BJC OPEN

British Journal of Cancer (2012) 106, 883 – 888

www.bjcancer.com

Immunohistochemical discrimination of wild-type EGFR from EGFRvIII in fixed tumour specimens using anti-EGFR mAbs ICR9 and ICR10

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BACKGROUND: The human epidermal growth factor receptor (EGFR) is an important therapeutic target in oncology, and three different types of EGFR inhibitors have been approved for the treatment of cancer patients. However, there has been no clear association between the expression levels of EGFR protein in the tumours determined by the FDA-approved EGFR PharmDx kit (Dako) or other standard anti-EGFR antibodies and the response to the EGFR inhibitors.

METHOD: In this study, we investigated the potential of our anti-EGFR monoclonal antibodies (mAbs; ICR9, ICR10, ICR16) for immunohistochemical diagnosis of wild-type EGFR and/or the type-III deletion mutant form of EGFR (EGFRvIII) in formalin-fixed, paraffin-embedded human tumour specimens.

RESULTS: We found that the anti-EGFR mAb in the EGFR PharmDx kit stained both wild-type and EGFRvIII-expressing cells in formalin-fixed, paraffin-embedded sections. This pattern of EGFR immunostaining was also found with our anti-EGFR mAb ICR16. In contrast, mAbs ICR10 and ICR9 were specific for the wild-type EGFR.

CONCLUSION: We conclude that mAbs ICR9 and ICR10 are ideal tools for investigating the expression patterns of wild-type EGFR protein in tumour specimens using immunohistochemistry, and to determine their prognostic significance, as well as predictive value for response to therapy with EGFR antibodies.

British Journal of Cancer (2012) **106,** 883 – 888. doi:10.1038/bjc.2012.27 www.bjcancer.com
Published online 7 February 2012

Keywords: ICR10; EGFR; EGFRvIII; EGFR PharmDx; immunohistochemistry

The epidermal growth factor receptor (EGFR)/HER1 is a 170-KDa transmembrane glycoprotein with tyrosine kinase activity and the prototype of the type-I growth factor receptor (also called HER/ErbB) family, which can be activated following the binding of several ligands (EGF, TGFα, amphiregulin, HB-EGF, betacellulin and epiregulin) to its extracellular domain (Carpenter, 1987; Modjtahedi and Dean, 1994; Salomon *et al*, 1995; Gullick, 2001; Singh and Harris, 2005). Ligand binding induces EGFR homo-dimerisation or heterodimerisation with other members of the ErbB family, autophosphorylation of tyrosine residues and ultimately, activation of several intracellular signalling pathways (Yarden and Sliwkowski, 2001). Signalling via the EGFR has been associated with increased cell proliferation, reduced apoptosis, angiogenesis, invasion and metastasis, and all of which are hallmarks of cancer (Yarden, 2001; Lui and Grandis, 2002; Hanahan and Weinberg, 2011). Aberrant expression and activation of EGFR have been reported in a wide range of epithelial tumours, and in several studies, have been associated with poor prognosis and resistance to therapy (Modjtahedi and Dean, 1994; Salomon *et al*, 1995; Nicholson *et al*, 2001).

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Received 16 September 2011; revised 15 November 2011; accepted 16 January 2012; published online 7 February 2012

