIP-AdBoard) of the QuIP GmbH with the request for an EGFR −IHC ring trial (RT). In October 2016, the AdBoard nominated two lead institutes at the Jena University (PI: Petersen) and the Charité in Berlin (PI: Jöhrens). The panel institutes were selected from Heidelberg, Cologne, Munich (TU, LMU), Hannover, Hamburg and Göttingen and are represented by the coauthors of the study. The lead institutes were responsible for organization and realization of the trial. This included the application of the scoring-criteria as well as selection and validation of appropriate cases. Lead and panel institutes together conducted the internal RT. The panel institutes were responsible for validation of the testing condition within the frame of the internal ring trial.

The RT was set up by a three-step approach

1. Both lead institutes screened sqNSCLC cases for EGFR expression from their archives, selected appropriate samples and validated all cases. The blocks of suitable cases were processed by the Jena institute of pathology.
2. To validate the suitability of the material and to check for all conditions of the planned open ring trial, an “internal ring trial” was performed between 7 experienced German institutes (panel institutes) in November 2016.
3. As third step, a set of 10 suitable and validated sqNSCLC cases were selected for the open ring trial which was announced by the German Society of Pathology (DGP) and the Association of German Pathologists (BDP) in November 2016. Institutes that had registered to QuIP and RfB (Reference Institute of Bioanalytics, Bonn, the logistic partner of QuIP GmbH) for being informed on new QuIP initiatives or the repetition of already existing round-robin tests received an official invitation to participate in the first nationwide trial for immunohistochemical EGFR testing in sqNSCLC. Participants could register until December 2nd. Samples were shipped on December 7th, results had to be submitted until December 22nd. The evaluation of the results and the certificates for the participants were provided in January 2017.

Each participating institute was free to select its preferred antibody and detection systems. Nonetheless, they were asked to provide the information on the antibody and detection system to the principle investigators of the lead institutes within the internal ring trial and the QuIP/RfB in the open trial. Antibodies used in the open ring trial are listed in Table 1. The information in Table 1 was retrieved from the Internet by using Google and PubMed (search terms: “EGFR + clone

Table 1
EGFR antibodies used in the open ring trial.

Clone	Source[a]	Recognized epitope/Immunogen	Suppliers	References	Results[d]
E30	Mouse	wild-type EGFR, EGFRvIII/purified, denatured EGFR	Dako	[14,27]	6 of 8
EGFR.25	Mouse	200 aa, cytoplasmic domain	Novocastra	[26]	1 of 2
31G7	Mouse	extracellular domain (full length EGFR + EGFRvIII)	Zymed, Ventana, ThermoFisher[b], Zytomed. Abcam	[1,2,4,6,7]	3 of 4
3C6	Mouse	extracellular domain (full length EGFR + EGFRvIII)	Ventana/Roche	[7]	3 of 3
SP84	Rabbit	synthetic peptide from C-terminus EGFR protein	Cell Marques, ThermoFisher, Menarini	[7]	3 of 3
H11	Mouse	14 aa EGFRvIII-specific synthetic peptide	Dako	[1,13]	1 of 2
111.6	Mouse	extracellular domain	Diagnostic BioSystems, Zytomed, ThermoFisher	[4]	4 of 4
EP22 (=EP38Y)	Rabbit	residues surrounding Tyr1068	Abcam[c], Quartett, Zeta Corporation	[3,25]	1 of 1
2-18C9	Mouse	extracellular domain	Dako (pharmDx)	[1,2,4,7]	2 of 2
5B7	Rabbit	internal domain (carboxy terminal)	Ventana/Roche	[17]	1 of 1

[a] all monoclonal antibodies.
[b] formerly Invitrogen.
[c] formerly Epitomics.
[d] number of successful participants/total number of participants using this antibody.

name" or "EGFR + clone name + supplier name") and is derived from datasheets of the antibody suppliers and the cited References

In addition, the participants were asked to analyze the cases according to the H-score system providing the percentages of cells with the reactivity scores negative (0), slightly positive (1+), moderately positive (2+) and strongly positive (3+) and to calculate the H-score ranging from 0 (all tumor cell negative) to 300 (all tumor cells strongly positive). The H-score was applied as described [21].

Within the instructions for the open trial, a reference was provided to the article of Mathieu et al., 2010 in which 4 different antibodies were tested for their usability and reproducibility for EGFR immunohistochemistry [18]. Similar to the procedure of this study, the participants were asked to evaluate positive membranous EGFR staining of the tumors cells (without the need for full circumferential positivity) in relation to the total number of tumor cells [18].

3. Results

3.1. Selection of cases by the lead institutes

Since about 95% of squamous cell carcinomas of the lung show EGFR expression [8,19], the key aim of this step was to identify EGFR-negative carcinomas. It was important to evaluate the reproducibility of the EGFR staining in different institutes on the same panel of cases and to establish consent reliable criteria of EGFR negative staining.

