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CLINICAL STUDY

High levels of c-Met is associated with poor prognosis in glioblastoma

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Abstract The tyrosine kinase receptor c-Met has been suggested to be involved in crucial parts of glioma biology like tumor stemness, growth and invasion. The aim of this study was to investigate the prognostic value of c-Met in a population-based glioma patient cohort. Tissue samples from 238 patients with WHO grade I, II, III and IV tumors were analyzed using immunohistochemical staining and advanced image analysis. Strong c-Met expression was found in tumor cells, blood vessels, and peri-necrotic areas. At the subcellular level, c-Met was identified in the cytoplasm and in the cell membrane. Measurements of high c-Met intensity correlated with high WHO grade ($p = 0.006$) but no association with survival was observed in patients with WHO grade II ($p = 0.09$) or III ($p = 0.17$) tumors. High expression of c-Met was associated with shorter overall survival in patients with glioblastoma multiforme ($p = 0.03$). However the prognostic effect of c-Met in glioblastomas was time-dependent and only observed in patients who survived more than 8.5 months, and not within the first 8.5 months after diagnosis. This was significant in multivariate analysis (HR 1.99, 95 % CI 1.29–3.08, $p = 0.002$) adjusted for treatment and the clinical variables age (HR 1.01, 95 % CI 0.99–1.03, $p = 0.30$), performance status (HR 1.34, 95 % CI 1.17–1.53, $p < 0.001$), and tumor crossing midline (HR 1.28, 95 % CI 0.79–2.07, $p = 0.29$). In conclusion, this study showed that high levels of c-Met holds unfavorable prognostic value in glioblastomas.

Keywords c-Met · Glioblastoma · Image analysis · Prognosis · Biomarker

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Introduction

Gliomas are the most frequent type of primary brain tumors in adults. Primary brain tumors are classified and graded after the World Health Organization (WHO) classification with glioblastoma multiforme (GBM) being the most malignant glioma having a median survival range from 12 to 15 months [1]. The treatment includes surgery, radiotherapy and chemotherapy, but most patients experience relapse. Due to a limited effect of standard treatment new therapeutic targets are highly needed.

c-Met, a membrane receptor tyrosine kinase, is expressed in epithelial cells, liver, pancreas, prostate, kidney and in bone marrow [2]. c-Met deregulation is implicated in the

cancer biology of many cancer types such as small cell lung cancer [3], breast-carcinoma [4], prostatic carcinoma [5], and hepatocellular carcinoma [6]. In gliomas, c-Met has been suggested to be important for tumor cell survival, angiogenesis and invasion [7–9]. c-Met has also been connected to the stem cell phenotype in glioma by regulating sphere formation, cell proliferation, and differentiation [9, 10]. Additionally it has been suggested that c-Met confers resistance to radiation therapy in GBM patients [11], and c-Met inhibition with imatinib significantly inhibited growth of GBM cell lines in vitro [12]. Based on immunohistochemical investigations recurrent GBMs expressed higher c-Met levels than primary tumors [13]. Furthermore, radiographic and clinical improvement in one GBM patient using a small molecule c-Met inhibitor has been observed [14].

Due to the importance of c-Met in glioma biology and the proposed clinical potential of c-Met inhibitors, the aim of the present study was to investigate the protein expression level and prognostic potential of c-Met in a large population-based cohort reflecting a complete population of patients. c-Met expression was investigated in 238 patients using immunohistochemistry and advanced quantitative image analysis producing continuous measurements of staining intensity reflecting the c-Met expression. Earlier glioma studies included 93 [9], 69 [15], and 62 patients [16] and evaluated the expression with manual subjective scoring. We have previously shown that the use of advanced image analysis is of great advantage when investigating biomarkers in gliomas [17–19]. Besides the benefit of obtaining continuous measurements this approach eliminates intraobserver variability. We found that c-Met expression increased with increasing WHO grade and that high c-Met expression was associated with a shorter overall survival in GBM patients. However c-Met was time-dependent and only had prognostic effect after 8.5 months. c-Met expression and age, performance status, tumor crossing midline and post-surgical treatment were found to be the most significant independent predictors of poor overall survival of GBM patients.

Materials and methods

Patients

We identified a population-based cohort of 433 patients in the Region of Southern Denmark. All patients underwent initial surgery between 01.01.2005 and 31.12.2009. Of these, 238 patients had a sufficient amount of viable tumor tissue for immunohistochemical analyses. Tumor tissue from GBM (n = 186), anaplastic astrocytomas (AA) (n = 15), anaplastic oligodendrogliomas (AOD) (n = 6), anaplastic oligoastrocytomas (AOA) (n = 4), diffuse astrocytomas (DA) (n = 12), oligodendrogliomas (OD) (n = 8), oligoastrocytomas (OA) (n = 4), and pilocytic astrocytomas (PA) (n = 3) were obtained. No treatment was received prior to surgical resection. All tumor samples were classified by two neuropathologist according to the WHO classification from 2007 [20]. If there was disagreement, the pathologists met and agreement was achieved. The patient cohort used is well described and investigated in several studies [17, 18, 21, 22]. Additionally, tissue microarrays with normal brain tissue from two autopsies were included.

