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Detection, characterization and inhibition of FGFR-TACC fusions in IDH wild type glioma

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Abstract

Purpose—Oncogenic fusions consisting of FGFR and TACC are present in a subgroup of glioblastoma (GBM) and other human cancers and have been proposed as new therapeutic targets. We analyzed frequency, molecular features of FGFR-TACC fusions, and explored the therapeutic efficacy of inhibiting FGFR kinase in GBM and grade-II–III glioma.

Experimental Design—Overall, 795 gliomas (584 GBM, 85 grade-II–III with wild-type and 126 with *IDH1/2* mutation) were screened for FGFR-TACC breakpoints and associated molecular profile. We also analyzed expression of the FGFR3 and TACC3 components of the fusions. The effects of the specific FGFR inhibitor JNJ-42756493 for FGFR3-TACC3-positive glioma were determined in preclinical experiments. Two patients with advanced FGFR3-TACC3-positive GBM received JNJ-42756493 and were assessed for therapeutic response.

Results—Three of 85 *IDH1/2* wild type (3.5%) but none of 126 *IDH1/2* mutant grade-II–III glioma harbored FGFR3-TACC3 fusions. FGFR-TACC rearrangements were present in 17 of 584 GBM (2.9%). FGFR3-TACC3 fusions were associated with strong and homogeneous FGFR3 immunostaining. They are mutually exclusive with *IDH1/2* mutations and *EGFR* amplification whereas co-occur with *CDK4* amplification. JNJ-42756493 inhibited growth of glioma cells harboring FGFR3-TACC3 *in vitro* and *in vivo*. The two patients with FGFR3-TACC3 rearrangements who received JNJ-42756493 manifested clinical improvement with stable disease and minor response, respectively.

Conclusions—RT-PCR-sequencing is a sensitive and specific method to identify FGFR-TACC-positive patients. FGFR3-TACC3 fusions are associated with uniform intra-tumor expression of the fusion protein. The clinical response observed in the FGFR3-TACC3-positive patients treated with a FGFR inhibitor supports clinical studies of FGFR inhibition in FGFR-TACC-positive patients.

Keywords

glioma; FGFR3-TACC3 fusion; *IDH* wild type; target therapy

INTRODUCTION

The history of successful targeted therapy of cancer largely coincides with the inactivation of recurrent, oncogenic and addicting gene fusions in hematological malignancies and recently in some types of epithelial cancer (1, 2). Glioblastoma multiforme (GBM) is among

the most lethal forms of human cancer and targeted therapies against common genetic alterations in GBM have not changed the dismal outcome of the disease (3, 4). Underlying biological features including infiltrative growth behavior, intratumoral heterogeneity, and adaptive resistance mechanisms coupled with the unique challenges of intracranial location present significant problems in its effective management. Despite surgery and chemoradiotherapy, most patients rapidly recur and no effective treatment options are available at that stage. Beside GBM, which features the highest grade of malignancy among glioma (grade IV), lower grade glioma which include grade II and grade III are a heterogeneous group of tumors in which specific molecular features are associated with divergent clinical outcome. The majority of grade II–III glioma (but only a small subgroup of GBM) harbor mutations in *IDH* genes (*IDH1* or *IDH2*), which confer a more favorable clinical outcome. Conversely, the absence of *IDH* mutations is associated with the worst prognosis (5).

We have recently identified *FGFR-TACC* gene fusions (mostly *FGFR3-TACC3*, and rarely *FGFR1-TACC1*) as the first example of highly oncogenic and recurrent gene fusions in GBM. The FGFR-TACC fusions that have been identified so far include the Tyrosine Kinase (TK) domain of FGFR and the coiled-coil domain of TACC proteins, both necessary for the oncogenic function of FGFR-TACC fusions. We also tested tumor dependency on FGFR-TACC fusions in preclinical mouse models of FGFR-TACC glioma and observed marked anti-tumor effects by FGFR inhibition (6). After our report, *FGFR3-TACC3* fusions have been identified in pediatric and adult glioma, bladder carcinoma, squamous lung carcinoma and head and neck carcinoma, thus establishing FGFR-TACC fusions as one of the chromosomal translocation most frequently found across multiple types of human cancers (6–15).

From a mechanistic standpoint, we discovered the unexpected capacity of FGFR-TACC fusions to trigger aberrant chromosome segregation during mitosis, thus initiating chromosome instability (CIN) and aneuploidy, two hallmarks of cancer. However, we still have an incomplete understanding of the full repertoire of the structural variants of FGFR-TACC fusions occurring in GBM and lower grade glioma. Furthermore, it remains unknown whether FGFR-TACC fusions mark distinct grades of glioma and GBM subtypes.

To date eight variants of the *FGFR3-TACC3* fusion have been reported that mostly differ for the breakpoint in the *TACC3* gene (6–15). Because of the close proximity of *FGFR3* and *TACC3* (the two genes map at a distance of 70 Kb on chromosome 4p16.3), detection of *FGFR3-TACC3* rearrangements by FISH is not a feasible option with the currently available methods. Here we report a screening method for FGFR-TACC fusions that includes a RT-PCR assay designed to identify the known and novel *FGFR3-TACC3* fusion transcripts, followed by confirmation of the *in-frame* breakpoint by Sanger sequencing. Using this assay, we have analyzed a dataset of 584 GBM and 211 grade II and grade III gliomas.

A crucial question with fundamental clinical relevance for any novel candidate target mutation is the frequency of the alteration in the cancer cell population, thus discriminating between a clonal or sub-clonal origin of the mutation. In fact, GBM is characterized by a formidable degree of subclonal heterogeneity, whereby neighboring cells display amplification and expression of different Receptor Tyrosine Kinase (RTK)-coding genes

(16–19). This notion poses major therapeutic challenges for targeting any individual RTK will result, at best, in the eradication of a limited tumor sub-clone. In this study, we determine that brain tumors harboring FGFR-TACC fusions manifest strong and homogeneous intra-tumor expression of the FGFR3 and TACC3 component invariably included in the fusion protein, when analyzed by immunostaining. We also report a significant clinical benefit following treatment with a specific inhibitor of FGFR-TK in two GBM patients who harbored FGFR3-TACC3 rearrangement.

