MET in gastric cancer – discarding a 10% cutoff rule

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Aims: We aimed to develop a putative predictive biomarker score for future hepatocyte growth factor receptor (MET)-targeted therapy of gastric cancer (GC).

Methods and results: MET expression and *MET* amplification were analysed by immunohistochemistry (IHC) and chromogenic *in-situ* hybridization (CISH) in 470 GC patients. Immunostaining was documented with the HistoScore. The percentage area of *MET*-amplified tumour cell clones was assessed by virtual microscopy. The expression of MET was heterogeneous in primary and metastatic GC. Immunostaining intensity (MET-IHC 2+/3+) correlated with *MET* amplification and a positive MET status was defined by a combination of MET-IHC 2+ or 3+ with *MET* amplification, or MET-IHC 3+ without *MET* amplification. The prognostic significance of the MET status was independent from the percentage area of positive tumour cells (e.g. <10 versus ≥10%). MET-positive GCs were microsatellite stable and of *KRAS/PIK3CA* wild-type. MET-positive GCs had a very poor prognosis, with a median survival of 5.4 months and a hazard ratio of 2.126.

Conclusions: A combination of immunohistochemistry and CISH is suitable to assess MET status. If MET status is used as a predictive biomarker, prospective studies should pay specific attention to adequate tissue sampling, should ignore cutoff values for tumour areas, may consider the *KRAS* and *PIK3CA* genotype as negative predictive markers and should carry out the analysis expeditiously.

Keywords: gastric cancer, immunohistochemistry, *in-situ* hybridization, MET, predictive biomarker, targeted therapy

Introduction

In recent decades we have witnessed major advancements in the understanding of the epidemiology, pathology and pathogenesis of gastric cancer (GC). Infection with *Helicobacter pylori* or Epstein–Barr virus (EBV), dietary and lifestyle factors contribute to the risk of developing GC. These advancements were accompanied by the introduction of chemotherapy for the treatment of GC, which is evolving continuously and improves patients' survival.[1–3] Evidence is increasing that patient prognosis and treatment response depends not only on tumour stage, but also on the expression and tumour-specific alteration of signalling pathways. A target currently explored in GC is the tyrosine kinase receptor hepatocyte growth factor receptor (MET) and its single ligand hepatocyte growth factor/scatter factor (HGF/SF). Phases I/II and III studies either explore antibodies directed against HGF/SF (e.g. onartuzumab, rilotumumab) or tyrosine-kinase inhibitors (e.g. crizotinib, foretinib, tivantinib).[4–9] However, previous studies targeting tyrosine kinase receptors in GC have shown that treatment response depends upon patient selection: trastuzumab is efficacious only in patients with a positive human epidermal growth factor receptor 2 (HER2)/*neu* status, i.e. strong HER2/*neu* protein expression or moderate HER2/*neu* protein expression in conjunction with *HER2* amplification.[10]

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In this retrospective observational study on a large GC patient cohort we tested the following hypotheses: (i) MET expression is heterogeneous in GC, (ii) immunostaining intensity of MET correlates with the *MET* amplification status and (iii) percentage area of *MET* amplification correlates with patient survival. Finally, we aimed to develop a putative predictive biomarker score, which could be tested and validated prospectively in clinical trials.

Materials and methods

ETHICS STATEMENT

This project was approved by the local ethics committee of the University Hospital in Kiel, Germany (reference number D 453/10). All patient data were pseudonymized prior to study inclusion.

STUDY POPULATION

From the archive of the Institute of Pathology, University Hospital Kiel, we identified all Caucasian patients who had undergone either total or partial gastrectomy for adenocarcinomas of the stomach or oesophago–gastric junction between 1997 and 2009 (GC cohort). The following patient characteristics were retrieved: type of surgery, age at diagnosis, gender, tumour localization and tumour size, tumour type, tumour grade, depth of invasion, number of lymph nodes resected and number of lymph nodes with metastases (Table 1). Date of patient death was obtained from the Epidemiological Cancer Registry of the state of Schleswig-Holstein, Germany. Follow-up data of patients still alive were retrieved from hospital records and general practitioners.

STUDY INCLUSION AND EXCLUSION CRITERIA

Inclusion and exclusion criteria for the gastric cancer cohort were defined as follows: patients were included if (i) histology confirmed an adenocarcinoma of the stomach or oesophago–gastric junction and (ii) the date of death or survival data were available. Patients were excluded if (i) histology identified a tumour type other than adenocarcinoma, (ii) patients had previously undergone a resection of a Billroth-II stomach with cancer in the gastric remnant and (iii) patients who had received perioperative chemotherapy.

HISTOLOGY AND TUMOUR–NODE–METASTASIS (TNM) CLASSIFICATION

Tissue specimens were fixed in 10% neutralized formalin for at least 24 h and embedded in paraffin. The formalin fixation was standardized during the study period. Sections taken from the tumour included luminal surface, tumour centre and invasion front. Deparaffinized sections were stained with haematoxylin and eosin. Tumours were classified according to the Laurén classification[11] and the mucin phenotype.[12] pTNM stage of all study patients was determined according to the 7th edition of the Union for International Cancer Control (UICC) guidelines,[13] and was based solely on surgical pathological examination.

IMMUNOHISTOCHEMISTRY

The hepatocyte growth factor receptor was detected by immunohistochemistry (IHC) using a rabbit monoclonal anti-MET antibody (dilution 1:50; clone SP44; Spring Bioscience, Pleasanton, CA, USA) and the autostainer Bond™ Max System (Leica-Menarini, Berlin, Germany). Antigen retrieval was carried out using the ER1 antigen retrieval solution for 20 min at pH 6.0 (Leica-Menarini).

