Brain Metastases of Gastro-oesophageal Cancer: Evaluation of Molecules with Relevance for Targeted Therapies

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Abstract. *Background: Brain metastases (BM) of gastro-oesophageal cancer are exceedingly rare and only limited data exist on their pathobiology. Materials and Methods: We identified tissue samples of BM of gastro-oesophageal cancer and analyzed the expression of human epidermal growth factor receptor-2 (HER2), phosphorylated signal transducer and activator of transcription-3 (pSTAT3), epithelial growth factor receptor (EGFR), V600E point mutation of the v-raf murine sarcoma viral oncogene homolog-B1 (BRAF V600E), cluster of differentiation molecule-34 (CD34), hypoxia inducible factor-1α (HIF 1-α) and Ki-67 by immunohistochemical methods. Results: Our series comprised of twenty adenocarcinomas and one oesophageal squamous cell carcinoma. Three (14%), 7 (33%), 9 (43%), 18 (86%) and 0 BM specimens were scored positively for HER2, EGFR, pSTAT3, HIF1-α and BRAF V600E expression. The median Ki-67 index was 59%. The microvascular density was moderate-to-high and active intratumoral microvascular sprouting was evident in 20/21 (95%) of BMs. The HER2 and EGFR expression status were consistent between primary tumors and BM in all three assessable cases. HIF1-α and pSTAT3 expression were significantly higher in HER2-positive cases. Conclusion: Therapeutic use of agents targeting HER2, pSTAT3, EGFR and angiogenesis may be feasible for selected BM of gastro-esophageal cancer. HER2 positivity does not seem to predispose to brain colonization in gastro-esophageal cancer.*

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Gastro-oesophageal cancer is a major cause of cancer-related mortality and is the second leading cause of death worldwide. The risk of local and distant recurrence is high, even for resectable disease at initial presentation. In approximately two-thirds of patients, metastatic disease is present at first diagnosis, and for such patients, palliative chemotherapy results in 5-year survival rates of below 15% (1, 2).

Brain metastases (BM) are rare in patients with gastro-oesophageal cancer, affecting fewer than 2% of patients (3). Since the incidence of gastro-esophageal junction (GEJ) cancer has seen a significant rise within the last decades, the total number of patients with this disease and BM is also expected to increase. BM are generally associated with high morbidity, high mortality and poor prognosis. Current treatment options are mainly based on local therapy approaches such as neurosurgery, radiosurgery and radiotherapy and symptomatic therapy with steroids (4-7). For some tumor types and molecular tumor sub-types, targeted agents have recently shown favourable efficacy and led to shrinking of BM, *e.g.* the v-Raf murine sarcoma viral oncogene homolog-B1 (BRAF) inhibitors vemurafenib and dabrafenib in *BRAF* V600E-mutated melanoma, the antibody against cytotoxic T-lymphocyte-associated antigen-4 (CTLA4) ipilimumab in melanoma, epithelial growth factor receptor (EGFR) inhibitors in non-small cell lung cancer, and human epidermal growth factor receptor (HER2) tyrosine kinase inhibitor lapatinib in HER2-overexpressing breast cancer (8). However, owing to the rarity of the disease, only very limited data on the pathobiology and particularly the expression of potential targets for novel drug compounds in BM of gastro-oesophageal carcinoma are available. We undertook the present study in order to analyze the expression of molecules relevant for targeted therapies, as well as angiogenic patterns and tumor cell proliferation, in a relatively large series of 21 cases of BM of gastro-oesophageal cancer.

Table I. *Antibodies and staining protocols used in this study.*

Antigen	Antibody	Manufacturer	Dilution
Human epidermal growth factor receptor-2 (HER2)	4B5	Ventana, Tucson, AZ, USA	Ready to use
Hypoxia induced factor-1α (HIF 1-α)	Clone 54	BD Transduction Laboratories, Sparks, MD, USA	1:10
Epithelial growth factor receptor (EGFR)	5B7	Ventana, Tucson, AZ, USA	Ready to use
V600E point mutation of the v-raf murine sarcoma viral oncogene homolog-B1 (BRAF V600E)	VE1	Provided by Andreas von Deimling, Heidelberg, Germany	Ready to use
Tyrosine-705 phosphorylated signal transducer and activator of transcription-3 (pSTAT3)	Clone D3A7	Cell Signaling Technology, Danvers, MA, USA	1:100
Cluster of differentiation molecule-34 (CD34)	Clone qbend10	Novocastra, Vienna, Austria	1:50
Ki-67	MIB-1	Dako, Glostrup, Denmark	1:50

Materials and Methods

Patients. The bio-banks of the Institute of Neurology (Neuropathology), Medical University of Vienna, and the Department of Neuropathology, University of Heidelberg, were searched from 1994-2012 for BM originating from gastro-oesophageal cancer. Cranial bone, dural or spinal metastases were not included in this study. If available, tissue specimens of corresponding primary tumors were also retrieved. The study was approved by the local Ethics Committee.