The EGFR is an important therapeutic target in patients with metastatic colorectal cancer (mCRC), head and neck cancer, non-small cell lung cancer and pancreatic cancer (Mendelsohn and Baselga, 2003; Zhang *et al*, 2007; Ciardiello and Tortora, 2008; Modjtahedi and Essapen, 2009). Currently, two classes of EGFR inhibitors namely monoclonal antibodies (mAbs) and small molecules, which bind to the extracellular ligand-binding domain and the intracellular tyrosine kinase domain of the EGFR, respectively, have been approved for the treatment of cancer patients (Modjtahedi and Essapen, 2009). Of these, the mAbs cetuximab and panitumumab have been approved for the treatment of patients with mCRC in combination with chemotherapy, and several other anti-EGFR antibodies are currently at different stages of clinical development (Wong, 2005; Wu *et al*, 2008). To aid in the identification of CRC patients who are eligible for treatment with cetuximab and panitumumab, the EGFR status of the tumours is determined using the FDA-approved EGFR PharmDx immunohistochemistry kit (Dako, Cambridge, UK; Mitchell, 2004; Bhargava *et al*, 2006; Buckley and Kakar, 2007; Ensinger and Sterlacci, 2008). However, although treatment with these agents improve survival in mCRC patients, the duration of response is often limited and is only seen in a subset of patients (Schrag, 2004). In addition, there has been no clear association between the expression levels of EGFR in the tumours determined by the FDA-approved PharmDx kit or other commercial anti-EGFR antibodies and the response to therapy or patient prognosis

(Cunningham *et al*, 2004; Schrag, 2004; Chung *et al*, 2005; Bhargava *et al*, 2006; Derecskei *et al*, 2006; Hebbar *et al*, 2006; Bralet *et al*, 2007; Buckley and Kakar, 2007; Ensinger and Sterlacci, 2008; Modjtahedi and Essapen, 2009).

The increased signalling and cellular responses via the EGFR may be mediated by several mechanisms, including overexpression of wild-type EGFR, overproduction of EGFR ligands, deletion of part or all of the extracellular domain, somatic mutations of its intracellular tyrosine kinase domain and heterodimerisation with other members of the EGFR family (Yarden, 2001; Modjtahedi and Essapen, 2009). For example, the type-III deletion mutant form of EGFR (EGFRvIII) is the most common variant form of EGFR and has been detected on both the cell surface and in the cytoplasm of many cancer types, including colorectal, glioma, breast, ovary, prostate and lung cancer (Olapade-Olaopa *et al*, 2000; Tang *et al*, 2000; Cunningham *et al*, 2005). EGFRvIII is characterised by deletion of 267 amino acids in the external domain of the receptor, which consequently is ligand-independent, constitutively active and highly transforming (Sugawa *et al*, 1990; Wong *et al*, 1992). In addition, several useful anti-EGFRvIII-specific mAbs have been developed for investigating expression levels, subcellular location, biological and prognostic significance, predictive value, as well as for targeting human cancers (Wikstrand *et al*, 1995; Omidfar *et al*, 2004; Heimberger *et al*, 2005; Gupta *et al*, 2010). Unfortunately, antibodies that are used in the immunohistochemical detection of EGFR and determining its prognostic significance and predictive value are not specific to the wild-type EGFR and can also bind to the EGFRvIII (Modjtahedi and Essapen, 2009). To our knowledge, there is currently no comprehensive study examining the expression pattern, prognostic significance and predictive value of the wild-type EGFR protein with mAbs, which discriminate against the common EGFRvIII mutant. Such studies may lead to identification of a more defined subpopulation of CRC patients whose tumours are EGFR-dependent and may therefore be more sensitive to therapy with the EGFR-blocking antibodies (Meropol, 2005; Khambata-Ford *et al*, 2007; Modjtahedi and Essapen, 2009).

We have extensively profiled our unique panel of high affinity rat anti-EGFR mAbs for use in the diagnosis and therapy of human cancers (Modjtahedi *et al*, 1993, 2003; Dean *et al*, 1994). In this study, we investigated the potential of some of our antibodies (ICR9, ICR10, ICR16) for immunohistochemical diagnosis of the wild-type EGFR and/or the EGFRvIII in formalin-fixed, paraffin-embedded human tumour specimens. We show that unlike the antibody in the EGFR PharmDx kit, mAbs ICR10 and ICR9 are specific for the wild-type EGFR, and therefore, these antibodies may form ideal tools for investigating the expression pattern, prognostic significance and predictive value of wild-type EGFR protein in human cancers.

