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METHODS

Protocol Modifications Influence the Result of EGF Receptor Immunodetection by EGFR pharmDx™ in Paraffin-Embedded Cancer Tissues

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EGF receptor (EGFR) became a useful target for several recently introduced therapies of various cancer types including colorectal, lung, head and neck cancers and glioblastoma. The successful clinical application of these novel molecularly targeted therapies requires the expression of their target, EGFR, determined by nucleic acid based or immunohistochemical techniques. However, until now, immunohistochemistry has not become a reliable diagnostic approach for this purpose. The golden standard for the determination of EGFR protein expression in paraffin-embedded cancer tissues is the EGFR pharmDx™ kit. Here we show that the recommended protocol may not be optimal for EGFR immunodetection. Microwave antigen retrieval and extended primary antibody incubation time converted four out of eight EGFR-negative tumors into EGFR-positive in a study of 50 lung adenocarcinoma cases. Accordingly, we recommend retesting cases negative for EGFR with EGFR pharmDx™ using protocol modifications optimizing antigen retrieval and the incubation periods. (Pathology Oncology Research Vol 12, No 4, 243–246)

Key words: EGF receptor immunodetection, EGFR pharmDx™, antigen retrieval, incubation time

Introduction

Targeted therapy is a rapidly evolving field in clinical oncology, changing the established therapeutic protocols. One of the most promising agent groups is the EGF receptor inhibitors each of which demonstrated significant clinical activity in a given cancer type, thereby providing new hope for patients.[1] Today there are two classes of anti-EGFR agents, monoclonal antibodies and tyrosine kinase inhibitors, having their primary target on the human EGF receptor, HER-1. Targeted therapy requires careful selection of cancer patients whose malignant tumor expresses the given target in at least a small proportion of the cell population. This low diagnostic level of EGFR expression is questionable compared to the rational application of anti-HER-2/neu antibody therapy in patients having HER-2/neu overexpressing breast cancer (2+/3+ HercepTest™ and/or HER-2/neu gene amplification). The initial enthusiasm among oncologists toward the „targeted" nature of these therapies has recently been declined since clinical trials frequently failed to connect unequivocally the efficacy of these new treatments to the expression of EGFR protein determined by immunhistochemistry.[2] Although there are several possible causes for this failure, among them non-selective (or multiple) targeting nature of the agents, one of the most straightforward problems could well be the efficacy of the pathological diagnostic procedure.

There are a couple of accepted diagnostic kits available worldwide to detect EGFR protein expression in cancer tissues for selecting of patients with the highest chance for clinical benefit from anti-EGFR therapy: the FDA-approved kit EGFR pharmDx™,[3] the CONFIRM anti-EGFR™ method,[4] and the widely used anti-EGFR antibody clone 31G7.[5] However, the results of EGFR expres-

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sion in cancer tissue greatly depend not only on the genotype of the tumor cells but also on the processing of the tissue sample for pathologic analysis. Since EGFR pharmDx™ is the FDA-approved diagnostic kit to determine the eligibility of colorectal cancer patients for anti-EGFR antibody (Cetuximab) therapy, this method has quickly become a „gold standard" of EGFR immunodiagnostics.

Here we report that protocol modifications involving antigen retrieval techniques and incubation times fundamentally affect the results obtained with the EGFR phamDx™ kit. Based on these data we recommend a large-scale re-evaluation of the FDA-approved protocol due to the clinical significance of its results.

Materials and Methods

Fifty paraffin-embedded surgical samples of lung adenocarcinoma were used in the study. Tissue samples were routinely fixed in 10% (v/v) neutral buffered or unbuffered formalin, dehydrated in a graded series of ethanol, infiltrated with xylene and embedded into paraffin at a temperature not exceeding 60ºC. Three to four micron thick sections were mounted on Superfrost slides (Thermo Shandon, Runcorn, UK), and were manually deparaffinized according to the manufacturer's protocol (EGFR pharmDx™, Dako, Glostrup, Denmark).