Methods. Immunohistochemistry and *in situ hybridization* were performed on 1 to 3-μm-thick slides of the paraffin-embedded specimens fixed in 4% buffered formalin.

Expression of HER2 protein, tyrosine-705 phosphorylated signal transducer and activator of transcription-3 (pSTAT3), EGFR, cluster of differentiation molecule-34 (CD34), BRAFV600E, hypoxia-inducible factor-1 (HIF1)-α and Ki-67 was detected applying the antibodies shown in Table I. A Dako (Dako, Glostrup, Denmark) autostainer was used for immunohistochemistry in the case of Ki-67 analysis and a Benchmark Ultra Immunostainer (Ventana, Tucson, AZ, USA) for all other antibodies. *HER2* gene status was investigated using a colorimetric double-color *in situ hybridization* system (INFORM; Ventana), according to the manufacturer's instructions with the Benchmark Ultra stainer.

Analysis of immunohistochemistry and FISH. HER2: Expression of HER2 was scored according to the evaluation scheme for gastric cancer outlined in the Trastuzumab for Gastric Cancer (ToGa) study (9). In brief, a specimen was regarded as being positive for HER2 expression if ≥10% of tumor cells showed strong (baso-)lateral expression of HER2 protein (3+) or weak/moderate expression (2+) in combination with *HER2* gene amplification. A specimen was regarded as positive for *HER2* gene amplification if in 20 analyzed cells the ratio of HER2 and centromere 7 signals was >2.

pSTAT3: A specimen was considered as being positive for pSTAT3 expression if ≥10% of tumor cells exhibited distinct nuclear staining (Figure 1).

EGFR: A specimen was considered as being positive for EGFR expression if the vast majority of cells exhibited strong cytoplasmic/membranous staining reaction (Figure 1) (10).

BRAFV600E: VE1 immunoreaction was scored positive, when viable tumor cells showed a unambiguous cytoplasmic staining with VE1. A faint diffuse staining, any type of isolated nuclear staining, weak staining of single interspersed cells or staining of monocytes/macrophages was scored as negative. VE1 immunostaining has been shown to have a high sensitivity and specificity for *BRAF V600E* gene mutations (11-16).

CD34: Anti-CD34 immunostained slides were used to assess the microvessel density (MVD) and angiogenic patterns. The MVD was assessed semi-quantitatively by visual impression in the tumor area of the highest number of microvessels ('hot spot') and was graded as low, moderate or high. In addition, the intratumoral vascularization pattern in each case was assessed and the frequency of 'silent vessels' and 'active microvascular sprouting' was graded as none, few or many. Silent vessels were characterized by thin and monolayered endothelial cells, and the microvascular sprouting was characterized by microvessels with activated broad and multilayered endothelial cells.

HIF1-α: The HIF1-α score was calculated as the sum of the percentage of cells with weak expression (×1), the number of cells showing moderate expression (×2), and of the number of cells with strong expression (×3), resulting in a possible score ranging from 0 to 300.

Ki-67: Five hundred cells in the tumor area with the highest proliferative activity were evaluated and the ratio between positive and negative cells was calculated (MIB1 index), as previously described (17).

Results

Patients. In total, 21 cases of BM from gastro-oesophageal cancer were identified for which sufficient BM tissue was available (Table II). Twelve patients underwent neurosurgical BM resection in Vienna, nine in Heidelberg. The mean patient age at the time of diagnosis of BM was 60±10 years. Sixteen (76.2%) patients were male, five (23.8%) female. Twenty BM were classified as metastases of adenocarcinomas and one metastasis originated from an oesophageal squamous cell carcinoma. In four adenocarcinomas, distal gastric cancer was identified as the primary tumor, all other 16 adenocarcinoma cases originated from primary tumors located in the distal oesophagus or GEJ. In three adenocarcinoma cases of the distal oesophagus, tissue from the primary tumor was available in addition to the BM tissue specimen.

