Novel targeted approaches to treating biliary tract cancer: the dual epidermal growth factor receptor and ErbB-2 tyrosine kinase inhibitor NVP-AEE788 is more efficient than the epidermal growth factor receptor inhibitors gefitinib and erlotinib

Marcus Wiedmann[a], Jürgen Feisthammel[a], Thilo Blüthner[a], Andrea Tannapfel[b], Thomas Kamenz[a], Annett Kluge[a], Joachim Mössner[a] and Karel Caca[a]

Aberrant activation of the epidermal growth factor receptor is frequently observed in neoplasia, notably in tumors of epithelial origin. Attempts to treat such tumors with epidermal growth factor receptor antagonists resulted in remarkable success in recent studies. Little is known, however, about the efficacy of this therapy in biliary tract cancer. Protein expression of epidermal growth factor receptor, ErbB-2, and vascular endothelial growth factor receptor-2 was assessed in seven human biliary tract cancer cell lines by immunoblotting. In addition, histological sections from 19 patients with extrahepatic cholangiocarcinoma were analyzed for epidermal growth factor receptor, ErbB-2 and vascular endothelial growth factor receptor-2 expression by immunohistochemistry. Moreover, we sequenced the cDNA products representing the entire epidermal growth factor receptor coding region of the seven cell lines, and searched for genomic epidermal growth factor receptor amplifications and polysomy by fluorescence in-situ hybridization. Cell growth inhibition by gefitinib erlotinib and NVP-AEE788 was studied *in vitro* by automated cell counting. In addition, the anti-tumoral effect of erlotinib and NVP-AEE788 was studied in a chimeric mouse model. The anti-tumoral drug mechanism in this model was assessed by MIB-1 antibody staining, terminal deoxynucleotidyl transfer-mediated dUTP nick end-labelling assay, von Willebrand factor staining, and immunoblotting for p-p42/44 (p-Erk1/2, p-MAPK) and p-AKT. Immunoblotting revealed expression of epidermal growth factor receptor, ErbB-2, and vascular endothelial growth factor receptor-2 in all biliary tract cancer cell lines. EGFR was detectable in six of 19 (32%) extrahepatic human cholangiocarcinoma tissue samples, ErbB-2 in 16 of 19 (84%), and vascular endothelial growth factor receptor-2 in nine of 19 (47%). Neither epidermal growth factor receptor mutations nor amplifications or polysomy were found in the seven biliary tract cancer cell lines. Gefitinib, erlotinib and NVP-AEE788 caused a significant growth inhibition *in vitro*; however, there was a significant difference in efficacy (NVP-AEE788 > erlotinib > gefitinib). After 14 days of in-vivo treatment, using the chimeric mouse model, tumors had a significantly reduced volume and mass after NVP-AEE788, but not after erlotinib treatment, as compared with placebo. Reduction of proliferation (signalling via the mitogen-activated protein kinase pathway), induction of apoptosis and inhibition of angiogenesis were the main mechanisms of drug action. No significant reduction of anti-apoptotic AKT phosphorylation, however, occurred, which may be a possible counter mechanism of the tumor. Epidermal growth factor receptor, ErbB-2, and vascular endothelial growth factor receptor-2 expression was detectable in biliary tract cancer, and receptor inhibition exerts marked effects on tumor growth *in vitro* and *in vivo*, which was strongest for the dual EGFR/ErbB-2 inhibitor NVP-AEE788. Therefore, further clinical evaluation of this new drug for the treatment of biliary tract cancer is recommended.

Anti-Cancer Drugs 2006, **17:**783–795

Keywords: cholangiocarcinoma, erlotinib, gefitinib, NVP-AEE788, tyrosine kinase inhibitor

[a]Department of Internal Medicine II and [b]Institute of Pathology, University of Leipzig, Leipzig, Germany.

Correspondence to M. Wiedmann, Department of Internal Medicine II, University of Leipzig, Philipp-Rosenthal-Strasse. 27, 04103 Leipzig, Germany.
Tel: +49 341 9712200; fax: +49 341 9712239;
e-mail: wiedm@medizin.uni-leipzig.de

Sponsorship: Study supported by grants 10-2106-Wi 1 from the Deutsche Krebshilfe (to M.W.) and BMBF NBL-3, 01ZZ0106 (to J.F).

Received 12 December 2005 **Revised form accepted** 12 April 2006

Introduction

The epidermal growth factor receptor (EGFR, or HER1, ErbB-1) was cloned in 1984 by Ullrich *et al.* [1] and is a close relative of v-*abl*. It belongs to a family of related receptor tyrosine kinases that includes HER2 (or NEU, ErbB-2), HER3 (or ErbB-3) and HER4 (or ErbB-4). Three classes of ligands bind the EGFRs: the first class

These authors contributed equally to the work.

0959-4973

includes EGF, amphiregulin and transforming growth factor-α; the second class includes β-cellulin, heparin binding EGF (HB-EGF) and epiregulin; and the third class is composed of the neuregulins [2]. When activated, the different receptors form either homodimers or heterodimers. They play an important role during embryogenesis and development by relaying signals for proliferation, survival, migration and differentiation from the extracellular environment into the cell. As signalling by EGFR is associated with cellular survival and proliferation, it is easy to understand why over-expression of EGFR and dysregulation of EGFR signalling pathways are frequently observed in many types of human neoplasias including most of the gastrointestinal malignancies [3,4]. Cells over-expressing EGFR display many characteristics associated with tumorigenesis. These characteristics include invasion of the cell into surrounding tissue, lymph vessels or blood vessels, metastasis and unregulated cell proliferation, late-stage disease, resistance to chemotherapeutic drugs, resistance to hormone therapy, and poor patient prognosis [5]. Oncogenic activation of EGFR signalling, however, can occur not only by increased expression of EGFR, but also by excessive ligand expression or transactivation through receptor dimerization [6] with the Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK and phophatidylinositol 3′-kinase (PI3K)/AKT signalling cascades as the main downstream pathways [7]. Whereas the first pathway influences cell proliferation, migration and differentiation, the second results in inhibition of apoptosis. Other pathways involved in EGFR signalling are phospholipase C, Ca^{2+}/calmodulin-dependent kinases, and the JAK/signal transducer and activator of transcription pathway. Furthermore, angiogenesis is enhanced by upregulation of vascular endothelial growth factor (VEGF) and interleukin-8 [8]. Finally, there is a crosstalk with other signalling molecules such as G-protein-coupled receptors and integrins.

The two main classes of EGFR inhibitors are monoclonal (mc) antibodies (such as cetuximab, matuzumab, panitumumab, ABX-EGF, EMD 72000 and h-R3) and tyrosine kinase inhibitors (TKIs). Some of the TKIs are selective for EGFR (such as gefitinib, erlotinib, AG1478 and PD153035), others inhibit both (dual) the EGFR and ErbB-2 [such as lapatinib (GW572016), BMS-599626, EKB-569 and PKI-166], a third class inhibits all (pan) EGFR family members (such as CI-1033 and PD183805), and a fourth class includes combined dual EGFR and VEGF receptor (VEGFR) inhibitors (such as NVP-AEE788 and ZD6474) [4,9,10]. Anti-EGFR monoclonal antibodies have shown antitumor activity in advanced colorectal carcinoma, squamous cell carcinomas of the head and neck, non-small cell lung carcinoma (NSCLC), and renal cell carcinomas. TKIs have a partially different activity profile. Gefitinib and erlotinib are approved for the treatment of NSCLC. NVP-AEE788 has been tested in preclinical studies for the treatment of lung cancer, mammary cancer, pancreatic cancer, colon carcinoma, bladder cancer, prostate cancer, ovarian carcinoma, anaplastic thyroid carcinoma, cutaneous squamous cell carcinoma and oral squamous cell carcinoma. It is now under trial in a phase I study in patients with solid tumors (NCT00118456) and in phase I/II studies in combination with everolimus for the therapy of patients with recurrent or relapsed glioblastoma multiforme (NCT00107237 and NCT000825215). Recently, mutations and amplifications of the *EGFR* gene have been identified in NSCLC and predict for enhanced sensitivity to anti-EGFR TKIs [11]. Little is known, however, about the situation for biliary tract cancer, a rare tumor with a grim prognosis and limited treatment options. Therefore, the objectives of the current study were to investigate EGFR, ErbB-2 and VEGFR-2 expression in biliary tract cancer cell lines and in histological sections from patients with extrahepatic cholangiocarcinoma (CC), and to evaluate the efficacy of in-vitro and in-vivo treatment with selective EGFR inhibitors gefitinib and erlotinib and the dual EGFR/ErbB-2 inhibitor NVP-AEE788.