Cell lines and Western blot

We validated our c-Met antibody using the commercial cell line U87 and a patient-derived GBM cell line T78 established in our laboratory. Both cell lines were grown as described by Jensen et al. [23]. Spheroids from each cell line were processed for Western blot and immunohistochemistry. Western blot was done according to the NuPage Technical Guide from Invitrogen [24]. The upper part of the membrane was incubated over night with mouse anti-c-Met (1:500, clone: 3D4, Invitrogen) and the lower part with mouse anti β-actin (1:10,000, clone: AC15, Sigma). The secondary antibody was in both cases mouse IgG HRP conjugated and visualization of the proteins was done by adding the chemiluminescent substrate RA and RB (1:1, ECL WP20005, Invitrogen) followed by film exposure. Immunohistochemistry was performed as described below using sections of formalin fixed paraffin embedded spheroids.

Immunohistochemistry

Immunohistochemical staining was carried out on a Dako Autostainer Universal Staining System (Dako, Denmark). The sections were dewaxed with xylene and rehydrated with ethanol. Endogen peroxidase activity was blocked by 1.5 % hydrogen peroxide. Heat induced epitope retrieval was performed using TEG-buffer in three steps using a microwave oven: (1) heating 9 min at 900 W, (2) boiling 15 min at 440 W, and (3) cooling in 15 min at room temperature. Afterwards, the sections were incubated with primary antibody diluted in antibody diluent S2022 (Dako, Denmark) for 60 min. The primary antibody used was Mouse anti-c-Met (1:400, clone: 3D4, Invitrogen). The sections were afterwards incubated with "Ready to use" Post-Blocking for 20 min, and further incubated with Powervision Polymer: Poly-HRP anti-Mouse/Rabbit IgG (PV-HRP) for 30 min. Finally, the sections were incubated with DAB+ as chromogen for 10 min and with Mayers Haematoxylin for 2 min. The slides were scanned on a Hamamatsu digital slide scanner. Omission of primary antibody abolished all staining reaction.

Image analysis

The stained sections were evaluated using the Visiopharm software module (Visiopharm, Hørsholm, Denmark). Sample images were collected using systematic uniform random sampling (meander fraction based), and a 20× objective. A classifier was trained to measure c-Met intensity levels, by growing a 3 µm perimeter around all detected nuclei in which intensity levels was measured. This provided continuous measurements based on intensity. The optimal sampling fraction was found to be 10 % (Fig S1.). To obtain a reliable estimation of mean intensity levels, sample images contained at least 50 % viable tumor tissue and at least five images per tumor were recorded. Areas containing staining artifacts, normal or tumor infiltrated brain tissue, unspecific background staining or necrotic areas were manually excluded. Three tumors were re-sampled at 20 % and three at 40 % to fulfill the criteria and avoid exclusion from the analysis.

Statistical analysis

Kaplan–Meier survival curves were constructed and compared using the log rank test. The median was pre-specified as the cutoff value. Multivariate Cox proportional hazard regression analysis was performed for patients with WHO grade II, III and IV tumors separately. Patients with WHO grade I tumors were not included. Due to a limited number of patients with WHO grade II and III tumors only age, and performance status were included in multivariate analysis. Previously identified prognostic variables were included in multivariate analysis for patients with GBMs. Subsequently, an exploratory optimal cut-point analysis was performed. The optimal cut-point was validated using receiver operating characteristic (ROC) analysis. Overall survival (OS) was defined from day of initial surgery until death or censoring, last evaluated in January 2014. An overall significance level of $p < 0.05$ was chosen. Statistical analysis was carried out using STATA version 11.

Results

Comparison of c-Met western blot and immunohistochemistry

In Western blot analysis c-Met was not detected for T78, whereas it was clearly detected for U87 (Fig. 1a). In line with this immunohistochemical staining showed negative staining of T78 spheroids (Fig. 1b) but a marked positive reaction for U87 spheroids (Fig. 1c).

