EGFR signaling regulates tumor cell migration in craniopharyngiomas

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EGFR signaling directs brain invasion of craniopharyngiomas

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Translational relevance

Craniopharyngiomas (CP) originate from the sellar region and present the most frequent non-neuroepithelial brain tumor in children. Tumor cell infiltration into adjacent brain tissue, i.e. pituitary stalk and hypothalamus renders complete surgical resection difficult, as it could produce severe neuroendocrinological 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 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, i.e. 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.
Abstract

Purpose: Constitutive Wnt-signaling caused by mutations in the β-catenin gene is a molecular hallmark of adamantinomatous craniopharyngiomas (adaCP), and promotes infiltration into adjacent brain tissue. Herein, we studied the pathogenic role of epidermal growth factor receptor (EGFR) activation in adaCP and whether tumor cell migration can be inhibited by the tyrosine kinase inhibitor Gefitinib.

Experimental Design: EGFR expression and activation (EGFR-P) was examined in a cohort of 25 surgical adaCP samples using PCR and Western blotting. Regional and cellular localization patterns of EGFR-P, β-catenin and its target gene Fascin were determined by immunofluorescence microscopy. Mutation analysis and gene copy number assay were performed to examine genetic alterations in the EGFR gene. The impact of EGFR signaling on tumor cell migration was studied in vitro using eleven primary human adaCP cultures treated with the EGFR ligand EGF and its inhibitor Gefitinib.

Results: Neither mutations nor amplifications in the EGFR gene were detected in our adaCP series. However, phosphorylated EGFR was detectable in tumor cell clusters located at the brain infiltration border and co-localized with nuclear β-catenin and Fascin. Activated EGFR significantly promoted tumor cell migration in vitro, whereas Gefitinib reduced both, tumor cell motility and Fascin expression.

Conclusion: Our data suggest EGFR signaling to play a role in cell migration and brain infiltration of adaCP. Targeting the EGFR signaling pathway by Gefitinib may present a promising pharmacological option in the treatment of this challenging tumor disease.
Introduction

Adamantinomatous craniopharyngiomas (adaCP) account for 5.6-15% of intracranial neoplasms in children and young adults (1). AdaCP are histopathologically classified as benign epithelial tumors (WHO grade I) but remain difficult to treat (2-4). Their close relation to surrounding brain structures, i.e. the pituitary stalk and the hypothalamus, render complete surgical resection difficult and presents a risk for tumor relapse (4, 5). Following partial tumor removal and radiotherapy, recent series reported a 10-year recurrence-free survival rate of 81% to 91%, with 46% to 58% of the survivors living a normal independent life (1). A major issue in current treatment modalities of craniopharyngioma (CP) patients remains, therefore, to achieve tumor-free survival without aggravating long-lasting morbid obesity and hormonal deregulations (2, 6). Available compounds for adjuvant chemotherapeutic treatment of CP are yet restricted. Intracystic injection of interferon-α and bleomycin are under clinical investigation and await further approval (4, 7-9).

