Brain Pathology ISSN 1015-6305

RESEARCH ARTICLE

MET Gain in Diffuse Astrocytomas is Associated with Poorer Outcome

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Keywords
diffuse astrocytoma, *MET*, primary glioblastoma, secondary glioblastoma.

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Received 10 April 2012
Accepted 11 May 2012
Published Online Article Accepted 1 June 2012

doi:10.1111/j.1750-3639.2012.00609.x

Abstract

Glioblastoma may develop rapidly without evidence for precursor lesions (primary glioblastomas), or progress from diffuse or anaplastic astrocytomas (secondary glioblastomas). Despite having distinct genetic profiles, these glioblastoma subtypes have similar histological features. We hypothesized that the highly malignant phenotype of glioblastoma may be attributable to genetic alterations that are common to both glioblastoma subtypes. In the present study, we first searched for commonly (>35%) amplified genes in glioblastomas with *IDH1* mutation (a hallmark of secondary glioblastoma) and those without *IDH1* mutation (typical for primary glioblastoma) in data from The Cancer Genome Atlas (TCGA). A total of 25 genes were identified, of which 21 were located at 7q31-34. We then screened 264 gliomas (70 glioblastomas, 112 diffuse astrocytomas, 82 oligodendrogliomas) for gain of the *MET* at 7q31.2 with quantitative polymerase chain reaction (PCR). *MET* gain was detected in primary glioblastomas (47%) and secondary glioblastomas (44%), suggesting that this genetic alteration plays a role in the pathogenesis of both glioblastoma subtypes. *MET* gain was also common in diffuse astrocytomas (38%), but less frequent in oligodendrogliomas (16%). *MET* gain in diffuse astrocytomas was associated with shorter survival (median, 43.0 vs. 70.7 months; $P = 0.004$), suggesting that *MET* gain is a useful prognostic marker for diffuse astrocytomas.

INTRODUCTION

Gliomas account for up to 70% of primary brain tumors in adults, with glioblastoma (WHO grade IV) being the most common and malignant histologic type (25). The 5-year survival of patients with glioblastoma is <3% (25), despite multimodal therapy with surgery, radiotherapy and chemotherapy. Most glioblastomas are considered to arise with a short clinical history, and in the absence of less malignant precursor lesions (primary glioblastoma). Other glioblastomas (secondary glioblastomas) develop slowly from diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). Primary and secondary glioblastomas carry distinct genetic alterations, with *IDH1* mutations being the most reliable genetic marker to distinguish between these glioblastoma subtypes (12, 51). On the other hand, the histological features of primary and secondary glioblastomas are similar, suggesting that they may share genetic alterations that play important roles in their development.

Diffuse astrocytomas tend to progress to secondary glioblastomas, but time to progression varies considerably among patients. In one study, time to progression of diffuse astrocytoma to glioblastoma ranged from 7 to 133 months (48), while in another study, the interval before malignant transformation of low-grade astrocytomas ranged from 39 to 119 months (45). Little is known about the molecular mechanisms underlying the rapid progression of diffuse astrocytomas. We hypothesized that this may be at least partly attributable to the presence of genetic alterations that are essential for the glioblastoma phenotype, and that such genetic alterations may be common to both primary and secondary glioblastomas.

The objective of this study was to identify molecular markers that are prognostic for unfavorable outcome of patients with diffuse astrocytoma. We first analyzed The Cancer Genome Atlas (TCGA) dataset and found frequent gain at 7q31 in both glioblastomas with *IDH1* mutation (typical of secondary glioblastoma) and those without *IDH1* mutation (typical of primary glioblastoma). We then decided to focus on the *MET* gene at 7q31.2, which is known to be involved in the pathogenesis of a variety of neoplasms (7, 50), and screened for *MET* gain in 112 diffuse astrocytomas, 82 oligodendrogliomas (WHO grade II), 34 primary glioblastomas and 36 secondary glioblastomas.

MATERIALS AND METHODS

Analysis of TCGA data

We used the TCGA data portal (http://cancergenome.nih.gov/), which contains copy number data for 372 glioblastomas (of these, eight glioblastomas had *IDH1* mutations). A log2 ratio of >0.5 was considered as copy-number gain. Furthermore, TCGA data were analyzed using cBio Cancer Genomics Portal (http://www.cbioportal.org/public-portal/index.do), provided by the Memorial Sloan-Kettering Cancer Center. For comparison of the results obtained, the Rembrandt database (http://rembrandt.nci.gov) was used.

