EGFR Signaling Regulates Tumor Cell Migration in Craniopharyngiomas

Annett Hölken, Matthias Gebhardt, Michael Buchfelder, Rudolf Fahrbusch, Ingmar Blümcke, and Rolf Buslei

Abstract

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Translational Relevance

Craniopharyngiomas (CP) originate from the sellar region and present the most frequent nonneuroepithelial brain tumor in children. Tumor cell infiltration into adjacent brain tissue, that is, pituitary stalk and hypothalamus, renders complete surgical resection difficult, as it could produce severe neuroendocrinologic deficits. Recurrent tumor growth requires adjuvant treatment modalities such as localized brain irradiation. Better understanding of molecular mechanisms driving tumor growth into surrounding brain structures will be mandatory to develop successful chemotherapeutic treatment strategies and to provide a better medical care for our young patients. In this study, we identified epidermal growth factor receptor (EGFR) as promoter of cell migration in the most frequent adamantinomatous CP variant (adaCP). Its activated form, phosphorylated EGFR (EGFR-P), was detected in tumor cells at the brain invasion zone. These tumor cell aggregates were also characterized by nuclear β-catenin accumulation and expression of target molecules of the Wnt signaling cascade, that is, Fascin. We were able to attenuate tumor cell migration in primary human adaCP cultures in vitro when inhibiting EGFR signaling with gefitinib. The tyrosine kinase inhibitor gefitinib is already approved as a chemotherapeutic drug in non–small-cell lung cancer and may present a promising chemotherapeutic option for adaCP treatment.

of multiple tyrosine (Y) residues (Y992, Y1045, Y1068, Y1086, Y1148, and Y1173). This process also initiates recruitment of other enzymes and adapter proteins acting as signal transducers and activators of different intracellular cascades (18), regulating proliferation, differentiation, apoptosis, and motility (21).

Aberrant EGFR signaling was identified in several cancers such as non–small-cell lung cancer (NSCLC), colorectal carcinoma, breast cancer, head and neck squamous cell carcinoma, bladder cancer, and pancreatic cancer (22). Various pathomechanisms lead to EGFR activation, that is, overexpression of ligands and receptors, EGFR gene amplification, or activating mutations. The mutation hotspot region ranges between exons 18 and 21, encoding the tyrosine kinase domain of the EGFR. Furthermore, truncating mutations affecting exons 2 to 7 (EGFRvIII), encoding the extracellular domain, lead to a constitutive activation of EGFR signaling. Such EGFRvIII deletions mostly concern gliomas and to a minor extent NSCLC (18). The EGFR cascade has been identified as an important therapeutic target in various tumors of epithelial origin (18). Over the last decade, several anticancer drugs targeting the EGFR signaling cascade have been developed. Amongst these compounds, gefitinib (Iressa) is a low-molecular-weight synthetic anilinoquinazoline-4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinoproxy)quinazoline and highly specific inhibitor of EGFR function (22). Gefitinib is approved as a chemotherapeutic agent for the treatment of NSCLC and currently also tested for efficacy in other solid tumors including head and neck cancer, breast cancer, or colorectal cancer (23).

Herein, we studied a cohort of surgical adaCP samples and primary in vitro cell culture assays to unravel the intriguing relationship between EGFR signaling and adaCP tumor cell migration and whether selective EGFR inhibition has any antimigratory action.

Materials and Methods

Craniopharyngioma collection

Native CP tissue was acquired from the Departments of Neurosurgery at the University Hospital of Erlangen and the International Neuroscience Institute in Hanover. Each specimen was classified according to WHO guidelines, using hematoxylin and eosin staining as well as immunohistochemistry. Beside paraffin processing (see later), unfixed native tumor samples were used for DNA and RNA extraction as well as establishment of primary cell cultures (n = 11).

Twenty-five surgical adaCP specimens from 16 male and 9 female patients were used for analyses of EGFR, β-catenin, and Fascin expression (cDNA and protein level), and screening for genetic alterations in the EGFR gene (Table 1). The age ranged between 3 and 66 years in the group of male patients (mean age = 31 years) and between 4 and 64 years in female patients (mean age = 32 years). Informed and written consent was given by all patients included in our study for additional scientific investigations approved by the local ethics committee of the Erlangen University. All procedures were conducted in accordance with the Declaration of Helsinki.