Our main interest is to identify molecular mechanisms and pathways involved in the pathogenesis of adaCP, and to unravel new targets for adjuvant chemotherapeutic treatment options. Recently, tumor cell migration in adaCP was linked to cells with an activated canonical Wnt signaling pathway, characterized by nuclear accumulation of its key player β-catenin (10, 11). In addition, activation of the β-catenin target gene Fascin, which is involved in cellular motility (12), is increased in the same adaCP cell clusters (10). The epidermal growth factor receptor (EGFR) cascade was very recently identified to also play a role in Wnt signaling activation, most likely due to a direct interaction between EGFR and β-catenin (13-17). The EGFR is a 170 kDa receptor tyrosine kinase (RTK) also referred to as HER1, ErbB1, mENA and PIG61. The transmembrane glycoprotein consists of an extracellular domain facilitating ligand-binding, a transmembrane region, and an intracellular region that comprises the tyrosine kinase domain and autophosphorylation sites (18). Numerous growth factors can activate EGFR, including the epidermal growth factor (EGF), transforming growth factor alpha (TGF-alpha), amphiregulin, epiregulin, heparin-binding EGF (HB-EGF) and betacellulin (19, 20). Ligand binding induces receptor dimerisation which is associated with
tyrosine kinase activation and subsequent phosphorylation 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 carcinoma, bladder cancer, and pancreatic cancer (22). Various pathomechanisms lead to EGFR activation, i.e. overexpression of ligands and receptors, EGFR gene amplification, or activating mutations. The mutation hotspot region ranges between exons 18 to 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 such as anti-EGFR antibodies and RTK inhibitors have been developed. Amongst these compounds, Gefitinib (Iressa™) is a low-molecular-weight synthetic anilinoquinazoline -4-(3-chloro-4-fluoroanilino)- 7-methoxy-6-(3-morpholinopropoxy) quinazoline and highly specific inhibitor of EGFR function (22). Gefitinib is approved as 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 anti-migratory 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 (INI) in Hanover. Each specimen was classified according to World Health Organization (WHO) guidelines using haematoxylin and eosin (HE) staining as well as immunohistochemistry. Beside paraffin processing (see below) 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 males and 9 females were used for analyses of EGFR, β-catenin and Fascin expression (cDNA and protein level) as well as screening for genetic alterations in the EGFR gene (Table 1). The age ranged between 3 and 66 years in the group of males (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, Hilden, Germany). 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 TRlzol reagent following the manufacturers’ instructions (Invitrogen, Carlsbad, USA). 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 RT-PCR (Invitrogen) with oligo (dT) primers.

**Gene expression analysis by PCR**

EGFR, β-catenin and fascin gene expression was analysed by PCR using cDNA generated from adaCP samples (n=25). CDK4 and B2M PCR were used to control cDNA application and to exclude DNA contamination, demonstrated 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 40s, annealing temperature for 40s and 72°C for 40s followed by a final extension at 72°C for 10 minutes.

**Immunohistochemistry**

Phosphorylated EGFR (EGFR-P) was detected using rabbit anti-EGFR, phospho (Tyr1068) monoclonal antibody (clone EP774Y, Abcam, Cambridge, UK, 1:250). Four µm thin sections were prepared from formalin fixed paraffin embedded (FFPE) tissue and developed using the 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/Beta-Catenin, BD-Biosciences, Franklin lakes, USA, 1:200), mouse anti-Fascin (clone FCN01, NeoMarkers, Fremont, USA, 1:200) and secondary antibodies cy2 (goat anti mouse; 1:100) and cy3 (goat anti rabbit, 1:200, both obtained from Dianova, Hamburg, Germany). Nuclei were counterstained with Hoechst 33342 (500ng/ml, Sigma-Aldrich, Steinheim, Germany).

**EGFR gene copy number variation analysis**

EGFR gene amplification was analyzed using EGFR TaqMan® copy number assay (Hs01426560_cn; Applied Biosystems, Foster City, USA) and TaqMan Copy Number Reference Assay RNase P according to the standard TaqMan® Genotyping Assay protocol. DNA from a patient with glioblastoma multiforme (GBM, WHO Grade IV) and proven EGFR
amplification as well as DNA from a healthy person served as control samples. Data was analyzed using the CopyCaller™ Software (Applied Biosystems, Foster City, USA).