Tumor samples

A total of 264 gliomas were obtained from the Department of Neuropathology, University Hospital Zurich; Switzerland, the Department of Neuropathology, University Frankfurt, Germany; the Departments of Neuropathology and Neurosurgery, University Hospital Essen, Germany; the Department of Pathology, Gunma University, Japan; the Institute of Neuropathology and Department of Neurosurgery, University Hospital Munster, Germany; Institute of Neuroscience, Bordeaux, France; and the Department of Neurosurgery, University Hospital Bern, Switzerland. Histologically, these tumors were classified as diffuse astrocytoma WHO grade II (112 cases; mean age, 39 years), oligodendroglioma WHO grade II (82 cases; mean age, 43 years), primary glioblastoma (34 cases; mean age, 59 years) and secondary glioblastoma (36 cases; mean age, 41 years). The diagnosis of secondary glioblastoma was made on the basis of clinical information. All primary glioblastomas lacked *IDH1* mutations, whereas 27 out of 36 (75%) secondary glioblastomas were found to have *IDH1* mutations. Median survival of patients with primary glioblastomas, secondary glioblastomas, diffuse astrocytomas or oligodendrogliomas was 8.0 months, 7.8 months, 70.7 months or 112.5 months, respectively. Mean survival of patients with primary and secondary glioblastoma was 9.7 $\pm$ 6.2 months and 13.7 $\pm$ 14.9 months, respectively ($P = 0.145$), and that of patients with glioblastoma without and with *IDH1* mutations was 9.2 $\pm$ 5.8 months and 15.9 $\pm$ 16.2 months, respectively ($P = 0.015$). Genetic alterations in these tumors have been reported previously (17, 34). Survival analyses were carried out for patients who were followed up for at least 80 months after the date of surgery. The study was approved by the International Agency for Research on Cancer Ethics Committee.

Quantitative polymerase chain reaction (PCR)

DNA was extracted from formalin-fixed and paraffin-embedded histological sections as previously reported (34). Quantitative PCR was carried out using an iCycler (Bio-Rad, Hercules, CA, USA) in a 20 µL reaction mixture composed of 10 µL 2× iQ SYBR Green Supermix, 2 µL H_2O, 1.6 µL template DNA (approx. 20 ng/µL) and primers. *MET* primers were designed for exon 20/21. The primer sequences were 5′-TCC TAC AAC CCG AAT ACT GC-3′ (sense) and 5′-GGT GCC AGC ATT TTA GCA TT-3′ (antisense; product, 155 bp). As a reference, beta-globin (at 11p15.5) was used as previously reported (24). Primer sequences for beta-globin were 5′-GTG CAC CTG ACT CCT GAG GAG A-3′ (sense) and 5′-CCT TGA TAC CAA CCT GCC CAG-3′ (antisense; product, 102 bp). The primers were designed using Primer3 online software (http://frodo.wi.mi.edu/primer3/input.htm). DNA was first denatured at 95°C for 12 minutes, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 45 s. The final extension step was at 72°C for 2 minutes. Each plate contained measurements for the target and the reference genes. Each reaction was carried out in triplicate. We calculated the PCR cycle umber (Ct) value, δ-Ct [Ct (MET) – Ct (beta-globin)] and δδ-Ct [δ-Ct (tumor) – δ-Ct (normal)] values, and the relative copy number ($2^{-\delta\delta\text{-Ct}}$) as previously reported (31). For normalization, DNA was extracted from paraffin-embedded sections of 10–11 samples of normal tissue. A tolerance interval (TI) with a confidence interval (CI) of 95% was determined from the standard deviation of normal DNA, as reported previously (31). Gain was considered as a copy number > 2.699 (Figure 1).