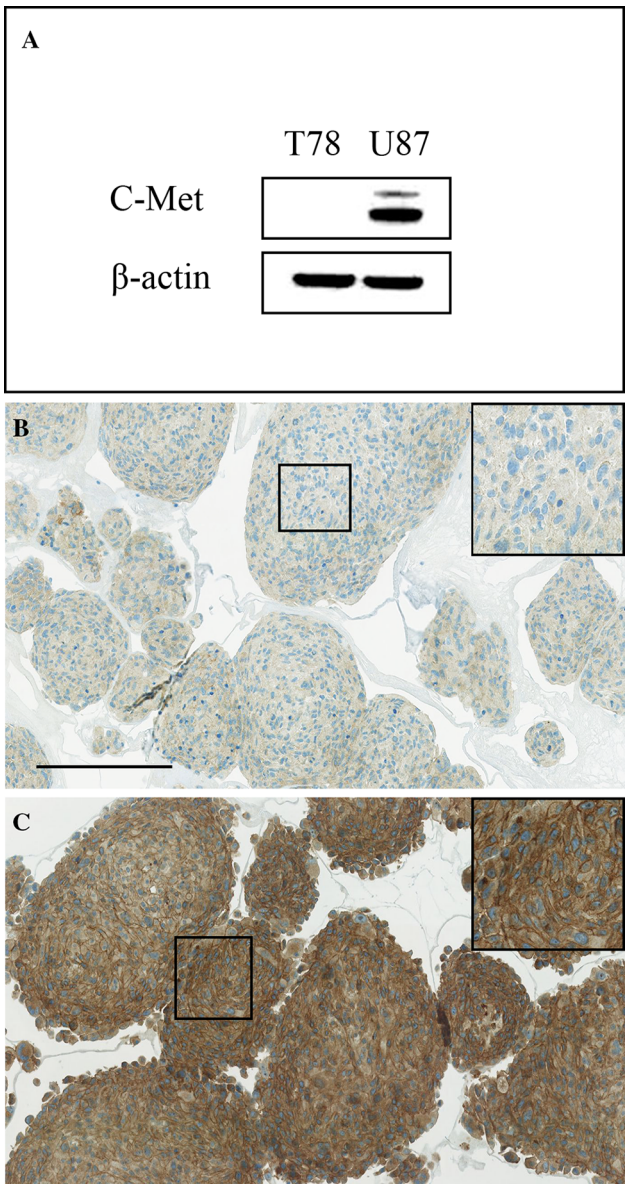


Fig. 1 c-Met antibody validation. Western blot (**a**) detected c-Met protein at 110 and 160 KD in U87 spheroids but not in T78 spheroids. Immunohistochemical staining using histological sections of spheroids showed no c-Met expression in T78 spheroids (**b**) but high c-Met expression in U87 spheroids (**c**). *Scalebar* 200 µm

c-Met staining patterns

Immunohistochemistry was performed on 238 gliomas (Table 1). In normal brain tissue without any pathology, c-Met was not identified (Fig. 2a, b), but neurons displayed positive c-Met staining, for instance in tumor invasion zones (Fig. 2c). High c-Met expression was found in tumor cells but also in blood vessel. At a subcellular level positive c-met staining was found in the cytoplasm and in the cell membrane of tumor cells. Low grade tumors displayed weak staining and PAs showed a diffuse staining pattern

Table 1 Clinicopathological characteristics of patient samples

Histological diagnosis	Number of patients	Median survival (months)	Age (median)	Gender (male/female)	Censored (alive/dead)
WHO grade I	3	38	50	1/2	3/0
WHO grade II	24	43	45	14/10	13/11
WHO grade III	25	14	58	18/7	6/19
WHO grade IV	186	10	65	108/78	15/171

without marked membrane staining (Fig. 2d). DAs with negative staining (Fig. 2e) as well as DAs with cytoplasmic and membrane (Fig. 2f) staining were observed. ODs as well as OAs in general showed a moderate expression of c-Met in the cell membranes (Fig. 2g).

All types of WHO grade III tumors showed marked c-Met staining of tumor cell membranes and cytoplasm compared to low grade tumors (Fig. 2h). Some of the AODs and AOAs showed especially strong c-Met staining. This was also seen in multinucleated giant cells (Fig. 2h, indicated by the arrow in the insert).

GBMs showed high c-Met expression localized to tumor cell membranes and cytoplasm. Gemistocytic tumor cells with enhanced c-Met membrane levels were found in several tumors (Fig. 3a). Furthermore, GBMs with more elongated tumor cells (Fig. 3b) with strong c-Met staining were found in some tumors. Large pleomorphic tumor cells displayed marked staining as well (Fig. 3c–d). c-Met staining was also observed close to areas with microvascular proliferation (Fig. 3e), and large necrotic areas were often surrounded by strongly stained tumor cells (Fig. 3f). Pseudopalisading tumor cells surrounding necrotic areas were found to be both c-Met positive and negative (Fig. 3g–h).

c-Met and tumor grade

The pixel based classifier successfully detected the nuclei for measurement of c-Met intensity in the surrounding cytoplasm/membrane (Fig. 4a–d). c-Met expression increased with WHO grade, but only the c-Met expression level of GBMs (WHO grade IV) was significantly higher compared to the level of WHO grade II tumors ($p < 0.05$) (Fig. 4e). The c-Met expression in histological subtypes was not found to be significantly different (Fig. 4f).

c-Met intensity and survival in WHO grade II and III tumors

Median c-Met intensity in WHO grade II tumors was 38 (range 12.7–104.6) and c-Met expression was not significantly associated with OS (HR = 3.14, 95 % CI 0.89–12.81, $p = 0.09$) in univariate analysis (Fig. 4G) or multivariate analysis (HR = 3.60, 95 % CI 0.92–14.13, $p = 0.07$). Median c-Met intensity in WHO grade III tumors was 58.8 (range 36.6–187.1) and not significantly associated with OS (HR = 1.92, 95 % CI 0.76–4.81, $p = 0.17$) in univariate analysis (Fig. 4h) or multivariate analysis (HR = 1.31, 95 % CI 0.49–3.50, $p = 0.59$).

