Simultaneous blockade of the epidermal growth factor receptor/mammalian target of rapamycin pathway by epidermal growth factor receptor inhibitors and rapamycin results in reduced cell growth and survival in biliary tract cancer cells

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Abstract

The prognosis of patients with biliary tract adenocarcinomas (BTA) is still poor due to lack of effective systemic treatment options. Knowledge of the molecular mechanisms involved in the pathogenesis of this disease is of importance for the development of new treatment strategies. We determined the expression of epidermal growth factor receptor (EGFR) and activated mammalian target of rapamycin (p-mTOR) in paraffin-embedded surgical specimens of BTA (n = 89) by immunohistochemistry. Overall survival was analyzed with Cox models adjusted for clinical and pathologic factors. Combined EGFR/p-mTOR expression was significantly associated with relapse-free survival (adjusted hazard ratio for relapse, 2.20; 95% confidence interval (95% CI), 1.45-3.33; P < 0.001) and overall survival (adjusted hazard ratio for death, 2.32; 95% CI, 1.50-3.58; P < 0.001) of the patients. The effect of the EGFR inhibitors erlotinib or cetuximab and the mTOR inhibitor rapamycin on growth and survival of five BTA cell lines was tested in short-term 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and long-term colony formation assays. Simultaneous blockade of EGFR and mTOR in biliary tract cancer cell lines results in a synergistic inhibition of both phosphatidylinositol-3-kinase and mitogen-activated protein kinase pathways, leading to reduced cell growth and survival. These results suggest that combined targeted therapy with EGFR and mTOR inhibitors may potentially benefit patients with BTAs and should be further evaluated in clinical trials. [Mol Cancer Ther 2009;8(6):1547–56]

Introduction

Prognosis of patients with adenocarcinoma of the biliary tract (BTA) is still poor. Due to lack of characteristic early symptoms, a definitive diagnosis is often established at an advanced stage in the majority of the patients (1). For patients with resectable BTA, surgery remains the only definitively curative therapy; however, even after complete resection, recurrence rates are high. In advanced BTA, systemic treatment is necessary but there are no effective systemic treatment options available at the moment. Knowledge of molecular pathways associated with the pathogenesis of BTAs may help to devise new treatment strategies to improve the clinical outcome of the patients. The epidermal growth factor receptor (EGFR, HER1), as well as the downstream pathway phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) are in the focus of interest to develop novel targeted therapies (2, 3).

EGFR as well as its ligands EGF and transforming growth factor α (TGFα) play an important role in cell proliferation, adhesion, angiogenesis, invasion, and survival. EGFR and TGFα expression is frequently increased in BTA and it has been shown that bile acids activate EGFR and cellular proliferation through a TGFα-dependent mechanism in human cholangiocarcinoma cells (4). mTOR, a Ser/Thr protein kinase, is a key factor in cellular growth and homeostasis (5, 6) and its overexpression is associated with poor outcome of BTA patients (7). mTOR is activated through the PI3K-AKT signaling pathway by phosphorylation at Ser2448 and by autophosphorylation at Ser2481 (8, 9). Because of their key functions in cellular growth and proliferation, EGFR and mTOR are currently under investigation as potential targets for anticancer therapy in various malignant diseases.

We determined the clinical relevance of combined EGFR and activated mTOR (p-mTOR) expression in patients with BTA and investigated the effect of simultaneous blockade of the EGFR/mTOR signaling pathway at two different sites. The results provide the rationale for targeted therapy of BTA using a combination of EGFR and mTOR inhibitors.
EGFR/mTOR Signaling Pathway in Biliary Tract Cancer

Patients and Methods

Patients

One hundred and two consecutive patients with BTA who underwent complete (R0) resection at the Department of Surgery, Medical University of Vienna, between 1993 and 2006 were identified from a database. Informed consent was obtained according to institutional guidelines. The study population consisted of patients with hilar, distal, and intrahepatic cholangiocarcinoma and gallbladder carcinoma. Clinical data of the patients were obtained from the database and supplemented with review of the medical record.