Statistical analyses

Statistical analyses were carried out using SPSS Statistics 15.0 Software for Windows (SPSS Inc., Chicago, IL, USA). The Fisher's exact test was applied for the evaluation of associations between clinical parameters and genetic alterations. The Cox proportional hazards model was implemented to examine the effect of different combinations of genetic alterations after adjusting for age at diagnosis (<40 years vs. ≥40 years) and sex (female vs. male). Factors with no significant association were eliminated ($P \geq 0.05$). The remaining factors in the multivariate analysis ($P < 0.05$) were assumed to be independent predictors of survival. Kaplan–Meier survival statistics were used to compare survival curves in the different patient groups. The log-rank test was used to compare the different survival curves. The figures were created using StatView software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Analysis of TCGA data

With the criterion log2 ratio of >0.5, we identified 25 genes that showed gain in >35% cases of glioblastomas with *IDH1/2* mutations (8 cases) and those without *IDH1/2* mutations (364 cases). Except for *CFHR3* (located at 1q31.3), *LCE3B* (1q21.3), *LCE3C* (1q21.3), *TARP* (7p14.1), all the other 21 genes (*ANKRD7, ASZ1, C7ORF58, CAPZA2, CFTR, CTTNBP2, ING3, KCND2, NAA38, MET, MGAM, PRSS2, PTPRZ1, ST7, ST7-AS1, ST7-AS2, ST7-OT3, ST7-OT4, TSPAN12, WNT16, WNT2*) were located at 7q31-34. The cBio Cancer Genomics Portal showed that 68% of glioblastomas showed gain of all 21 genes, and 71%–74% of glioblastomas showed gain in at least 1 of these 21 genes.

The *MET* gene at 7q31.2 was selected for further analysis, as it is a well-characterized oncogene implicated in the pathogenesis and progression of a variety of tumors (2, 36). In the cBio Cancer Genomics Portal, *MET* gain was present in 72% of glioblastomas, and was associated with significantly shorter patient survival ($P = 0.035$). The Rembrandt database also showed *MET* gain in 133 of 181 (74%) glioblastomas, and in 58 of 98 (59%) WHO grade II/III astrocytomas.

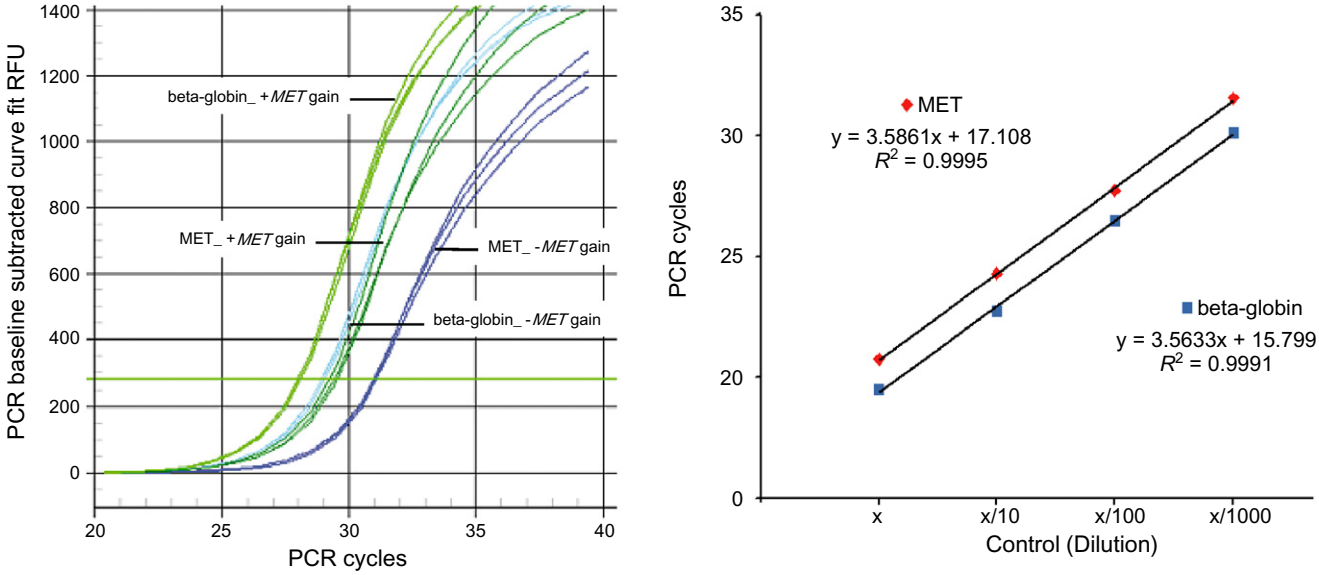

Figure 1. Quantitative PCR showing *MET* gain in low-grade diffuse gliomas. The left-hand figure shows the original quantitative PCR graph for low-grade diffuse gliomas with (+*MET* gain) and without (–*MET* gain) *MET* gain. Relative CT values for *MET* and *beta-globin* at different concentrations of normal control DNA are shown. The slopes of the curves are similar, suggesting equal efficiencies of the two PCR reactions (right).