Fig. 2 Immunohistochemical c-Met staining patterns in brain parenchyma and WHO grade I, II and III tumors. Normal *grey matter* (**a**) and *white matter* (**b**) without c-Met expression. In tumor infiltrated brain parenchyma, neurons expressed c-Met (*arrow* in **c**). Pilocytic astrocytomas had diffuse c-Met expression (**d**), whereas diffuse astrocytomas were found both without (**e**) and with (**f**) c-Met expression. Oligodendrogliomas showed moderate c-Met expression (**g**). Anaplastic oligoastrocytomas showed strong c-Met expression both in general and in multinucleated giant cells (*arrow* in **h**). *Scalebar* 200 μm ▶

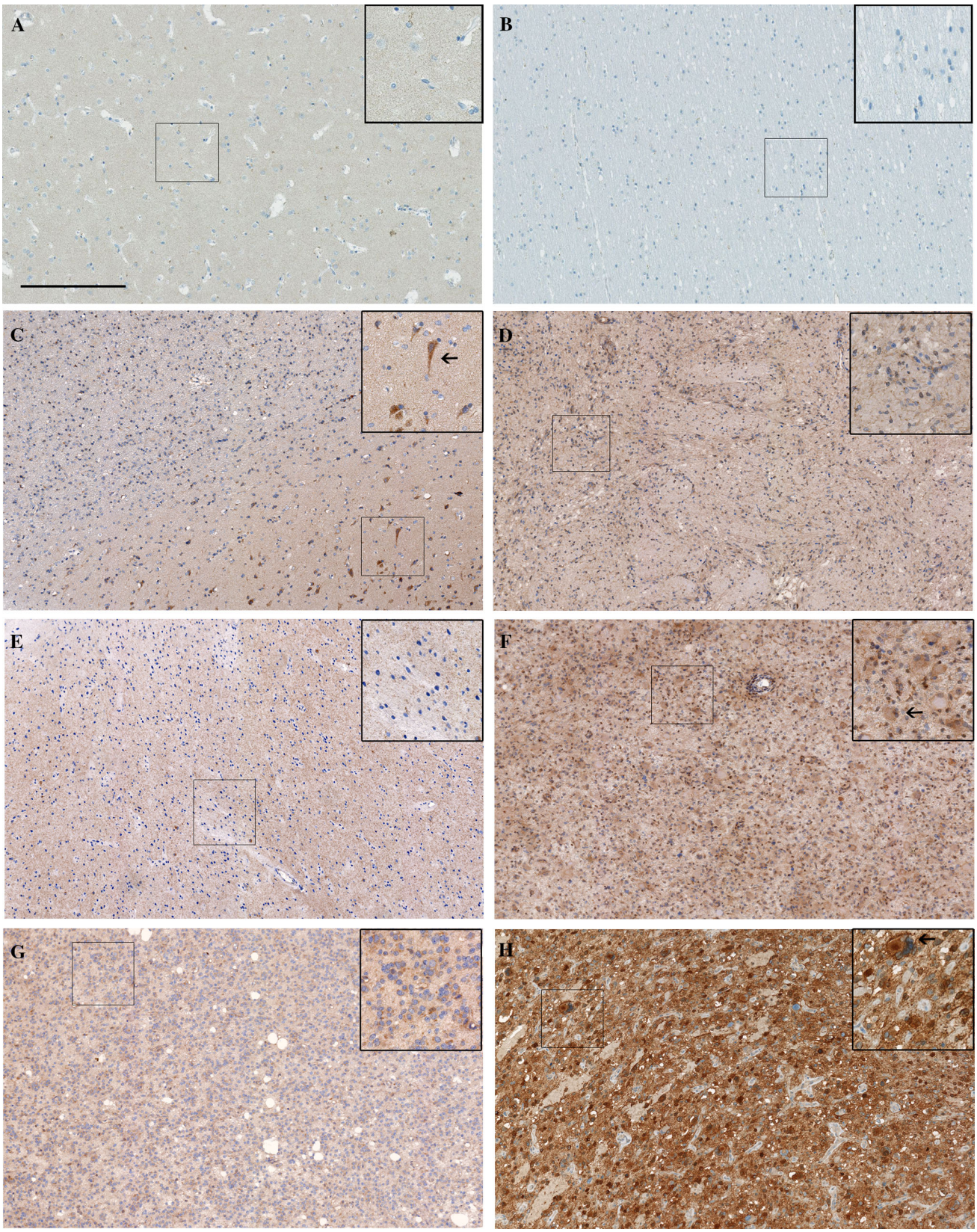
c-Met intensity and survival in GBM patients

Median c-Met intensity was 70.8 (range 15.5–200.1). When divided at the median c-Met intensity levels were not prognostic (HR = 1.03, 95 % CI 0.75–1.37, $p = 0.93$) in univariate analysis, or in multivariate analysis (HR = 1.02, 95 % CI 0.75–1.39, $p = 0.89$).