Immunostaining (MET-IHC) was evaluated according to the HistoScore (Hscore), as described previously.[14] The first parameter was based on the intensity of the stained cells. A score of 0 (no evidence of staining, MET-IHC 0) to 3 (strong staining reaction, MET-IHC 3+) was applied. The second parameter (P) estimated the distribution of the stained cells in percentage. The sum total of all staining intensities found in a single case always added to a total Hscore of 100, according to the following formula: P (MET-IHC 0) + P (MET-IHC 1+) + P (MET-IHC 2+) + P (MET-IHC 3+) = 100%. The entire series was screened and three representative cases were selected for the adjustment of MET-IHC 1+, 2+ and 3+ staining intensity (Figure 1). These cases were used subsequently as reference standard for the in-depth evaluation of the entire cohort. MET-IHC 0 was characterized by the complete lack of any membranous immunostaining. MET-IHC 1+ was characterized by faint, MET-IHC 2+ by moderate and MET-IHC 3+ by strong membranous staining. Membranous immunostaining was almost always associated with cytoplasmatic immunostaining (Figure 1). The localization of membranous immunostaining was variable, i.e. lateral, basolateral and circumferential, but

was not used to classify immunostaining or MET status, as it was highly variable within a single tumour (intratumour heterogeneity).

CHROMOGENIC IN-SITU HYBRIDIZATION (CISH)

Analysis of *MET* amplification was performed by CISH using the ZytoDot 2C SPEC MET/CEN7 Probe and the ZytoDot 2C CISH Implementation Kit (ZytoVision GmbH, Bremerhaven, Germany).

The results of CISH were evaluated by screening the entire tissue sections in order to find, where present, *MET*-amplified invasive cancer areas. Subsequently, *MET* and centromer 7 signals were counted in at least 20 representative adjacent cancer cell nuclei within the invasive region. Forty nuclei were

Table 1. Clinicopathological patient characteristics of the gastric cancer cohort

	Valid (*n*)	Missing (*n*)		Total (*n*)	MET negative [*n* (%)]	MET positive [*n* (%)]	*P*-value
Gender	470	0	Men	291	265 (91.1)	26 (8.9)	0.041*
			Women	179	172 (96.1)	7 (3.9)	
Age	470	0	<68 years	233	214 (91.8)	19 (8.2)	0.371*
			≥68 years	237	223 (94.1)	14 (5.9)	
Localization	470	0	Proximal stomach	143	128 (89.5)	15 (10.5)	0.075*
			Distal stomach	327	309 (94.5)	18 (5.5)	
Laurén phenotype	470	0	Intestinal	241	228 (94.6)	13 (5.4)	0.060*
			Diffuse	147	137 (93.2)	10 (6.8)	
			Mixed	31	25 (80.6)	6 (19.4)	
			Unclassified	51	47 (92.2)	4 (7.8)	
Mucin phenotype	412	58	Intestinal	117	113 (96.6)	4 (3.4)	0.237*
			Gastric	62	57 (91.9)	5 (8.1)	
			Mixed	169	153 (90.5)	16 (9.5)	
			Unclassified	64	60 (93.8)	4 (6.2)	
pT-category	470	0	pT1a/b	59	57 (96.6)	2 (3.4)	0.043†
			pT2	56	55 (98.2)	1 (1.8)	
			pT3	184	170 (92.4)	14 (7.6)	
			pT4a/b	171	155 (90.6)	16 (9.4)	
pN-category	469	1	pN0	134	131 (97.8)	3 (2.2)	0.004†
			pN1	65	64 (98.5)	1 (1.5)	
			pN2	85	74 (87.1)	11 (12.9)	
			pN3 (a/b)	185	167 (90.3)	18 (9.7)	
Lymph node ratio	468	2	<0.189	229	221 (96.5)	8 (3.5)	0.004*
			≥0.189	239	214 (89.5)	25 (10.5)	

Table 1. *(Continued)*

	Valid (*n*)	Missing (*n*)		Total (*n*)	MET negative [*n* (%)]	MET positive [*n* (%)]	*P*-value
Stage (UICC)	470	0	IA	46	45 (97.8)	1 (2.2)	<0.001†
			IB	36	36 (100.0)	0 (0.0)	
			IIA	55	54 (98.2)	1 (1.8)	
			IIB	46	43 (93.5)	3 (6.5)	
			IIIA	55	51 (92.7)	4 (7.3)	
			IIIB	80	73 (91.2)	7 (8.8)	
			IIIC	64	58 (90.6)	6 (9.4)	
			IV	88	77 (87.5)	11 (12.5)	
Lymphatic invasion	455	15	pL0	221	214 (96.8)	7 (3.2)	0.003*
			pL1	234	210 (89.7)	24 (10.3)	
Venous invasion	454	16	pV0	403	379 (94.0)	24 (6.0)	0.068*
			pV1	51	44 (86.3)	7 (13.7)	
Tumour grade	469	1	G1/G2	112	110 (98.2)	2 (1.8)	0.010*
			G3/G4	357	326 (91.3)	31 (8.7)	
R-status	470	0	pR0	410	387 (94.4)	23 (5.6)	0.005*
			pR1	60	50 (83.3)	10 (16.7)	
Survival	455	15	Total	455	425 (93.4)	30 (6.6)	
			Dead	354	325 (91.8)	29 (8.2)	
			Median (months ± SD; 95%CI)		16.69 ± 1.42 (13.90–19.48)	5.42 ± 0.97 (3.53–7.32)	<0.001‡
Helicobacter pylori	402	68	Positive	60	56 (93.3)	4 (6.7)	0.769*
			Negative	342	322 (94.2)	20 (5.8)	
EBV	453	17	Positive	17	15 (88.2)	2 (11.8)	0.356*
			Negative	436	405 (92.9)	31 (7.1)	
KRAS	470	0	Wild-type	453	420 (92.7)	33 (7.3)	0.622*
			Mutant	17	17 (100.0)	0	
PIK3CA	470	0	Wild-type	450	417 (92.7)	33 (7.3)	0.384*
			Mutant	20	20 (100.0)	0 (0.0)	
MSI status	441	29	MSS	407	376 (92.4)	31 (7.6)	0.155*
			MSI high	34	34 (100.0)	0	