Frequency of *MET* gain in glioma

We screened for *MET* gain in 112 diffuse astrocytomas, 82 oligodendrogliomas, 34 primary glioblastomas and 36 secondary glioblastomas with quantitative PCR. *MET* gain was detected at similar frequencies in primary (16/34; 47%) and secondary (16/36; 44%) glioblastomas, and in glioblastomas without and with *IDH1* mutations (20/43; 46.5% vs. 12/27, 44.4%). *MET* gain was significantly more frequent in glioblastoma patients aged >40 years than in younger patients [29/54 (54%) vs. 3/16 (19%); $P = 0.021$]. In the majority of cases, the level of *MET* gain was low (2.7–5.0 copies), except for four secondary glioblastomas and one primary glioblastoma, which showed 11–21 *MET* copies.

MET gain was also common in diffuse astrocytomas (43/112; 38%), but less frequent in oligodendrogliomas (13/82; 16%; $P = 0.001$; Table 1). Gain of more than five copies of *MET* was found in two diffuse astrocytomas, but in none of the oligodendrogliomas. *MET* gain was significantly more frequent in low-grade gliomas with *TP53* mutations than in those without *TP53* mutation (41% vs. 20%; $P = 0.002$). There was no significant difference in the frequency of *MET* gain in younger (<40 years) and older (>40 years) patients with diffuse astrocytoma (Fisher's exact test; $P = 0.41$).

Table 1. Frequency of *MET* gain in glioma

Tumor type	Tumors with *MET* gain (%)
Diffuse astrocytoma (WHO grade II)	43/112 (38)**
Oligodendroglioma (WHO grade II)	13/82 (16)
Primary glioblastoma (WHO grade IV)	16/34 (47)
Secondary glioblastoma (WHO grade IV)	16/36 (43)

**Significantly more frequent than in oligodendrogliomas ($P = 0.001$).

Timing of *MET* gain during astrocytoma progression

Pairs of tumor tissues of diffuse astrocytoma and the subsequently developing secondary glioblastoma were available for 14 patients. In one patient, *MET* gain was present both in the first biopsy (diffuse astrocytoma) and the second biopsy (secondary glioblastoma), whereas in five patients, *MET* gain was observed only in the secondary glioblastoma.

MET gain and clinical outcome

The median overall survival of patients with diffuse astrocytoma showing *MET* gain (10 cases) was significantly shorter [log-rank test; 43.0 months (95% CI, 42.5–43.5)] than that of patients without *MET* gain (53 cases) [70.7 months (95% CI, 55.0–86.4); $P = 0.004$] (Figure 1). In multivariate analysis after adjusting for patient age and sex, *MET* gain remained a significant prognostic factor for poorer survival among patients with diffuse astrocytoma [Cox-Regression; HR 2.96 (95% CI, 1.43–6.15); $P = 0.004$]. *MET* gain did not affect the survival of patients with all glioblastomas combined ($P = 0.39$), primary glioblastomas ($P = 0.89$), secondary glioblastomas ($P = 0.29$) (Figure 2), glioblastomas with *IDH1* mutations ($P = 0.26$) or those without *IDH1* mutations ($P = 0.9$)

DISCUSSION

To identify genes that are commonly amplified in both primary and secondary glioblastomas, we first analyzed TCGA data, using