DNA and cDNA preparation

DNA and RNA were extracted from snap-frozen adaCP tumor tissue or cells in culture. From all frozen tissues, instantaneous sections were prepared and microscopically reviewed to prove sufficient tumor content. DNA was extracted using the DNeasy tissue kit (Qiagen). Control DNA was obtained from peripheral blood leukocytes of 20 healthy persons. Blood leukocyte DNA was extracted using the Blood DNA kit according to the supplier’s protocols (Qiagen). RNA from tumor tissue was isolated using the RNeasy extraction kit (Qiagen), whereas RNA of cultured cells was extracted using TRIzol reagent following the manufacturers’ instructions (Invitrogen). Subsequently, digestion with RNase-free DNase I and purification via RNeasy columns (Qiagen) was followed by reverse transcription, using SuperScript first-strand synthesis system for reverse transcriptase PCR (Invitrogen), with oligo(dT) primers.

Gene expression analysis by PCR

EGFR, β-catenin, and Fascin gene expression was analyzed by PCR by using cDNA generated from adaCP samples (n = 25). CDK4 and B2M PCR were used to control cDNA
Table 1. Characterization of the CP series including results of EGFR and β-catenin screening

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gender</th>
<th>Age</th>
<th>Mutational analysis (exon 3 of the β-catenin gene)</th>
<th>EGFR expression (cDNA)</th>
<th>EGFR-P staining</th>
<th>EGFR amplification</th>
<th>Mutation analyses</th>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EGFRvIII</td>
</tr>
<tr>
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<td>3</td>
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<td>neg.</td>
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<td>pos.</td>
<td>pos.</td>
<td>neg.</td>
<td>+</td>
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</table>

NOTE: Patients’ information corresponding to each sample is listed. EGFR expression (pos.) and occurrence of activated EGFR (EGFR-P staining) were detected in all samples. Mutation analysis revealed known SNPs with a reference cluster ID (rs). Base substitutions are GAG(Glu) intron AAG(Leu) in exon 17/18; CAG(Gln)787CAA(Gln) in exon 20; ACC(Thr)903ACT(Thr) in exon 23; and GAC(Asp)994GAT(Asp) in exon 25. The EGFRvIII variant was absent. Primary cell cultures were generated from samples shown in bold. Abbreviations: m, male; f, female; neg., no EGFR amplification detectable.
application and to exclude DNA contamination, shown for each primer, using a genomic DNA template (DNA Co.). Primers used for PCR are listed in Table 2. Cycling conditions were as follows: an initial incubation at 94°C for 3 minutes, 40 cycles of 94°C for 40 seconds, annealing temperature for 40 seconds, and 72°C for 40 seconds, followed by a final extension at 72°C for 10 minutes.

**Immunohistochemistry**

Phosphorylated EGFR (EGFR-P) was detected using rabbit anti-EGFR, phospho-Tyr1068 (P-Y1068) monoclonal antibody (clone EP774Y; Abcam; 1:250). Four-micrometer thin sections were prepared from formalin-fixed paraffin-embedded tissue and developed using the 3,30-diaminobenzidine (DAB) method as previously described (24).

Double labeling of EGFR-P and β-catenin or Fascin were detected with following antibodies: mouse anti-β-catenin (clone 14/β-catenin; BD-Biosciences; 1:200), mouse anti-Fascin (clone FCN01; NeoMarkers; 1:200), and secondary antibodies cy2 (goat anti-mouse; 1:100) and cy3 (goat anti-rabbit, 1:200, both obtained from Dianova). Nuclei were counterstained with Hoechst 33342 (500 ng/mL; Sigma-Aldrich).

**EGFR gene copy number variation analysis**

EGFR gene amplification was analyzed using EGFR Taq-Man copy number assay (Hs01426560_cn; Applied Biosystems) and TaqMan Copy Number Reference Assay RNase P according to the standard TaqMan Genotyping Assay protocol. DNA from a patient with glioblastoma multiforme (WHO grade IV) and proven EGFR amplification as well as DNA from a healthy person served as control samples. Data were analyzed using the CopyCaller Software (Applied Biosystems).