MATERIALS AND METHODS

Tumour cell lines and cell culture

The human EGFR-overexpressing head and neck HN5 (1.4×10^7 EGFRs per cell) and breast carcinoma cell line MCF-7, which expresses undetectable levels of EGFR and EGFRvIII, were cultured routinely in Dulbecco's modified Eagle's medium (DMEM; Sigma, Poole, UK) supplemented with 10% fetal calf serum (Invitrogen, Loughborough, UK) and the antibiotics penicillin, streptomycin, neomycin as described previously (Cowley *et al*, 1984; Modjtahedi *et al*, 1993). As human cancer cell lines show loss of EGFRvIII expression under cell culture conditions (Lammering *et al*, 2004), HC2 20d2/c was generated by transfection of NIH3T3 cells with cDNA encoding EGFRvIII, and these cells were employed as EGFRvIII-positive tumour ($\sim 2 \times 10^6$ mutant receptors) cell line (Moscatello *et al*, 1995). HC2 20d2/c cells were maintained in DMEM supplemented with 10% fetal calf serum and 350 $\mu g\,ml^{-1}$ Geneticin (Invitrogen) as described previously (Moscatello *et al*, 1995). The paraffin-embedded primary human glioblastoma (GBM) tumour slides were acquired from the Neuropathology division of Stanford Medical Centre, with all patient identification removed.

Antibodies

The three anti-EGFR mAbs used in this study (ICR9, ICR10 and ICR16) were raised against three epitope clusters on the extracellular domain of the human EGFR, using the human head and neck carcinoma cell line HN5 as a source of immunogen (Modjtahedi *et al*, 1993). ICR9 binds to epitope 'A', ICR10 binds to epitope 'B', and ICR16 binds to epitope 'C' on the EGFR, respectively (Modjtahedi *et al*, 1993, 2003; Dean *et al*, 1994). The EGFR PharmDx kit, containing positive and negative control cell lines, was purchased from Dako. The mouse anti-human EGFRvIII mAb G100 was purchased from Zymed Life Technologies (Grand Island, NY, USA). The rabbit anti-rat HRP secondary antibody and FITC-conjugated F(ab′)2 goat anti-rat IgG secondary antibody was purchased from ABD Serotec Ltd (Oxford, UK).

Flow cytometry

The cell surface expression of the growth factor receptors was determined using FACS analysis as described previously (Khelwatty *et al*, 2011). Briefly, approximately 1×10^6 HN5 or HC2 20d2/c cells in 1 ml of DMEM/2% FBS were incubated with primary antibodies or control medium or for 1 h by rotation at 4 °C. Cells were washed three times by centrifugation for 5 min at 1000 r.p.m., and resuspended in DMEM/2% FBS before incubation with FITC-conjugated F(ab′)2 goat anti-rat IgG secondary antibody. Following rotation for 1 h at 4 °C, tumour cells were washed three more times, and the final cell pellet was resuspended in FACS Flow buffer (Becton Dickinson Ltd, Oxford, UK). A minimum of 10 000 events were recorded by excitation with an argon laser at 488 nm, and analysed using the FL-1 detector (FITC detector; 525 nm) of a BD FACScalibur flow cytometer (Becton Dickinson Ltd) using CellQuest Pro software (Becton Dickinson Ltd).

Cell pellet preparation, specimen fixation and paraffin embedding

Cells were grown to near confluence in 170-cm^2 Nunc cell-culture flask as described above. The cell monolayer were detached by a cell scraper, transferred to a volumetric tube and centrifuged at 1600 r.p.m. for 5 min. The resultant pellet was then fixed in 10% (v/v) neutral buffered formalin (Bios Europe Ltd, Skelmersdale, UK) for 45 min. Sequentially, the pellet was dehydrated in a series of alcohols and cleared in histoclear, and then paraffin embedded (Paramat, VWR International Ltd, East Grinstead, UK). Tissue specimens were cut into sections of 5 μm and mounted onto poly-L-lysine-coated slides (Polysine, 631-0107, VWR International Ltd) for immunohistochemistry.