Immunostaining and FISH. Table II shows the clinical data and results of immunostaining. At immunohistochemistry for HER2, three oesophageal/GEJ adenocarcinoma BM (15%) were scored

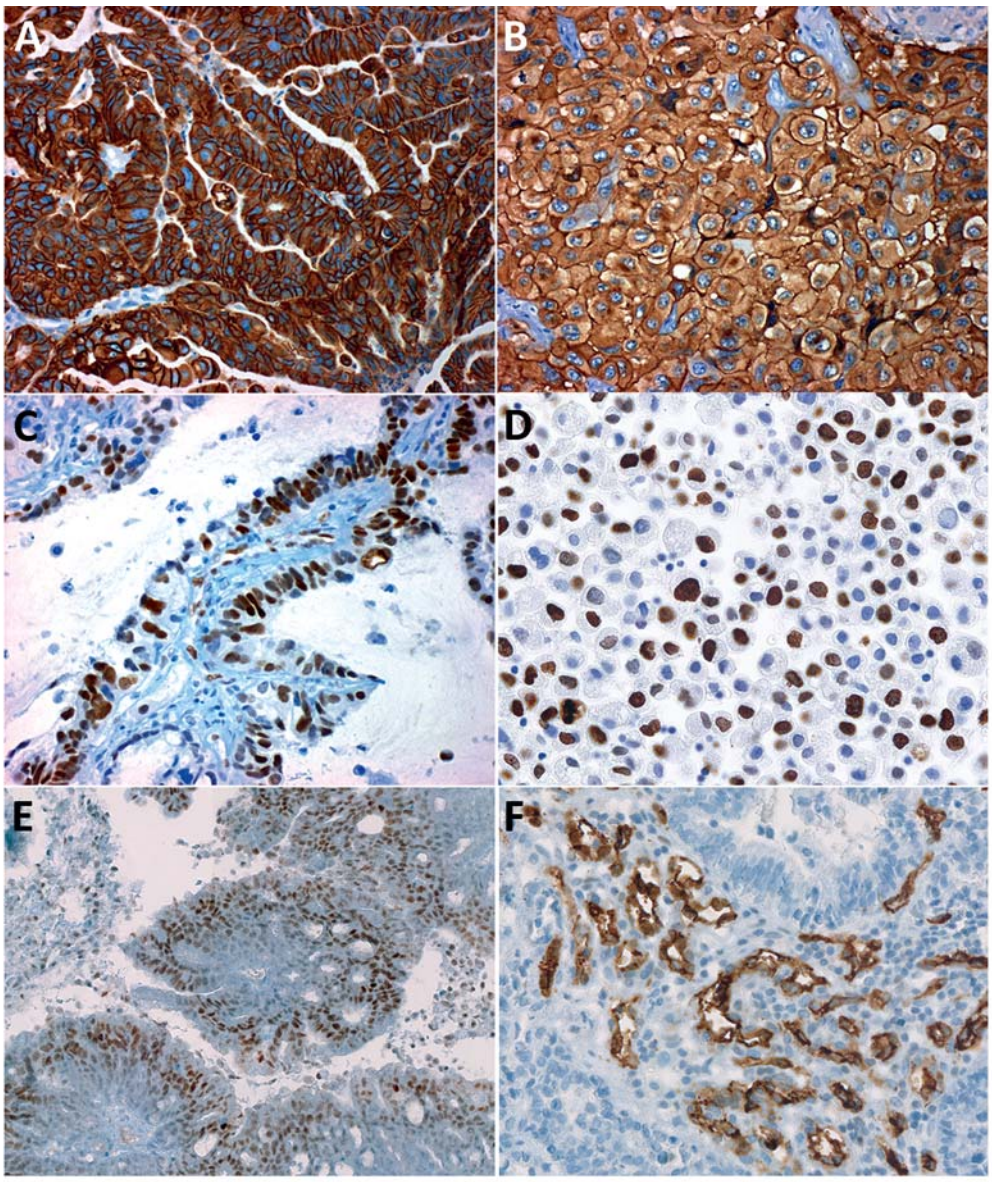

Figure 1. *Examples of immunostaining results for brain metastases of gastro-oesophageal cancer. A: Strong (3+) anti-human epidermal growth factor receptor-2 (HER2) immunostaining; B: prominent anti-epithelial growth factor receptor (EGFR) immunostaining; C: prominent nuclear expression of phosphorylated signal transducer and activator of transcription-3 (pSTAT3); D: anti-Ki67 immunostaining showing labeling of a high fraction of tumor cell nuclei; E: prominent nuclear expression of hypoxia induced factor-1α (HIF1-α); F: marked microvascular sprouting with microvascular clustering, proliferation and activated endothelial cells.*