The exploratory cutpoint analysis showed that when dichotomizing at an intensity of 75 (60 vs. 40 %) high levels of c-Met were associated with shorter OS in GBM patients (HR = 1.41, 95 % CI 1.01–1.86, $p = 0.03$) (Fig. 4i). However; c-Met appeared to be a time-dependent variable. When including the time-dependency in the multivariate analysis c-met was not prognostic in the first 8.5 months after diagnosis (HR = 0.97, 95 % CI 0.62–1.51, $p = 0.89$). After 8.5 months, patients with high levels of c-Met had a significant poorer survival as compared to patients with low levels of c-Met (HR = 2.06, 95 % CI 1.33–3.18, $p = 0.001$). This was significant in multivariate analysis (HR = 1.99, 95 % CI 1.29–3.08, $p = 0.002$) adjusted for the clinical variables, age (HR = 1.01, 95 % CI 0.99–1.03, $p = 0.30$), performance status (HR = 1.34, 95 % CI 1.17–1.53, $p < 0.001$), tumor crossing midline (HR = 1.28, 95 % CI 0.79–2.07, $p = 0.29$) and treatment (palliative, HR = 1.89, 95 % CI 1.28–2.70, p = 0.001; surgery only, HR = 13.21, 95 % CI 7.12–24.61, $p < 0.001$) (Table 2). Surprisingly; sub-analyses indicated that this was only observed in patients who received post-surgical treatment (radio- or chemotherapy) (HR = 1.82, 95 % CI 1.19–2.79, $p = 0.006$) (Fig. 4j). c-Met was not associated with shorter OS in patients who only underwent surgery (no radio-chemotherapy) (HR = 0.75, 95 % CI 0.47–1.19, $p = 0.23$).

Discussion

We investigated c-Met expression in 238 gliomas and found c-Met expression in both tumor cells and blood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vessels. At a subcellular level this staining was localized to cytoplasm and cell membranes. c-Met expression increased with tumor grade and high expression was independently associated with poor prognosis in GBM patients 8.5 months after diagnosis. Our data suggest that c-Met intensity level, as determined by chromogenic immunohistochemistry and digital image analysis, successfully identifies GBM patients with high c-Met levels and poor prognosis.

In our study, areas of necrosis were often surrounded by highly c-Met positive cells, suggesting that c-Met expression could be induced by hypoxia. This association has also been suggested in vitro with GBM cell lines showing increased c-Met levels after exposure to hypoxia [8, 25]. Interestingly, this is in line with results suggesting c-Met to be a key-player in maintaining the GBM stem cell population, since these cells are thought to reside in hypoxic niches [10, 26, 27]. Additionally, c-Met positive cells were often located near c-Met positive blood vessels, similar to what has been found in a previous study [9]. These results suggest that c-Met might be involved in angiogenesis, supported by a study showing significant lower vessel density in GBM when inhibiting c-Met [28]. The localization of c-Met near blood vessels may as well support the role of c-Met in tumor stemness, since tumor stem cells have been described to reside in perivascular niches [26, 29].

We found no significant association between high c-Met intensity and overall survival in WHO grade II and III tumors, although a trend towards poorer survival was observed in patients with high expression of c-Met. To our knowledge, no studies so far have investigated the prognostic potential of c-Met protein using immunohistochemistry in low grade gliomas. However, it is worth noticing that our results are based on a relatively small number of patients (WHO grade II n = 24, WHO grade III n = 25), and that a significant association between high c-Met intensity and overall survival may be obtained with a larger patient material. Previous immunohistochemical studies also identified c-Met expression in WHO grade I, II, and III tumors [30, 31] and concluded that expression levels correlated with tumor grade. None of these studies correlated c-Met expression with survival, probably due to a limited number of patients (n = 8 [31] and n = 27 [30]). In a recent study performed by Pierscianek et al. 194 WHO grade II tumors, including 112 DAs and 82 ODs were evaluated. c-Met was expressed in 38 % of the DAs and 16 % of ODs using quantitative PCR [32]. c-Met expression in the DAs was associated with shorter overall survival, suggesting c-Met to be a useful prognostic marker in these patients. Further studies including a higher number of low-grade tumors will be necessary to investigate whether c-Met protein has prognostic potential.