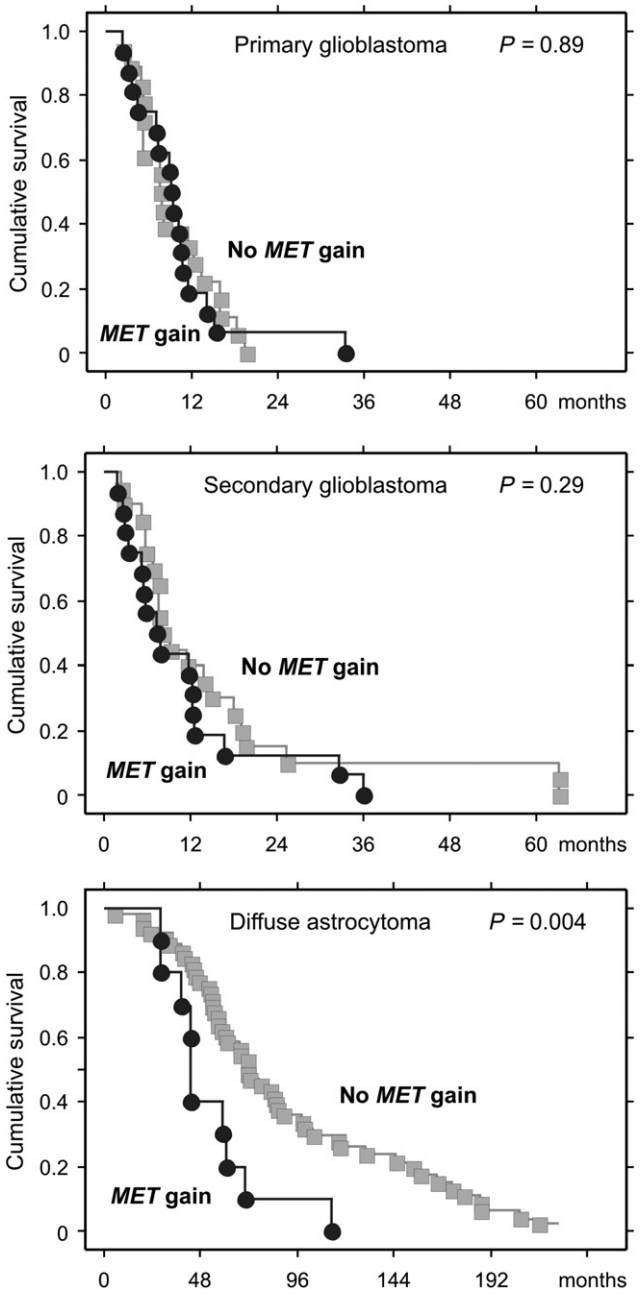

Figure 2. Cumulative survival of patients with diffuse astrocytoma is significantly shorter for tumors with *MET* gain than for tumors without *MET* gain (median overall survival, 43.0 months vs. 70.7 months; $P = 0.004$). There was no significant difference in patient survival for glioblastomas with or without *MET* gain.

IDH1 mutations as a molecular marker of secondary glioblastomas (32, 33). We found 25 genes that showed gene copy-number gain in more than one-third of glioblastomas with or without *IDH1* mutations. Interestingly, most of these genes (21/25; 84%) were located at 7q31-34, where gain has been observed frequently in a variety of human neoplasms, including gastric cancer (46%) (29) and fibrosarcoma (44%) (40). Gain at 7q was reported in 38%–79% of glioblastomas (4, 20) and in 21%–50% of low-grade gliomas (13, 42). Gain at 7q was associated with a shorter survival of patients with high-grade astrocytomas (20, 49).

We chose the *MET* gene at 7q31.2 for detailed analysis because this gene has been demonstrated to play important roles in pathogenesis and progression in a variety of human neoplasms (4, 7, 50). The *MET* gene encodes the receptor tyrosine kinase MET, comprising an α-chain linked by a disulfide bridge to a β-chain. The α-chain is located extracellularly and carries the binding site for the substrate, whereas the β-chain traverses the membrane and includes the cytoplasmic kinase domain and the carboxy-terminal, which is essential for downstream signaling (36). The MET receptor is the only known target of hepatocyte growth factor (HGF) (2). Binding of HGF leads to MET dimerization and the subsequent autophophorylation of tyrosine residues at the carboxy-terminal binding site. This is followed by the activation of downstream signaling cascades (9), including PI3K/Akt, Ras/MAPK and STAT pathways (5). MET signaling is therefore involved in a variety of cellular functions, such as cell proliferation, survival, apoptosis, invasion and angiogenesis (2). Dysregulation of the MET pathway may also play important roles during the epithelial–mesenchymal transition, tumor invasion, progression and metastasis (9).

Abnormal MET signaling may be caused by gene amplification, overexpression, missense mutations, translocations, and ligand-dependent autocrine or paracrine mechanisms (44). *MET* missense mutations have been detected in a small fraction of papillary renal cell carcinomas (13%) (41), lung cancer (13%) (19) and mesothelioma (9%) (15), but rarely in gliomas (<2%) (1). MET overexpression has been observed in a variety of human neoplasms, including ovarian cancer (30%–67%) (26, 50), colorectal cancer (78%) (26), chordomas (77%) (47). Immunohistochemistry has revealed MET overexpression in glioblastomas (34%–88%) (11, 30) and low-grade astrocytomas (21%) (30). In glioblastomas, MET expression was higher in recurrent tumors than in primary tumors, and glioblastoma patients with MET overexpression had a significantly shorter progression-free survival time (6.1 months vs. 11.5 months) (23). However, little is known about *MET* gain in glioma.