Materials and methods

Drugs, cells and animals

Seven biliary tract cancer cell lines – five extrahepatic bile duct cancer cell lines (EGI-1, TFK-1, CC-SW-1, CC-LP-1 and SK-ChA-1) [12–16] and two gallbladder cancer cell lines (Mz-ChA-1 and Mz-ChA-2) [15] – were examined. All cell lines were cultured in a 37°C incubator with 5–10% CO_2 in appropriate media. Human epidermoid carcinoma cells (A-431) and NIH3T3 murine fibroblasts were obtained from the Tumorbank of the German Cancer Research Center (DKFZ, Heidelberg, Germany) and were grown as monolayers in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The EGFR inhibitors gefitinib (ZD1839, Iressa) and erlotinib OSI-774, Tarceva) were provided by AstraZeneca (London, UK) and Roche Pharma (Reinach, Switzerland; originally Genentech, San Francisco, USA). NVP-AEE788 (targeting EGFR, ErbB-2 and VEGFR-2) was obtained from Novartis (Basel, Switzerland). Hoechst dye and mc β-actin antibody were purchased from Sigma-Aldrich Chemie (Munich, Germany), mc EGFR (ErbB-1) and polyclonal (pc) VEGFR-2 (KDR, Flk-1) antibodies from Santa Cruz Biotechnology (Santa Cruz, California, USA), mc MIB-1 and pc von Willebrand factor (vWF) antibodies from Dako (Glostrup, Denmark), and mc p-p42/44 (p-Erk1/2, p-MAPK), mc AKT, mc p-AKT and mc ErbB-2 antibodies from Cell Signaling Technology (Beverly, Massachusetts, USA). Six- to 8-week-old female athymic NMRI nude mice were supplied from Taconic Europe (Ry, Denmark) and held under pathogen-free conditions. Human care was administered and study protocols complied with the institutional guidelines.

Immunoblotting

Cell culture monolayers were washed twice with ice-cold phosphate-buffered saline and lysed with buffer containing Tris–HCl (20 mmol/l, pH 7.5), NP-40 (1%), Triton-X (0.5%), NaCl (250 mmol/l), ethylenediamine tetraacetic acid (1 mmol/l), 10% glycerol and one tablet of complete mini-ethylenediamine tetraacetic acid-free protease inhibitor cocktail (Boehringer, Mannheim, Germany) (in 10 ml buffer). Protein concentration was determined by the Bradford protein assay (Bio-Rad, Munich, Germany). Cell lysates (30 μg) were separated on sodium dodecyl sulphate polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution [5% dry milk in 10 mmol/l Tris–HCl, 140 mmol/l NaCl, 0.1% Tween-20 (TBS-T)], followed by incubation with the primary antibody at 4°C overnight (5% dry milk or 5% bovine serum albumin in TBS-T). The membranes were then washed in TBS-T and incubated with horseradish peroxidase (HRPO)-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was carried out with an enhanced chemoluminescence reaction.

Reverse transcription polymerase chain reaction and sequencing of epidermal growth factor receptor cDNA

Total RNA was extracted from 5×10^5 cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. First-strand cDNA synthesis was carried out with 1 μg of total RNA and Oligo $(dT)_{12-18}$ using the Superscript Kit (Gibco/BRL, Rockville, Maryland, USA). In order to detect EGFR mutations, cDNA products were amplified in six overlapping segments using the following primers: (1) sense, 5′-ATG CGA CCC TCC GGG A-3′, antisense 5′-GGC AGG TGT CCT TGC ACG-3′ [base pair (bp) 247–1039], (2) sense, 5′-GCC GCA AAT TCC GAG AC-3′, antisense 5′-GGA GCA CAA GGC ATG GC-3′ (bp 998–1779), (3) sense, 5′-TGG GAC CTC CGG TCA GA-3′, antisense 5′-CTG GGT GTA AGA GGC TCC AC-3′ (bp 1689–2330), (4) sense, 5′-TCG TTC GGA AGC GCA-3′, antisense 5′-TGG CTG AGG GAG GCG TTC T-3′ (bp 2263–3054), (5) sense, 5′-GGA ATC CCT GCC AGC-3′, antisense 5′-GAT TCT GCA CAG AGC CAG-3′ (bp 2995–3568) and (6) sense, 5′-ACA TAA ACC AGT CCG TTC CC-3′, antisense 5′-CAT GCT CCA ATA AAT TCA CTG C-3′ (bp 3521–3879). The polymerase chain reaction (PCR) was performed using 60 s for denaturation at 94°C, 1 min annealing at 56°C and 90 s for extension at 72°C for a total of 35 cycles. The amplified PCR products were gel purified (QIAquick Gel Extraction Kit; Qiagen), and directly sequenced from both directions. Results were compared with published wild-type sequence bp 247–3879 (amino acids 1–1210) using BLAST search program (Gen Bank accession no. NM_005228) covering exons 1–26 of the gene.

Fluorescence in-situ hybridization

Studies were carried out with the use of dual-color DNA fluorescence in-situ hybridization (FISH) probes containing the LSI EGFR (Vysis, Downers Grove, Illinois, USA) probe specific for the *EGFR* locus (7p12) labelled with Spectrum Orange (Vysis) and the *CEP*7 chromosome 7 centromere (7p11.1–q11.1) probe labelled with Spectrum Green (Vysis). We analyzed 33–100 non-overlapping tumor cell nuclei to determine the number of red (*EGFR*) and green (*CEP*7) signals observed as well as the pattern of distribution of signals. We also determined the number of copies of *EGFR* and classified them according to the six FISH categories defined by Cappuzzo *et al.* [17]. Samples with a high number of copies of *EGFR* (high degrees of polysomy or amplification) were considered to be FISH-positive.

Immunohistochemical staining

Paraffin-embedded tissue sections from resection specimens of 19 patients with extrahepatic CC were de-waxed, hydrated through graded alcohol, and immunostained with antibodies to EGFR, ErbB-2 and VEGFR-2 using the avidin–biotin–HRPO method as described previously [18]. Immunoreactivity was revealed using True Blue peroxidase substrate (KPL, Gaithersburg, Maryland, USA). The sections were counterstained with hematoxylin–eosin and mounted with Permount (Fisher, Pittsburgh, Pennsylvania, USA). For the tumor tissues, the percentage of positive cells was estimated and the staining intensity was semiquantitatively recorded as 1+, 2+ or 3+. For statistical analyses, the staining results were categorized into four groups according to Went *et al.* [19]. Tumors without any staining were considered negative. Tumors with 1+ staining intensity in less than 60% of cells and 2+ intensity in less than 30% of cells were considered weakly positive. Tumors with 1+ staining intensity in or more 60% of cells, 2+ intensity in 30–79% or 3+ intensity in less than 30% were considered moderately positive. Tumors with 2+ intensity in 80% or more or 3+ intensity in 30% or more of cells were considered strongly positive. Only membranous or membranous plus cytoplasmic staining was considered for analysis because cytoplasmic staining alone proved to be false-positive in all preabsorption control experiments. For MIB-1 staining we used paraffin sections and for vWF-staining cryosections from animal tumors following protocols that have been described elsewhere [20,21].

Inhibition of cell growth

The effect of gefitinib, erlotinib and NVP-AEE788 on cellular proliferation was assessed by automated cell counting (Schaerfe Casy 2.0 Cell Counter, Reutlingen, Germany) according to the manufacturer's instructions. Briefly, 2×10^5 cells were seeded in T-25 cell culture flasks. Twenty-four hours after incubation, cells were treated with gefitinib, erlotinib and NVP-AEE788 at five

different concentrations. After 6 days of incubation, cells were trypsinized, washed and analyzed in triplicates by automated cell counting.

Animal studies

Tumors were induced by injecting 5×10^6 Mz-ChA-2 or EGI-1 cells in 200 µl phosphate-buffered saline subcutaneously into the flank region of NMRI nude mice. Treatment was started when an average tumor volume of 200 mm^3 was reached (usually after 2 weeks). The verum group received either erlotinib (0.5% w/v methyl cellulose solution) or NVP-AEE788 [10% v/v *m*-methyl-pyrrolidone (NMP) and 90% v/v PEG300 solution] orally, whereas the control group received placebo (carrier solution without drug) only. Treatment was continued for 14 consecutive days, tumors were daily measured with a Vernier caliper and tumor volumes were calculated using the formula tumor volume $= 0.5 \times L \times W^2$, where L represents the length and W the width of the tumor. When treatment was finished, animals were killed and tumors excised and weighed.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling POD test

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) (in-situ cell death detection kit; POD) was used to detect apoptosis in paraffin sections from mouse tumor tissue. The TUNEL test was carried out following the manufacturer's instructions (Roche, Penzberg, Germany) as described before [22]. Apoptotic cells (red) were counted under a light microscope after fluorescence signal conversion using antibody with conjugated peroxidase and the substrate for peroxidase (DAB; Roche).

Statistical analysis

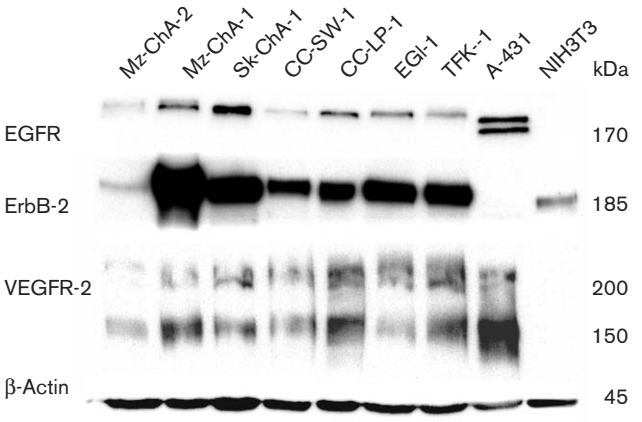
Statistical calculations were performed using SPSS, version 10.0 (SPSS, Chicago, Illinois, USA). Numeric data were presented as mean value with standard deviation (SD). Inter-group comparisons were carried out with the Student *t*-test. *P* values less than 0.05 were considered significant.