Therefore, each lead institute first selected 12 NSCLC cases from their archives and analyzed these for EGFR expression. Slides were then sent to the other lead institute. Evaluation was done according to the H-score. In addition, each institute had to define whether a tumor was EGFR negative or positive. To this end, a cut-off value of 1% positive tumor cells was used [12].

Staining results were comparable between the two lead institutes despite the use of different antibodies and detection systems (Jena: clone E30, Dako autostainer; Charité: clone 3C6, Ventana Benchmark) proving robustness of procedures. There were only single cases with no or minimal EGFR staining. Specifically, only one negative case was identified and also this sqNSCLC revealed a very discrete positivity within a focal area with lymph vessel invasion. Therefore it was decided to split this cancer sample eliminating the part with the lymph vessel invasion to generate two cancer samples without EGFR expression. In addition, positive samples with good reproducibility of EGFR expression according to the H-score were selected for the internal ring trial. Representative IHC results of 4 lung SqCC cases with different EGFR expression levels are shown in Fig. 1.

3.2. Internal ring trial by 7 panel institutes

The aim of the internal round-robin test was to recruit a total of ten well-suited cases for the open ring trial. Ten samples (8 positive and 2 negative ones) were distributed to the panel institutes. They were asked to determine the percentage of immunohistochemically negative (score 0) tumor cells as well as those with low (score 1), moderate (score 2) and strong (score 3) staining as well as the H-score being calculated on these figures ranging from 0 (all tumor cells negative) to 300 (all tumor are strongly positive). In addition, each case was expected to be classified as either negative or positive.

As for the two lead institutes, consistent results were obtained in spite of different antibodies and detection systems used (Dako mAb E10, Ventana/Roche 3C6, Zytomed clone 3167, Dako 2-18C3; Ventana Benchmark XT, Ventana Benchmark Ultra, Dako Autostainer Link 48,