MATERIALS AND METHODS

Patients and tissue samples

This study includes a cohort of 746 untreated patients with histologic diagnosis of glioma from 5 institutions. Forty-nine recurrent gliomas from Pitié-Salpêtrière Hospital and one recurrent glioma from the University of Calgary were also included. A summary of the patient cohort is provided in Table 1.

Tumor specimens, blood samples and clinico-pathological information were collected with informed consent and relevant ethical board approval in accordance with the tenets of the Declaration of Helsinki. For the samples from the Pitié-Salpêtrière Hospital, clinical data and follow-up are available in the neuro-oncology database (Onconeurotek, GH Pitié-Salpêtrière, Paris).

Two recurrent GBM patients harboring FGFR3-TACC3 were enrolled in the dose escalation part of JNJ-42756493 trial at the Gustave Roussy Institute.

Identification of fusion transcripts and analysis of genomic breakpoints

Total RNA was extracted from frozen tissues using Trizol (Invitrogen) according to manufacturer instructions. Two to three hundred nanograms of total RNA were retro-transcribed with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) or SuperScript II (Invitrogen). RT-PCR was performed using AccuPrime Taq DNA Polymerase (Invitrogen). Primer pairs used for the FGFR3-TACC3 fusions screening were: FGFR3ex12-FW: 5'-CGTGAAGATGCTGAAAGACGATG-3 and TACC3ex14-RV: 5'-AAACGCTTGAAGAGGTCGGAG; amplification conditions were 94°C-3min, (94°C-30sec/61°C-30sec/68°C-1min40sec) for 35 cycles, 68°C-7min. FGFR1-TACC1 fusions were amplified with FGFR1ex16-FW: 5'-TGCCTGTGGAGGAACCTTTCA-3' and TACC1ex13-RV: 5'-CCCAAACCTCAGCAGCCTAAG-3' primers (94°C-30sec/60°C-30sec/68°C-1min40sec for 35 cycles). PCR products were subjected to Sanger sequencing.

FGFR3-TACC3 genomic breakpoints were analyzed in 6 FGFR3-TACC3 positive samples, 5 of which from the Pitié-Salpêtrière Hospital and 1 from Montreal Neurological Institute. Three additional samples (MB-22, TCGA 27-1835 and TCGA 06-6390) available from our previous study (6) were also included in the analysis. Fifty nanograms of genomic DNA were used in the PCR reaction, performed with Accuprime Taq Polymerase (Invitrogen) and PCR products were Sanger sequenced. Primers used in genomic PCR were designed according to the breakpoint sequence in the mRNA; the list of primers used are: FGFR3ex17-FW 5'-TGGACCGTGTCTTACCGT-3' (PCR Samples 3048, 4373, 4867,

4451, MB-22, OPK-14, 06-6390, 27-1835 and Sequencing samples 3048, 4373, 4867, 4451, MB-22, OPK14, 06-6390, 27-1835); FGFR3ex16-FW 5'-GGTCCTTTGGGGTCCTGCT-3' (PCR and Sequencing Sample 3808); TACC3ex6-RV 5'-CCTCTTTTCAGTCCAAGGCA-3' (PCR and Sequencing Samples PCR 4451 and OPK-14); TACC3ex8-RV 5'-TCTACCAGGACTGTCCCTCAG-3' (Sequencing Samples 3048 and 4373); TACC3ex9-RV 5'-GGGAGTCTCATTTCACCGT-3' (PCR Samples 3048, 4373, 4867 and Sequencing Sample 4867); TACC3ex10-RV 5'-CTGCATCCAGGTCCTTCTGG-3' (PCR and Sequencing Samples MB-22 and 06-6390); TACC3ex11-RV 5'-CCAGTTCCAGGTTCTTCCCG-3' (Sequencing Samples 27-1837 and 3808); TACC3ex12-RV 5'-CAACCTCTTCGAACCTGTCCA-3' (PCR and Sequencing Samples 27-1837 and 3808). PCR conditions were 94°C-30sec/60°C-30sec/68°C-2min30sec for 40 cycles. For amplifications performed with the primer TACC3ex9-RV, the program was 94°C-30sec/56°C-30sec/68°C-2min30sec for 40 cycles.

Quantitation of FGFR3 and TACC3 transcripts in GBM

The relative expression of FGFR3 and TACC3 regions included in or excluded from the fusion transcript was assessed by qRT-PCR. Primer pairs with comparable efficiency of amplification were identified and efficiency was assessed using serial dilutions of cDNA (20) prepared from OAW28 ovarian carcinoma cells that contain wild type *FGFR3* and *TACC3* (21). Primers used are: N-terminal region of FGFR3, FGFR3-N: Forward 5'-AAGACGATGCCACTGACAAG-3', Reverse 5'-CCCAGCAGGTTGATGATGTTTTTG-3'; C-terminal region of TACC3, TACC3-C: Forward 5'-TCCTTCTCCGACCTCTTCAAGC-3', Reverse 5'-TAATCCTCCACGCACTTCTTCAG-3'. To amplify transcripts in regions excluded from FGFR3-TACC3 fusion, primers were designed in the C-terminal region of FGFR3, FGFR3-C: Forward 5'-TACCTGGACCTGTCGGCG-3', Reverse 5'-TGGGCAAACACGGAGTCG-3' and N-terminal domain of TACC3, TACC3-N: Forward 5'-CCACAGACGCACAGGATTCTAAGTC-3', Reverse 5'-TGAGTTTTCCAGTCCAAGGGTG-3'. All reactions were performed in triplicate and the data are reported as Fold Change $\pm$ Standard Deviation.