**Mutational analysis**

Single-strand conformation polymorphism (SSCP) analysis was applied to detect genomic alterations in tumor DNA (exons 17–28 of the EGFR) as previously described (25). All primers used for mutation analysis are listed in Table 2. DNA from shifted bands was extracted from the wet gel and directly sequenced after reamplification (GATC Biotech AG). To analyze whether a deletion of the extracellular domain (EGFRvIII) contributes to EGFR activation, cDNA prepared from native tumor tissue (n = 25) was analyzed. Amplicons span exon 1 to exon 8 of the EGFR gene. PCR products of wild-type (wt) EGFR mRNA consist

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### Table 2. Primers used for EGFR and β-catenin mutation screening and analysis of EGFR, Fascin, and β-catenin gene expression

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Exon</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 3'-5'</th>
<th>Template</th>
<th>Fragment length (bp)</th>
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<td>17</td>
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<td>actgctttccagcatggtga</td>
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<td>250</td>
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of 894 bp, whereas tumor probes with a deletion in the EGFR gene (exon 2 to exon 7) generate a fragment length of 93 bp (Table 2). wt cDNA extracted from the A172 glioma cell line and cDNA from F98 EGFRvIII (ATTAC) cells, which contain the truncating mutation, served as positive control samples, whereas genomic DNA and a nontemplate control were used as negative controls. Mutational analysis of exon 3 of the β-catenin gene was carried out as described previously (11, 24–26).

Cell culture

Eleven primary cell cultures of adaCPs were established from surgical tumor samples as previously described (10). The human glioma cell line A172 (generously supplied by U. Gaipl, Department of Radiation Oncology, University Hospital Erlangen, Germany) was cultured in Dulbecco’s modified Eagle’s medium supplemented with L-glutamine (2 mmol/L), penicillin/streptomycin (100 units/mL), and FCS (10%) at 37°C and 5% CO₂ atmosphere. The rat glioma cell line F98npEGFRvIII (ATTCC-CRL-2949; LGC-Standards GmbH) was cultured as recommended by American Type Culture Collection (ATCC).

Wound healing assay

Cells were cultured on 6-well plates until they reached confluence. An approximately 400-μm large scratch was applied to the monolayer cultures using a 1,000-μL pipette tip (27). All lesions were documented at 4-fold microscopic magnification and evaluated twice within 24 hours, using an IX70 microscope (Olympus GmbH) equipped with a CCD camera (F-View II; Soft Imaging System) and respective imaging software (Cell; Soft Imaging System). Four images were evaluated for each experiment. The migratory capacity of all cells was evaluated comparing the lesion size at 0 hours relative to the area measured 24 hours later.

Boyden chamber assay

To minimize that migration/invasion effects are mediated by excessive tumor cell proliferation, we carried out the Boyden chamber migration assay (QCM 24-well Colorimetric Cell Migration Assay; Chemicon) according to the manufacturer’s protocol. Briefly, 1.5 × 10⁵ cells were transferred into each Boyden chamber and incubated with EGF, gefitinib, or dimethyl sulfoxide (DMSO) as solvent control for 24 hours. Each experiment was repeated 3 times.
Statistical analysis
The value of migration for each CP sample after treatment (EGF alone, EGF and gefitinib, or gefitinib alone) was determined in relation to the corresponding untreated control that was set to 100%. The calculated values were analyzed statistically by a 1-way ANOVA with Bonferroni’s post hoc test for multiple comparisons by using GraphPad Prism version 4.00 for Windows (GraphPad Software). Differences were considered significant with \( P < 0.05 \).