**Mutational analysis**

Single-strand conformation polymorphism (SSCP) analysis was applied to detect genomic alterations in tumor DNA (exons 17 to 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, Konstanz, Germany). To analyze whether a deletion of the extracellular domain (EGFRvIII) contributes to EGFR activation, cDNA prepared from native tumor tissue (n=25) was analysed. Amplicons span exon 1 to exon 8 of the EGFR gene. PCR products of wild type (wt) EGFR mRNA consist 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). Wild type (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 non template control (NT) were used as negative controls. Mutational analysis of exon 3 of the β-catenin gene was performed 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 DMEM medium supplemented with L-glutamine (2mM), penicillin/streptomycin (100 units/ml), FCS (10%) at 37°C and 5% CO₂ atmosphere. The rat glioma cell line F98npEGFRvIII (ATTC-CRL-2949, LGC-Standards GmbH, Wesel, Germany) was cultured as recommended by ATCC.
Woundhealing 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 1000 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, Hamburg, Germany) equipped with a CCD camera (F-View II; Soft Imaging System, Münster, Germany) and respective imaging software (Cell, Soft Imaging System). Four images were evaluated for each experiment (n=4). 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 performed the Boyden chamber migration assay (QCM™24-well Colorimetric Cell Migration Assay, Chemicon, Temecula, USA) according to the manufacturer’s protocol. Briefly, 1.5 x 10^5 cells were transferred into each Boyden chamber and incubated with EGF, Gefitinib or DMSO as solvent control for 24 hours. Each experiment was repeated 3 times (n=3).

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 one-way ANOVA with Bonferroni’s posthoc test for multiple comparisons using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). Differences were considered significant with p<0.05.
Activation and inhibition of the EGFR

All cell cultures were treated with EGF (R&D Systems, Minneapolis, USA) added to the media in a concentration of 10ng/ml. The tyrosine kinase inhibitor Gefitinib (Iressa™; Biaffin GmbH & Co KG, Kassel, Germany) was dissolved in DMSO and used in a concentration of 500nM as described elsewhere (28, 29). Eleven different primary cell cultures were treated either with EGF, Gefitinib, both substances together or only with a solvent solution (medium plus DMSO). The latter were 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 performed as previously described (10). For detection of EGFR-P in cultured cells additionally phosphatase inhibitors NaF (10 mM, Sigma-Aldrich) and Na_3VO_4 (1 mM, Sigma-Aldrich) were supplemented to the lysis buffer. Equal protein loading (10µg per lane) was estimated using monoclonal mouse anti β-actin antibody (1:10,000, Sigma-Aldrich). Membranes were incubated with Fascin antibody (1:200) or EGFR (phospho Y1068) antibody (1:1000) and thereafter with HRP linked sheep anti mouse and rabbit secondary antibodies (1:5000, GE-Healthcare, Buckinghamshire, UK). Protein detection was facilitated by the Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad, Hercules, USA).

TaqMan gene expression assay

TaqMan® gene expression assays (Applied Biosystems, Darmstadt, Germany) were employed to determine Fascin expression after modulation of EGFR activity using EGF (10 µg/ml) and Gefitinib (500nM). Analyses were performed with the Applied Biosystems 7500 Fast Real-Time-PCR System (Applied Biosystems, Foster City, USA). Relative Quantification analysis of Fascin (Hs00602051_mH) was performed using cDNA from cultured adaCP when
enough material was available (n=8). Beta-2-microglobulin (B2M) (Hs99999907_m1) was used as endogenous control. All analyses were performed in triplicates and evaluated statistically by Student’s \( t \) test.

**Results**

**EGFR-P expression in adaCP**

EGFR mRNA encoding intracellular as well as extracellular domains was detected in all adaCP samples from this series (suppl. Figure 1A 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 tyrosine residue 1068 (EGFR-P, Figure 1A, Table 1). Microscopic evaluation localized activated EGFR-P to whirl-like structures as well as palisading cell layers of tumor protrusions infiltrating into brain parenchyma (Figure 1A). EGFR-P was also visible within the nucleus and, thereby, reminiscent to a similar pattern described for \( \beta \)-catenin (10). Double-immunofluorescence staining confirmed co-localization between activated EGFR in cells exhibiting nuclear \( \beta \)-catenin accumulations in our present series (Figure 1B).