Thirty-nine patients (44%) were treated with chemotherapy (10–12). Thirteen patients received gemcitabine monotherapy and 15 patients were treated with gemcitabine-based combination chemotherapy. Capecitabine monotherapy was given in two patients and two patients received capecitabine-based combination chemotherapy. Five patients were treated with 5-fluorouracil-based chemotherapy; one patient received chemotherapy consisting of cyclophosphamide, doxorubicin, and vincristine; and one patient received cisplatin plus pemetrexed.

Some of these patients had been included in previous studies evaluating the clinical role of various molecular biomarkers (7, 13, 14).

Specimen Collection

For the current research project, the participating pathologist from our institution was asked to provide a representative formalin-fixed, paraffin-embedded tumor block from each patient. All tumor specimens were obtained at the time of surgery before adjuvant therapy. Paraffin blocks were stored at room temperature and were identified only by an identification number. A H&E-stained section of each tumor block was prepared and used for pathologic confirmation of present BTA. Tumor blocks were available from 95 of the 102 patients with BTA. Among these 95 blocks, six contained no tumor material and were excluded from our study. The remaining 89 blocks were of sufficient amount and quality for sectioning. Further 4-μm sections were obtained for the immunohistochemical analysis.

Cell Lines

The human biliary tract cancer cell lines TKF-1 (derived from extrahepatic biliary tract cancer), Mz-ChA-2 (derived from extrahepatic BTA; ref. 15), CC-LP-1 (derived from intrahepatic cholangiocarcinoma), and GBC (derived from gallbladder cancer) were used for this study. TKF-1 and B2-2 cells were grown in RPMI 1640 supplemented with 10% FCS. Mz-ChA-2, CC-LP-1, and GBC cells were grown in DMEM supplemented with 10% FCS. All cell cultures were checked for contamination (Mycoplasma). Further 4-μm sections were obtained for histologic analysis.

Drugs

Erlotinib (Tarceva) was provided by Roche Austria GmbH. Rapamycin (Sirolimus, Rapamune) was provided by Wyeth Pharma GmbH. Cetuximab (Erbitux) was provided by Merck KGaA. Recombinant EGF was purchased from Sigma and used for stimulation experiments at a concentration of 10 ng/mL.

Cell Viability Assay

In vitro drug sensibility was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based survival assays as previously published (17). Briefly, cells were trypsinized, seeded at 5 × 10³ per well in 96-well plates, and allowed to grow for 24 h before treatment with exponentially increasing concentrations of drugs (erlotinib, rapamycin, cetuximab; either as a single agent or in combination) in the presence of 10% FCS. After a 72-h period of treatment, cell viability was checked by the Easy-for-You kit (Biomedica) following the instructions of the manufacturer. Each experiment was done in triplicate for each drug concentration and repeated at least three times.

Assessment of Clonogenic Survival

For determination of clonogenic survival, cells were seeded in six-well plates and exposed to increasing doses of drugs (erlotinib, rapamycin; either as a single agent or in combination). After an incubation period of 10 d, cultures were stained with crystal violet and colonies of >20 cells were counted at low magnification. Experiments were done at least twice in duplicates.

Western Blot and Immunoprecipitation

Western blot analysis and immunoprecipitation were done as described (17, 18). Briefly, total protein extracts were prepared and protein concentrations were measured with BCA Protein Assay (Pierce). For immunoprecipitation in each case, 3 × 10⁶ cells were lysed in RIPA buffer and processed as described (18). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Hybond ECL). Blots were probed with the antibodies listed in Supplementary Table S1. Visualization and quantification were done using the ChemiDoc System (Bio-Rad).

Cytogenetic Analyses

Preparation of genomic DNA, comparative genomic hybridization (CGH), and CDD banding were done as described previously (19). FISH was done using the EGFR/CEN-7 FISH Probe Mix and the DakoCytomation Cytology FISH Accessory Kit according to the manufacturer’s recommendations (DakoCytomation).