Activation and inhibition of the EGFR
All cell cultures were treated with EGF (R&D Systems) added to the media at a concentration of 10 ng/mL. The tyrosine kinase inhibitor gefitinib (Iressa; Biaffin GmbH & Co KG) was dissolved in DMSO and used at a concentration of 500 nmol/L as described elsewhere (28, 29). Eleven different primary cell cultures were treated with EGF, gefitinib, both substances together, or only with a solvent solution (medium plus DMSO). The latter was used to determine the basic value for relative quantification. Cells were exposed 24 hours for subsequent gene expression analyses, protein isolation, and migration assays. The status of EGFR phosphorylation was examined by immunoblotting after treatment duration lasting 1 hour (unicates).

Protein preparation and immunoblotting
Protein extraction and immunoblotting were carried out as previously described (10). For detection of EGFR-P in cultured cells, additionally, phosphatase inhibitors NaF (10 mmol/L, Sigma-Aldrich) and Na3VO4 (1 mmol/L, Sigma-Aldrich) were supplemented to the lysis buffer. Equal protein loading (10 \( \mu \)g per lane) was estimated using monoclonal mouse anti–β2-microglobulin (B2M; Hs99999907_m1) was carried out using cDNA (from cultured adaCP when enough material was available (n = 8). Beta-2-microglobulin (B2M; Hs99999907_m1) was used as an endogenous control. All analyses were carried out in triplicates and evaluated statistically by Student’s \( t \) test.

Results
EGFR-P expression in adaCP
EGFR mRNA encoding intracellular and extracellular domains was detected in all adaCP samples from this series (Supplementary Fig. S1A and B, Table 1). In addition, all adaCP showed an activation of the EGFR signaling pathway as determined by immunohistochemical staining with an antibody directed against the auto-phosphorylated Tyr1068 (EGFR-P, Fig. 1A, Table 1). Microscopic evaluation localized activated EGFR-P to whirl-like structures and palisading cell layers of tumor protrusions infiltrating into brain parenchyma (Fig. 1A). EGFR-P was also visible within the nucleus and, thereby, reminiscent to a similar pattern described for β-catenin (10). Double-immunofluorescence staining confirmed colocalization between activated EGFR in cells exhibiting nuclear β-catenin accumulations in our present series (Fig. 1B).

Genetic analysis of the EGFR and β-catenin in adaCP
To analyze whether EGFR activation is caused by genetic alterations, copy number variation analyses and mutation analyses of the entire cytoplasmic region (exons 17–28) were carried out. Thereby, we were not able to identify amplification of the EGFR gene in our series (Table 1). The tumor collection screened by SSCP analysis comprised DNA of 25 adaCP samples and was compared with 20 samples of healthy individuals. Aberrant conformations of PCR product were observed during gel electrophoresis for exons 17, 20, 23, and 25 (Table 1). The same frequency of aberrant shift patterns was observed in control DNA (data not shown). Sequencing of all cases revealed single nucleotide polymorphisms (SNP) with an attributive reference SNP cluster ID (rs). Results are listed in Table 1.

We also analyzed whether a deletion within the extracellular ligand-binding domain of the EGFR (EGFRvIII), reaching from exon 2 to exon 7, could be detected in adaCP. The cDNA of the tumor collection was screened with a primer pair generating a PCR product of 93-bp fragment length for EGFRvIII and a 894-bp product for wt EGFR. As shown in Supplementary Figure S1B, we were not able to detect deletions in the EGFR gene.

Mutation analysis of the β-catenin gene was carried out in all 25 tumor samples and revealed activating mutations in exon 3 in 76% of cases (Table 1), which corresponds to results described previously (11, 24–26).

EGFR modulation in primary adaCP cell cultures
EGF ligand activation of EGFR in adaCP was functionally studied in 11 primary adaCP cell cultures. Increased EGFR phosphorylation (EGFR-P) was confirmed already after 1 hour of EGF exposure and quantified in relation to untreated cells by immunoblotting and densitometric assessment (Fig. 2A). The specific EGFR inhibitor gefitinib was used to counteract EGFR activation. Gefitinib treatment of primary adaCP cells showed inhibition of EGF-induced EGFR phosphorylation in comparison with untreated tumor cells. Tumor cells without previous EGF stimulation exhibited in 8 of 11 cases a reduction of EGFR-P content when treated only with gefitinib (Fig. 2A).
EGFR Signaling Directs Brain Invasion of Craniopharyngiomas