Immunofluorescence and immunohistochemistry

For immunofluorescence (IF) staining of FGFR3, 5µm FFPE sections were subjected to antigen retrieval with citrate buffer for 8 min. Primary antibodies were: FGFR3-N (1:400, sc-13121, Santa Cruz Biotechnology), FGFR3-C (1:2000, sc-123, Santa Cruz Biotechnology), TACC3-N (1:600, ab134153, Abcam), and TACC3-C (1:300, NBP1-01032, Novus Biological). Secondary biotinylated antibodies were used at 1:50,000 followed by streptavidin and TSA Cy3-conjugated. Nuclei were counterstained with DAPI. For immunohistochemical analysis (IHC) of FGFR3 expression, antigen retrieval was performed for 12 min and FGFR-3 antibody (sc-13121, Santa Cruz Biotechnology) was diluted 1:500. Biotinylated anti-mouse antibody (1:30,000) and streptavidin were added before incubation with the chromogen. Nuclei were counterstaining with hematoxylin.

Molecular characterization of tumor samples

Mutational status of *IDH1*, *IDH2*, *TERT* promoter, as well as the methylation status of the *MGMT* promoter was analyzed in the Pitié-Salpêtrière cohort. Expression of IDH1-R132H mutant was analyzed by IHC in 500 cases as previously described (22). *IDH1* and *IDH2* gene mutations were identified by Sanger sequencing in 464 and 388 gliomas, respectively (5). *IDH* wild-type tumors are defined according to the absence of IDH1-R132H immunopositivity and/or mutations in *IDH1* and *IDH2* genes. *TERT* promoter status was determined by the same technique in 277 samples (23). Hyper-methylation of the *MGMT* promoter was tested in 242 samples by bisulfite pyro-sequencing (24). The presence of EGFRvIII was evaluated by RT-PCR in 118 samples using EGFR-FW 5'-CTTCGGGGAGCAGCGATGCGAC-3' and EGFR-RV 5'-CTGTCCATCCAGAGGAGGAGTA-3' primers (25).

Copy number variations analyses have been performed on 192 tissue samples using CGH arrays using BAC arrays (N=187), Agilent 4×180K (N=2), Nimblegen 3×720K (N=2), Agilent 8×60K (N=1). Results were normalized using control DNA from matched blood samples as previously described (26). Additional analyses of 193 tumor specimens were performed by SNP array, using Illumina Omni (N=110), Illumina HumCore (N=32), Illumina 370K (N=27), or Illumina 610K (N=24), as previously described (27). Array processing was outsourced to Integrigen. Raw copy numbers were estimated at each of the SNP and copy-number markers. Biodiscovery property SNP-FASST2 algorithm was then used to segment copy number data. Segments were mapped to hg18 genome assembly (28). Copy number alterations (CAN) magnitudes called log-R ratio (LRR) were classified using simple thresholds: deletion ($x < -1$), loss ($-1 < x < -0.2$), gain ($0.2 < x < 1$) or amplification ($x > 1$) according to default Nexus 7.5 software. For additional 56 gliomas, 10q loss was assessed on tumor and blood DNA by microsatellite analysis, while amplification of *EGFR*, *MDM2* and *CDK4*, and deletion of *CDKN2A* gene, were determined by qPCR, as previously reported (29, 30).

The molecular profiles obtained in Pitié-Salpêtrière dataset were combined with those available in the TCGA dataportal. TCGA GBM segmented copy number variation profile was downloaded from The UCSC Cancer Genomics Browser (31). Copy Number Variations (CNVs) were measured experimentally using the Affymetrix Genome-Wide Human SNP Array 6.0 platform at the Broad TCGA genome characterization center (32). Raw copy numbers were estimated at each of the SNP and copy-number markers. Circular binary segmentation was then used to segment the copy number data (28). Segments are mapped to hg18 genome assembly at Broad.

For CNV analysis of the regions across *FGFR3* and *TACC3* genes, we considered samples for which RNAseq and CNV data were available or samples for which only CNV data were available and RT-PCR-sequencing of *FGFR3-TACC3* fusion had been performed. Overall, 158 GBM (all with a wild type *IDH1* gene) satisfied these criteria. Among them, 5 harbored an *FGFR3-TACC3* fusion whereas 153 were FGFR-TACC-negative. The CNV magnitudes, called log-R ratio (LRR), were classified using the following thresholds: deletion ($x < -1$), loss ($-1 < x < -0.2$), gain ($0.2 < x < 1$) or amplification ($x > 1$), according to the Atlas-

TCGA (32). The analysis of the genomic regions encompassing *EGFR*, *MDM2*, *CDK4*, *CDKN2A*, *7p*, *10q*, according to *hg18* genome assembly, was performed to evaluate their CNV. *EGFRvIII* mutation status was inferred according to Brennan et al. (32). The frequencies of the aberrations of these genes in *FGFR3-TACC3* positive and negative samples were calculated and the obtained data were then combined with the Pitié-Salpêtrière Hospital dataset.

Statistical Analysis

Differences in the distribution on categorical variables were analyzed using Fisher Exact test. The *p*-values were adjusted for multiple testing according to Benjamini and Hochberg false discovery rate (FDR). A *q*-value = 0.05 (two-sided) was considered to be statistically significant.

Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Patients who were still alive at the last follow-up were considered as censored events in the analysis. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at the last follow-up were considered as censored events in the analysis. Survival curves were calculated by the Kaplan-Meier method and differences between curves assessed using the Log-Rank test. A Log-Rank test *p*-value = 0.05 (two-sided) was considered to be statistically significant.

Cell culture and cell growth assay

GIC-1123 gliomaspheres were cultured in neurobasal medium (Invitrogen) supplemented with B27, N2 (Invitrogen), EGF and FGF2 (20 ng/ml, PeproTech). Mouse astrocytes *Ink4A-Arf*^{-/-} were cultured in DMEM supplemented with 10% Fetal Bovine Serum. Cells were seeded at 1,000 cells/well in a 96-well plate and treated with JNJ-42756493 (Active Biochem, #A-1278). After 72 hours cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Data are mean ± SEM of six replicates. Experiments were performed three times.