Mutational Analyses of EGFR and KRAS

For mutational analysis, genomic DNA was prepared from all cell lines. Amplifications of exons 18 through 21 of EGFR and exon 2 of KRAS were carried out as previously described (20). Briefly, PCR fragments were sequenced and analyzed in both sense and antisense directions for the presence of heterozygous mutations. Primer sequences were as follows:

EGFR exon 18 forward 5′-CTGAGGTGACCTTGTTCTG-3′
EGFR exon 18 reverse 5′-CCAACACTCGTGAACAAAGAG-3′
EGFR exon 19 forward 5′-TGCCAGTAAACGTCCTCCT-3′

Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
EGFR exon 19 reverse 5′-CAGGGTCTAGAGCAGAGCAG-3′
EGFR exon 20 forward 5′-CATTCACTGCCCTCCTCCTG-3′
EGFR exon 20 reverse 5′-TATCTCCCCCTCCCCGATAC-3′
EGFR exon 21 forward 5′-CTTCCCATGATCCTTGCTCC-3′
EGFR exon 21 reverse 5′-TATCTCCCCCTCCCCGATAC-3′
KRAS forward 5′-ACCTTATGTGTGACATGTTC-3′
KRAS reverse 5′-CTATTGTGGATCATATTCC-3′.

**Immunostaining for p-mTOR and EGFR**

Immunohistochemistry for p-mTOR was done as previously described (7). Briefly, for epitope retrieval, specimens were exposed to 10 mmol/L citrate buffer (pH 6.0) and heated for 10 min in a pressure cooker. Tumor sections were incubated overnight at 4°C in a rabbit monoclonal antibody specific for p-mTOR (Phospho-mTOR, Ser 2448; 49F9; dilution 1:100; Cell Signaling Technology). Antibody binding was detected by means of the UltraVision LP detection system (Lab Vision Corporation) with 3,3′-diaminobenzidine as the substrate and hematoxylin as the counterstain.

Immunohistochemistry for EGFR was done using the EGFR pharmDX test according to the manufacturer’s recommendations (DakoCytomation). EGFR pharmDX control slides and sections of gallbladder cancer specimens known to express EGFR served as positive controls. EGFR expression was examined by an investigator who was blinded to clinical data of the patients. Immunostaining was classified according to the EGFR pharmDX scoring guidelines. Absence of membrane staining above background in all tumor cells was scored as EGFR negative. EGFR-positive staining was defined as any immunohistochemical staining of tumor cell membranes above background level, whether it is complete or incomplete circumferential staining.

**Statistical Analyses and Calculation of Synergism**

The calculations of combination effects between EGFR inhibitors and rapamycin were analyzed using the CalcuSyn software (Biosoft) and were expressed as the combination index (21). A combination index of 1 indicates an additive effect, <1 synergism, and >1 antagonism.

The primary end point of the clinical part of the study was overall survival. Relapse-free survival was analyzed as a secondary end point. To identify any selection bias, the baseline characteristics of patients with or without tumor blocks were compared using \( \chi^2 \) tests and the overall rates of survival were compared with the use of a Cox model. Baseline data according to EGFR status were compared in univariate analyses with the use of \( \chi^2 \) tests. Survival time was defined as the period between the time of surgery and death (overall survival) or the period between the time of surgery and relapse or tumor-related death (relapse-free survival). Survival rates were estimated by means of the Kaplan-Meier method. Differences between survival curves were analyzed by means of the log-rank test. The independent prognostic value of EGFR was studied with the use of a Cox model, which was adjusted for age (<65 years or ≥65 years), gender, anatomic location ( hilar cholangiocarcinoma, distal cholangiocarcinoma, intrahepatic cholangiocarcinoma, or gallbladder carcinoma), tumor stage (T1, T2, T3, or T4), lymph node status (negative or positive), tumor grade (G1, G2, or G3), chemotherapy (yes or no), and EGFR/p-mTOR (double negative, single positive, or double positive). All reported \( P \) values are two-sided. All analyses were done with the use of SPSS software, version 15.0 (SPSS).