EGFR PharmDx immunohistochemical staining

EGFR PharmDx immunohistochemical staining was carried out manually according to the manufacturer's (Dako) protocol. Briefly, before staining, slides were deparaffinised and rehydrated. Protein K proteolytic digestion was then carried out and endogenous activity was blocked. Subsequently, slides were treated with primary antibody or negative-control reagent and incubated for 30 min in a humid chamber. After rinsing in wash buffer, the slides were then treated with labelled polymer, HRP, according to the manufacturer's instructions. Finally, the slides were treated with diaminobenzidine substrate-chromogen solution (Dako), followed by counterstaining with haematoxylin and mounting in DPX mounting medium.

Immunostaining with anti-EGFR mAbs ICR9, ICR10 and ICR16

As before, the slides were deparaffinised in histoclear and rehydrated in series of graded alcohols. The endogenous peroxidase activity was then blocked by incubation in 3% hydrogen peroxide. Non-specific binding was blocked by incubating the slides in normal rabbit serum for 20 min in a humidity chamber. After blotting off the excess serum, each slide was incubated with 100 μl primary antibody or TBS for 1 h at room temperature. Slides were then rinsed with TBS and incubated with rabbit anti-rat antibody HRP secondary antibody for 30 min at room temperature. Finally, the slides were treated with diaminobenzidine substrate-chromogen solution, followed by counterstaining with haematoxylin and mounting in DPX mounting fluid.

RESULTS

The anti-EGFR antibody clone 2-18C9 from the FDA-approved EGFR PharmDx kit was used for immunohistochemical staining of the human head and neck carcinoma cell line HN5 pellet (1.4×10^7 wild-type EGFR per cell) and HC2 20d2/c (1.2×10^6 EGFRvIII per cell) cell pellets. As shown in Figure 1, the antibody in the EGFR PharmDx kit binds strongly to both wild-type EGFR on HN5 cells and EGFRvIII on HC2 20d2/c cells in formalin-fixed, paraffin-embedded sections. There was no staining of EGFR-negative CAMA-1 cells with antibody clone 2-18C9 (data not shown).

Next, we performed immunohistochemical staining of HN5 and HC2 20d2/c cell pellets using three rat anti-EGFR mAbs: ICR9, ICR10 and ICR16 (Modjtahedi *et al*, 1993, 2003). Unlike the anti-EGFR antibody 2-18C9 in the EGFR PharmDx kit (Figure 1), mAbs

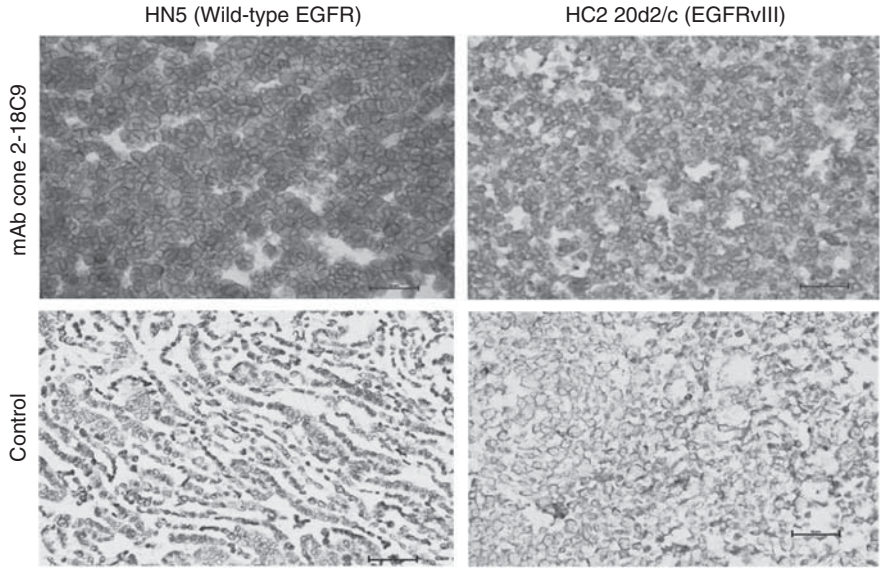

Figure 1 Immunohistochemical staining of formalin-fixed, paraffin-embedded HN5 and HC2 20d2/c cells, using the EGFR PharmDx primary antibody Clone 2-18C9 and negative control. EGFR staining was carried out manually according to the manufacturer's (Dako) protocol.