Subcutaneous xenografts and drug treatment

GIC-1123 cells (5×10^5) were injected subcutaneously in the flank of athymic nude (Nu/Nu) mice (Charles River Laboratories). Mice carrying ~200 mm³ subcutaneous tumors were randomized to receive 12 mg/kg JNJ-42756493 (Active Biochem, #A-1278) or DMSO in 1% Tween 80 by oral gavage. Tumor diameters were measured with caliper and tumor volumes estimated using the formula: $0.5 \times \text{length} \times \text{width}^2$. Data are mean ± SD of nine mice in each group. Mice were sacrificed when tumors in the control group reached the maximal size allowed by the IACUC Committee at Columbia University.

MRI imaging and evaluation of clinical response to JNJ-42756493

Baseline and follow-up imaging assessments were performed on 1.5 Tesla MR imaging systems, including at least axial T1 weighted images before gadolinium injection, Axial or 3D FLAIR (Fluid-Attenuated Inversion-Recovery), dynamic susceptibility contrast MR perfusion (0.1 mmol/kg of gadobutrol), axial and 3D T1 weighted images after gadolinium

injection. Tumor response was assessed according to the RANO criteria (33). Contrast-enhancing lesion volume was assessed with the help of a semi-automated volumetry tool (SegmentiX), based on shape-detection and thresholding, with control and manual correction of edges when necessary. Since exclusion of cystic or necrotic portions of the lesion may be affected by operator subjectivity, we included them both for volumetric and axial measurements.

DSC (dynamic susceptibility contrast) perfusion datasets were processed with vendor's software suite (Neuroperfusion, Philips), including coregistration and rCBV (relative cerebral blood volume) parametric maps generation with 3 different algorithms (Gamma-variate fitting, Arterial Input Function based deconvolution and Model Free).

RESULTS

Detection of FGFR1-TACC1 and FGFR3-TACC3 fusions in GBM and grade II–III glioma

To determine the frequency and molecular features of FGFR-TACC fusions in human glioma patients, we screened a cohort of 584 GBM and 211 grade II–III glioma treated at five Neuro-oncology centers (Table 1). 108 were grade III (49 *IDH* wild type, 52 *IDH1* mutant and 7 *IDH2* mutant) and 103 were grade II (36 *IDH* wild type, 63 *IDH1* mutant and 4 *IDH2* mutant). We also established the *IDH* mutational status of 333 GBM and determined that 303 harbored wild type *IDH1/2* and 30 were mutated at codon 132 of *IDH1*. We designed a RT-PCR assay for the detection of all known and possibly new variants of FGFR1-TACC1 and FGFR3-TACC3 fusions that retain the mRNA sequences coding for the key FGFR-TK and TACC domains required for the oncogenic activity of the fusion protein (Fig. 1 and Fig. 2A–D). Overall, we found 20 tumors with an FGFR3-TACC3 fusion, of which 17 were GBM (2.9% positives) and 3 lower grade glioma harboring wild type *IDH1/2* genes (3.5% positives). The size of the FGFR3-TACC3 RT-PCR amplicons ranged from 928 bp (for *FGFR3ex18-TACC3ex13*) to 1706 bp (for *FGFR3ex18-TACC3ex4*). The FGFR1-TACC1 fusion was detected in one grade II *IDH* wild type glioma (Fig. 1). Conversely, we did not find any *IDH1/2* mutant glioma harboring FGFR-TACC fusions ($p < 0.02$). Sanger sequencing of the fusion amplicons revealed that each FGFR-TACC cDNA joined *in-frame* the sequence coding for the entire TK domain upstream of TACC-coding sequences that invariably include the coiled-coil TACC domain (Fig. 1). However, we detected a notable variability among FGFR3-TACC3 fusion isoforms, whereby 5 of the identified variants occurred only in individual cases (Fig. 1). Furthermore, 6 fusion transcripts emerged as new variants that have not been reported before in human cancer (marked in red in Fig. 1).

Next, we designed suitable PCR primers to map the genomic breakpoint coordinates for 9 FGFR3-TACC3-positive samples for which genomic DNA was available (Supplementary Figs 1 and 2). We successfully reconstructed the genomic breakpoints by Sanger sequencing and found that they differ for each of the 9 positive cases. Interestingly, even cases harboring the same *FGFR3-TACC3* transcript splice variants (#4451 and #OPK-14 joining exon 17 of *FGFR3* to exon 6 of *TACC3*; #3048 and #4373 joining exon 17 of *FGFR3* to exon 8 of *TACC3*; #3808 and #27-1835 joining exon 17 of *FGFR3* to exon 11 of *TACC3*) had different genomic breakpoints (Supplementary Fig. 2). Taken together, the above

findings indicate that the noticeable variability among FGFR3-TACC3 fusion transcripts and genomic breakpoints is efficiently resolved by the RT-PCR screening assay.