Table 1. *(Continued)*

	Valid (*n*)	Missing (*n*)		Total (*n*)	MET negative [*n* (%)]	MET positive [*n* (%)]	*P*-value
HER2	443	27	Positive	36	35 (97.2)	1 (2.8)	0.497*
			Negative	407	377 (92.6)	30 (7.4)	

EBV, Epstein–Barr virus; MSI, Microsatellite instability; HER2, Human epidermal growth factor receptor 2; SD, Standard deviation; MET, Hepatocyte growth factor receptor; UICC, Union for International Cancer Control.
*Fisher's exact test.
†Kendall's tau test.
‡Log-rank test.

counted when the *MET*/centromer 7 ratio ranged from 1.8 to 2.2. The presence of CISH clusters was noted and the ratio of *MET*/centromer 7 signals was calculated. The gene count was calculated by dividing the number of *MET* gene signals by the number of cancer cell nuclei studied.

ASSESSMENT OF PHENOTYPE, GENOTYPE AND INFECTIOUS STATUS

The *KRAS* genotype, *PIK3CA* genotype, mucin phenotype and the *Helicobacter pylori*, Epstein–Barr virus, microsatellite and HER2/*neu* status were assessed as described in detail previously.[15]

VIRTUAL MICROSCOPY

Tissue sections were scanned using a Leica SCN400 microscopic whole-slide scanner (Leica Biosystems, Nussloch, Germany) at its maximum, nominally ×40 magnification. In the scanned images, pixel-to-pixel distance represents 0.26 micrometres. Images were exported from the scanner system into files of Leica SCN format. For performing the computer-assisted parts of the study, a viewer program was written to display images of the Leica SCN file format, as described in detail previously.[16] This provided the flexibility to create the screen layout, user interaction, assistance tool and calculation routines we needed for our study.

STATISTICS

Statistical analyses were performed using SPSS version 20.0 (IBM Corporation, Armonk, NY, USA). For continuous variables, cases were divided into two groups by splitting at the median value. Median overall survival was determined using the Kaplan–Meier method, and the log-rank test was used to determine significance. For comparison purposes, the median survival time, its standard deviation and 95% confidence interval (CI) were calculated. To investigate prognostic relevance, we included all variables having $P < 0.10$ into a Cox regression model and used the backward logistic regression (LR) method ($P_{in} = 0.05$ and $P_{out} = 0.10$) to reduce the model to the independent variables. The significance of correlation between clinicopathological parameters and biomarker expressions was tested using Fisher's exact test. For parameters of ordinal scale (T-category, N-category, tumour stage), we applied Kendall's tau test instead. $P \leq 0.05$ was considered statistically significant. To account for the effects of multiple testing, we applied the explorative Simes (Benjamini–Hochberg) procedure. *P*-values are given unadjusted, but are marked where they lose significance under the explorative Simes procedure.