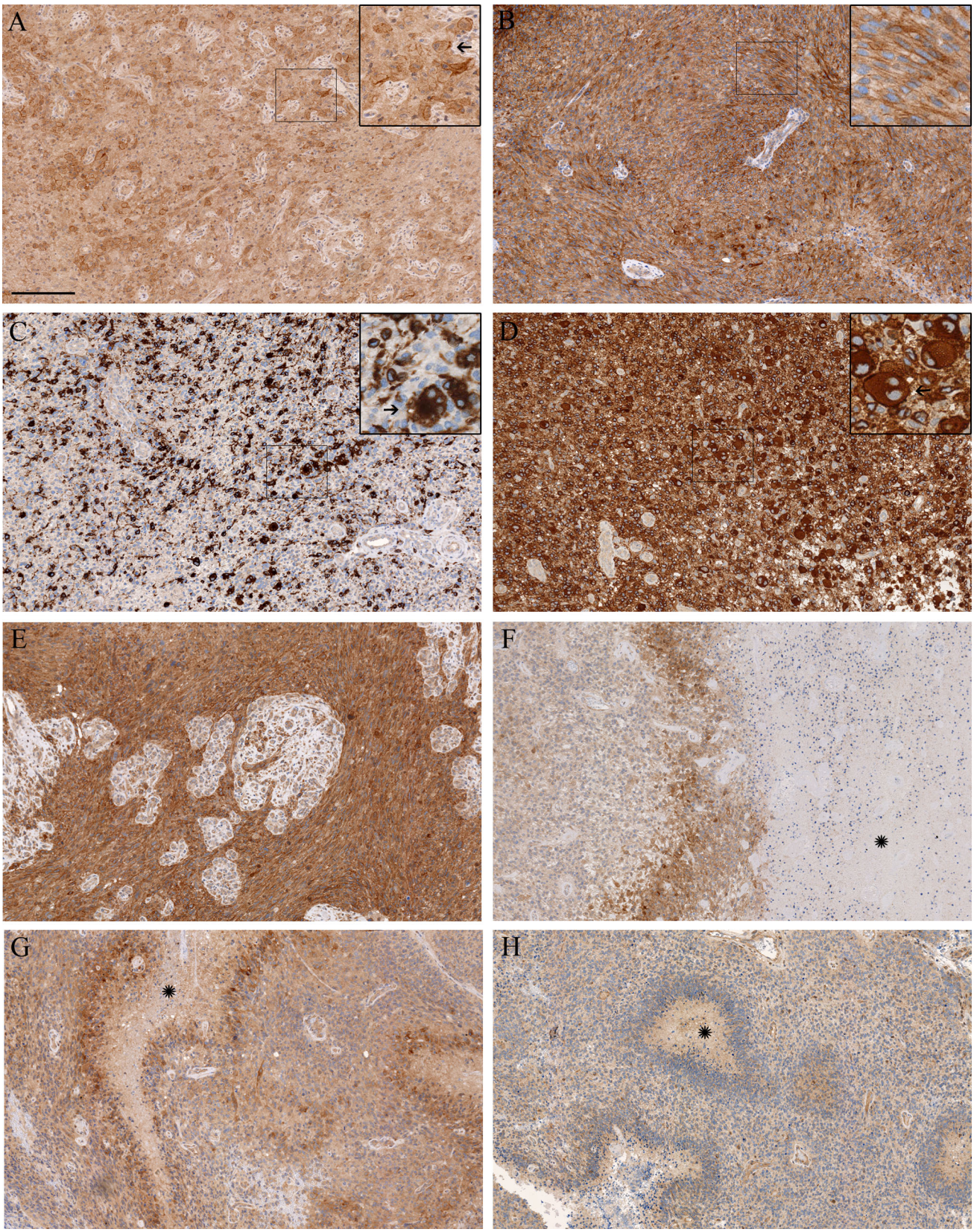
Fig. 3 Immunohistochemical c-Met staining patterns in glioblastoma multiforme (GBM). Most GBMs showed strong c-Met expression. Both positive gemistocytic tumor cells (**a**), and more elongated tumor cells (**b**) were found. Very strong c-Met expression was only observed in few tumor cells including large, pleomorphic and multinucleated cells (**c**, **d**). Glomeruloid vessels (**e**) and large necrotic areas (**f**) were surrounded by tumor cells with strong c-Met expression. Pseudopalisading tumor cells surrounding necrotic areas were found to be both c-Met positive (**g**) and negative (**h**). Necrotic areas are indicated with *asterisk*. *Scalebar* 200 μm ▶

We showed that high expression of c-Met was associated with poor survival in GBM patients, which is consistent with three previous studies [9, 15, 16], based on manual scoring and including 93, 62 and 69 GBM patients, respectively. In contrast to these studies we demonstrated the association to prognosis using multivariate analysis adjusting for clinical variables and post-surgical treatment. Furthermore we demonstrated the prognostic value of c-Met in a large cohort including 186 GBMs using advanced image analysis thereby replacing subjective scoring.