Immunostaining analysis of FGFR3-TACC3-positive tumors

We analyzed the expression of the FGFR3 fusion protein by IHC or IF using an antibody that recognizes the N-terminal region of FGFR3 (FGFR3-N) in 12 GBM and 3 lower grade glioma harboring FGFR3-TACC3 fusions for which sufficient tissue was available. Remarkably, each of the 15 positive tumors but none of those that had scored negative in the RT-PCR assay, displayed strong positivity for FGFR3 in the vast majority of tumor cells but not endothelial cells throughout the analyzed tumor section (Fig. 2A–H). Notably, IF using an antibody that recognizes an epitope at the C-terminus of TACC3, which is invariably retained within FGFR3-TACC3 variants (TACC3-C), reproduced the staining pattern of the FGFR3-N antibody in FGFR3-TACC3 positive tumors. Conversely, negative or very weak staining was obtained in FGFR3-TACC3-positive tumors with antibodies recognizing the regions of FGFR3 (FGFR3 C-terminal region, FGFR3-C) and TACC3 (TACC3 N-terminal region, TACC3-N) constantly excluded from FGFR3-TACC3 fusion proteins (Supplementary Fig. 3A). Consistently, quantitative RT-PCR of GBM harboring FGFR3-TACC3 fusions showed that the expression of the N-terminal coding region of FGFR3 and the C-terminal coding region of TACC3 (which are included in the fusion genes) is markedly higher than the expression of the C-terminal coding region of FGFR3 and the N-terminal coding region of TACC3, which are excluded from the fusion transcripts (Supplementary Fig. 3B). We also analyzed one recurrent GBM from a patient whose tumor had been found positive for FGFR3-TACC3 at the initial diagnosis and who had recurred after concurrent radiotherapy and temozolomide treatment. The recurrent tumor retained the same *FGFR3-TACC3* fusion gene and protein that was present in the untreated GBM as determined by RT-PCR-sequencing and FGFR3 IF, respectively (Supplementary Fig. 4). Although this requires additional evaluation, the retained uniform positivity for FGFR3 in this recurrent GBM suggests that targeting the FGFR3-TACC3 fusion protein at relapse is a valid therapeutic strategy.

Clinical and molecular characteristics of glioma patients with FGFR3-TACC3 fusions

Clinical and molecular profiling data were available for 591 patients including 380 GBM (9 with FGFR3-TACC3 fusions) and all 211 lower grade glioma (3 with FGFR3-TACC3 fusions). Of these 12 patients 5 are males and 7 females, aged 48y to 82y (median= 61y). We sought to determine the molecular profile of FGFR3-TACC3-positive glioma. To do so, we combined the analysis of CNVs and somatic mutations of key GBM genes in our dataset with the SNP6.0 high-density genomic array analysis of 158 TCGA-derived GBM samples fully annotated for *FGFR3-TACC3* fusion genes (the RNA-seq and/or RT-PCR analysis of these samples had revealed that 5 of them harbor FGFR3-TACC3 fusions) (6). Patients with FGFR3-TACC3 fusions displayed unique characteristics (Table 2). FGFR3-TACC3 fusions were mutually exclusive with *EGFR* amplification (0/16 vs. 166/411; $p=0.0004$, FDR q -value corrected for multiple comparisons=0.0012) and showed a clear trend against the presence of the *EGFRvIII* transcript variant (0/16 vs. 37/219; $p=0.083$). Conversely, *CDK4* amplification was significantly more frequent in FGFR3-TACC3-positive tumors (7/16 vs 41/408, $p=0.0008$; FDR q -value= 0.0024). A less significant association of FGFR3-TACC3

fusions was also seen with amplification of *MDM2*, which as *CDK4*, maps to chromosome 12q (4/16 vs 24/408, $p=0.016$; FDR q -value= 0.048). We found no statistical association between *FGFR3*-*TACC3* fusions and other genetic and epigenetic alterations that commonly occur in gliomas harboring wild type *IDH* genes (*CDKN2A* deletion, *TERT* promoter mutations, gain of chromosome 7p, loss of chromosome 10q and methylation of the *MGMT* promoter, Table 2). When compared with the *IDH* wild type patient population of grade II and grade III glioma and GBM, there was no significant difference in progression free survival (PFS) or overall survival (OS) between patients positive or negative for *FGFR3*-*TACC3* (Supplementary Fig. 5A–B). Finally, we sought to establish whether the CNV analysis of the *FGFR3* and *TACC3* genomic loci could be used to predict positivity for *FGFR3*-*TACC3* fusions. The analysis of high-density SNP6.0 arrays of the 158 GBM samples from the Atlas-TCGA revealed that 10 samples displayed different degrees of copy number gains encompassing the entire *FGFR3* and *TACC3* loci (Supplementary Fig. 6). However, none of them harbored *FGFR3*-*TACC3* fusions. Conversely, the 5 *FGFR3*-*TACC3*-positive samples in the dataset harbor micro-amplification events involving only the exons of the *FGFR3* gene that are included in the fusion breakpoint. This finding suggests that any CNV survey that is less accurate than high-density SNP arrays, could fail to identify the genomic marks associated with true *FGFR3*-*TACC3*-positive cases.

Preclinical and clinical relevance of targeting *FGFR3*-*TACC3* fusions

JNJ-42756493 is a potent, oral pan-FGFR tyrosine kinase inhibitor with IC_{50} values in the low nanomolar range for all members of the FGFR family. It has demonstrated potent antitumor activities in nonclinical models with FGFR aberrations including squamous non-small cell lung cancer, gastric, breast, hepatocellular cancer (HCC), endometrial, and bladder (34, 35). To ask whether JNJ-42756493 is effective in targeting specifically *FGFR3*-*TACC3*-positive cells, we treated with JNJ-42756493 mouse astrocytes expressing *FGFR3*-*TACC3*, *FGFR3*-*TACC3* containing a mutation that inactivates the kinase activity of *FGFR3* (*FGFR3*-*TACC3*-KD), or the empty vector. We also studied the effect of JNJ-42756493 on human glioma stem cells GIC-1123 that harbor the *FGFR3*-*TACC3* gene fusion (6). These experiments revealed that both mouse astrocytes and GIC-1123 that express *FGFR3*-*TACC3* but not cells expressing the KD mutant fusion or the empty vector are highly sensitive to FGFR inhibition by JNJ-42756493 with an IC_{50} of 3.03 nM and 1.55 nM, respectively (Fig. 3A–B). Next, we tested whether oral treatment with JNJ-42756493 of mice bearing xenografts of human GIC-1123 affects tumor growth. Mice were randomized to receive vehicle or JNJ-42756493 (12 mg/kg). Mirroring the *in vitro* results, JNJ-42756493 elicited a potent growth inhibition of GIC-1123 tumor xenografts (Fig. 3C–D) with a statistically significant tumor regression after two weeks (p -value of the slope calculated from the treatment starting point = 0.04). The above findings provide a strong foundation for the treatment of GBM patients harboring FGFR-TACC rearrangements with JNJ-42756493.