Results

MET EXPRESSION IN GASTRIC CANCER

First we examined the expression of MET in GC by immunohistochemistry (MET-IHC). MET-IHC was available from 470 GC cases. Weak immunostaining (MET-IHC 1+) was found in 184 (39.1%) cases, moderate (MET-IHC 2+) in 51 (10.9%) cases and strong (MET-IHC 3+) in 27 (5.7%) cases. Immunonegative tumour cells (MET-IHC 0) were found in 460 (97.9%) cases. The percentage area of the four immunostaining categories, i.e. MET-IHC 0, 1+, 2+ and 3+, ranged from 0% to 100% (see Supporting information, Figure S1) and the combination of the staining categories in each individual case varied: 272 (57.9%) GCs were completely devoid of any MET expression. One case showed 100% MET-IHC 3+; 197
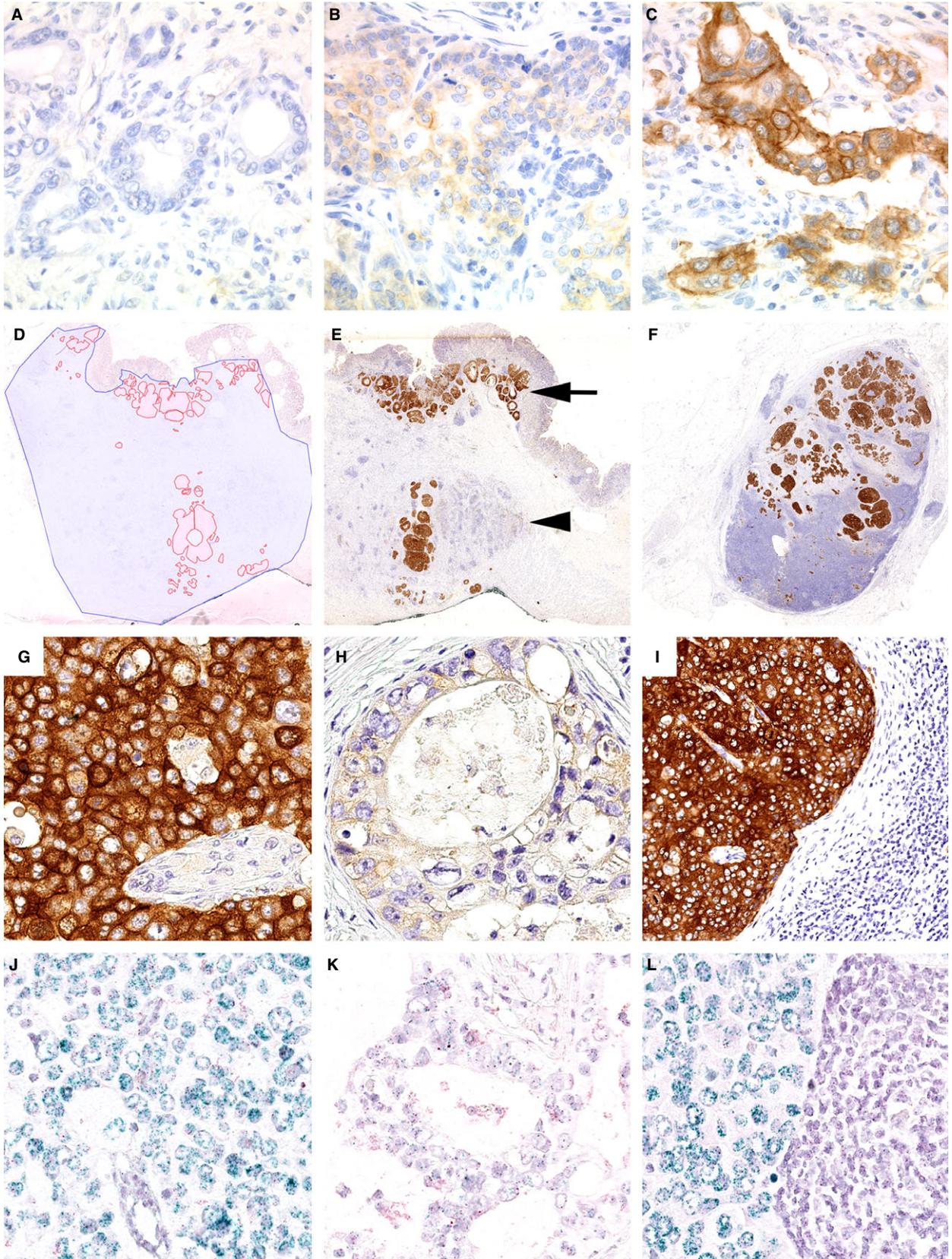
Figure 1. Hepatocyte growth factor receptor (MET) protein expression in gastric cancer. The expression of MET was studied by immunohistochemistry (IHC) (**A–C, E–I**) and chromogenic *in-situ* hybridization (**D, J–L**). The entire gastric cancer cohort was screened and three representative cases were selected for MET IHC 0 (**A**), 1+ (**B**), 2+ (**C**) and 3+ (**G**). Using a viewer program with a polygon line drawing function we traced manually the outlines of the total tumour tissue area (blue) and the *MET*-amplified tumour area (red; **D**). This illustrates the heterogeneity of MET expression and *MET* amplification. Less than 10% of the entire tumour area showed MET-IHC 3+ (**E, G**), while other areas were MET-IHC 0 or 1+ (**H**). *MET* was amplified (**J**) or not (**K**). A corresponding lymph node metastasis of the same patient showed MET-IHC 3+ (**F, I**) and *MET* amplification of the metastatic tumour cells (**L**). The spatial distribution of MET status-positive tumour cell clones is also illustrated in (**E**). Immunostaining was localized at the cell membrane as well as in the cytoplasm. MET-positive tumour cell clones were found near the mucosal surface (arrow) and in the tumour centre (arrowhead; **E**).

cases showed a combination of two or three staining intensities, i.e. MET-IHC 0/1+ [143 cases (30.4%)], 0/2+ [2 (0.4%)], 1+/2+ [2 (0.4%)], 0/3+ [1 (0.2%)], 2+/3+ [4 (0.9%)], 0/1+/2+ [24 (5.1%)], 0/1+/3+ [2 (0.4%)], 0/2+/3+ [6 (1.3%)], 1+/2+/3+ [3 (0.6)] or 0/1+/2+/3+ [10 (2.1)] (also see Figure 2). These data show that the expression (=combination of intensity of immunostaining and amount of immunopositive tumour areas) of MET is heterogeneous in GC, including 'grey-scale' and 'black-and-white' immunostaining patterns (Figures 1 and 2).

CHROMOGENIC IN-SITU HYBRIDIZATION

All cases ($n = 55$) with MET-IHC 2+ and/or MET-IHC 3+ tumour areas were forwarded to CISH. Overall, 25 of 55 (45.5%) were reported positive, i.e. 19 of 27 (70.4%) cases with MET-IHC 3+ and six of 28 (21.4%) cases with MET-IHC 2+ and without MET-IHC 3+ staining showed *MET* amplification (Figure 2). The gene ratio ranged from 2.07 to 6.26 (median 3.82) and the gene count ranged from 4.55 to 14.10 (median 8.70). *MET* gene clusters were found in 24 cases. In summary, 5.3% of the entire GC cohort showed a *MET* amplification (25 of 470 cases).

Gene amplification was heterogeneous (see below), in that amplified and unamplified tumour areas were demarcated sharply on a cell-by-cell basis (Figure 1). Amplification correlated spatially with the MET-IHC 3+ or MET-IHC 2+ areas. *MET* amplification was not found in MET-IHC 0 or MET-IHC 1+ tumour areas (Figure 1).

CORRELATION OF PATIENT SURVIVAL WITH *MET*-AMPLIFIED TUMOUR AREAS AND MET IMMUNOSTAINING

Next we tested the hypothesis that the percentage area of *MET*-amplified tumour cells and intensity of MET immunostaining correlates with patient survival. For this purpose, we scanned CISH-labelled tumour-bearing tissue sections harbouring *MET* amplification (ratio ≥2.0) with a microscopic whole-slide scanner.