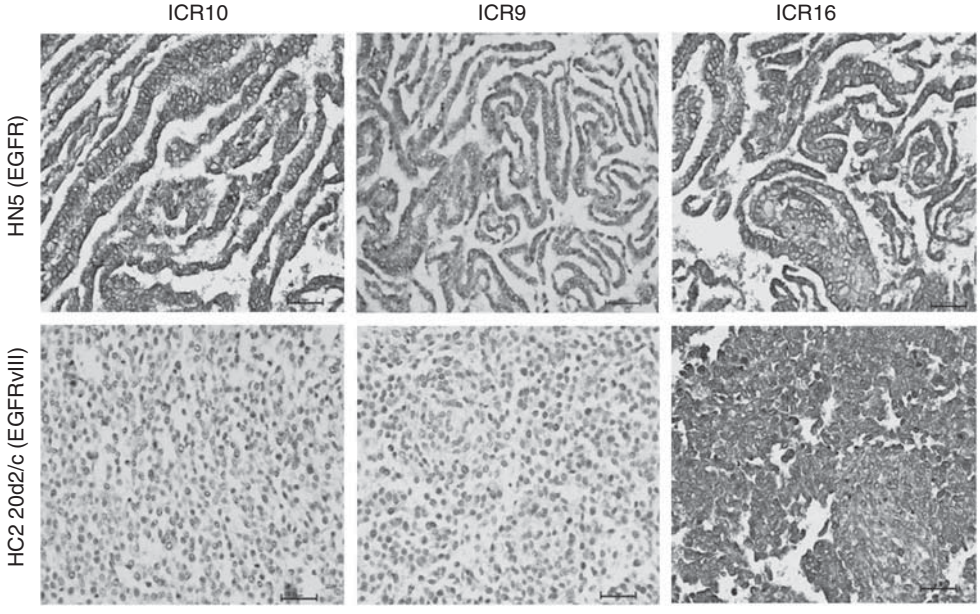

Figure 2 Immunohistochemical staining of formalin-fixed, paraffin-embedded HN5 and HC2 20d2/c cell pellets, using our anti-EGFR mAbs ICR10, ICR9 and ICR16, and negative control, as described in the 'Materials and Methods' section.

ICR9 and ICR10 are specific for the wild-type EGFR and do not stain the EGFRvIII in formalin-fixed, paraffin-embedded tumour specimens (Figure 2). However, like antibody clone 2-18C9, mAb ICR16 stained both HN5 and HC2 20d2/c cells and can therefore be used for immunohistochemical detection of both wild-type EGFR and EGFRvIII in paraffin-embedded fixed tissues (Figure 2). The specificity of ICR10 for the wild-type EGFR was also demonstrated by flow cytometry and the results as presented in Table 1.

Finally, we examined the diagnostic potential of mAb ICR10 for immunohistochemical detection of wild-type EGFR in formalin-fixed, paraffin-embedded tissue. We stained tissue from a tumour that was EGFRvIII positive, but wild-type EGFR-negative with ICR10 and an antibody specific for EGFRvIII (G100; Figure 3). This revealed staining by the G100, but not the ICR10. On the other hand, using these same antibodies to stain a tumour that is EGFRvIII-negative but wild-type EGFR-positive showed labelling of the tumour by ICR10, but not G100. These results demonstrate that ICR10 can be used to specifically label wild-type EGFR in pathology specimens.