We have used the antigen retrieval technique suggested by the instructions to the EGFR pharmDx™ kit: sections were exposed for 5 min at room temperature to 100 µl 0.1% proteinase K diluted in TRIS-HCl buffer containing 0.015 mol/L sodium azide, followed by HQ water washings (3+2 min). Alternatively, slides were immersed in 0.05 mM citrate buffer (pH=6), and exposed to 750 W microwave for 3x5 min (MFX-800-3 automatic microwave, Meditest, Budapest, Hungary). To block endogenous peroxidase activity, slides were treated for 5 min at room temperature with 3% H_2O_2, diluted either in distilled water in the case of protease digestion (post-treat-

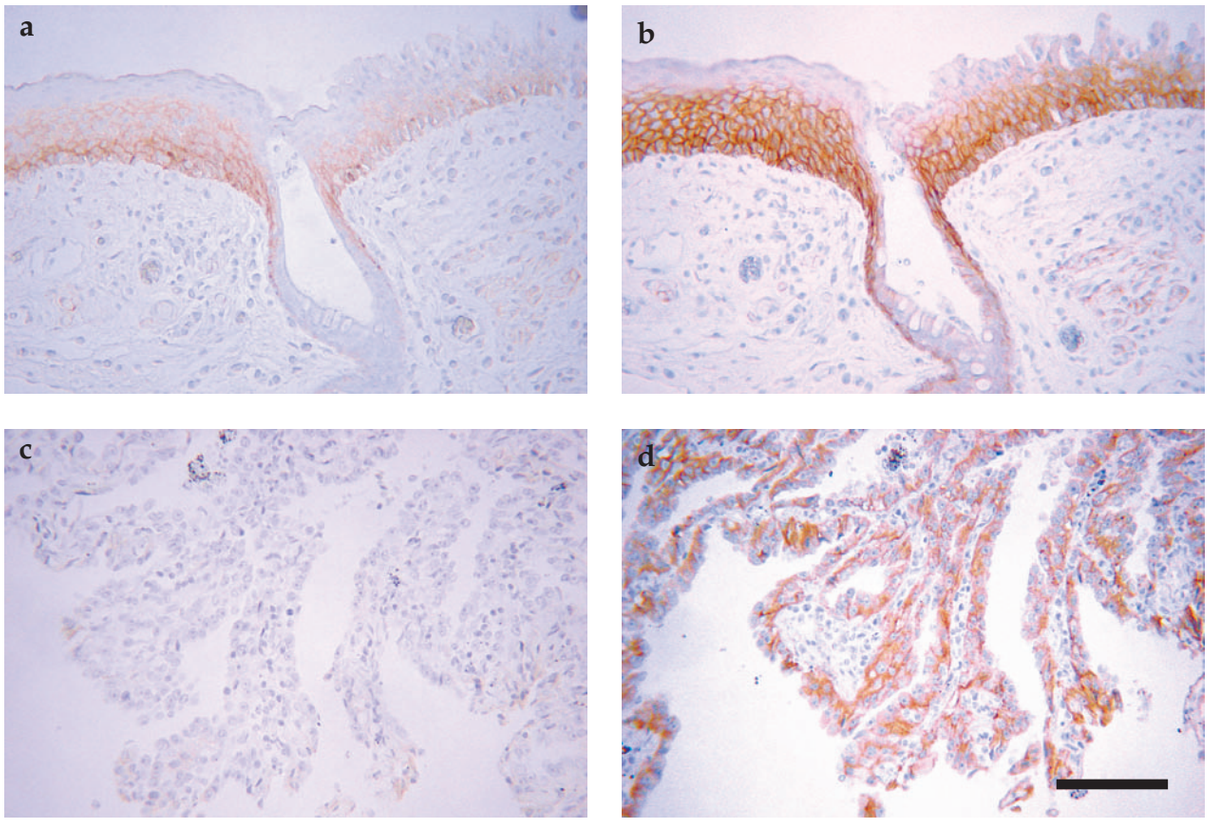

Figure 1. *Expression of EGFR protein in normal laryngeal epithelia (positive control,* ***a,b****) and lung adenocarcinoma cases* ***(c,d)*** *using EGFR pharmDx™ protocol or its modification. Antigen retrieval: a,c, protease K digestion and FDA protocol; b,d, microwave treatment and extended incubation with the primary antibody, LSAB developer. Note the significantly more intensive immunoreaction on normal epithelial cells in* ***b*** *(+++) compared to* ***a*** *(++). Lung bronchoalveolar carcinoma classified EGFR-negative* ***(c)*** *following the original FDA protocol of EGFR pharmDx™. Note the almost undetectable reaction in tumor cells.* ***(d)*** *shows the same case as in (c) but after microwave antigen retrieval, extended incubation with the primary antibody and using LSAB developer. Note that more than 50% of tumor cells demonstrated intense membrane labeling. Bar is 100 µm.*

ment), or in methanol in the case of microwave antigen retrieval (pretreatment), respectively.