as 3+ (Table II, Figure 1), and one case as 2+. Subsequent *in situ hybridization* in the HER2 2+ case revealed a trisomy of chromosome 7, but no gene amplification was found, so it was scored as HER2-negative. Of one of the three HER2-positive BM cases, a tissue specimen of the primary tumor was available, which was also scored as HER2 3+ at immunohistochemistry. The two other primary tumors available were negative for HER2 expression, as were their corresponding BM.

A total of 7/21 (33%) BM were scored as being positive for EGFR expression. Primary tumor specimens were available from three EGFR-negative BM cases, which also showed no EGFR expression.

pSTAT3 expression was observed in 9/21 (42.9%) patients and HIF1-α expression in 18/21 (86%) samples (Table I). None of the cases showed expression of the BRAF V600E protein. The median MIB-1 index was 59% (range 2-89%).

Table II. *Clinical data and results of immunostaining of brain metastases (BM) and their corresponding primary tumors.*

ID	Specimen type	Tumor type	Age at BM diagnosis	Gender	HER2 status	pSTAT3 score	EGFR	BRAF V600E	HIF1-alpha index	Ki-67 index (%)	MVD	Silent MV	Sprouting MV
1	BM	AC esophagus/ GEJ	65	M	Pos	30	Neg	Neg	110	56	Moderate	Many	Few
1	Primary tumor				Pos	50	Neg	Neg	90	79	High	Few	Many
2	BM	AC esophagus/ GEJ	54	M	Neg	0	Neg	Neg	0	46	High	Many	Few
2	Primary tumor				Neg	10	Neg	Neg	80	60	Moderate	Few	Many
3	BM	AC esophagus/ GEJ	64	M	Neg	0	Neg	Neg	30	80	Moderate	Few	Many
3	Primary tumor				Neg	0	Neg	Neg	80	50	Moderate	Few	Many
4	BM	AC esophagus/ GEJ	64	M	Neg	20	Neg	Neg	30	54	Low	Many	Few
5	BM	AC esophagus/ GEJ	46	M	Neg	10	Neg	Neg	90	38	High	Few	Many
6	BM	AC esophagus/ GEJ	67	M	Neg	0	Neg	Neg	40	64	High	Many	Few
7	BM	AC esophagus/ GEJ	65	M	Neg	0	Neg	Neg	70	57	Moderate	Many	Few
8	BM	AC esophagus/ GEJ	59	M	Neg	0	Pos	Neg	80	65	Moderate	Few	Few
9	BM	AC esophagus/ GEJ	62	M	Neg	40	Neg	Neg	0	79	Moderate	Few	Few
10	BM	AC esophagus/ GEJ	62	M	Neg	0	Neg	Neg	30	86	High	Few	Few
11	BM	AC esophagus/ GEJ	48	M	Neg	0	Pos	Neg	10	76	Moderate	Few	Few
12	BM	AC esophagus/ GEJ	34	F	Neg	5	Pos	Neg	50	62	Moderate	Many	Few
13	BM	AC esophagus/ GEJ	58	M	Neg	20	Pos	Neg	90	9	Moderate	Few	Few
14	BM	AC esophagus/ GEJ	61	F	Neg	20	Neg	Neg	40	39	Moderate	Many	Few
15	BM	Esophageal SCC	52	F	Neg	0	Neg	Neg	10	34	High	Few	Many
16	BM	AC esophagus/ GEJ	65	M	Pos	40	Pos	Neg	90	82	Moderate	Few	Few
17	BM	AC esophagus/ GEJ	65	M	Pos	5	Pos	Neg	70	89	High	Few	Many
18	BM	Distal gastric AC	42	F	Neg	0	Neg	Neg	10	59	Moderate	Many	Few
19	BM	Distal gastric AC	69	F	Neg	0	Neg	Neg	70	44	Moderate	Many	Few
20	BM	Distal gastric AC	74	M	Neg	0	Pos	Neg	20	59	Moderate	Many	Few
21	BM	Distal gastric AC	72	M	Neg	0	Pos	Neg	0	2	Low	Many	None