DISCUSSION

Despite the approval of several EGFR inhibitors for the treatment of human cancers, there has been no clear association between the expression levels of EGFR in the tumours determined by the FDA-approved EGFR PharmDx kit (Dako) or other standard anti-EGFR antibodies and the response to the EGFR inhibitors (Arteaga, 2002; Cunningham *et al*, 2004; Chung *et al*, 2005; Tos and Ellis, 2005; Derecskei *et al*, 2006; Hebbar *et al*, 2006; Bralet *et al*, 2007; Buckley and Kakar, 2007; Modjtahedi and Essapen, 2009; Hecht *et al*, 2010). In some studies, the presence of somatic mutations of *KRAS, BRAF, PI3KCA* and loss of PTEN in tumours (i.e., EGFR-independent tumours) were associated with primary resistance, whereas other studies found an association between the *EGFR* gene amplification, high levels of EGFR ligands such as amphiregulin and epiregulin, and sensitivity to therapy with anti-EGFR mAbs (Amado *et al*, 2008; Cappuzzo *et al*, 2008; Di Nicolantonio *et al*, 2008; Jacobs *et al*, 2009; Modjtahedi and Essapen, 2009; Bardelli and Siena, 2010; De Roock *et al*, 2010; Oliveras-Ferraros *et al*, 2010). Of all the markers, only KRAS genotyping is performed routinely to exclude patients, whose tumours contain KRAS mutations, from receiving anti-EGFR therapy (Allegra *et al*, 2009; Siena *et al*, 2009). In addition, clinical benefit is not seen in all mCRC patients with wild-type KRAS, and there is a need for the identification of more reliable predictive markers for use in the selection of patients whose tumours are EGFR-dependent and will therefore benefit from therapy with EGFR-blocking antibodies.

In CRC, the percentage of EGFR-positive cases reported in the literature ranged from 8 to 100% of the cases examined. In addition, there are also conflicting data regarding the correlation between the EGFR protein expression in the primary tumour and its related metastases, and their prognostic significance and predictive value (Scartozzi *et al*, 2004; Chung *et al*, 2005; Ljuslinder *et al*, 2009). This wide variation may be due to the usage of different antibodies, tumour specimen source (e.g., primary tumour and/or related metastasis), scoring system, choice of fixative, storage time and patient subpopulations (Atkins *et al*, 2004; Scartozzi *et al*, 2004; Cunningham *et al*, 2005; Meropol, 2005; Perez-Soler and Saltz, 2005; Hebbar *et al*, 2006; Penault-Llorca *et al*, 2006; Bralet *et al*, 2007; Buckley and Kakar, 2007; Ljuslinder *et al*, 2009; Modjtahedi and Essapen, 2009; Bardelli and Siena, 2010; Yarom *et al*, 2010). In addition, antibodies which are

Table 1 MFI for binding of anti-EGFR mAbs ICR10 and ICR16 to the EGFR-overexpressing HN5 and EGFRvIII-overexpressing (HC2 20d2/c) cell lines

	MFI	
Antibody	**HN5**	**HC220d2/c**
Control	6.87	5.04
ICR10	1092.45	3.55
ICR16	1189.75	593.05

Abbreviations: EGFR = epidermal growth factor receptor; EGFRvIII = type-III deletion mutant form of EGFR; mAbs = monoclonal antibodies; MFI = mean fluorescence intensity.

