

MET Tyrosine Kinase Receptor Expression and Amplification as Prognostic Biomarkers of Survival in Gastroesophageal Adenocarcinoma

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BACKGROUND: *MET* gene amplification and Met protein overexpression may be associated with a poor prognosis. The *MET*/Met status is typically determined with fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC), respectively. Targeted proteomics uses mass spectrometry-based selected reaction monitoring (SRM) to accurately quantitate Met expression. FISH, IHC, and SRM analyses were compared to characterize the prognostic value of *MET*/Met in gastroesophageal adenocarcinoma (GEC). **METHODS:** Samples from 447 GEC patients were analyzed for *MET* gene amplification (FISH) and Met protein expression (IHC and SRM). Cox proportional hazards models and Kaplan-Meier estimates were applied to explore relations between Met, overall survival (OS), and clinical/pathological characteristics. Spearman's rank coefficient was used to assess the correlation between parameters. **RESULTS:** Patients with *MET*-amplified tumors had worse OS when: the *MET*/centromere enumeration probe for chromosome 7 FISH ratio was ≥ 2 (hazard ratio [HR], 3.13; 95% confidence interval [CI], 1.84-5.33), the *MET* gene copy number was ≥ 5 (HR, 2.51; 95% CI, 1.45-4.34), or $\geq 10\%$ of the cells had ≥ 15 copies (HR, 4.28; 95% CI, 2.18-8.39). Similar observations were made with Met protein overexpression by IHC ($\geq 1+$ intensity in $\geq 25\%$ of the tumor cell membrane: HR, 1.39; 95% CI, 1.04-1.86) or SRM (≥ 400 amol/ μg : HR, 1.76; 95% CI, 1.06-2.90). A significant correlation was observed between *MET* FISH/Met IHC, *MET* FISH/Met SRM, and Met IHC/Met SRM; only *MET* FISH and Met SRM were independent negative prognostic biomarkers in multivariate analyses. **CONCLUSIONS:** *MET* amplification and overexpression, assessed by multiple methods, were associated with a worse prognosis in univariate analyses. However, only *MET* amplification by FISH and Met expression by SRM were independent prognostic biomarkers. Compared with IHC, SRM may provide an added benefit for informed decisions about Met-targeted therapy. *Cancer* 2017;123:1061-70. © 2016 The Authors. *Cancer* published by Wiley Periodicals, Inc. on behalf of *American Cancer Society*. This is an open access article under the terms of the Creative Commons Attribution NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

KEYWORDS: fluorescence in situ hybridization, gastroesophageal adenocarcinoma, gene amplification, immunohistochemistry, MET tyrosine kinase, negative prognostic biomarker, protein overexpression, selected reaction monitoring mass spectrometry.

INTRODUCTION

The Met receptor tyrosine kinase (c-Met or hepatocyte growth factor [HGF] receptor) is a single-pass transmembrane receptor that undergoes homodimerization and activation upon the binding of HGF, its only known ligand.¹ Numerous signaling pathways are activated by the interaction of HGF and Met²; thus, Met plays a critical role in many biological functions ranging from embryogenesis to wound healing. Mechanisms for Met-induced oncogenesis include constitutive activation of the kinase domain and/or dysregulated paracrine and/or autocrine signaling.^{1,3,4} The underlying activating mechanism typically involves *MET* gene amplification, Met and/or HGF protein overexpression, or, rarely, domain-specific sequence mutations/translocations,^{2,3} including the recently observed Met exon 14-skipping mutations in non-small cell lung cancer.^{5,6}

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Aberrant Met activity has been ubiquitously reported across cancers.^{1,2,7,8} For gastroesophageal adenocarcinoma (GEC), *MET* amplification and Met protein overexpression are found in 4% to 10% and 25% to 70% of GEC cases, respectively; the rates depend on the definition of *positive*, and the cohort studied.⁹⁻¹⁶ As with other malignancy types,^{17,18} patients with Met-positive GEC (as defined by various criteria) reportedly have a worse prognosis.^{10,12,15,16,19-21} Because of the clinical impact and relevance of Met, the diagnostic determination of its expression and/or mutational/amplification status can provide physicians with critical information for understanding a patient's prognosis, identifying clinical trials for which the patient may be a candidate, and, importantly, making a determination about whether the patient may benefit from Met-targeted therapies.