Results

Epidermal growth factor receptor, ErbB-2 and vascular endothelial growth factor receptor-2 protein expression in biliary tract cancer cell lines

The expression of EGFR, ErbB-2 and VEGFR-2 in all seven human biliary tract cancer cell lines was assessed by immunoblotting. Cell lysates of cell lines A-431 and NIH3T3 served as positive controls, and showed bands at 170 (EGFR), 185 (ErbB-2) and 150/200 kDa (VEGFR-2). Staining with β-actin-antibody confirmed equal protein loading. All seven cell lines demonstrated EGFR, ErbB-2 and VEGFR-2 expression at different levels (Fig. 1). Sk-ChA-1 and Mz-ChA-1 showed the highest expression level for EGFR and ErbB-2; CC-LP-1, TFK-1 and Mz-ChA-1 the highest level for VEGFR-2; CC-SW-1 showed the lowest expression level for EGFR, Mz-ChA-2 for ErbB-2, and EGI-1 and Mz-ChA-2 the lowest level for VEGFR-2.

Fig. 1



The expression of EGFR, ErbB-2, and VEGFR-2 in all seven human biliary tract cancer cell lines was assessed by immunoblotting. Cell lysates of cell lines A-431 and NIH3T3 served as positive controls. Staining with β-actin antibody confirmed equal protein loading. All seven cell lines demonstrated EGFR, ErbB-2 and VEGFR-2 expression at different levels. EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

Reverse transcription polymerase chain reaction and sequencing of epidermal growth factor receptor cDNA

Mutational analysis in the EGFR-positive cell lines by direct sequencing of polymerase chain reaction (PCR) products did not reveal any mutations.

Fluorescence in-situ hybridization

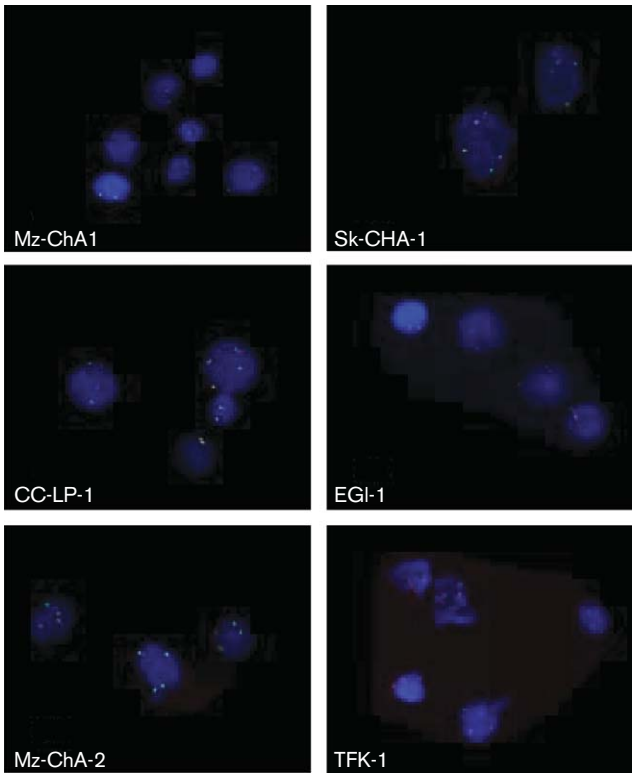
We searched for genomic EGFR amplifications and polysomy by FISH. FISH of diploid tumor cells showed one or two red (EGFR) and one or two green (CEP7) signals, and no excess of red signals. Therefore, no amplifications and polysomy were found in the seven biliary tract cancer cell lines (Fig. 2).

Epidermal growth factor receptor, ErbB-2 and vascular endothelial growth factor-2 protein expression in human biliary tract cancer tissue

In order to analyze the expression of EGFR, ErbB-2 and VEGFR-2 in human biliary tract cancer, paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC were immunostained with EGFR, ErbB-2 and VEGFR-2 antibodies using the avidin–biotin–HRPO method. The clinical characteristics of the study population are summarized in Table 1 [23,24]. As a result, three of the 19 tissues samples displayed a strong EGFR immunostaining (+ + +), three a rather weak immunostaining (+) and 13 were negative (–). Thus, six of 19

(32%) of the histological sections of extrahepatic hilar CC tested showed strong to weak expression of EGFR. In addition, two of the 19 tissue samples displayed a strong ErbB-2 immunostaining (+ + +), two an intermediate immunostaining (+ +), 12 a rather weak immunostaining (+) and three were negative (–). Therefore, ErbB-2 expression was detected in 16 of 19 (84%) samples. Finally, nine of 19 (47%) tissue samples showed a rather weak immunostaining for VEGFR-2 (+), whereas 10 samples were negative (–). The immunoreactivity of EGFR, ErbB-2 and VEGFR-2 was found mainly in the cell membrane (Fig. 3).

Fig. 2



We searched for genomic EGFR amplifications and polysomy by FISH. No amplifications and polysomy were found in the seven biliary tract cancer cell lines. FISH of diploid tumor cells showed one or two red (EGFR) and one or two green (CEP7) signals. EGFR, epidermal growth factor receptor; FISH, fluorescence in-situ hybridization.

Inhibition of cell growth

After 6 days of incubation, three cell lines were intermediate sensitive to gefitinib [1 μmol/l $< IC_{50}$ (inhibitory concentration 50%) $\leq$ 10 μmol/l] and three were resistant ($IC_{50} > 10$ μM) (Fig. 4a). In contrast, three cell lines were sensitive to erlotinib ($IC_{50} \leq 1$ μmol/l), four intermediate sensitive and no cell line resistant (Fig. 4b). The efficacy of NVP-AEE788 treatment, however, was the highest (four cell lines were sensitive, three were intermediate sensitive and no cell line was resistant) (Fig. 4c). The mean IC_{50} value was 15.29 ± 9.71 μmol/l for gefitinib, 2.68 ± 2.32 μmol/l for erlotinib and 1.01 ± 0.70 μmol/l for NVP-AEE788 (Table 2). In addition, dimethyl sulfoxide, the solvent of gefitinib, erlotinib and NVP-AEE788, alone had no influence on cell growth (data not shown).

Chimeric mouse model

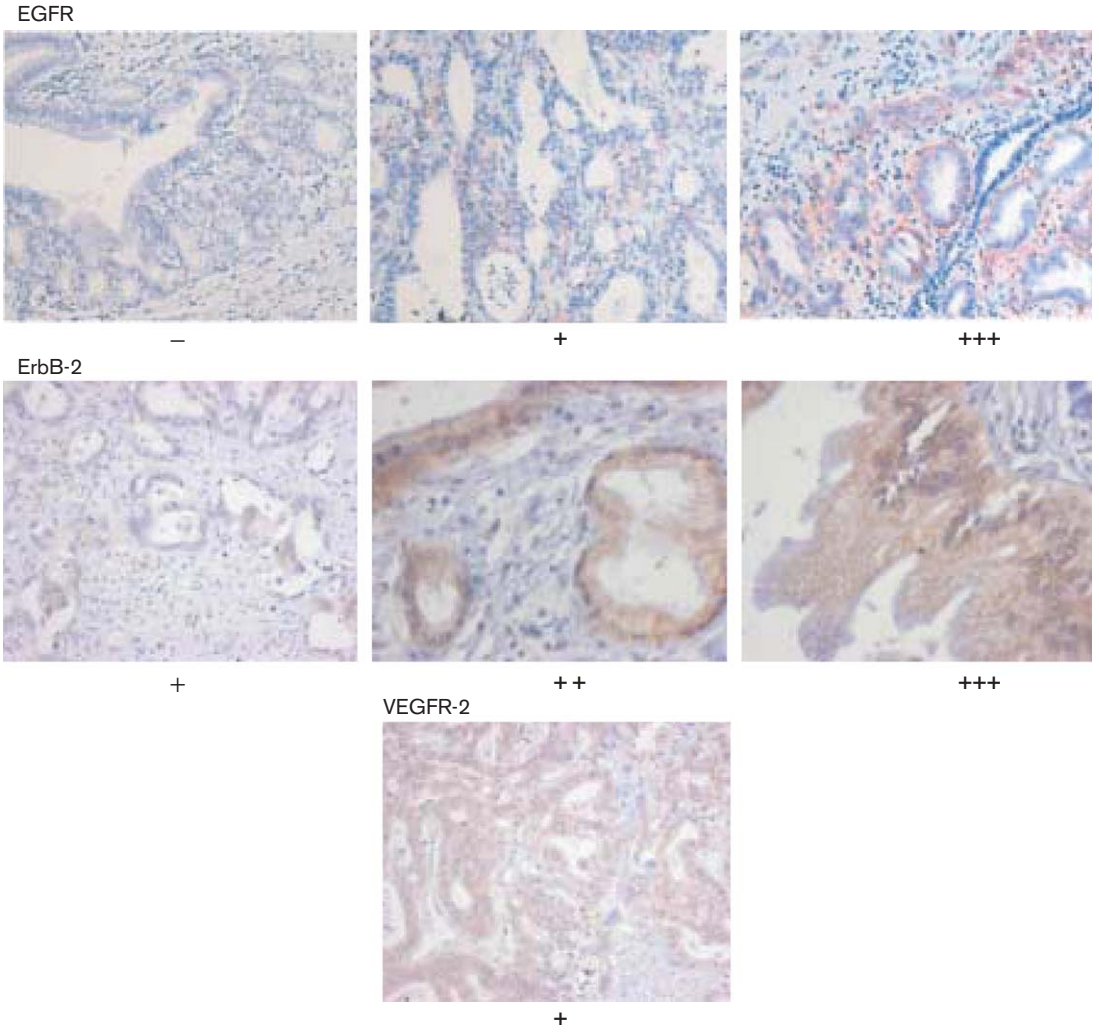
Tumors were induced in nude mice by subcutaneous injection of Mz-ChA-2 and EGI-1 cells. These cell lines were selected because they had shown the best growth capability in nude mice in our previous studies [25]. Treatment of mice consisted of oral gavage of erlotinib, NVP-AEE788, or placebo. Five and 6 days after beginning erlotinib treatment [20 mg/kg body weight (BW) daily], EGI-1 cell tumors had a significantly reduced volume in comparison with control ($n = 10$ for each group) (Fig. 5a).