EGFR protein expression was detected by the EGFR pharmDx™ kit using mouse monoclonal anti-human EGFR (clone 2-18C9),[6] goat anti-mouse IgG and a dextran polymer conjugated with HRP, and DAB substrate-chromogen, applied following the manufacturer instructions rigorously. As a positive control, slides provided by the manufacturer (formalin-fixed and paraffin-embedded pellet of HT29 human colorectal carcinoma cell line) as well as human head and neck carcinoma tissue samples previously diagnosed 3+ for membrane EGFR by using EGFR pharmDx™ and CONFIRM anti-EGFR (Ventana, BioMarker, Gödöllõ, Hungary) were used. For negative control, slides were exposed to the diluent instead of the primary antibody and were processed in the same way as other slides. We have also tested modifications of this protocol: the incubation time of the slides with the primary antibody was extended to overnight at 4°C and the dextran polymer conjugate was replaced by the LSAB kit (Dako). Nuclear counterstaining and mounting was performed according to the manufacturer's protocol.

Table 1. **Effect of technical modifications on the EGFR immunohistochemical reaction performed by EGFR pharmDx™ on lung adenocarcinoma cases**

	Protocol			
Case N°	*FDA*	*MW-FDA*	*FDA-long*	*MW-long*
1	0	0	>50	>50
2	0	0	<10	>50
3	0	0	>20	<10
4	0	1	>10	>20
5	0	nt	nt	0*
6	0	nt	nt	0
7	0	nt	nt	0
8	0	nt	nt	0

Data are expressed in % of EGFR+ cancer cells. FDA= original FDA-approved protocol. MW-FDA= FDA protocol, but the antigen retrieval was replaced my microwave cooking. FDA-long= FDA protocol, but the incubation with the primary antibody was extended to overnight at 4°C. MW-long= similar protocol to FDA-long, but the antigen retrieval was switched to microwave cooking. MW-long used LSAB developing reagent. *=fixation problem

Results and Discussion

During a retrospective analysis of EGFR protein expression in fifty lung adenocarcinomas, we found 8 completely negative cases using the EGFR pharmDx™ kit. Since the inner positive control elements (peripheral nerves, bronchial epithelium) were weakly positive and the control slide provided by the manufacturer was positive, we have initially classified these tumors EGFR-negative. Since antigen retrieval techniques can fundamentally affect the efficacy of antigen detection, at first we have tested EGFR pharmDx™ kit on EGFR-overexpressing head and neck cancer (HNCC) tissue after microwave antigen retrieval. Data indicated that the specific immunoreaction became stronger not only on EGFR membrane positive squamous cancer cells (data not shown) but on the adjacent normal squamous and columnar epithelium as well (*Figure 1a,b*), suggesting a better performance of the kit when using this alternative antigen retrieval instead of protease digestion. It is of note that this modification did not affect the background of the staining. We have also tested if the extended incubation time of the primary antibody and the use of an alternative detection system, LSAB kit, affect the EGFR reaction. Our data indicated that on the HNCC test slides these modifications did not influence the percentage or intensity of the EGFR-reaction (data not shown).

In the followings we have retested the eight EGFR-negative lung adenocarcinoma cases using the FDA protocol of EGFR pharmDx™ after microwave antigen retrieval, or using either antigen retrieval technique with extending the incubation period of the primary antibody to overnight incubation and replacing the detection system by the LSAB kit. In the case of one sample there was no positive reaction detectable for EGFR in normal bronchial epithelium even after alternative antigen retrieval or other protocol modifications and the tumor tissue remained repeatedly negative. This result suggested a fixation problem of the tissue sample which rendered the case unclassifiable. Out of the seven remaining cases, four became EGFR positive using the combination of microwave antigen retrieval technique and extended incubation with the primary antibody (*Table 1*). The four positive cases exhibited highly heterogeneous EGFR protein expressions after protocol modifications from less than 10% positive tumor cells to more than 50% (*Table 1, Figure 1c,d*). Comparing the percentage of positive tumor cells after various protocol modifications, it became evident that the major factor affecting the EGFR reaction is the incubation period which converted four previously negative cases strongly positive (*Table 1*). On the other hand, changing the antigen retrieval method further influenced the efficiency of the EGFR protein detection (*Table 1*). Replacement of the EGFR pharmDx™ developer by LSAB kit did not change the reaction specificity or intensity (data not shown).