AC: Adenocarcinoma; GEJ: gastro-esophageal junction; MV: microvessel; MVD microvascular density; SCC: squamous cell carcinoma; HER2: human epidermal growth factor receptor-2; pSTAT3: phosphorylated signal transducer and activator of transcription-3; EGFR: epithelial growth factor receptor; BRAF V600E: V600E point mutation of the v-raf murine sarcoma viral oncogene homolog B1; CD34: cluster of differentiation molecule 34; HIF1-α: hypoxia induced factor-1α.

Among the BM specimens, six showed high, 13 cases moderate and two cases low MVD. Silent tumor microvessels were found in all 21 BM cases and in 20/21 (95%) BMs, intratumoral microvascular sprouting was evident.

Correlation of protein expression. In HER2-positive cases, the HIF1-α score was significantly higher (median=90, range=0-110) than in HER2-negative ones (median 30, range 0-90) (p=0.024, Fisher's exact test). In addition, the pSTAT3 score was higher in HER2-positive cases (median=30, range=5-40 *vs*. median=0, range=0-40; p=0.047, Fisher's exact test). Furthermore, a significant correlation between HIF1-α and pSTAT3 scores (p=0.028, Spearman's coefficient of correlation 0.48) was observed.

Discussion

In the present study, we investigated HER2 status and expression of pSTAT3, EGFR, BRAF V600E, CD34, HIF1-α and Ki-67 in BM of gastro-oesophageal cancer.

The tyrosine kinase receptor HER2 is overexpressed in about 15% of gastro-esophageal carcinomas and is an important novel drug target for this tumor type. The antibody against HER2, trastuzumab has shown clinically meaningful activity in HER2-positive advanced gastric and GEJ cancer in a recent phase III trial (9). Interestingly, in breast cancer, HER2 expression is associated with an increased incidence of BM. However, it is unclear whether HER2 expression itself or an effect of HER2-targeted therapy with trastuzumab leads to the increased frequency of central nervous system (CNS) involvement. Trastuzumab does not cross the blood-brain barrier due to its high molecular weight and exiled of HER2-positive tumor cells into the 'sanctuary' of the brain has been suggested as a potential reason for the brain-tropism of HER2-positive disease (9). We found HER2 positivity in only three cases of our series of 21 patients with gastro-oesophageal cancer who developed BM without having been treated with anti-HER2 therapy, which corresponds to a similar rate of HER2-positive cases described for primary tumors. However, we found HER2

status to be consistent in the three available cases with matched primary tumors and BM, as is also evident between primary tumors and lymph node and non-brain visceral metastases (18). Thus, our data provide indirect evidence against brain-tropism of gastro-oesophageal cancer cells conferred by HER2 expression-alone. However, a recent smaller study reported a higher frequency of HER2 overexpression, with 5/9 BM samples of oesophageal cancer showing HER2 3+ positivity (19). A selection bias may explain the differing results between our study and the investigation by Hejleh *et al*. and the characterization of a larger independent series would be of interest to clarify the definite proportion of HER2-expressing gastro-oesophageal cancer BM. In any case, HER2-targeted therapeutics crossing the blood-brain barrier such as lapatinib, or other novel drugs may be a feasible treatment option for selected patients with BM of gastro-oesophageal cancer. Furthermore, future studies will show whether the use of trastuzumab leads to an increase of BM in patients with gastro-oesophageal cancer patients, as has been described for breast cancer (8).