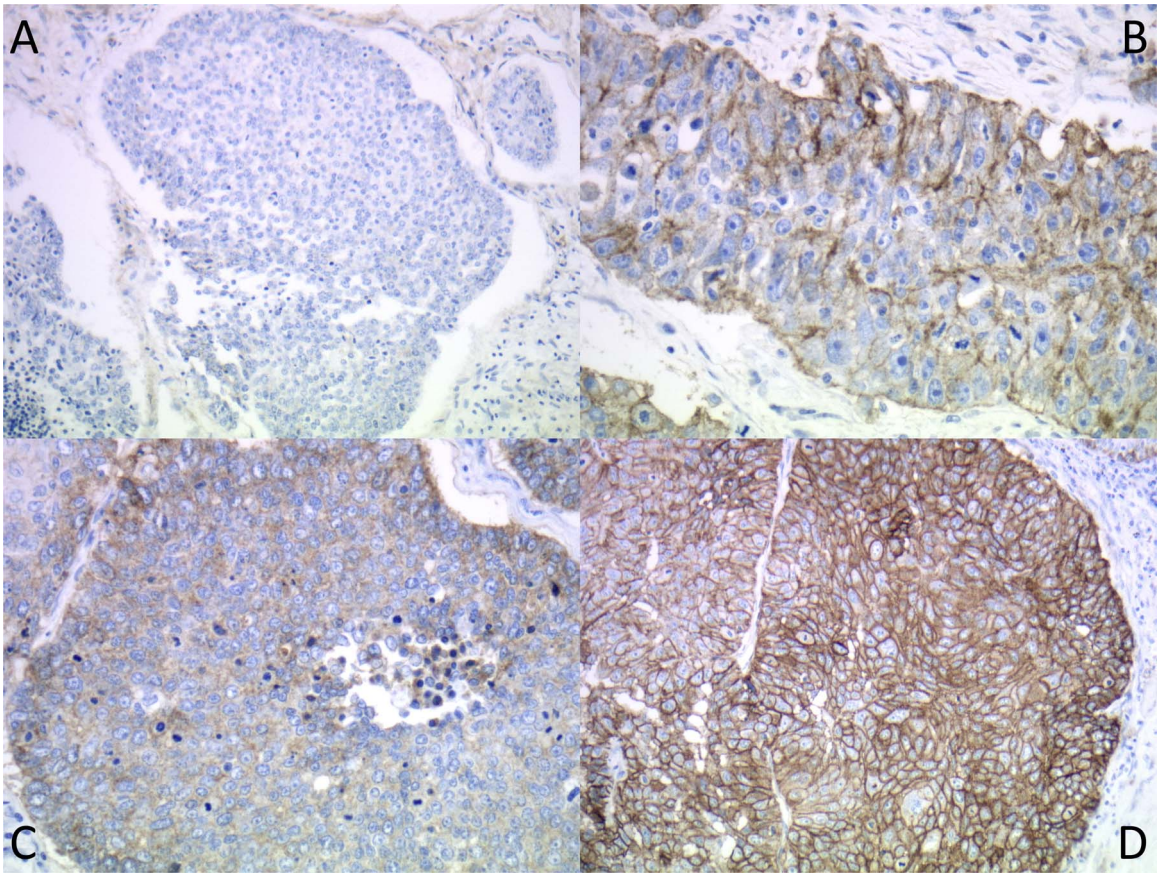

Fig. 1. Representative examples of EGFR protein expression in lung squamous cell carcinoma showing a negative case (A), two cases with heterogeneous membranous (B) and cytoplasmic plus focal membranous staining (C) and one case with strong ubiquitous membranous staining. The antibody clone E30 was used for immunohistochemistry which recognizes the extracellular domain of the EGFR protein.

Leica Bond). In 5 of the 7 panel institutes all 8 positive and 2 negative cases were correctly identified. Only one panel institute considered a negative case as positive with an H score of 2. Another panel institute classified a positive case as negative, but at the same time a cytoplasmic background staining was reported for this case. Therefore all panel institutes had no or maximum 1 failure case. According to the general rule of QuIP allowing for 1 failure out of 10 cases, each panel institute successfully completed the EGFR evaluation procedure. One case was assessed with an H-score 2 by one panel institute which led to recommendation for the open ring trial to score a case only as positive if the cut-off values of ≥ 1% positive tumor cells and an H-score ≥ 3 are met.

3.3. QuIP open ring trial

The test kits were sent by the RfB together with the evaluation instructions. Two tissue sections per case were provided to all participating institute. Participants had 14 workdays to perform and interpret the EGFR immunostains in their institutes, and to report the results online to the RfB.

In the evaluation of the 10 cases – according to the established QuIP ring trial criteria – two points were scored for each correct case. Thus, the participating institutes could reach a maximum of 20 points. In order to successfully pass the test, 18 (of 20 possible) points had to be reached. A correctly assessed case was scored with two points, a wrongly assessed case yielded 0 points. In the case of technical problems which rendered a sample non-evaluable (for example, problems during the staining process), the case was scored with one point.

Overall, test kits were ordered by 37 institutes of which 34 participants provided their results. Of these, 28 (82.4%) were successful, while 6 participants (17,6%) achieved less than 18 points. Five out of the six institutes only failed by reaching sixteen points. In 4 institutes, 2 cases were wrongly classified. In one institute, only one case was misjudged, but there were two technical problems, so that only 16 points were scored. Only one institute had a very clear deviation with a total of 8 incorrectly assessed cases (score 4). The two negative cases were correctly identified by this participant, but at the same time all positive cases were classified as negative. This might be due to a misinterpretation of immunohistochemistry and not a technical error.

Of the 28 successful participants, 18 reached the maximum score of 20, while 10 participants wrongly scored one case achieving 18 points. The wrong assessments were essentially related to 4 cases (1 negative, 3 positive ones), which were incorrectly evaluated by at least 3 participants. This accumulation of wrong results to single cases suggests that despite the variability of the antibodies and detection systems used, EGFR immunohistochemistry provides fairly reproducible results.

In total, 30 institutes provided information on the antibodies and staining platform/detection system. There was no association between a specific antibody or detection system and failure in passing the RT. The participants used a divergent panel of antibodies (Table 1). Similarly, the detection systems were divers and included mainly the ones employed by the panel institutes.

4. Discussion

4.1. EGFR as target in cancer therapy and biomarker for EGFR inhibition

The expression of EGFR is a valuable biomarker for indicating survival and response benefit of an anti-EGFR antibody therapy in conjunction with standard chemotherapy. In lung cancer, this has been convincingly shown for cetuximab in NSCLC and necitumumab in squamous lung SCC [23,29]. However, the correlation between EGFR expression and therapy response is not perfect. While 94–100% of head and neck squamous cell carcinomas show EGFR expression, only 10–13% of cases responded to cetuximab as single agent or combined with either cisplatin or carboplatin [31]. The relatively poor correlation between EGFR expression and therapy response is surprising and far from being fully understood [10].

Until recently, immunohistochemical analysis of EGFR has not become a routine marker for any entity in which anti-EGFR therapy has been approved. This is surprising given the fact that the EMA and FDA approval of panitumumab and cetuximab in colorectal cancer mentioned tumor surface positivity of EGFR together with the exclusion of RAS mutations. In this setting at least a weak membranous staining (1+) in ≥ 1% of tumor cells is required for eligibility [9]. In daily practice, however, only RAS mutation analysis has become a routine biomarker in Germany. Similarly, the detection of EGFR expression is not required in most countries for any entity in which receptor tyrosine kinase inhibitors have been approved for therapy, i.e. NSCLC and pancreas cancer.