Table 1 Clinical characteristics of the study population

Patient initials	Patient age (years)	Tumor stage (UICC 2002) [23]	Bismuth–Corlette classification [24]	Tumor grade	EGFR staining	ErbB-2 staining	VEGFR-2 staining
D.K.	56	IIB	IV	G2	–	–	+
G.E.	72	IIB	IIIa	G2	–	–	+
R.E.	46	IIB	IIIa	G2	–	+	+
H.H.	59	IIA	IV	G2	–	+ +	–
E.K.	43	IA	IIIa	G1	–	+	–
A.K.	46	IIA	IV	G2	–	–	–
L.S.	61	III	IIIb	G2	+	+	+
C.B.	74	IIA	IIIa	G3	+ + +	+ + +	–
B.K.	72	IIA	II	G2	–	+	+
R.M.	61	IB	IV	G2	–	+	–
H.M.	61	IIB	IIIa	G2	+	+ +	–
B.P.	39	IV	IV	G2	+ + +	+	–
C.S.	42	IIA	IIIa	G2	+	+	+
B.S.	68	IIA	IIIb	G2	–	+	+
G.T.	59	IIB	IIIa	G2	+ + +	+ + +	+
M.T.	65	IIA	IV	G2	–	+	–
M.U.	74	IB	I	G3	–	+	–
U.W.	44	IIB	IIIa	G3	–	+	+
E.D.	67	IB	IV	G2	–	+	–

EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

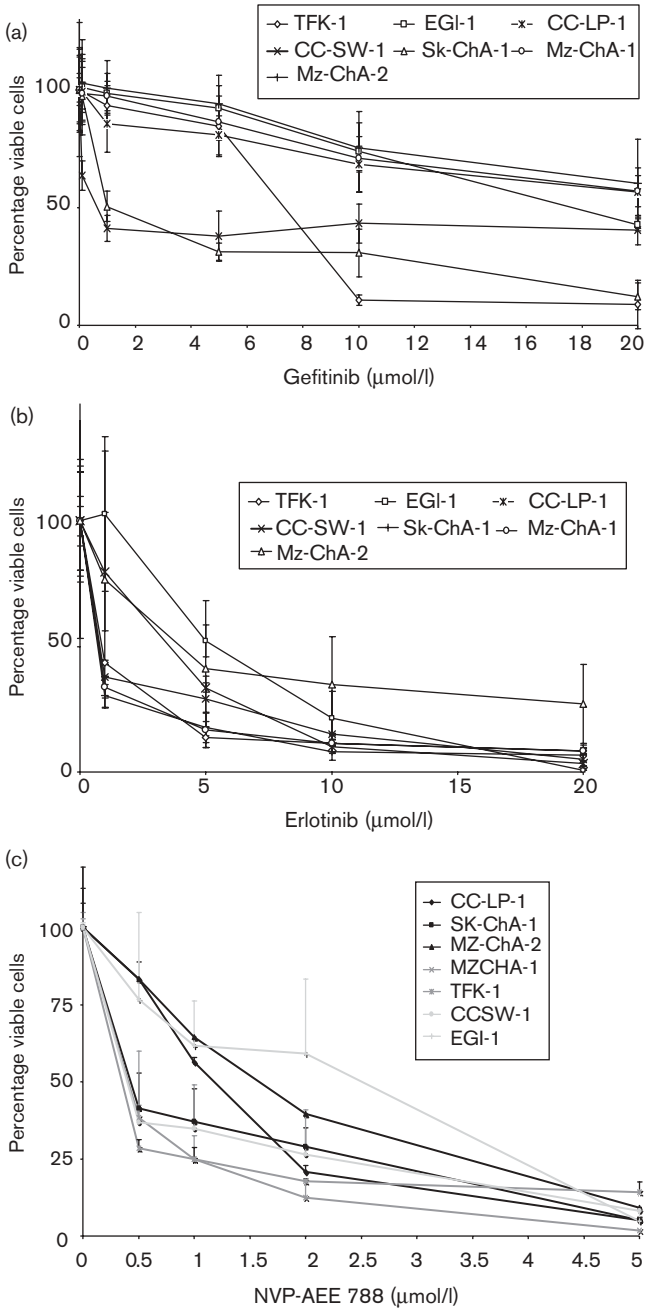
Fig. 3



Paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC were immunostained with EGFR, ErbB-2 and VEGFR-2 antibodies by using the avidin–biotin–horseradish peroxidase method. (a) As a result, three of the 19 tissues samples displayed a strong EGFR immunostaining, three a rather weak immunostaining and 13 were negative. (b) In addition, two of the 19 tissues samples displayed a strong ErbB-2 immunostaining, two an intermediate immunostaining, 12 a rather weak immunostaining and three were negative. (c) Finally, nine of 19 (47%) tissue samples showed a rather weak immunostaining for VEGFR-2, whereas 10 samples were negative (magnification × 40). CC, cholangiocarcinoma; EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

In contrast to that, daily erlotinib treatment of mice bearing Mz-ChA-2 cell tumors did not result in any change of tumor growth compared with control ($n = 10$ for each group) (Fig. 5b). Treatment of Mz-ChA-2 tumors with NVP-AEE788 resulted in a reduced volume in comparison with control ($n = 4$ for each group). This effect was stronger for 30 mg/kg BW daily than 50 mg/kg BW every 3 days (Fig. 5c). Differences, however, were not statistically significant [$P = 0.45$ for NVP-AEE788 (50 mg/kg BW every 3 days) vs. placebo and $P = 0.15$ for NVP-AEE788 (30 mg/kg BW daily) vs. placebo]. In contrast, EGI-1 cell tumors had a significantly reduced volume in comparison with control ($n = 10$ for each group, $P < 0.01$) from 2 days after beginning treatment with NVP-AEE788 (30 mg/kg BW daily) (Fig. 5d). At the end of the experiment (day 15), tumor mass was significantly diminished as compared with control after daily treatment with 30 mg/kg BW NVP-AEE788 ($P < 0.01$), which was not observed for erlotinib (Fig. 5a–e). In order to assess the anti-tumoral drug mechanism, paraffin sections of mouse tumors were stained with hematoxylin–eosin, MIB-1 (proliferation marker), TUNEL (apoptosis marker) and vWF (angiogenesis marker). Treatment with erlotinib and NVP-AEE788 reduced proliferation (reduced MIB-1 staining), induced apoptosis (increased TUNEL-staining) and inhibited angiogenesis (reduced

Fig. 4



Inhibition of cell growth. (a) After 6 days of incubation, three cell lines were intermediate sensitive to gefitinib (1 μmol/l$<IC_{50} \leq$ 10 μmol/l) and four were resistant ($IC_{50}>10$ μmol/l). (b) In contrast, four cell lines were sensitive to erlotinib ($IC_{50} \leq 1$ μmol/l) and three were intermediate sensitive. (c) The efficacy of treatment with NVP-AEE788, however, was the highest (five cell lines were sensitive and two were intermediate sensitive).

vWF staining) (Fig. 6a). The expression of p-p42/44 (p-Erk1/2, p-MAPK) and p-AKT was assessed by immunoblotting. NIH3T3 cells without and with EGF stimulation served as negative and positive control. Whereas signalling via the MAPK pathway was diminished by erlotinib and NVP-AEE788, there was no significant reduction of PI3K/AKT signalling, which may be a possible counter mechanism of the tumor.