**Results**

**Clinical Relevance of Combined EGFR/p-mTOR Expression**

We assessed EGFR expression using standard immunohistochemistry (Fig. 1A and B). Only membranous immunostaining of EGFR was scored as positive (Fig. 1A). Immunostaining ranged from 0% to 80% (median 30%) of the tumor cells. Comparisons of EGFR expression with clinical parameters, including survival of the patients, were done with EGFR expression as a continuous variable and as a dichotomized variable classified as positive (immunohistochemistry score of >1%) or negative (immunohistochemistry score of ≤1%). Of the 89 tumors, 47 (64%) were EGFR positive. Supplementary Table S2 compares the characteristics of the patients according to EGFR expression in univariate analyses. EGFR expression was significantly associated with intrahepatic cholangiocarcinoma compared with other anatomic locations \( (P < 0.001) \) and with G2 tumors \( (P = 0.05) \). Multivariate Cox regression analyses revealed that EGFR positivity is associated with a shorter relapse-free survival (adjusted hazard ratio for relapse, 2.65; \( P = 0.001 \)) and overall survival (adjusted hazard ratio for death, 2.51; \( P = 0.004 \)) of the patients (Supplementary Table S3).

Because both EGFR and p-mTOR were independent prognostic factors (Supplementary Table S3), we combined the staining results. The clinical significance of p-mTOR protein expression has already been shown (7). Examples of p-mTOR immunostaining are shown in Fig. 1C and D. Thirty-two (36%) patients were positive for both factors, 40 patients (45%) were positive for either EGFR or p-mTOR, and 17 (19%) patients were negative for both parameters. Combined EGFR/p-mTOR expression was more frequently observed in intrahepatic cholangiocarcinoma compared with other anatomic locations \( (P = 0.002) \) but no significant associations between combined EGFR/p-mTOR expression and the remaining clinical and pathologic parameters listed in Supplementary Table S4 was observed. At a median follow-up of 5 years, 54 of 89 (61%) patients had died. Twenty-six deaths (81%) occurred in patients with EGFR/p-mTOR double-positive tumors, 22 deaths (55%) were observed in patients with EGFR/p-mTOR single-positive tumors, and 6 deaths (35%) were seen in patients with EGFR/p-mTOR double-negative tumors. The results of the univariate survival analyses are listed in Supplementary Table S5. Patients with combined EGFR/p-mTOR expression had a significantly shorter relapse-free survival (hazard ratio for relapse, 2.18; \( P < 0.001 \)) and overall survival (hazard ratio for death, 2.36; \( P < 0.001 \); Supplementary Table S5 Fig. 1E and F). The independent effect of combined EGFR/p-mTOR expression on relapse-free and
overall survival was assessed by Cox proportional hazard regression models adjusted for age, gender, anatomic location, tumor stage, lymph node status, tumor grade, and chemotherapy (Table 1). In these analyses, combined EGFR/p-mTOR expression was significantly associated with relapse-free survival (adjusted hazard ratio for relapse, 2.20; \(P < 0.001\)) and overall survival (adjusted hazard ratio for death, 2.32; 95% confidence interval, 1.50-3.58; \(P < 0.001\)). Thus, combined EGFR/p-mTOR expression is an independent poor prognostic factor for recurrence and death in patients with BTA.