Using a viewer program with a polygon line-drawing function we traced the outlines of the total tumour tissue area and the *MET*-amplified tumour area, which was demarcated sharply (on a cell-by-cell basis) from unamplified tumour areas. The percentage of *MET*-amplified tumour area was subsequently computed and ranged from 0.4% to 100.0% (Figure 2). Thereafter, GCs were categorized into five groups: ≥10% *MET*-amplified tumour area (group A; Figure 2), <10% *MET*-amplified tumour area (group B), MET-IHC 3+ without *MET* amplification (group C), MET-IHC 2+ without *MET* amplification (group D) and MET-IHC 1+ or MET-IHC 0 (group E; Table 2). Interestingly, the median survivals of groups A, B and C were not significantly different from each other (A versus B: $P = 0.438$; A versus C: $P = 0.463$; B versus C: $P = 0.811$). To the contrary, patients of group D had lived significantly longer compared with groups A ($P = 0.001$), B ($P < 0.035$) or C ($P = 0.031$). The median survival between groups D and E was not significantly different. Thus, independently from the *MET*-amplified tumour area, any *MET* amplification and MET-IHC 3+ without amplification is significantly prognostically unfavourable. The Kaplan–Meier plots further illustrate the survival of the different patient groups (Figure 3, Table 2) and the effect any percentage area of *MET* amplification has on patient survival.

CORRELATION OF MET STATUS WITH CLINICOPATHOLOGICAL PATIENT CHARACTERISTICS

Next, we correlated the MET status with various clinicopathological patient characteristics. To this purpose, and based on the aforementioned results, we categorized all patients of groups A–C as MET-positive, thereby discarding the 10% cutoff value used for HER2/*neu*-scoring,[10] and all GCs of groups D and E as MET-negative (Table 2). The MET status correlated significantly with lymphatic invasion, N category, lymph node ratio, UICC tumour stage, tumour grade and R-status (Table 1). MET-positive GCs were insignif-
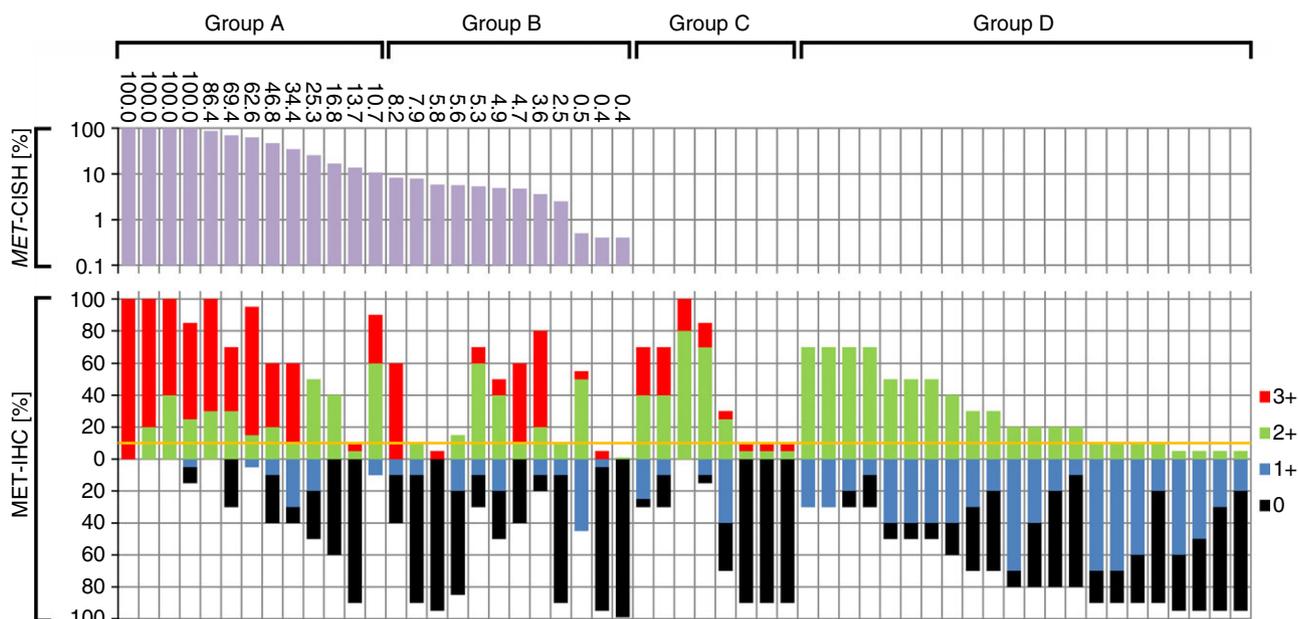
Figure 2. Waterfall-plot illustrating the distribution of *MET* amplification and hepatocyte growth factor receptor (MET) immunostaining among 55 patients. The waterfall plot shows case-by-case the results of chromogenic *in-situ* hybridization (*MET*-CISH; logarithmic data presentation), and immunohistochemistry (MET-IHC; liner data presentation) for patients belonging to group A (≥10% *MET* amplification), group B (<10% *MET* amplification), group C (MET-IHC 3+ without *MET* amplification) and group D (MET-IHC 2+ without *MET* amplification). Note that the different colours at the bottom illustrate the different staining intensities: MET-IHC 3+ (red), MET-IHC 2+ (green), MET-IHC 1+ (blue) and MET-IHC 0 (black). The sum total of all staining intensities found in a single case always added to a total HistoScore (Hscore) of 100% according to the following formula: P (MET-IHC 0) + P (MET-IHC 1+) + P (MET-IHC 2+) + P (MET-IHC 3+) = 100%. The yellow line marks a 10% cutoff, which is used, for example, to classify HER2/*neu* as either positive (above) or negative (below).