**Genetic analysis of the EGFR and \( \beta \)-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 to 28) were performed. 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 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 (SNPs) with an attributive reference SNP cluster ID (rs). Results are listed in Table 1.
We additionally 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 as well as a 894 bp product for wild type (wt) EGFR. As shown in supplementary Figure 1B, we were not able to detect deletions in the EGFR-gene. Mutation analysis of the β-catenin gene was performed 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 eleven primary adaCP cell cultures. Increased EGFR phosphorylation (EGFR-P) was confirmed already after one hour of EGF exposure and quantified in relation to untreated cells by immunoblotting and densitometric assessment (Figure 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 to untreated tumor cells. Tumor cells without previous EGF stimulation exhibited in eight out of eleven cases a reduction of EGFR-P content when treated only with Gefitinib (Figure 2A).

EGFR activation promotes cell motility in adaCP

We then analyzed the effect of EGFR activation on cell motility in eleven primary adaCP cell cultures. In all experiments, tumor cells revealed increased migration and invasion capacity with activation of the EGFR compared to untreated cells (Figure 2B). In Boyden Chamber experiments, EGF treatment increased cell migration by 150% (CP13) to 350% (CP18) compared to control levels (Figure 2B). Corresponding results were obtained in wound healing assays showing elevated levels ranging from 140% (CP20) to 280% (CP18) (Figure 2B). The mean values of both assays (Boyden chamber: 206% and wound healing assay: 161.5%) reached statistical significance (p<0.001).
To evaluate whether inhibition of EGFR signaling is capable to counteract migration, Boyden chamber assays and wound healing assays were conducted after both, EGF and Gefitinib administration (EGF+) or after Gefitinib 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 (Figure 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 induced 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 supplement data Figure 2). Double immunofluorescence unraveled co-expression of Fascin and EGFR-P (Figure 3A). In order to examine the impact of EGFR signaling on Fascin expression, TaqMan gene expression analyses were performed in eight primary adaCP cell cultures after modulation with EGF and/or Gefitinib. In six out of eight experiments, EGFR activation significantly enhanced Fascin expression levels (Figure 3B), whereas EGFR inhibition by Gefitinib treatment significantly inhibited Fascin expression in seven out of eight samples. Interestingly, Fascin levels were also decreased following Gefitinib administration without previous EGF stimulation (Figure 3B). Protein analysis confirmed the data obtained from TaqMan fascin expression analysis in one case (Figure 3C), from which sufficient material was available to also perform 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 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, i.e. hypothalamus or pituitary stalk, as this growth pattern represents the main cause of neuroendocrinological 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 Tyr-1068) was not uniformly distributed in the tumor sample. EGFR-P 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 demonstrate nuclear co-localization of activated EGFR and β-catenin which suggests 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 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 tumour protrusions exhibiting activated EGFR. Our results thus corroborate findings in esophageal squamous cell carcinoma suggesting that regulation of Fascin expression is depending on EGFR signaling (32). The fact that adaCP exhibit nuclear co-expression 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 ≤1µM (28, 29). In our human cell culture assay, sufficient inhibition of EGF induced EGFR activation by Gefitinib was achieved at a concentration of 500nM 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 intracranial into the tumor cyst after fluid aspiration or using an Ommaya-Reservoir to bypass systemic exposition. A similar strategy is currently performed for interferon alpha treatment in patients with cystic craniopharyngiomas (7).

Activation of the EGFR signaling pathway was identified in different neoplasms including epithelial, neuroepithelial and embryonal tumors (19, 34, 35). Truncating EGFRvIII, point or deletion mutations 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 single nucleotide polymorphisms (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).

To summarize, our data shows commonly activated EGFR signaling in adaCP independent from genetic alterations affecting the EGFR gene. Activated EGFR is co-expressed with β-catenin and Fascin in tumor cell clusters located at the tumor brain infiltration zone. EGFR signaling promotes adaCP tumor cell migration as well as the expression of the motility factor Fascin. Gefitinib treatment was sufficient to counteract EGFR activity, and reduced tumor cell migration as well as Fascin expression. Our data point to the feasibility of adjuvant chemotherapeutic EGFR inhibition by Gefitinib for the control of long-term complications in craniopharyngioma patients.