Discussion

Non-resectable biliary tract cancer is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy. It is therefore essential to search for new therapeutic approaches. It has been shown that the EGF superfamily of transmembrane receptors is involved in the pathogenesis of CC [26]. Transformed cholangiocytes, but not normal or fetal biliary epithelial cells, over-express ErbB-2 as determined by immunohistochemical staining of tumor specimens [27–30]. A strong positive correlation was observed between increased ErbB-2 immunoreactivity measured in the tumors and risk conditions like hepatolithiasis and primary sclerosing cholangitis [28,30–32]. Enhanced immunoreactivity for ErbB-2 correlated directly with tumor differentiation and was highest in well-differentiated tumors [31,32]. A rat model of CC induced by chemical carcinogens showed clear development of c-*neu* (rat homolog of human ErbB-2) expression and activation, particularly in regions of the tumor that contain dysplastic and neoplastic cells [33]. ErbB-2 is able to form homodimers or heterodimers with other ErbB receptor family members, resulting in an unregulated proliferative signal. Thus, a transgenic mouse model that over-expresses ErbB-2 was found to rapidly develop gallbladder adenocarcinomas in the context of increased ErbB-2/EGFR heterodimer formation, activation of MAPK and up-regulation of cyclooxygenase-2 levels [34]. Recently, the EGFR has also been linked with the pathogenesis of CC. Bile acids, including deoxycholic acid, a potent tumor promotor in the hamster model of cholangiogenesis [35], were shown to induce cyclooxygenase-2 in cultured immortalized human H69 cholangiocyte and KMBC cell lines through a mechanism involving transactivation of the EGFR [36,37]. Bile acids further have been shown to block protein degradation of myeloid cell leukemia protein 1, a potent anti-apoptotic protein of the B-cell leukemia/lymphoma-2 (Bcl-2) family, via activation of EGFR signalling [38]. Considering these data, there are a number of reasons why EGFR is such an attractive target for therapy of biliary tract cancer. First, pathological examination of diagnostic tissue taken from patients can be used to identify patients with EGFR-positive tumors. Second, clinical trials for other tumors have demonstrated that interruption of the EGFR signalling using specific EGFR inhibitors inhibits tumor proliferation and viability. Finally, in adults, EGFR does not appear to play a crucial physiological role. Moreover, it is hoped that by targeting this receptor it will be possible to reduce the harmful side effects caused by chemotherapy.

Table 2 Inhibition of cell growth by gefitinib, erlotinib and NVP-AEE788

Cell line	IC_{50}(μmol/l)		
	Gefitinib	Erlotinib	NVP-AEE788
TFK-1	5.09	0.90	0.49
EGI-1	17.57	4.22	2.31
CC-LP-1	23.20	3.10	1.10
CC-SW-1	6.64	1.67	0.56
Sk-ChA-1	4.27	0.90	0.72
Mz-ChA-1	23.79	0.90	0.36
Mz-ChA-2	26.47	7.05	1.51

Therefore, in a first step, we evaluated the expression of EGFR and ErbB-2 in seven human biliary tract cancer cell lines by immunoblotting. In addition, VEGFR-2 status was assessed. As a result, all examined cell lines demonstrated EGFR, ErbB-2 and VEGFR-2 expression, at different levels, however.

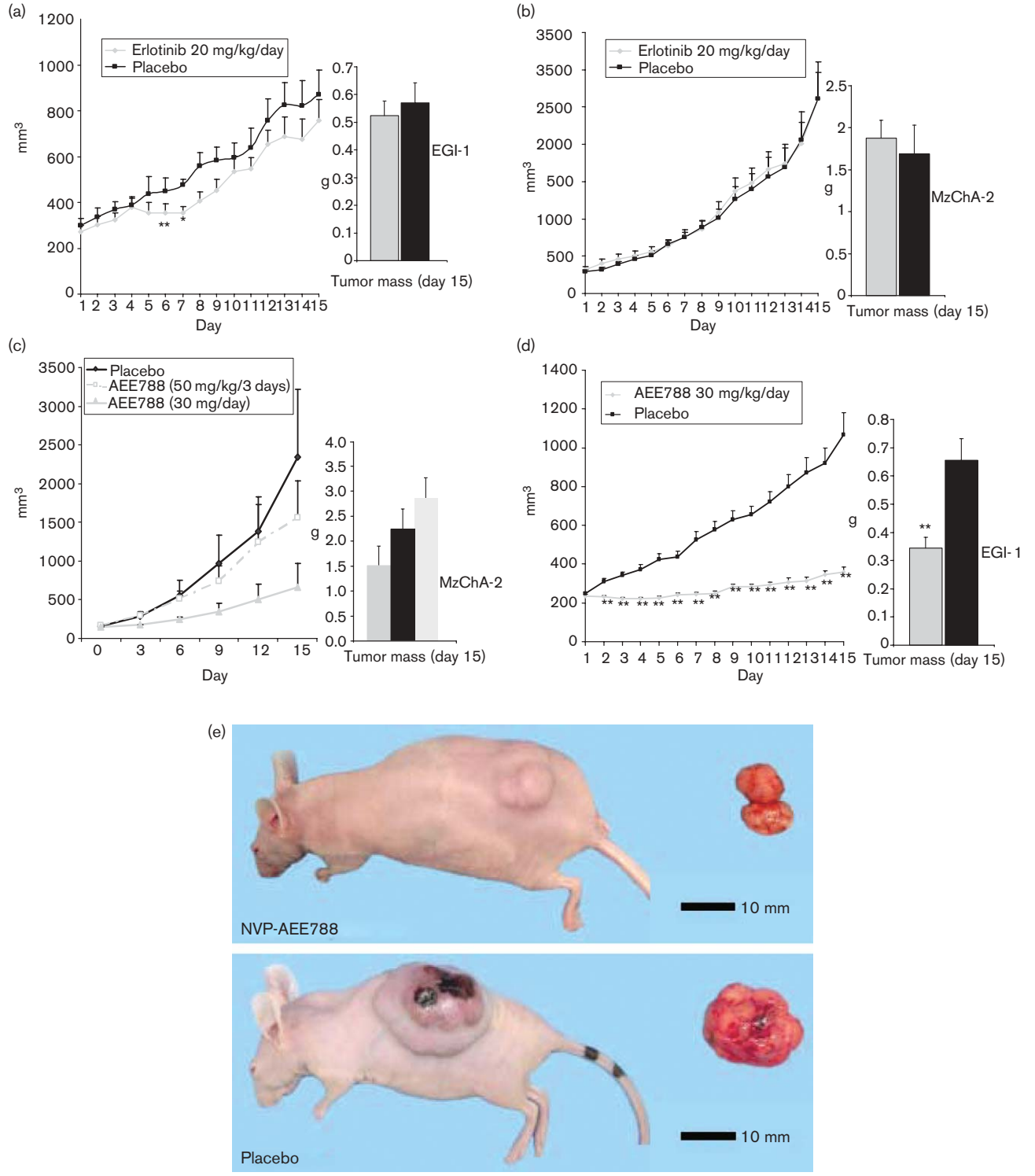
In certain tumors, including some glioblastomas and, as shown recently, breast cancer, a truncated EGFR lacking a portion of the extracellular domain (exons 2–7) is expressed (EGFRvIII) [39–42]. EGFRvIII appears to be constitutively active and is oncogenic in NIH3T3 cell transformation assays [40,43–47]. Detection of EGFRvIII, which is one of a number of EGFR splice variants that have been observed in human cells (e.g. EGFRvI, EGFRvII and others), is associated with a poor patient prognosis. In addition, data from three recent studies strongly suggest that other gain-of-function somatic mutations affecting the catalytic domain (specifically the ATP binding site, exons 18–21) of the gene encoding EGFR are strongly associated with response of NSCLC to gefitinib and erlotinib, two EGFR TKIs [48–50]. These mutations appear to enhance the ability of the ligand to induce EGFR activity and also prolong the binding of the EGFR inhibitor to the kinase domain. Secondary mutations, however, can also lead to resistance in initially treatment-sensitive tumors, as shown recently for gefitinib treatment of NSCLC [51]. As there is no sufficient data for the situation in biliary tract cancer, we performed reverse transcriptase PCR and sequencing of EGFR cDNA in our seven EGFR-positive cell lines. Mutational analysis by direct sequencing of PCR products did not reveal any mutations. This is consistent with the results of a study that examined exons 18–21 only in 40 hepatomas and biliary tract cancers [52]. Another alteration is amplification of the EGFR gene, a phenomenon that has been found in 40–50% of glioblastomas, and also in other tumors such as NSCLC and colorectal cancer [53,54]. It is related to a shorter interval to relapse and poorer survival in human glioblastoma, and an increased likelihood of mutations occurring in the EGFR gene, but a better response to anti-EGFR treatment in colorectal cancer and NSCLC [50,54–57]. We searched for genomic EGFR amplifications and polysomy by FISH, but results were negative for our seven biliary tract cancer cell lines.

In a next step, we analyzed the expression of EGFR, ErbB-2 and VEGFR-2 in paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC by immunohistochemistry. No data exist for VEGFR-2 expression in biliary tract cancer and only a few data for EGFR expression in intrahepatic CC, whereas several studies have examined ErbB-2 expression in intrahepatic CC and gallbladder cancer [28–31,58,59]. Thus, Ito *et al.* [29] found 45% of intrahepatic CC positive for EGFR and other groups of ErbB-2 expression between 0 and 70% in intrahepatic CC and 64% in gallbladder cancer [28–31,58,59]. As a result of our study, 32% of the histological sections of extrahepatic hilar CC tested showed strong to weak expression of EGFR, 84% of ErbB-2 and 47% of VEGFR-2.