Two patients with recurrent GBM harboring *FGFR3*-*TACC3* fusions were treated with JNJ-42756493 in a first-in-man phase I trial. Patient 1, male aged 52, underwent partial surgical resection of a right parietal GBM, followed by fractionated radiotherapy and concomitant temozolomide (TMZ) as first line treatment (36). The RT-PCR-sequencing

analysis of the GBM specimen revealed positivity for the FGFR3-TACC3 fusion (*FGFR3-exon17-TACC3-exon 6*, sample 4451, Supplementary Figs 1 and 2) and the immunostaining using FGFR3 antibody on paraffin embedded sections showed strong positivity in a large fraction of tumor cells (not shown). After 5 cycles of TMZ, the patient presented with dizziness and headache and brain MRI revealed tumor progression (Fig. 4A). At this time the patient was enrolled in the JNJ-42756493 trial and received JNJ-42756493 (12 mg/day administered in cycles of 7 days followed by 7 days off treatment). After 3 weeks the patient reported a marked clinical improvement (complete regression of dizziness and headache). On MRI, the sum of product diameters (RANO criteria, Fig. 4B) and volumetry (Fig. 4C) measured without excluding cystic and necrotic components showed disease stabilization. However, the tumor mass underwent significant decrease of the enhancing parenchyma (–44%) with formation of a cystic portion in the central core (33). The objective response was further corroborated by the marked reduction of the extent of tumor vascularity estimated by quantitative analysis of rCBV (relative cerebral blood volume) from dynamic susceptibility MR perfusion maps (37) (Fig. 4D). Stabilization lasted for 115 days. During JNJ-42756493 treatment mild and manageable toxicity was observed (grade I hyperphosphatemia, asthenia, dysgueusia, dry mouth, keratitis, and grade II nail changes). After 4 months, tumor progressed on MRI locally both on T1 contrast-enhanced area and T2/FLAIR hypersignal. The patient was re-operated and subsequently treated with CCNU. He is still alive, but in progression after 21 months from diagnosis and 287 days from the start of the anti-FGFR therapy.

Patient 2 is a 64 years old woman, affected by left parietal GBM, diagnosed by stereotactic biopsy. The tumor was positive for FGFR3-TACC3 gene fusion by RT-PCR-sequencing and showed diffuse FGFR3 expression in most tumor cells (Figure 2A, 2C, 2E, sample 4620). The patient received as first line treatment fractionated radiotherapy and TMZ according to the Stupp protocol (36), but after 2 cycles of monthly TMZ she presented with clinical deterioration including progressive headaches, right homonymous hemianopsia and memory impairment. Brain MRI performed 3 and 4 months after the completion of concomitant chemo-radiotherapy revealed tumor progression with increase of the left parietal mass and the appearance of a small contralateral lesion (Fig. 4E). The patient was thus enrolled in the JNJ-42756493 trial (12 mg/day administered in cycles of 7 days followed by 7 days off treatment) and showed clinical improvement after 4 weeks (regression of headaches, visual field defect and memory impairment). Best response was observed after 104 days of treatment with a 22% reduction of tumor size according to the RANO criteria (Fig. 4F) and 28% according to volumetry (Fig. 4G). Grade I hyperphosphatemia, nail changes, and mucositis were observed. Clinical status remained stable until disease progression occurring 134 days after the start of the anti-FGFR therapy. The patient is still alive and is receiving a third-line chemotherapy with nitrosoureas and bevacizumab.

DISCUSSION

FGFR-TACC fusions are potent oncogenic events that when present in brain tumor cells confer sensitivity to FGFR inhibitors (6). Since our original identification of recurrent FGFR-TACC fusions in GBM, small subgroups of patients harboring FGFR-TACC translocations have been identified in several other tumor types (7–15). Here, we report an

unbiased RT-PCR-sequencing analysis for the identification of all possible functional FGFR-TACC fusion transcripts. The screening of a large glioma dataset from multiple Institutions not only confirmed that FGFR-TACC rearrangements occur in ~3% of human GBM but also revealed that FGFR-TACC fusions are present in the subgroup of *IDH* wild type lower grade glioma (grade II–III) with prevalence similar to that of GBM. *IDH* wild type grade II and III glioma have a significantly worse clinical outcome than the *IDH* mutant glioma and manifests molecular and clinical features that resemble GBM (5). Our finding that FGFR-TACC fusions occur in *IDH* wild type but not *IDH* mutant glioma provides an important clue for the molecular characterization of this glioma subtype. Furthermore, the clustering of such potent oncogenic events in *IDH* wild type glioma underscores the particularly aggressive nature of this group of glioma. While we showed that FGFR-TACC fusions cluster within the poor clinical outcome subgroup of *IDH* wild type glioma, these translocations do not seem to carry prognostic value within the *IDH* wild type subgroup of glioma patients. However, the sample size of patients harboring FGFR-TACC fusions is too small to draw definitive conclusions with respect to the impact on survival and larger studies will be necessary to clarify the prognostic role of FGFR-TACC fusions in *IDH* wild type glioma.

Beside mutual exclusivity between *IDH1* mutations and FGFR-TACC fusions, our results showed that patients with FGFR3-TACC3 rearrangements lack *EGFR* amplification and *EGFRvIII* but are significantly enriched for amplification of *CDK4* (and *MDM2* to a lesser extent). Knowledge of these molecular characteristics will help select those patients who most likely harbor *FGFR-TACC* rearrangements and design combinatorial targeted therapies that might be more effective in the *FGFR-TACC*-positive glioma subgroup.