Role of EGFR and mTOR Signaling in Growth and Survival of BTA Cells

To study the molecular basis of the observed prognostic value of combined EGFR/p-mTOR expression, a panel of five BTA cell lines was investigated. Comparable with the clinical results, expression of both EGFR and mTOR was variable in the cell models analyzed (Fig. 2A). The PI3K pathway inhibitor phosphatase PTEN (phosphatase and tensin homologue) was expressed in all five cell lines. Phosphorylation of extracellular signal-regulated kinase (ERK), which was used as an indicator for mitogen-activated protein kinase (MAPK) activity, was variable. In contrast, AKT, S6 ribosomal protein, and 4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) were almost generally (hyper)phosphorylated, indicating constitutive activation of the PI3K pathway in BTA cells. EGFR phosphorylation was moderate to low under standard culture conditions (Fig. 2A and B). However, addition of recombinant EGF led to rapid and distinct phosphorylation of EGFR predominantly at tyrosine 845, followed by activation of downstream signal pathways even in cell lines with low endogenous EGFR expression (CCLP1 cells are representatively shown in Fig. 2C). Activation was pronounced in case of the MAPK (ERK1/2 phosphorylation) and the PI3K (S6K and S6 phosphorylation) pathways but only moderate with respect to signal transducer and activator of transcription 5 and glycogen synthase kinase 3\(\beta\) (Fig. 2C). None of the cell lines had mutations in the \(EGFR\) gene and/or the \(KRAS\) gene. Fluorescence in situ hybridization (FISH) analysis indicated no specific amplification in the \(EGFR\) locus (ratios \(EGFR\) to centromeres of chromosome 7 ranged between 0.99 and 1.01). Nevertheless, CGH revealed, in the case of B2-2 cells, strong gain of whole chromosome 7 corresponding to seven signals for \(EGFR\) and centromere 7 in FISH analysis (data not shown).
The effect of the EGFR tyrosine kinase inhibitor erlotinib and the mTOR inhibitor rapamycin on BTA cell growth and survival was tested in 72-hour exposure assays (Fig. 3A). Whereas rapamycin mainly caused moderate growth retardation, roughly correlating with p-mTOR expression levels, the response to erlotinib varied and was widely independent of the EGFR expression levels (Fig. 2). When both agents were combined, strongly synergistic anticancer activities were observed in both EGFR inhibitor–sensitive and EGFR inhibitor–resistant BTA cell lines, as shown for B2-2 and TKF-1 cells, respectively (Fig. 3B). Combination indices <1 indicate synergisms in Fig. 3B. As a second EGFR inhibitor, the monoclonal antibody cetuximab was investigated. Although only moderately active against BTA cells as a single agent in vitro, again a strong synergism was obtained by combination with rapamycin indicated by very low combination index shown for B2-2 cells (Fig. 3C).

In addition to the short-term MTT assays, colony formation assays were used for long-term exposure studies (Fig. 4). In contrast to the short-term experiments, rapamycin at 1 nmol/L inhibited clone formation by >50% in all BTA cell lines. In contrast, erlotinib responsiveness was again variable. Interestingly, erlotinib at 1 μmol/L significantly reduced colony formation in all BTA cell lines except those two cell lines harboring the lowest EGFR expression in Western analysis (Fig. 2). Combination of both drugs led to a significant synergistic activity in all BTA cell models, strongly reducing to almost completely inhibiting clone formation ability at relatively low and clinically achievable doses (1 nmol/L rapamycin, 1 μmol/L erlotinib).

To assess the molecular mechanisms underlying the synergistic activity of EGFR/mTOR inhibition, the effect on basal (10% FCS) and EGF-stimulated MAPK and PI3K activities were determined using phosphorylation of ERK and S6 as downstream markers, respectively (Fig. 5). Under full serum conditions, synergistic inhibition of p-S6 was more pronounced as the one of p-ERK (representatively shown for GBC cells in Fig. 5A). In contrast, rapamycin alone distinctly blocked S6 phosphorylation under serum-starved and EGF-stimulated conditions (Fig. 5B). Whereas erlotinib as a single agent was almost unable to reduce ERK phosphorylation under these conditions, the drug combination surprisingly reduced MAPK activity distinctly (Fig. 5B). However, it has to be mentioned that ERK phosphorylation in BTA cells was generally almost insensitive to serum starvation, leading to

![Figure 2](http://mct.aacrjournals.org/)