Table 2. Correlation of patient survival with hepatocyte growth factor receptor (MET)-immunostaining and *MET*-amplified tumour areas

Final MET status	Group	Immunohistochemistry	Chromogenic *in-situ* hybridization	Number of patients	Median survival ± SD (months)	95% CI
Positive	A	MET-IHC-2+ and/or 3+	*MET* amplified area ≥10%	13	3.81 ± 1.71	0.47–7.16
Positive	B	MET-IHC-2+ and/or 3+	*MET* amplified area <10%	12	4.27 ± 0.57	3.15–5.39
Positive	C	MET-IHC-3+	*MET* unamplified	8	5.85 ± 6.27	5.43–6.27
Negative	D	MET-IHC-2+	*MET* unamplified	22	18.76 ± 8.82	1.47–36.05
Negative	E	MET-IHC1+ or 0	*MET* unamplified	415	16.53 ± 1.45	13.69–19.37

MET, Hepatocyte growth factor receptor; IHC, Immunohistochemsitry; CISH, Chromogenic *in-situ* hybridization; CI, Confidence interval; SD, Standard deviation.

icantly more prevalent in men, in mixed-type GCs and locally more advanced tumours (8.2% pT3/4 versus 2.6% pT1/2; Table 1). GCs with venous invasion overexpressed MET more commonly compared with GCs without venous invasion. No correlation was found between MET status and infection with either *H. pylori* or EBV. Interestingly, all MET-positive GCs were microsatellite stable, and of *KRAS* and *PIK3CA* wild-type. *MET* and *HER2* status were mutually exclusive, except for a single case, which showed both, *MET* and *HER2* amplification in spatially distinct tumour areas (Figure 4).
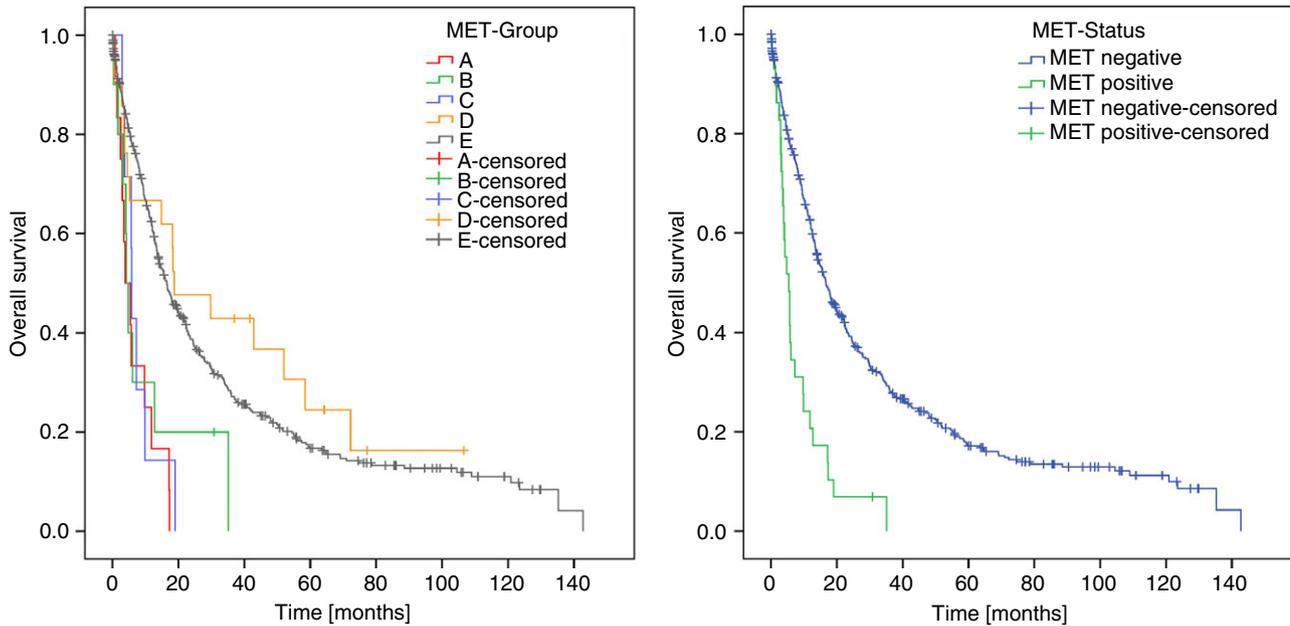
Figure 3. Kaplan–Meier curves depicting patients' survival. Patients were categorized into five hepatocyte growth factor receptor (MET) groups (**A**): ≥10% *MET*-amplified tumour area (group A), <10% *MET*-amplified tumour area (group B), MET-IHC 3+ and -unamplified (group C), MET-IHC 2+ and unamplified (group D), and MET-IHC 1+ or MET-IHC 0 (group E). Subsequently, groups A–C were classified as MET-positive and groups D–E as MET-negative (**B**).