The molecular screen uncovered 6 new *FGFR3-TACC3* fusion events. Together with the previously identified variants, others and we have reported 12 distinct isoforms of FGFR3-TACC3, thus revealing a remarkable variability of *FGFR3-TACC3* transcripts in human cancer (see Supplementary Table 1 summarizing the structure of all the FGFR-TACC variants identified to date). The structural heterogeneity of *FGFR3-TACC3* fusions is yet more pronounced at the genomic level, whereby each fusion event harbors distinct genomic breakpoints, even for identical fusion transcripts. This finding underscores the notion that targeted genomic analyses are unlikely to be suitable approaches for the molecular diagnosis of *FGFR3-TACC3* positivity. Conversely, the unbiased identification of *FGFR3-TACC3*-positive tumors with the RT-PCR-sequencing assay reported here overcomes the limitations of screening only for previously identified *FGFR3-TACC3* fusions and provides a simple molecular diagnostic assay.

Rather than displaying uniform amplifications of the *FGFR3* and *TACC3* genomic loci, *FGFR3-TACC3*-positive samples harbor small, intragenic micro-amplification events typically encompassing only the exons of the *FGFR3* and *TACC3* genes included in the breakpoint (6). This finding is consistent with the notion that a “fusion breakpoint principle” sustains the CNVs of driver gene fusions such as *FGFR3-TACC3* in which local CNVs target exclusively the breakpoint region (38). We note that such small and irregular CNVs may easily go undetected from CNV analyses performed using platforms less sensitive than the high-density SNP6.0 genomic arrays. Furthermore, the notion that *FGFR3-TACC3*-

negative GBM may harbor uniform amplifications across the *FGFR3* and *TACC3* loci argues against the standard analysis of *FGFR3* and/or *TACC3* CNVs as a method for the selection of FGFR3-TACC3-positive tumors.

There is a growing body of evidence supporting the notion that GBM is a markedly heterogeneous tumor. The formidable degree of intra-tumor heterogeneity of GBM is a potential cause of failure of targeted therapies in these tumors. In particular, the intra-tumor heterogeneity of GBM has previously been recognized in light of the mosaic expression of the RTK genes *EGFR*, *PDGFRA* and *MET* by neighboring cells (16–19). Thus, in the majority of GBM, amplification or overexpression of individual RTK genes are present in a sub-clonal fraction of tumor cells and co-exist with amplification/expression of other RTK-coding genes within the tumor mass. Therefore, it was essential to determine whether such heterogeneity was also present in gliomas harboring *FGFR-TACC* translocations. The immunostaining of FGFR3-TACC3-positive tumors revealed that positive specimens manifest strong and uniform expression of the fusion protein, which is also retained after recurrence. This behavior is reminiscent of other driver chromosome translocations (BCR-ABL, EML4-ALK) and is compatible with the glioma-initiating functions of FGFR-TACC fusions (6). It is also the scenario expected for a driver oncogene whose activity remains essential for tumor maintenance regardless of secondary genetic alterations that occur during tumor progression.

The strong antitumor effects obtained with JNJ-42756493 in glioma cells harboring *FGFR3-TACC3* fusions have built a compelling rationale for the treatment of glioma patients positive for *FGFR-TACC* rearrangements. JNJ-42756493 is an oral ATP-competitive pan-FGFR selective inhibitor that inhibits tyrosine phosphorylation of activated FGFR at nanomolar concentrations (34, 35). The enrollment of two patients with recurrent FGFR3-TACC3-positive GBM in a phase I trial with JNJ-42756493 showed that this treatment has tolerable toxicity and clear anti-tumor activity, thus validating FGFR-TACC as a therapeutic target. Therefore, targeted inhibition of FGFR-TK in preselected *IDH* wild type FGFR-TACC-positive glioma may provide clinical benefits for patients with recurrent glioma who currently lack valuable therapeutic options. In conclusion, we have shown the importance and feasibility of prospective genotyping for *FGFR-TACC* fusions in glioma patients and provided a preliminary evidence of clinical response that warrants the investigation of the sensitivity of gliomas harboring *FGFR-TACC* rearrangements to FGFR kinase inhibition in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STATEMENT OF TRANSLATIONAL RELEVANCE

This manuscript reports an unbiased screening assay for FGFR-TACC fusions in glioma that overcomes the great variability of variants that are generated by FGFR-TACC chromosomal translocation in human cancer. FGFR-TACC fusions occur in grade II and III glioma harboring wild-type IDH1 with frequency similar to glioblastoma (GBM), therefore providing a clue to the aggressive clinical behavior of this glioma subtype. The molecular characterization of fusion-positive glioma revealed that FGFR-TACC is mutually exclusive with *EGFR* amplification but co-occurs with *CDK4* amplification. FGFR-TACC-positive glioma displays strikingly uniform and strong expression of the fusion protein at the single cell level. Preclinical experiments with FGFR3-TACC3-positive glioma cells treated with the FGFR inhibitor JNJ-42756493 showed strong antitumor effects and treatment of two patients with recurrent GBM harboring FGFR3-TACC3 resulted in clinical improvement and radiological tumor reduction. These findings validate the treatment with FGFR inhibitors of glioma patients harboring FGFR-TACC chromosomal translocations.

Figure 1. Structure of *FGFR-TACC* gene fusions identified by RT-PCR-Sanger sequencing
Predicted FGFR-TACC fusion proteins encoded by the transcripts identified by RT-PCR. Regions corresponding to FGFR3 or TACC3 are shown in red or blue, respectively. FGFR1 and TACC1 corresponding regions are shown in yellow and green. On the left are indicated the *FGFR* and *TACC* exons joined in the fused mRNA; the presence of *TACC3* introns is also reported when they are spliced in the fusion cDNA. On the right, the number of patients harboring the corresponding fusion variant is indicated. The novel transcripts discovered in this study are highlighted in red. Black arrows indicate the position of the primers used for the *FGFR-TACC* fusions screening.