STAT3 is a transcription factor involved in physiological cellular response to cytokines and growth factors. Following activation by phosphorylation, dimerized STAT3 translocates to the cell nucleus and regulates transcription of several genes involved in growth, differentiation, proliferation and apoptosis including cyclin-D1, B-cell lymphoma/leukemia-2 (BCL2), vascular endothelial growth factor (VEGF), nuclear factor kappa-B, interleukin-6 and metalloproteinases, among others (20). Activation and overexpression of STAT3 is in involved in malignant transformation and has been found in various tumor types such as breast, prostate, gliomas, bladder, ovarian and head and neck cancer (21-23). A recent study by our group documented pSTAT3 expression in approximately 45% of esophageal carcinomas and found overexpression to be associated with an unfavorable prognosis; STAT3 signaling also correlates with epigenetic aberrance during gastrocarcinogenesis (24, 25). In line with our previous findings, pSTAT3 overexpression was observed in 43% of BM in our current series of BM from gastro-oesophageal cancer (24). The similar rate of pSTAT3 overexpression between primary tumors and BM seems to suggest that this molecule is not *per se* involved in brain colonization. However, as discussed previously, it may serve as a therapeutic target as some agents such as sunitinib, BP-1-102, STA-21 and S31-201 have shown significant antineoplastic activity *via* STAT3 inhibition and novel STAT3 inhibitors are currently under development (26-30). In good correlation with our findings in primary esophageal cancer, we found a significant association of pSTAT3 expression with HER2 expression in BM from gastro-oespohageal carcinoma. Our results thus support our previous data suggesting a relevant pathobiological connection between these two molecules and their potential feasibility as targets for multi-targeted drugs (24).

EGFR is overexpressed in many cancer types and has been effectively targeted by monoclonal antibodies (*e.g.* cetuximab, panitumumab) and tyrosine kinase inhibitors (*e.g.* gefitinib, erlotinib) in several tumor types including colorectal and lung cancer. EGFR overexpression is found in more than half of patients with gastro-oesophageal cancer and several clinical trials are investigating anti-EGFR drugs for this indication, although it must be noted that some agents such as panitumumab have not shown favorable antitumor activity in oesophago-gastric cancer (31-33). We found EGFR overexpression in one-third of our BM cases. While most monoclonal antibodies are too large to cross the blood–brain/blood–tumor barrier, small molecules such as tyrosine kinase inhibitors may be effective. Of note, EGFR tyrosine kinase inhibitors have been shown to induce tumor regression in BM of non-small cell lung cancer (NSCLC), particularly in cases with activating *EGFR* mutations, with erlotinib achieving higher cerebrospinal concentrations and allegedly higher response rates than gefitinib (8). However, while inhibition of EGFR-alone may not be sufficient in halting tumor growth due to redundant pro-neoplastic signaling pathways, novel multi-tyrosine kinase inhibitors targeting several signaling cascades in parallel may show more therapeutic efficacy. In any case, more research is needed to fully understand the role of the EGFR pathway in oesophago-gastric cancer and to develop effective inhibiting compounds.

We found a positive correlation of HER2 and HIF1-α expression, an observation which is well in keeping with previous findings in breast and ovarian cancer (34, 35). HER2 has in fact been shown to up-regulate HIF1-α expression by downstream signalling *via* the AKT pathway and may induce angiogenesis, even independently of hypoxia (36, 37). We found evidence for significant angiogenic activity in BM from gastro-esophageal cancer, with moderate-to-high microvascular density and active intravascular sprouting in most cases. Thus, antiangiogenic agents such as bevacizumab and cediranib, for which emerging data from large trials have shown high antitumor and anti-edematous symptomatic efficacy in primary brain tumors, may be a feasible treatment option for patients with BM of gastro-oesophageal cancer (38, 39). Antiangiogenic agents have been shown to be safe for patients with BM (40) and results of a large phase III study have shown evidence of bevacizumab activity in advanced gastric cancer, particularly in patients identified as having high plasma-VEGF levels and tissue neuropilin-1 expression (41).

BRAF V600E mutations are common in some cancer types, such as melanoma, papillary thyroid cancer and hairy cell leukemia and clinically active inhibitors do exist, which are also effective for mutation-bearing BM (42). Among gastrointestinal tumors, *BRAF* mutations have been detected in a fraction of colorectal carcinoma cases and the *BRAF*

V600E mutation status seems to be associated with metastatic patterns (43). In our series of BM from gastro-esophageal cancer cases, we detected no *BRAF* V600E aberrations, a finding which is in line with previous studies showing a low frequency of *BRAF* mutations in oesophago-gastric cancer (44-48). Our data support the notion that BRAF mutations are not a predisposing factor for CNS spread to this tumor type.

In conclusion, our study provides some insight into the pathobiology of BM of gastro-oesophageal carcinoma and may help to define treatment strategies and further research possibilities for this rare cancer manifestation.

Conflicts of Interest

Andreas von Deimling and David Capper declare shared inventorship of BRAF antibody clone VE1. A patent for diagnostic application of VE1 has been applied for. All terms are being managed by the German Cancer Research Center in accordance with its conflict of interest policies. All other Author have no conflicts of interest.

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