Figure 4. HER2/*neu*- and hepatocyte growth factor receptor (MET) overexpression in a single patient. A single patient showed overexpression of HER2/*neu* (**A**, **B**) and MET (**C**, **D**) in spatially distinct tumour areas. Four serial sections were stained immunohistochemically (**A**, **C**) and by chromogenic *in-situ* hybridization (**B**, **D**). Note the distinct immunoreactions of HER2/*neu* and MET. HER2/*neu* showed a characteristic delicate basolateral staining of the cell membrane, while MET immunostaining was also strong within the cytoplasm.
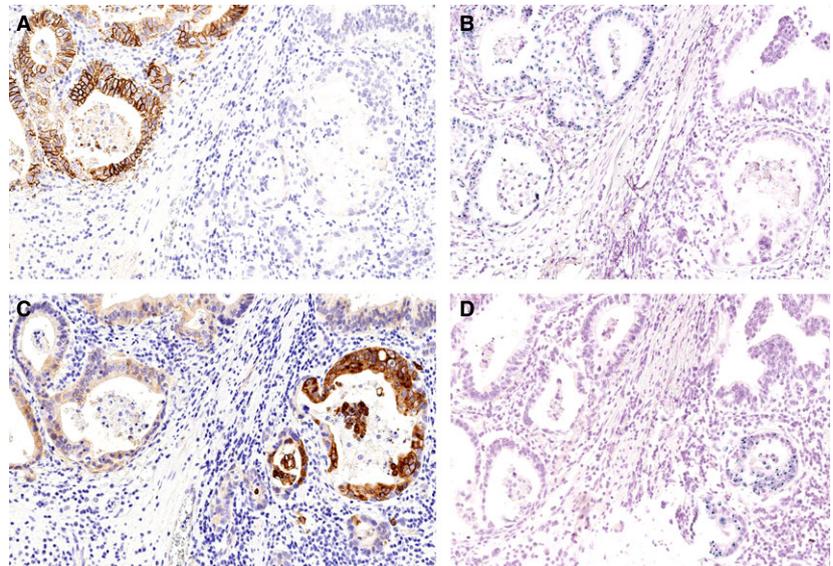
Finally, we correlated MET status with patient survival. MET-positive GCs had a highly significantly worse prognosis (Table 1, Figure 3). Patient prognosis also depended highly significantly upon patient age, Laurén phenotype, tumour grade, T-category, N-category, lymph node ratio, R-status, as well as UICC stage (data not shown).

MULTIVARIATE SURVIVAL ANALYSIS (COX REGRESSION)

A Cox regression was carried out on all parameters, which had a $P < 0.10$ in univariate survival analysis, i.e. Laurén phenotype, T-category, N-category, UICC-stage, lymph node ratio, lymphatic and venous

invasion, R-status and MET status. Four parameters remain in the Cox model after running the backward LR method with P_{in} = 0.05 and P_{out} = 0.10. These were R-status [hazard ratio (HR) = 2.208 (1.573–3.099); P < 0.001], MET status [HR = 2.126 (1.386–3.261); P = 0.001], lymph node ratio [HR = 1.633 (1.197–2.228); P = 0.002] and UICC stage [HR = 1.055 (95% CI: 1.034–1.077); P < 0.001].

MET STATUS IN LYMPH NODE METASTASES

We then explored the MET status in lymph node metastases of MET-positive GCs, i.e. MET-IHC 2+ and/or 3+ with *MET* amplification or MET-IHC 3+ without *MET* amplification (groups A–C). Lymph node metastases were available from 26 (78.8%) MET-positive cases. MET-IHC and *MET* amplification of the lymph node metastases was also heterogeneous with MET-IHC 0, 1+, 2+ and 3+ as well as *MET*-amplified and *MET*-unamplified tumour cell clones within the same lymph node. A comparison of the primary tumours with the corresponding lymph node metastases showed a concordant MET status in 15 (58%) cases (Figure 1E,F). In 11 (42%) cases, the lymph node metastases were MET-negative. The overall survival between concordant (n = 15) and discordant (n = 11) cases was not significantly different (P = 0.280). In cases with MET-positive lymph nodes metastases, the lymph node ratio, i.e. number of MET-positive lymph node metastases divided by the total number of lymph node metastases, did not correlate with the overall survival (P = 0.079). Finally, we explored MET status in all lymph node metastases of MET-negative GCs (group D). Lymph node metastases were available from 18 (81.8%) MET-negative cases. None of these showed a MET-positive lymph node metastasis.

SPATIAL LOCALIZATION OF MET-POSITIVE TUMOUR CELL CLONES

In a palliative setting, only tumour biopsies may be available for testing of a predictive biomarker. Finally, we explored the spatial distribution of MET-positive tumour cell clones in the primary tumour. The MET-positive tumour cells were close to the mucosal surface in 26 (78%) cases (Figure 1D). In seven (21%) cases, MET-positive tumour cell clones were localized only in the tumour centre or near the invasion front.

Discussion

Hepatocyte growth factor receptor is a tyrosine kinase receptor with pleiotropic effects. It is essential for embryonic development and regeneration, placenta and liver development, liver regeneration and wound repair. MET is activated in tumorigenesis, e.g. by HGF/SF overexpression, autocrine signalling, *MET* mutation or gene amplification. In a context-dependent manner it induces proliferation, survival, motility, cell scattering, angiogenesis and tubulogenesis and drives epithelial–mesenchymal transition as well as invasion.[4,17] Deng *et al.*[18] showed recently that 37% of the GCs harbour receptor tyrosine kinase gene amplifications affecting *FGFR2*, *EGFR*, *HER2* and *MET*, whose transcripts are promising therapeutic targets.[10,19] MET is currently explored for the treatment of GC,[4–9] and targeting MET may depend upon its expression pattern explored by immunohistochemistry and/or CISH.[4] Many studies have explored the tumour–biological and clinicopathological characteristics of MET-positive GCs. The expression has been studied by immunohistochemistry in several studies,[6,20–35] and the number of MET-IHC-positive GCs ranged from 3.8%[20] to 85%.[36] In our cohort, any immunostaining of MET (MET IHC 1+ to 3+) was found in 192 (42.1%) cases. This range of immunopositivity is due to the usage of different types of antibodies and different, non-standardized scoring systems. Seven studies used the rabbit monoclonal SP44 antibody applied in our study, which was found to be the most reliable antibody.[21,23,24,27,28,30,31] The positivity rate was more homogeneous, but still ranged from 9[24] to 41%.[21] This relates to the different scoring systems and cutoff values applied. However, none of these studies provided a rational explanation for the application of a certain cutoff value or scoring system. Until now the significance of the diverse scoring systems has not been explored systematically. In our study we carried out the first systematic analysis, to our knowledge, on MET expression using the HScore. This demonstrates a substantial intra- and intertumour variability of MET immunostaining with regard to the combination of staining intensities (MET-IHC 0, 1+, 2+, 3+) and the percentages of positive tumour areas (see Figure 2 and Supporting information, Figure S1). This is particularly difficult with regard to the application of immunostaining as a predictive biomarker.[16] Recently we have shown that pathologists have good ability to estimate ratios of clearly demarcated areas, but gradients in staining intensities (such as a combination of two or three different immmunostaining intensities in a single case) hinder reproducible visual demarcation of positive tumour areas.[16] However, for HER2/*neu* it has been demonstrated in breast and stomach cancer that a combination of immunohistochemistry and *in-situ* hybridization may improve the specificity of the predic-