EGFR-targeted therapies have changed the standard care of colorectal- and non-small cell lung (NSCL) cancer patients, and provided a promising alternative for the treatment of glioblastoma and head and neck cancer. The success of clinical trials on EGFR-targeted therapies imposes a great demand on pathologists to identify patients who could benefit most of these new regimens. Molecular diagnostics of these EGFR-targeted therapies still lags behind the clinical developments,[7,8] and the clinical utility

of nucleic acid-based and protein-based techniques are in the center of debate.[9] Recently the controversy over EGFR immunohistochemistry resulted in trials where EGFR-targeted therapies were introduced without the determination of the expression of EGFR protein in the given tumor type. On the other hand, other trials revealed that EGFR-negative colorectal cancer patients responded to EGFR-targeted antibody therapy,[10] supporting the critiques' opinion. However, at several instances the methodology used to define EGFR protein expression was not presented in details in the trial reports,[11,12] to be able to judge the performance of immunohistochemistry. Since the gold standard of EGFR immunohistochemistry, EGFR pharmDx™ kit, is the only FDA-approved test and therapeutic decisions are frequently based on its use, it would be necessary to re-evaluate the reliability and efficacy of its protocol. Based on our observations we suggest that in case of cancer tissues of low or negative EGFR protein expression determined by the protocol of the EGFR pharmDx™, it may be necessary to confirm the data by using extended incubation with the primary antibody and/or microwave antigen retrieval instead of the recommended protease digestion.

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References

1. *Baselga J and Arteaga CL:* Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. J Clin Oncol 23:2445-2459, 2005
2. *Meropol NJ:* Epidermal growth factor receptor inhibitors in colorectal cancer: it's time to get back on target. J Clin Oncol 23:1791-1793, 2005
3. http://www.fda.gov/cdrh/mda/docs
4. http://www.ventanamed.com/catalog/antibody
5. *Nguyen PL, Swanson PE, Jaszcz W, et al:* Expression of epidermal growth factor receptor in invasive transitional cell carcinoma of the urinary bladder. A multivariate survival analysis. Am J Clin Pathol 101:166-176, 1994
6. *Pii K, Andersen FG, Jensen S, Spaulding B:* Characterization of a new monoclonal antibody, clone 2-18C9, for the measurement of epidermal growth factor receptor expression in solid tumor. Proc. 95th AACR, Abstr #5029, 2004
7. *Harari PM and Huang S-M:* Searching for reliable epidermal growth factor receptor response predictors. Clin Cancer Res 10:428-432, 2004
8. *Dancey JE:* Predictive factors for epidermal growth factor receptor inhibitors. The bull's-eye hits the arrow. Cancer Cell 5:411-415, 2004
9. *Bunn PA Jr, Dziadziuszko R, Varella-Garcia M, et al:* Biological markers for non-small cell lung cancer patient selection for epidermal growth factor receptor tyrosine kinase inhibitor therapy. Clin Cancer Res 12:3652-3656, 2006
10. *Chung KY, Shia J, Kemeny NE, et al:* Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 23:1803-1810, 2005
11. *Baselga J, Trigo JM, Bourhis J, et al:* Phase II multicenter study of the antiepidermal growth factor receptor monoclonal antibody cetuximab in combination with platinum-based chemotherapy in patients with platinum-refractory metastatic and/or recurrent squamous cell carcinoma of the head and neck. J Clin Oncol 23:5568-5577, 2005
12. *Robert F, Blumenschein G, Herbst RS, et al:* Phase I/IIa study of cetuximab with gemcitabine plus carboplatin in patients with chemotherapy-naïve advanced non-small-cell lung cancer. J Clin Oncol 23:9089-9096, 2005