c-Met only had prognostic value after 8.5 months and patients with high levels of c-Met had a significantly poorer survival as compared to patients with low levels. Interestingly c-Met expression only seemed to affect survival in patients who received post-surgical treatment. The significant influence on GBM patient survival identified in our studies suggests that c-Met plays an important role in tumor progression, which could be explained by experimental studies proposing that c-Met increases invasiveness [33] and cellular proliferation [34] but also that c-Met has anti-apoptotic effects [35]. Multivariate analysis showed that high expression of c-Met, age, tumor crossing midline and performance status were found to be the most significant independent predictors of poor overall survival in GBM patients. Consistent with this, another c-Met study also found that age and high expression of c-Met were independent predictors of poor overall survival in GBM patients [16].

C-Met seems to hold great potential as a new biomarker in GBM, due to the association of high c-Met levels with poor prognosis and the general high expression levels found in GBMs. Previous results identified c-Met up regulation after radiation treatment [9] emphasizing c-Met inhibitors as new promising drug candidates. c-Met small molecule kinase inhibitors have shown evidence of antitumor activity, by reducing tumor cell proliferation, migration, invasion, and decreasing brain tumor volume in vivo [36, 37]. c-Met has in fact been identified in tumor-initiating stem cells and c-Met levels correlated with stem cell marker expression [10].

At the moment, the majority of clinical trials with various inhibitors targeting c-Met are currently in progress.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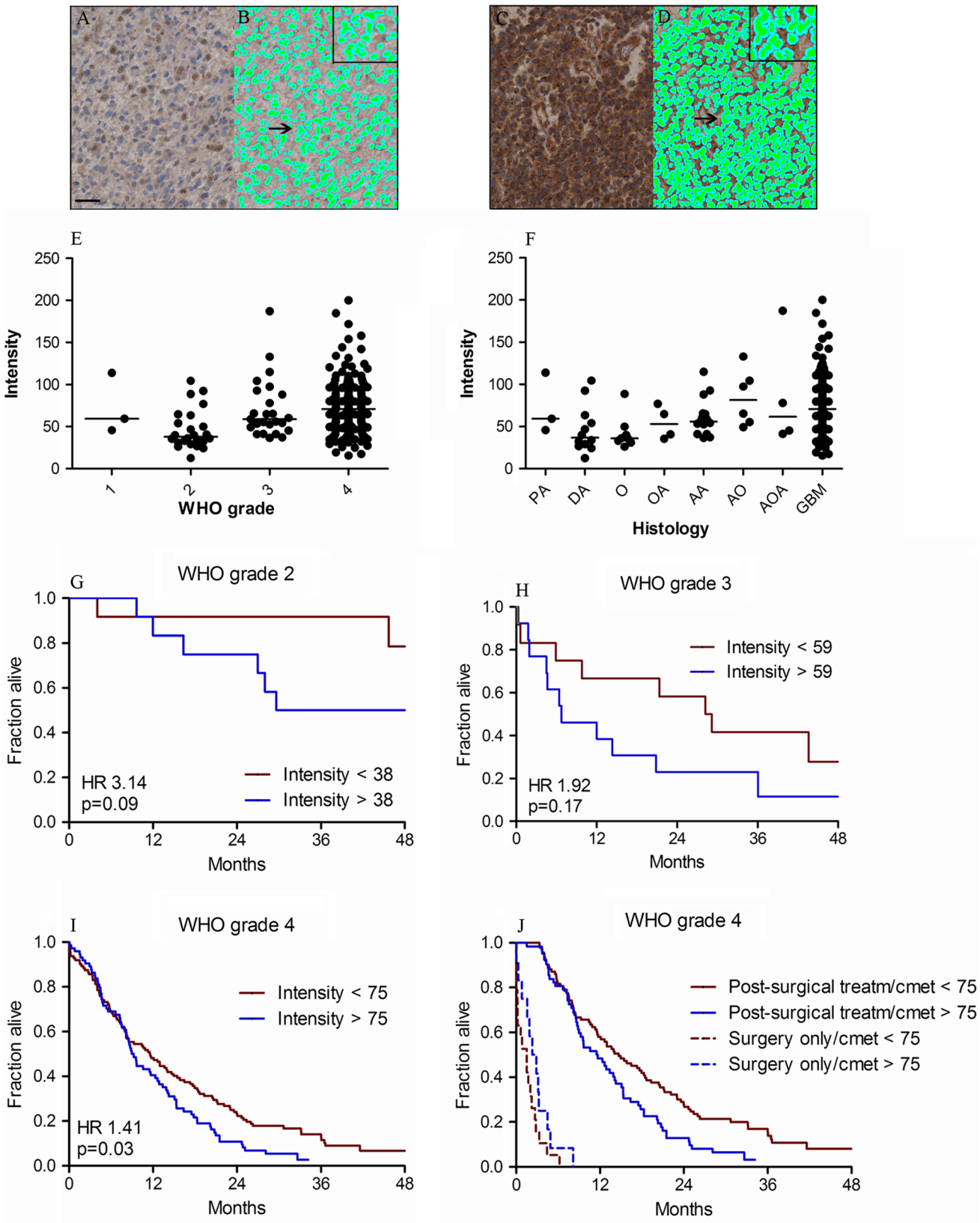
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WHO grade 4
Post-surgical treatm/cmet < 75
Post-surgical treatm/cmet > 75
Surgery only/cmet < 75
Surgery only/cmet > 75