tive biomarker. Using CISH, we show that only 5.3% of our GCs are *MET*-amplified. This low prevalence is in line with previous findings, where the number of *MET*-amplified GCs ranged from 1.3% to 12%.[5–7,23,28,30,31,37,38] Overall, *MET* amplification is rare in GC, and is also heterogeneous with amplified and unamplified tumour cell clones occurring in the same primary GC, which was reported occasionally,[31,39] and never explored systematically.

In the tissue-based HER2-scoring system for GC, GCs are classified as HER2/*neu*-positive when ≥10% of the tumour area shows strong immunostaining (3+) for HER2/*neu*, or moderate immunostaining (2+) in conjunction with *HER2* amplification (ratio ≥ 2.0). A 10% cutoff rule has also been applied to MET scoring.[20,21,28,31] Next we explored the putative significance of cutoff values and calculated the percentage area of MET-positive tumour cells with greater accuracy using virtual microscopy. We show that even a small subpopulation (0.4%) of *MET*-amplified tumour cells already indicates a poor prognosis. We provide further evidence that patient prognosis can be used to test and develop a rational classifier of MET status, i.e. positive or negative, by using a combination of immunostaining and CISH and by discarding any cutoff value for positive tumour areas. Using patient survival as a surrogate marker for the development of a predictive biomarker is sensible, as therapeutic efficacy of targeted therapy is assessed by the improvement of, for example, overall survival.[10]

Our study also supports the contention that tumour heterogeneity is a major issue with regard to analysing the MET status in GC. In a previous study we demonstrated the risk of false positive and negative ratings of HER2/*neu* status in GC due to sampling errors.[40] The spatial distribution of MET expression was highly variable. In seven (21%) cases, MET-positive tumour cell clones were localized only in the tumour centre or near the invasion front, and superficial tumour biopsies may carry the risk of a false negative test result (Figure 1). This may explain the low prevalence of MET amplification in studies utilizing only biopsy specimens (2%)[7] or tissue microarrays (1.3–3.4%).[28,31] Thus, assessment of the MET status carries the same risk of false positive and negative ratings due to a sampling error, as was shown previously for HER2/*neu*.[40,41] This also applies to the examination of lymph node metastases. MET status is heterogeneous between lymph node metastases of the same patient. While we did not find MET-positive lymph node metastases in MET-negative primary GCs, 11% of MET-positive primary GCs harboured MET-negative lymph node metastases.

Tumour heterogeneity may be an intrinsic problem of GCs with receptor tyrosine kinase gene amplifications. Recently, whole-genome sequencing and comprehensive molecular profiling of 100 tumour-normal pairs of GC have shown that GC with receptor tyrosine kinase gene amplifications are specifically enriched in the subgroup of genomic unstable GCs, which also show activation of the rat sarcoma (RAS) signalling pathway.[42,43] Therefore, our findings are in line with recent findings on the molecular biology of GC. More interestingly, in our cohort, *MET* amplification and *KRAS*/*PIK3CA* mutations were mutually exclusive. MET signalling is mediated primarily through the RAS–mitogen-activated extracellular kinase (MAPK) and phosphoinositide 3-kinase (PI3K)–protein kinase B (Akt) pathways. This observation leads to the conjecture that receptor tyrosine kinase amplification and RAS/PIK3CA mutations may be mutually exclusive. Interestingly, we have shown previously that *KRAS* mutant GCs have a worse prognosis compared with their *KRAS* wild-type counterparts (3.5 ± 3.1 versus 12.7 ± 0.7 months),[15] being in the range of *MET*-amplified GCs. Together, *MET*-amplified and *KRAS* mutant GCs account for 10% of our patient cohort, and seem to specify a unique subgroup with a very poor prognosis. Further studies into this topic are warranted.

In conclusion, MET-positive GCs define a small aggressive subgroup of genetically unstable GCs with a very poor prognosis. A combination of MET immunostaining and *in-situ* hybridization is suitable to assess MET status. If MET is used as a predictive biomarker, prospective studies should pay specific attention to adequate tissue sampling, should ignore cutoff values for tumour areas, may consider the *KRAS* and *PIK3CA* genotypes as negative predictive markers and should carry out the analysis expeditiously, as these patients have a very poor prognosis.

Acknowledgements

Conflicts of interest

The authors declare that no competing interests exist.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Hepatocyte growth factor receptor (MET)-immunostaining in gastric cancer. Immunostaining [MET immunohistochemistry (IHC)] was evaluated according to the HistoScore (Hscore). The first parameter documented the intensity of the stained cells: no evidence of immunostaining (MET-IHC 0; **A**), mild (cMET-IHC1+; **B**), moderate (MET-IHC 2+; **C**) or strong immunostaining (MET-IHC 3+; **D**). The second parameter (P) estimates the distribution of the stained cells in percentage (x-axis). The sum total of all staining intensities found in a single case always added to a total Hscore of 100% according to the following formula: P (MET-IHC 0) + P (MET-IHC 1+) + P (MET-IHC 2+) + P (MET-IHC 3+) = 100% (also see Figure 2). The prevalence of the percentage areas of the four different immunostainings found in our gastric cancer cohort is shown on the y-axis. The figure also demonstrates the heterogeneity of MET immunostaining in gastric cancer.