We then tested seven biliary tract cancer cell lines for the in-vitro effect of gefitinib, erlotinib and NVP-AEE788 by automated cell counting. Whereas gefitinib and erlotinib are pure EGFR inhibitors, NVP-AEE788 represents a dual EGFR/ErbB-2 inhibitor with additional anti-VEGFR-2 activity. It belongs to the class of the 7*H*-pyrrolo-[2,3-*d*]-pyrimidines. After 6 days of incubation, gefitinib, erlotinib and NVP-AEE788 caused a significant growth inhibition *in vitro*, but there was a significant difference in efficacy (NVP-AEE788 > erlotinib > gefitinib). The mean IC_{50} value was 15.29 ± 9.71 μmol/l for gefitinib, which is much higher than IC_{50} values reported for human breast, ovarian, gastric and colon cancer cell lines ranging from 0.1 to 0.9 μmol/l [60,61]. In contrast, the mean IC_{50} value was 2.68 ± 2.32 μmol/l for erlotinib and even lower for NVP-AEE788 (1.01 ± 0.70 μmol/l). The last value was quite promising as it is known that MCF-7 human breast cancer and T24 bladder carcinoma cells have values of 2.5 and 4.5 μmol/l [62]. In addition, we assessed apoptosis by the annexin V/propidium iodide staining kit (Annexin Apoptosis Detection Kit I; BD Biosciences, Heidelberg, Germany) and cell proliferation by the In-Situ Cell Proliferation Kit, FLUOS (Roche, Mannheim, Germany) after treatment with the three different drugs *in vitro*. Both an induction of apoptosis and an inhibition of cell proliferation were detectable (data not shown).

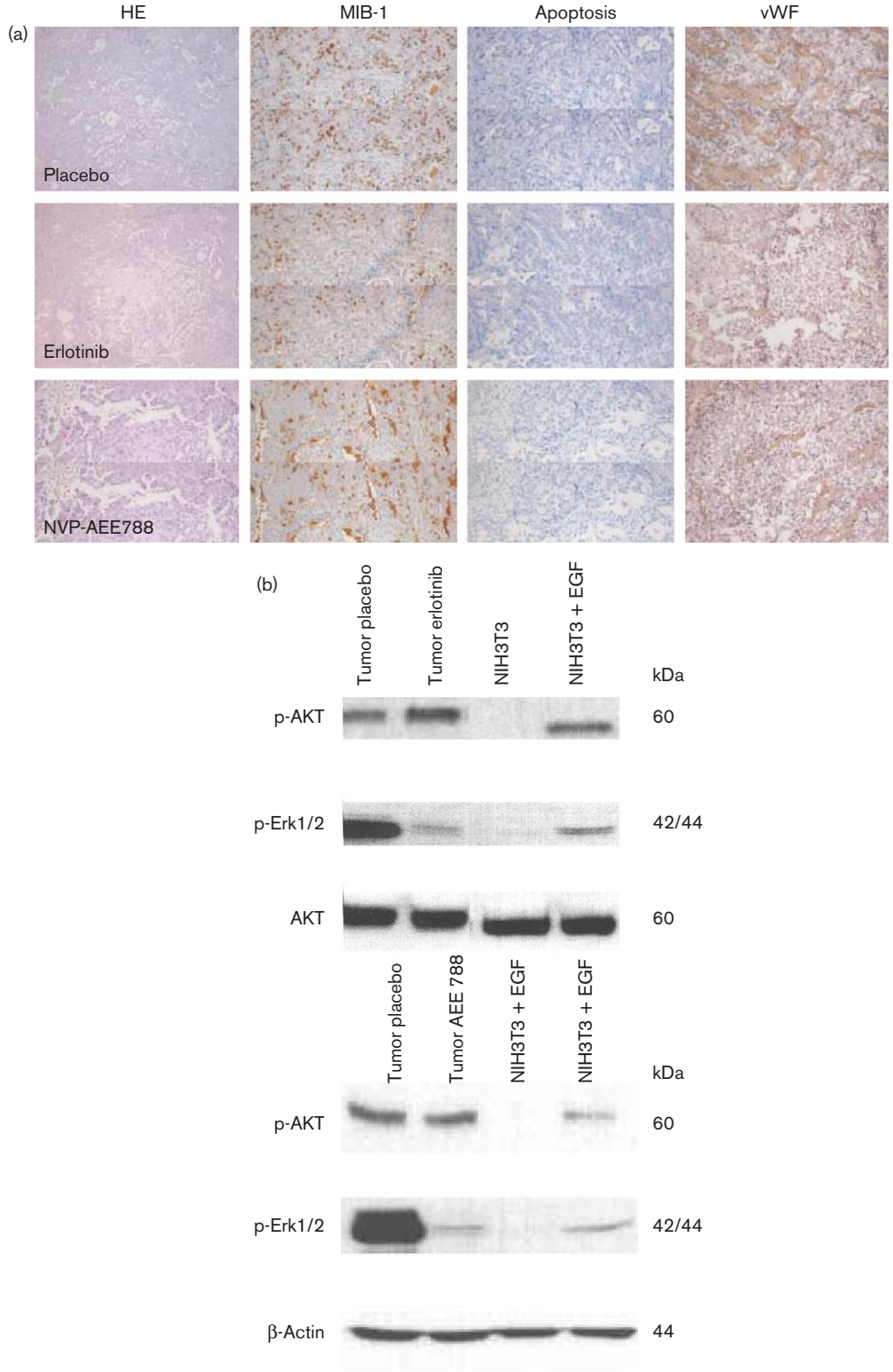
Considering these in-vitro results, we decided to test the two most effective drugs erlotinib and NVP-AEE788 *in vivo* in comparison with placebo using the chimeric mouse model. Five and 6 days after beginning daily oral erlotinib treatment with 20 mg/kg BW, a dose that was effective in a head and neck cancer model [63], EGI-1 cell tumors had a significantly reduced volume in comparison with control, whereas Mz-ChA-2 cell tumors had not. One limitation of our study may be the short course of treatment and surveillance in our mice, which

Fig. 5



Chimeric mouse model. Tumors were induced in nude mice by subcutaneous injection of Mz-ChA-2 and EGI-1 cells. Treatment of mice consisted of oral gavage of erlotinib, NVP-AEE788 or placebo. (a) Five and 6 days after beginning erlotinib treatment (20 mg/kg BW daily), EGI-1 cell tumors had a significantly reduced volume in comparison with control ($n=10$ for each group). (b) In contrast to that, daily erlotinib treatment of mice bearing Mz-ChA-2 cell tumors did not result in any change of tumor growth compared with control ($n=10$ for each group). (c) Treatment of Mz-ChA-2 tumors with NVP-AEE788 resulted in a reduced volume in comparison with control ($n=4$ for each group). This effect, however, was stronger for 30 mg/kg BW daily than 50 mg/kg BW every 3 days. (d) From 1 day after beginning treatment with NVP-AEE788 (30 mg/kg BW daily) EGI-1 cell tumors had a significantly reduced volume in comparison with control. (d and e) At the end of the experiment (day 15), tumor mass was significantly diminished as compared with control ($n=10$ for each group). *$P<0.05$, **$P<0.01$. BW, body weight.

Fig. 6



Chimeric mouse model. (a) Treatment with erlotinib and NVP-AEE788 reduced proliferation (reduced MIB-1 staining), induced apoptosis (increased TUNEL staining) and inhibited angiogenesis (reduced vWF staining) (magnification × 40). (b) The expression of p-p42/44 (p-Erk1/2, p-MAPK) and p-AKT was assessed by immunoblotting. NIH3T3 cells without and with EGF stimulation served as negative and positive control. Whereas signalling via the MAPK pathway was diminished by erlotinib and NVP-AEE788, there was no significant reduction of PI3K/AKT signalling, which may be a possible counter-mechanism of the tumor. EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; PI3K, phophatidylinositol 3′-kinase.

was much longer in the above-mentioned head and neck cancer study, in which mice were observed up to 60 days. Tumor size extent beyond 800 mm^3 in the placebo group, however, ended our experiment for ethical reasons. Treatment of Mz-ChA-2 tumors with NVP-AEE788 resulted in a reduced volume in comparison with control. The difference, however, was not significant, which might have been caused by the limitation of the small number of animals in each group ($n = 4$). The effect was stronger for the dose of 30 mg/kg BW daily than for the dose of 50 mg/kg BW every 3 days, confirming the data of Traxler *et al.* [62] for DU145 human prostate cancer cells. Therefore, in a second experiment with EGI-1 cell tumors we used NVP-AEE788 at a daily oral concentration of 30 mg/kg BW. From 1 day after beginning treatment with NVP-AEE788, tumors had a significantly reduced volume in comparison with control. At the end of all animal experiments (day 15), tumor mass was significantly diminished as compared with control after treatment with NVP-AEE788. This may be explained by the fact that each member of the EGFR family has differing kinase activity with ErbB-2, which is the preferred binding partner for EGFR, possessing the greatest [64,65]. Interestingly, the heterodimer between EGFR and ErbB-2 may transmit a more potent signal than an EGFR homodimer [66]. In addition, recent theories suggest that ErbB-2 may not need a ligand to be activated; in fact, it may not bind ligands at all [66]. This may explain the more potent effect of NVP-AEE788 than of erlotinib given the fact that CCs frequently co-express both receptors as demonstrated for our cell lines and patients' specimens. Finally, VEGFR-2, which is usually expressed in vascular endothelial cells to mediate the major growth and permeability action of VEGF, was found in our biliary tract cancer cell lines and patients' specimens' too. This discovery implies an additional direct inhibition of VEGFR-2 signalling by NVP-AEE788 on the tumor cell surface.