A

Rel. EGFR-P protein quantity (%)

B

Boyden chamber assay

Wound healing assay

C

EGF

Rel. migration (%)
EGFR activation promotes cell motility in adaCP

We then analyzed the effect of EGFR activation on cell motility in 11 primary adaCP cell cultures. In all experiments, tumor cells revealed increased migration and invasion capacity with activation of the EGFR compared with untreated cells (Fig. 2B). In Boyden chamber experiments, EGFR treatment increased cell migration by 150% (CP13) to 350% (CP18) compared with control levels (Fig. 2B). Corresponding results were obtained in wound healing assays showing elevated levels ranging from 140% (CP20) to 280% (CP18; Fig. 2B). The mean values of both assays (Boyden chamber: 206%; wound healing assay: 161.5%) reached statistical significance \( P < 0.001 \).

To evaluate whether inhibition of EGFR signaling is capable of counteracting migration, Boyden chamber assays and wound healing assays were conducted after both EGF and gefitinib administration (EGF+) or after gefitinib treatment alone (EGF−). In the latter paradigm, gefitinib treatment reduced migration only in moderate levels. In contrast, EGFR activation (EGF+) exhibited reduced motility when also exposed to gefitinib (Fig. 2C). Notably, both assays revealed comparable and statistical significant \( P < 0.001 \) results for migration inhibition in our cell culture assays (mean values Boyden chamber: 61.2%; wound healing assay: 68.7%). Our results indicate that efficacy of gefitinib treatment is likely to depend on the EGFR activation status.

EGFR signaling induces Fascin expression in adaCP

Previously, we identified that Fascin plays a role in adaCP cell migration (10). In the present study, we were able to show Fascin mRNA expression in all tumors under study (presented as Supplementary Fig. S2). Double immunofluorescence unraveled coexpression of Fascin and EGFR-P (Fig. 3A). To examine the impact of EGFR signaling on Fascin expression, TaqMan gene expression analyses were carried out in 8 primary adaCP cell cultures after modulation with EGF and/or gefitinib. In 6 of 8 experiments, EGFR activation significantly enhanced Fascin expression levels (Fig. 3B), whereas EGFR inhibition by gefitinib treatment significantly inhibited Fascin expression in 7 of 8 samples. Interestingly, Fascin levels were also decreased following gefitinib administration without previous EGF stimulation (Fig. 3B). Protein analysis confirmed the data obtained from TaqMan Fascin expression analysis in 1 case (Fig. 3C), from which sufficient material was available to carry out Western blotting after different treatment modalities.

Discussion

Although histologically benign (WHO grade I), adaCP represent one of the most challenging intracranial lesions (2, 6). Microsurgery and localized irradiation are standardized treatment modalities, but they also account for most patients’ long-term morbidity. Understanding the natural course of the disease will be most helpful to develop adjuvant chemotherapeutic treatment strategies. From clinical perspectives, it is important to target adaCP infiltration into surrounding brain structures, that is, hypothalamus or pituitary stalk, as this growth pattern represents the main cause of neuroendocrinologic symptoms and tumor recurrence.

We identify for the first time activated EGFR in adaCP cells to be involved in migration which could be suppressed by its inhibition using the tyrosine kinase inhibitor gefitinib in vitro. Interestingly, the cellular distribution of activating EGFR phosphorylation (at position Tyr1068) was not uniformly distributed in the tumor sample. EGFRP−positive cells aggregated in whirls at the tumor brain invasion border. It has been previously shown that these adaCP tumor cell clusters are also characterized by constitutive activation of canonical Wnt signaling and target gene expression, promoting epithelial differentiation and morphogenesis (11, 24–26). Here, we were able to show nuclear colocalization of activated EGFR and β-catenin, which suggests an interplay of both pathways in adaCP. This finding is in accordance with recent reports showing that EGFR signaling regulates localization, stability, and transcriptional activity of β-catenin in oral cancer cells (17). In addition, EGFR activation induces the expression of low-to intermediate-molecular-weight cytokeratins CK8 and CK18 (30). This peculiar cytokeratin profile has also been described in adaCP tumor cell whirls (24).