**Figure 2.** Characterization of the investigated biliary tract cancer cell lines with regard to endogenous or stimulated expression/phosphorylation of EGFR and downstream pathway molecules. A, endogenous expression of EGFR and the indicated downstream pathway molecules. B, EGFR was immunoprecipitated (IP) from cells grown as described in A, C. CCLP1 cells were grown under full serum conditions for 24 h and EGF (10 ng/mL) was added at the indicated time points before protein extraction and immunoblot analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Adjusted hazard ratio for relapse (95% CI)</th>
<th>P</th>
<th>Adjusted hazard ratio for death (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.48 (0.85-2.58)</td>
<td>0.17</td>
<td>1.94 (1.11-3.39)</td>
<td>0.02</td>
</tr>
<tr>
<td>Gender</td>
<td>0.81 (0.44-1.49)</td>
<td>0.50</td>
<td>0.78 (0.41-1.47)</td>
<td>0.44</td>
</tr>
<tr>
<td>Anatomic location</td>
<td>1.01 (0.70-1.44)</td>
<td>0.98</td>
<td>0.77 (0.50-1.18)</td>
<td>0.23</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>0.91 (0.66-1.25)</td>
<td>0.55</td>
<td>1.23 (0.89-1.70)</td>
<td>0.21</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>1.82 (0.93-3.57)</td>
<td>0.08</td>
<td>1.69 (0.87-3.28)</td>
<td>0.12</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>0.96 (0.48-1.91)</td>
<td>0.91</td>
<td>0.79 (0.40-1.54)</td>
<td>0.48</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>3.25 (1.69-6.25)</td>
<td>&lt;0.001</td>
<td>0.94 (0.52-1.68)</td>
<td>0.82</td>
</tr>
<tr>
<td>EGFR/p-mTOR</td>
<td>2.20 (1.45-3.33)</td>
<td>&lt;0.001</td>
<td>2.32 (1.50-3.58)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NOTE: Variables were coded as described in Patients and Methods. Abbreviation: 95% CI, 95% confidence interval.
weak stimulation by EGF only. Nevertheless, these data indicate that mTOR-mediated signals together with those derived from the EGFR are contributing to MAPK activity in BTA cells under serum-starved conditions.

Discussion

To date, the predominant genetic aberration suitable as therapeutic target in patients with BTA has not been identified. However, several observations indicate that EGFR-mediated signals and the PI3K-AKT-mTOR signaling pathway could play a key role in the pathogenesis of this disease (22, 23). In the present study, we showed that combined expression of EGFR/p-mTOR is an independent poor prognostic factor in patients with BTA. Overall survival is worst in patients with double-positive tumors, which comprise 36% of BTA in our study cohort. These data strongly suggest that the two signal molecules or pathways cooperatively support the aggressiveness of BTA in vivo. Furthermore, this assumption is supported by our in vitro analyses demonstrating synergistic growth inhibitory activity of EGFR-targeting agents with the mTOR inhibitor rapamycin in both EGFR inhibitor-sensitive and EGFR inhibitor-resistant BTA cell lines.

Hyperactivation of EGFR in BTA cells might be caused by several mechanisms. At the genomic level, occasional amplifications of the EGFR gene and activating EGFR mutations have been reported (24, 25). Moreover, bile acids were shown to cause a potent activation of EGFR and its downstream signaling pathways through a TGF-α-dependent
mechanism (4). In addition, EGFR internalization was shown to be defective in cholangiocarcinoma cells, leading to enhanced receptor tyrosine kinase signaling (22). Based on these observations, EGFR has already been considered as a therapeutic target in BTA. In other carcinomas such as non-small-cell lung cancer, colorectal cancer, head and neck cancer, and pancreatic cancer, EGFR inhibitors are already approved for clinical use (3). Generally, blockade of EGFR

Figure 4. Impact of erlotinib and rapamycin as single agents or in combination on the colony-forming activity of the indicated biliary tract cancer cell lines. Photomicrographs of a representative experiment for B2-2 cells (A) are opposed to the evaluations for all investigated cell lines (B). Columns, mean of four experiments; bars, SD. Groups were compared by Student's t tests: *P < 0.05, **P < 0.01, ***P < 0.001.
can be achieved by tyrosine kinase inhibitors or monoclonal antibodies (3) and both strategies have been tested in BTA patients. In a phase II study, Philip et al. (26) recently suggested a therapeutic benefit for EGFR blockade with erlotinib in patients with BTA. In addition, the EGFR-directed monoclonal antibody cetuximab either as single agent or in combination with chemotherapy demonstrated activity (27, 28). However, the currently available data suggest that comparable with other cancers, only a subgroup of BTA patients benefit from treatment with EGFR inhibitors. Several in vitro and in vivo studies in various cancer types suggested that insensitivity of the PI3K-AKT-mTOR pathway to upstream EGFR inhibition might be responsible for resistance to EGFR inhibitors (29, 30). Activation of the respective downstream pathway may be due to mutations in PI3K and/or loss or inactivation of the tumor-suppressor PTEN, a negative regulator of the PI3K pathway, as recently shown for colon cancer (31). These data suggest that downstream blockade of this cell survival pathway, for example, at the level of mTOR, could potentially synergize with EGFR inhibition.