Figure 2. Identification and immunostaining of FGFR3-TACC3-positive tumors

Results from RT-PCR screening in representative samples from the Pitié-Salpêtrière Hospital (A, C) and the Besta (B, D) datasets. M, DNA ladder. Schematic representation of the FGFR3-TACC3 fusion transcripts identified in samples GBM-4620 (C) and GBM-021 (D). The junction sequences on the mRNA and the reading frame at the breakpoint are reported. Representative microphotographs of H&E and FGFR3 immunostaining in the FGFR3-TACC3 positive samples GBM-4620 (E) and GBM-021 (F) and two FGFR3-TACC3 negative samples (panels G and H); a, H&E, 10X magnification; b, H&E, 40X magnification; c, FGFR3, 10X magnification; d, FGFR3, 40X magnification.

Figure 3. Pre-clinical evaluation of FGFR3-TACC3 inhibition by JNJ-42756493

(A) Mouse astrocytes expressing FGFR3-TACC3 (F3T3), FGFR3-TACC3-KD (F3T3-KD) or the empty vector (Vector) were treated with the indicated concentration of JNJ-42756493. Cell viability was determined by the MTT assay. Error bars show mean $\pm$ SEM (n=6). (B) Survival analysis of GIC-1123 treated with JNJ-42756493. (C) The FGFR-TK inhibitor JNJ-42756493 suppresses tumor growth of subcutaneous tumors generated by GIC-1123. After tumor establishment (arrow) mice were treated with vehicle or JNJ-42756493 (12 mg/kg) for 14 days. Values are mean tumor volumes $\pm$ SD, (n=9 mice per group). *P*-value of the slope calculated from the treatment starting point (arrow) is 0.04. (D) Photograph showing the tumors dissected from vehicle or JNJ-42756493 treated mice after two weeks of treatment.

Figure 4. Baseline and post-treatment Magnetic Resonance Imaging (MRI) of patients treated with JNJ-42756493

Patient 1 (Panels A–D). (A) Post-gadolinium T1 weighted images show the target lesion on the right parietal lobe. The interval (days) from the beginning of follow-up is indicated above each MRI. (B) Analysis of sum of product diameters (SPD) before and during the anti-FGFR treatment (RANO criteria). (C) Analysis of tumor volume (cm^3) before and during the anti-FGFR treatment. During anti-FGFR treatment a stabilization of the tumor was observed according to RANO criteria and volumetry. (D) Perfusion images at baseline and after 20 days of anti-FGFR treatment. rCBV (relative cerebral blood volume). Post-gadolinium T1 weighted images with color overlay of rCBV are shown. Patient 2 (Panels E–G). (E) Two different MRI slice levels of superior and middle part of the lesion are presented. (F) Analysis of sum of product diameters (SPD) before and during the anti-FGFR treatment. During the anti-FGFR treatment a reduction of 22% of tumor size was observed. (G) Volumetric evaluation showed a 28% tumor reduction. Vertical red arrow indicates the start of anti-FGFR treatment (baseline).

Table 1

Frequency of FGFR3-TACC3 Fusions in GBM and Grade II–III glioma.

Tumor sample source	No of case (GBM)	No of detected fusions	Immunostaining FGFR3 positive /Sample analyzed
Pitié-Salpêtrière Hospital	380	9	9/9
Besta Neurological Institute	85	5	2/2
University of Calgary	60 + 1R [§]	2 + 1R [§]	1/1 + 1/1R [§]
Montreal Neurological Institute	51	1	-
University of British Columbia	8	0	-
Total	584 (100%) [£]	17 (2.9%)	
Tumor sample source	IDH Status	No of detected fusions	Immunostaining FGFR3 positive /Sample analyzed
Pitié-Salpêtrière Hospital	IDH wt	3 (3.5%)	3/3
	IDH1/IDH2 Mut	0 (0%)	0

Distribution of the FGFR3-TACC3 fusions in GBM (upper panel) and lower grade glioma (lower panel) samples stratified according to the Institution of origin. The table reports number of cases analyzed, number of tumors harboring FGFR3-TACC3 fusion transcripts, and results of FGFR3 immunostaining. Lower grade glioma samples are further classified according to IDH status (IDH1 and IDH2). The respective frequency of FGFR3-TACC3 in GBM, Glioma grade II–III IDH wild type (wt), and IDH mutant (Mut) glioma is reported in parentheses.

R[§] Recurrent GBM.

£ Recurrent GBM from the University of Calgary Dataset is not included in the total count of GBM.

* 25 cases out of 85 are unknown for IDH2 status.

Table 2

Molecular alterations in *IDH* wild type glioma harboring *FGFR3-TACC3* fusions.

	N of FGFR3-TACC3 Positive	% of FGFR3-TACC3 Positive	N of FGFR3-TACC3 Negative	% of FGFR3-TACC3 Negative	P-value (Fisher test)	q-value (FDR)
EGFR amplification	0/16	0.0 %	166/411	40.4 %	4.E-04	0.0012
CDK4 amplification	7/16	43.7 %	41/408	10.0 %	8.E-04	0.0024
MDM2 amplification	4/16	25.0 %	24/408	5.9 %	0.016	0.048
EGFRvIII	0/16	0.0 %	37/219	16.9 %	0.083	0.25
CDKN2A deletion	4/16	25.0 %	188/411	45.7 %	0.13	0.39
Chr. 7p gain	12/15	80.0 %	242/374	64.7 %	0.28	0.84
Chr. 10q deletion	12/16	75.0 %	253/420	60.2 %	0.3	0.9
TERT promoter mutation	9/11	81.8 %	128/163	78.5 %	0.8	1
MGMT promoter hypermethylation	6/12	50.0%	73/160	45.6 %	0.7	1

The table reports the absolute number and frequency (percentage) of individual glioma-specific molecular alterations in tumors scoring positive or negative for *FGFR3-TACC3* fusions. The analysis is done on the Union dataset (TCGA and "Pitié-Salpêtrière Hospital" datasets, see methods for details). Statistically significant associations are indicated in bold (Fisher Exact test, *q*-values adjusted with FDR).