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Table 1

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<tr>
<th>Sample</th>
<th>Gender</th>
<th>Age</th>
<th>Mutation analysis (Exon 3 of the β-catenin gene)</th>
<th>EGFR expression (cDNA)</th>
<th>EGFR-P staining</th>
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Table legends

Table 1

**Characterization of the CP series including results of EGFR and β-catenin screening**

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 single nucleotide polymorphisms 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 marked with a gray background. Abbreviations: m= male; f= female; neg.= no EGFR amplification detectable; SSCP= single strand conformation polymorphism; ex= exon; wt= wild type.

Table 2

**Primers used for EGFR and β-catenin mutation screening and analysis of EGFR, fascin and β-catenin gene expression**

Figure legends

**Figure 1**

(A) Activation of the EGFR was analyzed by immunohistochemistry using an antibody directed against phosphorylated Tyrosine 1068 (EGFR-P). EGFR-P (CP12 arrowhead, CP13 and CP20 arrow) was identified in tumor regions invading adjacent brain parenchyma (#). Some cells revealed nuclear localization of EGFR-P (CP12 arrowhead and CP16 asterisk). Scale bars in CP12, CP16 and CP20 = 50μm, in CP13 = 100μm. (B) Double immunofluorescence staining of EGFR-P (green) and β-catenin (red) demonstrated co-localization in cells with activated EGFR and nuclear β-catenin accumulations (yellow). Nuclei (blue) were counterstained with Höchst. Scale bar = 50μm.
Figure 2

(A) Activated EGFR (EGFR-P) content was analyzed by immunoblotting in cells treated with EGF and/or its inhibitor Gefitinib (500nM) as shown for sample CP18 in relation to untreated cells. Full length blots are presented in Supplementary Figure 3. For densitometric evaluation protein values were normalized with β-actin content and correlated to untreated cells (bars represent unicates). Activation of the EGFR by EGF treatment (EGF+) enhanced tumor cell migration in Boyden chamber (right panel) and wound healing (left panel) assays (mean values are indicated) (B) and was inhibited by Gefitinib administration (C). The percentage of migrating cells (EGF+) was related to untreated (EGF-) controls. Each experiment was done in triplicate (p<0.001). (C) The impact of Gefitinib administration on EGF- and EGF+ cells was evaluated using also Boyden chamber and wound healing assays. Treatment of Gefitinib (Gefitinib+) inhibited migration in CP cultures (mean: 94% in Boyden chamber and 89% in wound healing assay) but this effect reached significance only in experiments with previous EGF (EGF+) stimulation (mean: 61% in Boyden chamber and 69% in wound healing assay) (p<0.001).

Figure 3

(A) Double immunofluorescence analyses revealed co-expression of Fascin and activated EGFR. TaqMan gene expression assays for Fascin were conducted from eight 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). (C) Fascin protein content was analyzed in untreated cells and after EGF and/or Gefitinib (500nM) treatment by immunoblotting. For densitometric evaluation protein values were normalized with β-actin content and related to untreated cells. Full length blots are presented in Supplementary Figure 3.
Figure 1

A

CP12  CP16

CP13  CP20

B

EGFR-P  β-catenin  merge

CP20
Figure 2

A

B

C

170 kd
42 kd
EGFR-P
Gefitinib

EGFR
CP18

TaqMan Gene Expression Assays

Boyden chamber assay

wound-healing assay

EGFR
CP18

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Figure 3

A

EGFR-P
Fascin
merge

B

C

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
EGFR signaling regulates tumor cell migration in craniopharyngiomas

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Clin Cancer Res Published OnlineFirst May 11, 2011.

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