◀**Fig. 4** Quantitation of c-Met intensity levels was carried out using a trained pixel classifier. The trained classifier detected all nuclei and the cytoplasm/membrane was identified by growing a 3 μm perimeter (*light blue*) around the detected nuclei (*green*) in both tumor areas with low (**a**, **b**) and high cellularity (**c**, **d**), *Scalebar* 100 μm. c-Met intensity levels (mean with SD) for WHO grade I, II, III and IV, showed an overall increase in c-Met expression together with tumor grade (**e**). The tumors were further divided into histological subtypes, and no significant difference was found between the subtypes (**f**). Kaplain–Meier curves based on the c-Met intensity level, when divided at the median c-Met expression was not associated with survival in WHO grade II (**g**), or WHO grade III tumors (**h**). When divided at the median, c-Met was not prognostic in WHO grade IV (data not shown). Optimal cutpoint analysis revealed that dichotomizing at an intensity of 75, high levels of c-Met was associated with poor overall survival ($p = 0.03$) (I). c-Met only showed prognostic effect after 8.5 months in patients receiving post-surgical treatment ($p = 0.006$) (J). Abbreviations: Pilocytic astrocytoma (PA), Oligodendroglioma (OD), Oligo-astrocytoma (OA), Diffuse astrocytoma (DA), Anaplastic astrocytoma (AA), Anaplastic oligodendroglioma (AOD), Anaplastic oligo-astrocytoma (AOA), Glioblastoma (GBM)

Table 2 Multivariate analysis of c-Met expression

WHO grade IV	HR (95 % CI)	*P* value
Age (continuous)	1.01 (0.99–1.03)	0.30
Tumor crossing midline		
No	1.00	
Yes	1.28 (0.79–2.07)	0.29
Performance status		
0–1	1.00	
2–4	1.34 (1.17–1.53)	<0.001
Treatment		
Stupp regimen	1.00	
Palliative	1.89 (1.28–2.70)	0.001
Surgery only	13.21 (7.12–24.61)	<0.001
c-Met intensity first 8.5 months		
Low	1.00	
High	0.75 (0.47–1.19)	0.21
c-Met intensity after 8.5 months		
Low	1.00	
High	1.99 (1.29–3.08)	0.002

Stupp regimen is defined as radiotherapy with concomitant and adjuvant temozolomide. Palliative treatment includes either radiotherapy or chemotherapy

HR hazard ratio, *CI* confidence interval

However, one completed clinical study found the monoclonal neutralizing antibody rilotumumab (AMG-102) directed against the c-Met ligand hepatocyte growth factor to cause no antitumor effect in patients with recurrent GBM [38]. This may be related to trial inclusion criteria. The c-Met ligand hepatocyte growth factor (HGF) has been suggested to be responsible for autocrine activation in some GBMs [39]. Thus HGF expression level may identify GBM patients with an active c-Met pathway and these patients may especially benefit from c-Met inhibitor therapy [40].

The inclusion criteria in one successful GBM clinical case testing a c-Met inhibitor (crizotinib), was c-Met amplification, determined by a FISH assay [14]. Previous reports suggested only 3, 9–5 % of the GBMs to contain c-Met amplification [41, 42]. However, additional work by Pierscianek et al. suggested increased c-Met expression in 47 % of the primary GBMs, detected by quantitative PCR [32]. This is better in line with our results. We found, that c-Met expression detected by immunohistochemistry identifies a much higher frequency of patients that potentially could be candidates for inhibitors targeting c-Met than the frequency identified with c-Met amplification based on a FISH assay. In fact successful lung cancer trials, testing the c-Met inhibitors (MetMAb and ARQ 197) included their patients based on c-Met expression determined by immunohistochemistry, and Spiegel et al. identified 50 % of the total population to be c-Met positive [43, 44]. Future GBM research should identify optimal methods and c-Met levels for selecting the right patients for treatment with c-Met inhibitors. Successful clinical trials with c-Met inhibitors may accordingly reveal an additional potential for c-Met as a predictive marker.

In conclusion, we demonstrated that c-Met expression levels increased with tumor grade. We found no significant association between c-Met and overall survival in WHO grade II and III gliomas. However, c-Met is prognostic in GBM patients, independent of clinical parameters. This suggests a clinical potential of c-Met but it needs further validation in an independent cohort.

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