We show here that *MET* gain is common in both primary and secondary glioblastomas (47% vs. 44%), indicating that abnormal MET signaling is involved in the pathogenesis of both glioblastoma subtypes. This was consistent with the TCGA data, in which 41% (151/372) of glioblastomas showed low-level amplification determined by the criterion of a log2 ratio > 0.5. It is of interest to note that *MET* gain was more frequent in patients aged ≥40 years (29/54; 54%) than younger patients with glioblastoma (3/16; 19%; $P = 0.021$). This is consistent with previous findings that 7q gain was associated with older age in patients with astrocytic tumors (4, 20).

Diffuse astrocytomas tend to progress to a more malignant histologic type, that is, anaplastic astrocytomas and eventually secondary glioblastomas (13). However, time until progression varies significantly between patients (18, 38, 48). Several clinical prognostic factors for poorer survival of patients with diffuse astrocytoma have been reported, including old age at diagnosis (35, 43), incomplete resection (37, 46) and predominant presence of gemistocytes (35). The genetic hallmark of diffuse astrocytomas is co-presence of *IDH1* mutations and *TP53* mutations (17).

Several studies have shown that *IDH1* mutations are a significant prognostic marker for favorable outcome of patients with diffuse astrocytoma (6, 12, 39). In contrast, the prognostic value of *TP53* mutations has been controversial. In a study of astrocytomas and oligoastrocytomas ($n = 159$), cumulative progression-free survival was significantly shorter for patients with tumors with *TP53* mutation, but there was no impact on overall survival (38). Other studies have shown that *TP53* mutations were marginally associated (14) or not associated (35) with poorer patient outcome. In the present study, we show that *MET* gain was also common (38%) in diffuse astrocytomas, and importantly, the presence of *MET* gain was significantly associated with poor clinical outcome.

MET gain and MET overexpression are associated with higher tumor grades of ovarian clear cell adenocarcinomas (50). In gastric cancer, MET expression correlates with advanced tumor stage with liver metastasis (3). MET overexpression was significantly associated with rapid tumor recurrence in patients with non-small cell lung carcinoma (NSCLC) (8). In astrocytomas, MET expression levels increased with higher WHO grades (2, 28, 30). In the present study, biopsies with diffuse astrocytoma and secondary glioblastoma were available for 14 patients. In one patient, *MET* gain was found in both the biopsies with diffuse astrocytoma and secondary glioblastoma, whereas in five cases, *MET* gain was observed only in secondary glioblastoma. Thus, *MET* gain is associated with astrocytoma progression, and is usually a late event; but if present in diffuse astrocytomas, this predicts poorer clinical outcome of patients.

The level of *MET* gain detected in the present study was relatively low (three to five copies) in the majority of gliomas. However, low-level gain may also have biologically significant effects. *MET* gain (four to five copies), detected by quantitative PCR and fluorescence *in situ* hybridization (FISH) in 10%–21% of gastric cancer, was significantly associated with a poorer prognosis (10, 22). In patients with NSCLC with an increased copy-number (more than five copies) of *MET* detected by FISH, survival was significantly shorter than in patients without *MET* gain (7).

The targeting of receptor tyrosine kinases is a promising therapeutic strategy for glioblastomas. MET inhibitors are emerging drugs for the treatment of different cancers and are currently undergoing phase I, II and III clinical trials in multiple types of tumors (44). MET monoclonal antibody inhibits MET phosphorylation, cell proliferation, migration and apoptosis in U87 glioblastoma cells (27). As MET activation is involved in the development of resistance to epidermal growth factor receptor (EGFR) inhibitors in glioblastoma (16), and inhibition of the MET pathway can overcome resistance to EGFR pathway inhibition in human EGFR-hyperactivated glioblastoma xenografts (21), combined treatment with EGFR inhibitors and MET inhibitors may be a promising additional treatment strategy. The present study provides evidence that both primary and secondary glioblastomas, as well as a fraction of diffuse astrocytomas with *MET* gain, may benefit from such treatment.

ACKNOWLEDGMENTS

Daniela Pierscianek was supported by the Fritz-Thyssen Foundation.

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