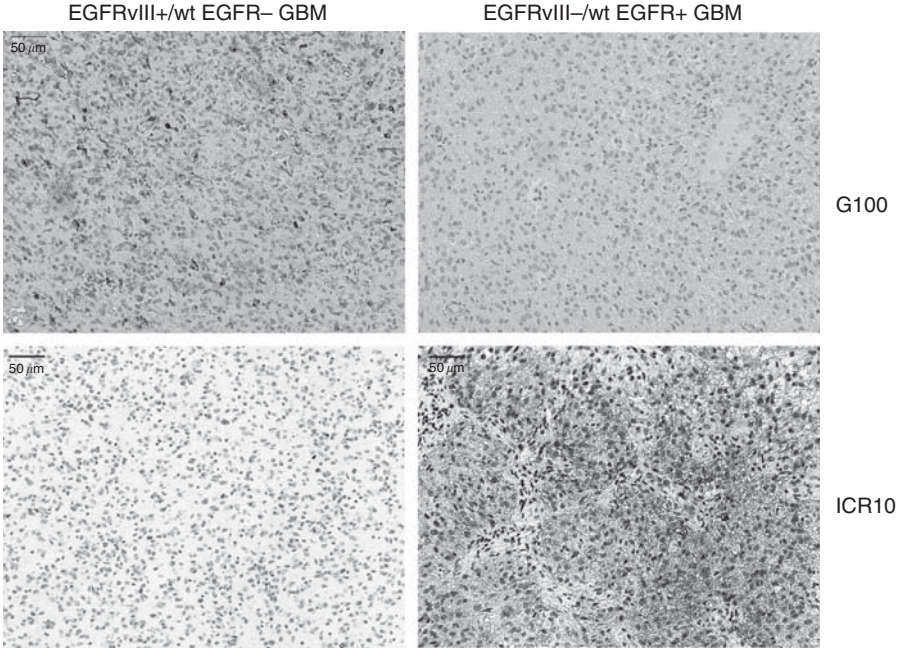

Figure 3 Differential staining of tumours containing wild-type EGFR *vs* EGFRvIII by ICR10. A primary human glioblastoma tumour positive for EGFRvIII, but lacking EGFR expression (EGFRvIII + /wt EGFR−GBM) was stained by immunohistochemistry using either G100 (specific for EGFRvIII only) or ICR10. A second glioblastoma tumour that was negative for EGFRvIII, but positive for wt EGFR (EGFRvIII−/wt EGFR +) was also stained with G100 and ICR10.

routinely used in the immunohistochemical detection of EGFR and to determine its prognostic significance and predictive value for response to therapy with the EGFR inhibitors are not specific to the wild-type EGFR and can also bind to the EGFRvIII. Indeed, in this study, we have shown that the FDA-approved antibody in the EGFR PharmDx kit (Dako), which is used in the selection of EGFR-positive cancer patients for therapy with anti-EGFR mAbs, is not specific to the wild-type EGFR protein and can also bind to the EGFRvIII in formalin-fixed, paraffin-embedded tissues (Figure 1). Our anti-EGFR mAb ICR16 can also bind to both EGFR and EGFRvIII in formalin-fixed, paraffin-embedded tissues. We have reported previously that in comparison with mAbs ICR9 and ICR10, mAb ICR16 was very effective in (1) blocking the binding of ligands to the EGFR, (2) inhibiting the tyrosine phosphorylation of EGFR and downstream cell signalling molecules, and (3) inhibiting the growth of *in vitro* and *in vivo* of EGFR-overexpressing tumour cell lines (Modjtahedi *et al*, 1993, 2003; Dean *et al*, 1994; Modjtahedi and Dean, 1994). Interestingly, unlike mAbs clone 2-18C9 and ICR16, we have shown here that mAbs ICR10 and ICR9 are specific for the wild-type EGFR and can therefore be used for immunohistochemical detection of wild-type EGFR in formalin-fixed, paraffin-embedded tissues (Figures 1–3).

In summary, at present, eligible patients for therapy with cetuximab and panitumumab, in addition to wild-type KRAS, should express cell surface EGFR in their tumours, using the FDA-approved Dako EGFR PharmDx immunohistochemistry kit or other anti-EGFR antibodies. However, such antibodies do not discriminate between the ligand-independent and constitutively active EGFRvIII, and wild-type EGFR and somatic mutation of the EGFR intracellular tyrosine kinase domain is rare in patients with mCRC (Lee *et al*, 2005). We conclude that mAbs ICR9 and ICR10 are ideal tools for investigating the expression level and the cellular location of wild-type EGFR in formalin-fixed, paraffin-embedded tumour specimens using immunohistochemistry, and to unravel its prognostic significance as well as predictive value for response to therapy with the EGFR-blocking antibodies in future studies.

ACKNOWLEDGEMENTS

We acknowledge the support of MRC (UK), Cancer Research UK, BRIGHT Charity (UK), Institute of Cancer Research (UK) and Kingston University London (UK).

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