EGFR signaling and β-catenin were also linked to facilitate epithelial cell migration in a synergistic manner (17, 31). To address this hypothesis, we modulated EGFR activity in a primary human cell culture assay of adaCP and observed significantly enhanced adaCP tumor cell motility after EGFR activation through its ligand EGF. AdaCP tumor cell migration was shown to be influenced by the actin-bundling protein Fascin (10). Here, we were able to show that activation of the EGFR pathway increases Fascin expression on mRNA and protein levels. This is supported by the notion that Fascin is elevated in whirl-like cell clusters of tumor protrusions exhibiting activated EGFR. Our results thus...
corroborate findings in esophageal squamous cell carcinoma, suggesting that regulation of Fascin expression is dependent on EGFR signaling (32). The fact that adaCP exhibit nuclear coexpression of activated EGFR, β-catenin, and Fascin in infiltrating cell aggregates suggests gefitinib as a promising target-oriented chemotherapeutic option. The small molecule gefitinib is known to inhibit the intracytoplasmic EGFR tyrosine kinase activity in an ATP-competitive manner. Gefitinib has a good bioavailability and is considered as highly selective for activated EGFR (23, 33). In previous studies, cancer cells were shown to be sensitive to gefitinib at concentrations of 1 μmol/L or less (28, 29). In our human cell culture assay, sufficient inhibition of EGF-induced EGFR activation by gefitinib was achieved at a concentration of 500 nmol/L as disclosed by immunoblotting. Interestingly, we were able to show that not only EGFR activation but also Fascin mRNA expression significantly decreased after gefitinib treatment. The drug has reached clinical phase II trials for colorectal cancer, breast cancer, gastrointestinal cancer, prostate cancer, and esophageal cancer and clinical phase III trials for head and neck cancer (23). In July 2009, gefitinib received the marketing authorization from the European Commission for the treatment of non–small lung cancer with activating mutations in the EGFR. Further studies in animal models will be needed to verify the benefit of gefitinib treatment on adaCP growth in vivo. Thereby, the application of gefitinib could occur directly into the intracranial tumor cyst after fluid aspiration or using an Ommaya-Reservoir to bypass systemic exposition. A similar strategy is currently carried out for IFN-α treatment in patients with cystic CPs (7).

Activation of the EGFR signaling pathway was identified in different neoplasms including epithelial, neuroepithelial, and embryonal tumors (19, 34, 35). Point mutations or truncated EGFRvIII cause EGFR activation in most of these tumor entities (36, 37). In our present series of adaCP, we were not able to detect EGFR gene amplification or mutations and the incidence of SNPs was similar between tumor and control samples. The silent GAC(D)903GAT(D) base substitution was previously described as rather protective in a case–control study for primary lung cancer risk in a Korean population (38). The ACC(T)903ACT(T) SNP was also characterized as a benign polymorphism (39).

Figure 3. A, double immunofluorescence analyses revealed coexpression of Fascin and activated EGFR. TaqMan gene expression assays for Fascin were conducted from 8 different primary adaCP cell cultures (B). Gene expression was evaluated from cells treated 24 hours with EGF (red), gefitinib (blue), or with both substances (yellow). The values of untreated cells (purple) were used as reference (set to 1) for all other treatment modalities (P < 0.05; t test; *, statistical significance). C, Fascin protein content was analyzed in untreated cells and after EGF and/or gefitinib (500 nmol/L) treatment by immunoblotting. For densitometric evaluation, protein values were normalized with β-actin content and related to untreated cells. Full-length blots are shown in Supplementary Figure S3.
To summarize, our data show commonly activated EGFR signaling in adaCP independent from genetic alterations affecting the EGFR gene. Activated EGFR is coexpressed with β-catenin and Fascin in tumor cell clusters located at the tumor brain infiltration zone. EGFR signaling promotes adaCP tumor cell migration and the expression of the motility factor Fascin. Gefitinib treatment was sufficient to counteract EGFR activity and reduced tumor cell migration and Fascin expression. Our data point to the feasibility of adjuvant chemotherapeutic EGFR inhibition by gefitinib for the control of long-term complications in CP patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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