Several signal transduction pathways have been implicated in EGFR-dependent cell survival. Primarily, these include the Ras/Raf/MEK/MAPK cascade and the PI3K/AKT-dependent signalling events. At least two MAPK targets relevant for cell survival in the anchorage-independent state have been identified. Specifically, EGFR-mediated MAPK activation is required for high-level expression of Bcl-X_L, an antiapoptotic member of the Bcl-2 family of proteins, as shown in keratinocytes and MDCK cells (human breast epithelial cell line) [67,68]. It also enhances phosphorylation of the pro-apoptotic Bcl-2 family member BAD on serines 112 and 155 in mammary epithelial cells [69]. Phosphorylation is a prerequisite for sequestration and functional inactivation of BAD [70,71], thus preventing apoptosis. In certain experimental settings, EGFR activation is also associated with activation of the PI3K/AKT survival pathway or nuclear factor κB [72,73]. In our study, the expression of p-p42/44 (p-Erk1/2, p-MAPK) and p-AKT was assessed by immunoblotting. Whereas signalling via the MAPK pathway was diminished by erlotinib and NVP-AEE788, there was no significant reduction of PI3K/AKT-signalling, which may be a possible counter mechanism of the tumor, induced by other events such as K-Ras, PI3K or PTEN mutations [74–77]. Finally, inhibition of angiogenesis by erlotinib and NVP-AEE788 was observed in our animal study (as demonstrated by reduced vWF staining). This phenomenon may be due to direct or indirect effects on endothelial cells. As EGFR stimulates transcription of VEGF through the MAPK pathway [78], erlotinib and NVP-AEE788 may inhibit VEGF transcription, thus diminishing the availability of ligand for endothelial cell-based VEGFR-2. In addition, NVP-AEE788 may directly inhibit endothelial cell-based VEGFR-2.

In conclusion, EGFR, ErbB-2 and VEGFR-2 expression was detectable in biliary tract cancer and receptor inhibition exerts marked effects on tumor growth *in vitro* and *in vivo*, which was strongest for the dual EGFR/ErbB-2 inhibitor NVP-AEE788. Therefore, further clinical evaluation of NVP-AEE788 for the treatment of biliary tract cancer is recommended.

Acknowledgements

The authors thank Mrs Ines Sommerer and Mrs Ulrike Haugwitz for technical assistance, and Astra Zeneca, OSI Pharmaceuticals and Novartis Pharma for the provision of gefitinib, erlotinib and NVP-AEE788.

References

1 Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, *et al.* Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 1984; **309**:418–425.
2 Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005; **5**:341–354.
3 Raymond E, Faivre S, Armand JP. Epidermal growth factor receptor tyrosine kinase as a target for anticancer therapy. *Drugs* 2000; **60** (**Suppl 1**):15–23; discussion 41–42.
4 Laskin JJ, Sandler AB. Epidermal growth factor receptor: a promising target in solid tumours. *Cancer Treat Rev* 2004; **30**:1–17.
5 Ritter CA, Arteaga CL. The epidermal growth factor receptor-tyrosine kinase: a promising therapeutic target in solid tumors. *Semin Oncol* 2003; **30**:3–11.
6 Hackel PO, Zwick E, Prenzel N, Ullrich A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol* 1999; **11**:184–189.
7 Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2000; **103**:211–225.
8 Rak J, Yu JL, Klement G, Kerbel RS. Oncogenes and angiogenesis: signaling three-dimensional tumor growth. *J Investig Dermatol Symp Proc* 2000; **5**:24–33.
9 Wiedmann MW, Caca K. Molecularly targeted therapy for gastrointestinal cancer. *Curr Cancer Drug Targets* 2005; **5**:171–193.
10 Baselga J, Arteaga CL. Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. *J Clin Oncol* 2005; **23**: 2445–2459.
11 Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. *N Engl J Med* 2005; **353**:172–187.
12 Saijyo S, Kudo T, Suzuki M, Katayose Y, Shinoda M, Muto T, *et al.* Establishment of a new extrahepatic bile duct carcinoma cell line, TFK-1. *Tohoku J Exp Med* 1995; **177**:61–71.

13 International Conference on Tumor Necrosis Factor and Related Cytotoxins. 14–18 September 1987, Heidelberg, Federal Republic of Germany. Abstracts. *Immunobiology* 1987; **175**:1–143.

14 Shimizu Y, Demetris AJ, Gollin SM, Storto PD, Bedford HM, Altarac S, *et al.* Two new human cholangiocarcinoma cell lines and their cytogenetics and responses to growth factors, hormones, cytokines or immunologic effector cells. *Int J Cancer* 1992; **52**:252–260.

15 Knuth A, Gabbert H, Dippold W, Klein O, Sachsse W, Bitter-Suermann D, *et al.* Biliary adenocarcinoma. Characterisation of three new human tumor cell lines. *J Hepatol* 1985; **1**:579–596.

16 Caca K, Feisthammel J, Klee K, Tannapfel A, Witzigmann H, Wittekind C, *et al.* Inactivation of the INK4a/ARF locus and p53 in sporadic extrahepatic bile duct cancers and bile tract cancer cell lines. *Int J Cancer* 2002; **97**:481–488.

17 Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, *et al.* Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* 2005; **97**:643–655.

18 Woo M, Hakem A, Elia AJ, Hakem R, Duncan GS, Patterson BJ, *et al.* In vivo evidence that caspase-3 is required for Fas-mediated apoptosis of hepatocytes. *J Immunol* 1999; **163**:4909–4916.

19 Went PT, Dirnhofer S, Bundi M, Mirlacher M, Schraml P, Mangialaio S, *et al.* Prevalence of KIT expression in human tumors. *J Clin Oncol* 2004; **22**:4514–4522.

20 Gadducci A, Baicchi U, Marrai R, Facchini V, del Bravo B, Fosella PV, *et al.* Pretreatment plasma levels of fibrinopeptide-A (FPA), D-dimer (DD), and von Willebrand factor (vWF) in patients with operable cervical cancer: influence of surgical–pathological stage, tumor size, histologic type, and lymph node status. *Gynecol Oncol* 1993; **49**:354–358.

21 Tannapfel A, Hahn HA, Katalinic A, Fietkau RJ, Kuhn R, Wittekind CW. Prognostic value of ploidy and proliferation markers in renal cell carcinoma. *Cancer* 1996; **77**:164–171.

22 Tannapfel A, Geissler F, Kockerling F, Katalinic A, Hauss J, Wittekind C. Apoptosis and proliferation in relation to histopathological variables and prognosis in hepatocellular carcinoma. *J Pathol* 1999; **187**:439–445.

23 Sobin LH, Wittekind C, editors. *UICC: TNM classification of malignant tumors*. 6th ed. New York: Wiley-Liss; 2002. pp. 74–85.

24 Bismuth H, Corlette MB. Intrahepatic cholangioenteric anastomosis in carcinoma of the hilus of the liver. *Surg Gynecol Obstet* 1975; **140**:170–178.

25 Kamenz T, Caca K, Bluthner T, Tannapfel A, Mossner J, Wiedmann M. Expression of c-kit receptor in human cholangiocarcinoma and *in vivo* treatment with imatinib mesilate in chimeric mice. *World J Gastroenterol* 2006; **12**:1583–1590.

26 Gores GJ. Cholangiocarcinoma: current concepts and insights. *Hepatology* 2003; **37**:961–969.

27 Radaeva S, Ferreira-Gonzalez A, Sirica AE. Overexpression of C-NEU and C-MET during rat liver cholangiocarcinogenesis: a link between biliary intestinal metaplasia and mucin-producing cholangiocarcinoma. *Hepatology* 1999; **29**:1453–1462.

28 Aishima SI, Taguchi KI, Sugimachi K, Shimada M, Tsuneyoshi M. c-erbB-2 and c-Met expression relates to cholangiocarcinogenesis and progression of intrahepatic cholangiocarcinoma. *Histopathology* 2002; **40**:269–278.

29 Ito Y, Takeda T, Sasaki Y, Sakon M, Yamada T, Ishiguro S, *et al.* Expression and clinical significance of the erbB family in intrahepatic cholangiocellular carcinoma. *Pathol Res Pract* 2001; **197**:95–100.