Data concerning the PI3K-AKT-mTOR pathway in BTA cells are relatively sparse. Loss/inactivation of PTEN has not been reported thus far but activating occasional mutations in PIK3CA, the p110 catalytic subunit of PI3K, have very recently been implicated in pathway hyperactivation (32). Based on an array approach, Hansel et al. (23) have shown that two mTOR downstream targets (p70S6 kinase and eukaryotic translation initiation factor 4E) are overexpressed in BTA tissues. Accordingly, in our analysis, S6 and 4EBP1 as downstream targets of p70S6 kinase were constitutively phosphorylated in BTA cells in vitro.

Within the PI3K-AKT pathway, currently mTOR represents the most promising target in anticancer therapy based on the use of rapamycin, an immunosuppressive agent that arrests cells in the G1 phase of the cell cycle and induces apoptosis. Rapamycin (sirolimus) or its analogues CCI-779 (temsirolimus), RAD-001 (everolimus), and AP23573 are specific small-molecule inhibitors of mTOR (2). They inhibit proliferation and induce apoptosis in cell lines derived from several human cancers, including small-cell lung cancer, prostate cancer, breast cancer, glioblastoma, osteosarcoma, pancreatic carcinoma, and renal cell carcinoma (33). The clinical relevance of mTOR inhibitors in multiple cancer types is currently under investigation in clinical trials but data on BTA have not been published thus far. However, several phase II studies evaluating the effects of temsirolimus in patients with renal cell carcinoma, mantle cell
lymphoma, breast cancer, and glioblastoma have been completed (34–37).

One might assume that phosphorylation of mTOR might be a result of either EGFR hyperactivation and/or RAS mutations frequently present in BTA. However, no EGFR or KRAS mutations were detected in the five BTA cell lines and blockade of mTOR exhibited synergistic cytotoxic/cytostatic activities with EGFR inhibitors against all these cell models. This data together with the clinical observations strongly suggest activation of the PI3K-AKT-mTOR pathway in BTA cells independently of EGFR. Our observations on BTA cells are in accordance with those obtained by the combination of everolimus and EGFR inhibitors, also against EGFR inhibitor–resistant colon cancer cell lines and xenografts (38). Bianco et al. showed a synergistic inhibition of both the PI3K and the MAPK pathway in their model. This observation is surprising and implicates a cross-talk from mTOR to the MAPK pathway upstream of the read-out molecule ERK. Whether a blockade of vascular endothelial growth factor/vascular endothelial growth factor receptor transactivation is responsible for this observation remains to be determined (38).

The feasibility of combining EGFR inhibitors and mTOR inhibitors has been evaluated in clinical trials. In patients with advanced non–small-cell lung cancer previously treated with chemotherapy, the combination of erlotinib and everolimus was evaluated in a phase I/II study (Papadimitrioupolou et al., ASCO 2006, abstract 17039). Of five evaluable patients, one had a partial response and three had stable disease as their best response based on investigator’s evaluation. Despite this promising efficacy, the optimal doses remain to be determined due to dose-limiting toxicity. In another phase I trial of cetuximab in combination with everolimus in patients with advanced solid tumors, everolimus at low doses does not increase cetuximab toxicity (Avadhani et al., ASCO Gastrointestinal Cancers Symposium 2007, abstract 14075). Taken together, these studies suggest that the combination of EGFR inhibitors and mTOR inhibitors is feasible and safe. In addition, these data are also in line with several other publications, reporting the efficacy of concomitant blocking of receptor tyrosine kinase and downstream signals (39, 40).

In summary, we showed that patients with EGFR/p-mTOR coexpressing BTAs have a significantly shorter overall survival compared with patients expressing only one of the factors or whose tumors were negative for both parameters. We observed that simultaneous treatment of BTA cells with erlotinib or cetuximab plus rapamycin led to synergistic inhibition of both PI3K and MAPK pathways and as a consequence to reduced cell growth and survival. These findings suggest that patients with BTA may benefit from targeted therapy with EGFR inhibitors combined with mTOR inhibitors, which should be further evaluated in clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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