In addition, the use of EGFR immunohistochemistry seems to diverge in different countries. The study of Mathieu et al., 2010 which compared 4 different EGFR antibodies for evaluation was inspired by the Belgian reimbursement criteria for erlotinib in NSCLC which required at least 10% of cells showing membranous staining. As “gold standard”, the 2-18C9 clone was compared to 3 other monoclonal antibodies. The outcome of staining was comparable, a positivity rate of > 80% of patients eligible for the erlotinib therapy was reported for the antibodies 2-18C3, 31G7 and EGFR.25 while a fourth antibody (Ab-10) yielded a positivity rate of < 70% [18]. Similarly, another study comparing the 2-18C3 and 31G7 clones reported a correlation coefficient of 0,96 [12]. These results are supported by our study which revealed no major differences between antibodies and detection procedures used by the participants of the internal and open ring trials.

4.2. EGFR subcellular localization and functional relevance of EGFR in cancer therapy

Our study revealed as potential pitfall in the interpretation of the immunostaining with regard to the subcellular localization of EGFR. In particular, cytoplasmic expression was not considered as a positive result by single participants. It is obvious that many carcinomas not only reveal membranous staining but also cytoplasmic positivity (Fig. 1) and the latter can be even more prominent. The subcellular localization of EGFR protein expression is probably dependent on the antibody used for immunohistochemistry (Table 1). In this context it must be mentioned that the recognized epitopes are localized within the extracellular or intracellular domains of the EGFR protein and that cytoplasmic staining is reported by many suppliers as specific expression pattern of their antibodies. Thus, cytoplasmic staining can be considered as specific expression and it is thus advisable to score it as positive if at least a focal membranous staining is also detectable.

EGFR is known of a long time and it is a well characterized protein in cancer biology and beyond. In addition, there is a range of antibodies available against normal and mutant forms of EGFR as well as isoforms with specific protein modifications. Despite the large variety of antibodies used in the open ring trial, the results were comparable and quite consistent. This strengthens the notion that different materials can be reliably used for protein biomarker analysis in cancer therapy. In general, it can be expected that the most valuable diagnostic tools will be selected by the pathologists in charge of performing predictive tests. As medical doctors, they are not only responsible for the accuracy of their results but also for the clinical consequences. And they have access to the patienťs follow up information by their tumor board colleagues and the local cancer centers which enables the ultimate feedback of a correct biomarker analysis.

The influence of the subcellular localization of EGFR with regard to its potential predictive value as biomarker for therapy response still needs to be studied and has to our knowledge not been comprehensively investigated. However, it clear that the subcellular localization of EGFR is relevant for its biological functions [11]. Interestingly, EGFR overexpression and nuclear translocation was associated with

resistance to chemotherapy and radiotherapy [15,16,33]. In general, EGFR is mainly considered to be active as a cell surface receptor tyrosine kinase. However, this is a simplistic view because the receptor itself is shuttled to the cell surface during biosynthesis and its activity is regulated by internalization, post-endocytic sorting, recycling and degradation. All these trafficking processes depend on distinct pathways and molecules [30]. EGFR seems to have kinase-independent functions and exhibits a nuclear signaling network by which it is implicated in a number of physiological and pathological processes as proliferation, inflammation, metastasis, DNA repair and resistance to DNA-damaging and alkylating anti-cancer agents [11]. Thus, alterations of EGFR signaling needs to be considered within the cellular context, i.e. the origin of the EGFR expressing cell, its cellular microenvironment and the biological mechanism being affected, e.g. proliferation [32].

EGFR and its downstream signaling pathways, in general, are highly relevant for cancer biology and likely to remain essential therapeutic targets. However, the biological functions of EGFR expressing, non-mutated tumors are not fully understood. In addition, there is a need for biomarkers predicting therapy response. The immunohistochemical analysis of EGFR expression is highly relevant in this context. And this is probably true not only for tumor samples with abundant protein expression but also those few cases with negative EGFR staining as it may represent a specific molecular state and pathway vulnerability.

5. Conclusions

The first nationwide ring test for the determination of the expression of EGFR in squamous cell carcinoma of the lung could be successfully performed in a very tight time frame in a multi-step process (internal and open ring trials). Of the 34 participating institutes, 27 succeeded to correctly identify 10 cases with positive and negative results. A wide range of primary antibodies were used by the participating institutes, the success did not depend on the type of antibody or the detection system used. The German pathology community is prepared to incorporate this marker in the panel of predictive cancer tests and to initiate and accompany future studies on EGFR pathway pathology.

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