30 Terada T, Ashida K, Endo K, Horie S, Maeta H, Matsunaga Y, *et al.* c-erbB-2 protein is expressed in hepatolithiasis and cholangiocarcinoma. *Histopathology* 1998; **33**:325–331.

31 Endo K, Yoon BI, Pairojkul C, Demetris AJ, Sirica AE. ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. *Hepatology* 2002; **36**:439–450.

32 Terada T, Nakanuma Y, Sirica AE. Immunohistochemical demonstration of MET overexpression in human intrahepatic cholangiocarcinoma and in hepatolithiasis. *Hum Pathol* 1998; **29**:175–180.

33 Sirica AE, Radaeva S, Caran N. NEU overexpression in the furan rat model of cholangiocarcinogenesis compared with biliary ductal cell hyperplasia. *Am J Pathol* 1997; **151**:1685–1694.

34 Kiguchi K, Carbajal S, Chan K, Beltran L, Ruffino L, Shen J, *et al.* Constitutive expression of ErbB-2 in gallbladder epithelium results in development of adenocarcinoma. *Cancer Res* 2001; **61**:6971–6976.

35 Kinami Y, Ashida Y, Gotoda H, Seto K, Kojima Y, Takashima S. Promoting effects of bile acid load on the occurrence of cholangiocarcinoma induced by diisopropanolnitrosamine in hamsters. *Oncology* 1993; **50**:46–51.

36 Yoon JH, Higuchi H, Werneburg NW, Kaufmann SH, Gores GJ. Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. *Gastroenterology* 2002; **122**:985–993.

37 Werneburg NW, Yoon JH, Higuchi H, Gores GJ. Bile acids activate EGF receptor via a TGF-alpha-dependent mechanism in human cholangiocyte cell lines. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**:G31–G36.

38 Yoon JH, Werneburg NW, Higuchi H, Canbay AE, Kaufmann SH, Akgul C, *et al.* Bile acids inhibit Mcl-1 protein turnover via an epidermal growth factor receptor/Raf-1-dependent mechanism. *Cancer Res* 2002; **62**: 6500–6505.

39 Ekstrand AJ, Sugawa N, James CD, Collins VP. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proc Natl Acad Sci U S A* 1992; **89**:4309–4313.

40 Wong AJ, Ruppert JM, Bigner SH, Grzeschik CH, Humphrey PA, Bigner DS, *et al.* Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A* 1992; **89**:2965–2969.

41 Humphrey PA, Wong AJ, Vogelstein B, Zalutsky MR, Fuller GN, Archer GE, *et al.* Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. *Proc Natl Acad Sci U S A* 1990; **87**:4207–4211.

42 Malden LT, Novak U, Kaye AH, Burgess AW. Selective amplification of the cytoplasmic domain of the epidermal growth factor receptor gene in glioblastoma multiforme. *Cancer Res* 1988; **48**:2711–2714.

43 Moscatello DK, Montgomery RB, Sundareshan P, McDanel H, Wong MY, Wong AJ. Transformational and altered signal transduction by a naturally occurring mutant EGF receptor. *Oncogene* 1996; **13**:85–96.

44 Moscatello DK, Holgado-Madruga M, Emlet DR, Montgomery RB, Wong AJ. Constitutive activation of phosphatidylinositol 3-kinase by a naturally occurring mutant epidermal growth factor receptor. *J Biol Chem* 1998; **273**:200–206.

45 Nishikawa R, Ji XD, Harmon RC, Lazar CS, Gill GN, Cavenee WK, *et al.* A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Natl Acad Sci U S A* 1994; **91**:7727–7731.

46 Antonyak MA, Moscatello DK, Wong AJ. Constitutive activation of c-Jun N-terminal kinase by a mutant epidermal growth factor receptor. *J Biol Chem* 1998; **273**:2817–2822.

47 Huang HS, Nagane M, Klingbeil CK, Lin H, Nishikawa R, Ji XD, *et al.* The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem* 1997; **272**:2927–2935.

48 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**:1497–1500.

49 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, *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**:2129–2139.

50 Tsao MS, Sakurada A, Cutz JC, Zhu CQ, Kamel-Reid S, Squire J, *et al.* Erlotinib in lung cancer-molecular and clinical predictors of outcome. *N Engl J Med* 2005; **353**:133–144.

51 Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, *et al.* EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005; **352**:786–792.

52 Bekaii-Saab TS, Sawada T, Williams N, Frankel W, Plass C, Villalona-Calero M, *et al.* Intragenic EGFR and EGFR2 mutations in hepatobiliary tumors and potential role in predicting response to agents that target EGFR. *J Clin Oncol (ASCO 2004)* 2005; abstr 4037

53 Frederick L, Wang XY, Eley G, James CD. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res* 2000; **60**:1383–1387.

54 Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, *et al.* Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 2005; **6**:279–286.

55 Schlegel J, Merdes A, Stumm G, Albert FK, Forsting M, Hynes N, *et al.* Amplification of the epidermal-growth-factor-receptor gene correlates with different growth behaviour in human glioblastoma. *Int J Cancer* 1994; **56**:72–77.

56 Hurtt MR, Moossy J, Donovan-Peluso M, Locker J. Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis. *J Neuropathol Exp Neurol* 1992; **51**:84–90.

57 Jaros E, Perry RH, Adam L, Kelly PJ, Crawford PJ, Kalbag RM, *et al.* Prognostic implications of p53 protein, epidermal growth factor receptor, and Ki-67 labelling in brain tumours. *Br J Cancer* 1992; **66**:373–385.

58 Chow NH, Huang SM, Chan SH, Mo LR, Hwang MH, Su WC. Significance of c-erbB-2 expression in normal and neoplastic epithelium of biliary tract. *Anticancer Res* 1995; **15**:1055–1059.

59 Collier JD, Guo K, Mathew J, May FE, Bennett MK, Corbett IP, *et al.* c-erbB-2 oncogene expression in hepatocellular carcinoma and cholangiocarcinoma. *J Hepatol* 1992; **14**:377–380.

60 Sirotnak FM. Studies with ZD1839 in preclinical models. *Semin Oncol* 2003; **30**:12–20.

61 Ciardiello F, Caputo R, Bianco R, Damiano V, Fontanini G, Cuccato S, *et al.* Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 2001; **7**:1459–1465.

62 Traxler P, Allegrini PR, Brandt R, Brueggen J, Cozens R, Fabbro D, *et al.* AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity. *Cancer Res* 2004; **64**:4931–4941.

63 Pollack VA, Savage DM, Baker DA, Tsaparikos KE, Sloan DE, Moyer JD, *et al.* Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358,774: dynamics of receptor inhibition in situ and antitumor effects in athymic mice. *J Pharmacol Exp Ther* 1999; **291**:739–748.

64 Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J* 1997; **16**:1647–1655.

65 Karunagaran D, Tzahar E, Beerli RR, Chen X, Graus-Porta D, Ratzkin BJ, *et al.* ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J* 1996; **15**:254–264.

66 Johannessen LE, Haugen KE, ostvold AC, Stang E, Madshus IH. Heterodimerization of the epidermal-growth-factor (EGF) receptor and ErbB2 and the affinity of EGF binding are regulated by different mechanisms. *Biochem J* 2001; **356**:87–96.

67 Jost M, Huggett TM, Kari C, Boise LH, Rodeck U. Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-x_L expression through a MEK-dependent pathway. *J Biol Chem* 2001; **276**:6320–6326.

68 Schulze A, Lehmann K, Jefferies HB, McMahon M, Downward J. Analysis of the transcriptional program induced by Raf in epithelial cells. *Genes Dev* 2001; **15**:981–994.

69 Gilmore AP, Valentijn AJ, Wang P, Ranger AM, Bundred N, O'Hare MJ, *et al.* Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. *J Biol Chem* 2002; **277**:27643–27650.

70 Fang X, Yu S, Eder A, Mao M, Bast RC Jr, Boyd D, *et al.* Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. *Oncogene* 1999; **18**:6635–6640.

71 Tan Y, Demeter MR, Ruan H, Comb MJ. BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. *J Biol Chem* 2000; **275**:25865–25869.

72 Okano J, Gaslightwala I, Birnbaum MJ, Rustgi AK, Nakagawa H. Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *J Biol Chem* 2000; **275**:30934–30942.

73 Habib AA, Chatterjee S, Park SK, Ratan RR, Lefebvre S, Vartanian T. The epidermal growth factor receptor engages receptor interacting protein and nuclear factor-kappa B (NF-kappa B)-inducing kinase to activate NF-kappa B. Identification of a novel receptor-tyrosine kinase signalosome. *J Biol Chem* 2001; **276**:8865–8874.

74 Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, *et al.* KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* 2005; **2**:e17.

75 Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, *et al.* High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004; **304**:554.

76 Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, *et al.* Mutation of the PIK3CA gene in ovarian and breast cancer. *Cancer Res* 2004; **64**:7678–7681.

77 She QB, Solit D, Basso A, Moasser MM. Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3′-kinase/Akt pathway signaling. *Clin Cancer Res* 2003; **9**:4340–4346.

78 Yen L, Benlimame N, Nie ZR, Xiao D, Wang T, Al Moustafa AE, *et al.* Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. *Mol Biol Cell* 2002; **13**:4029–4044.