This study sought to further evaluate Met's prognostic potential with a large, multi-institutional, clinically linked cohort of GEC patients and to compare various diagnostic platforms for identifying Met-positive tumors. Fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) were used to examine the frequency of *MET* gene amplification and Met protein overexpression, respectively. Because of the inherent limitations of FISH (time-consuming, labor-intensive, subjective scoring) and IHC (variability in tissue staining, lack of intra-sample reproducibility over time, and subjective scoring), mass spectrometry-based selected reaction monitoring (SRM)^{11,22} was also used to objectively quantitate Met protein expression.¹¹ The relation between the *MET*/Met status and overall survival (OS) was assessed with all 3 approaches in both univariate and multivariate analyses, with adjustments made for known prognostic covariates of GEC. In addition, the data from *MET* FISH, Met IHC, and Met SRM were compared for intermethod agreement.

MATERIALS AND METHODS

Patients and Samples

Formalin-fixed, paraffin-embedded GEC samples were obtained from the University of Chicago (Chicago, Ill) or the University of Urbino (Urbino, Italy) from 2000 to 2015. Samples were collected/annotated under institutional review board-approved protocols. The median follow-up time was 113.0 months (80% confidence interval [CI], 99.3-126.3 months); the median follow-up times for patients with and without curative-intent surgery were 115.3 months (80% CI, 99.3-129.0 months) and 31.6 months (80% CI, 14.5-31.6 months), respectively. The

clinical/pathological characteristics of the samples are included in Supporting Tables 1 and 2 (see online supporting information).

MET Analysis

A dual-color *MET* FISH assay was conducted with the *MET* IQFISH probe with centromere enumeration probe for chromosome 7 (*CEP7*; Agilent Technologies, Santa Clara, Calif) and a histology FISH accessory kit (Dako North America, Carpinteria, Calif); scoring was performed as previously described.^{11,23,24} An average *MET* gene copy number (GCN) ≥ 5 , a *MET/CEP7* ratio ≥ 2 , and $\geq 10\%$ of counted tumor nuclei with ≥ 15 gene copies were the 3 amplification scoring criteria considered positive, and each assessed independently.^{24,25}

Met IHC was performed with a horseradish peroxidase-labeled, dextrose-based polymer complex bound to a secondary antibody (Dako North America) as previously described.^{21,26} The intensity of cell membrane staining, scored by an experienced pathologist (0, none; 1, low; 2, intermediate; or 3, high), along with the percentage of tumor cells (extensity) for each sample was documented.^{11,21} The membrane max intensity parameter of a given tumor was obtained from the highest IHC score that had a nonzero percentage of staining. For example, if a tumor had a score breakdown of 10%, 65%, 25%, and 0% (for scores of 0, 1, 2, and 3, respectively), the membrane max intensity was 2. The predominant score parameter was obtained from the highest percentage-positive score; in the provided example, this was 1. IHC was defined as positive if $\geq 25\%$ of the tumor cell membranes were stained with an intensity $\geq 1+$; a second analysis was also performed at a higher extensity ($\geq 50\%$) of cells with an intensity $\geq 1+$.^{21,26} The pathologists performing FISH and IHC were blinded to the results of previously performed assays and clinical outcomes.

For Met protein quantification by mass spectrometry-based SRM, tissue sections (10 μm) were prepared as previously described.^{11,22,27} This tumor tissue was solubilized with Liquid Tissue, and the resulting tryptic peptides were analyzed with a TSQ Vantage triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, Calif) equipped with a nanoACQUITY LC system (Waters, Milford, Mass). The SRM conditions have been described previously.^{11,22}

Statistical Methods

Spearman's rank correlation coefficient was used to assess correlations between parameters. Cox proportional hazards models and Kaplan-Meier estimates were applied to

explore relations between Met, OS, and other clinical and pathologic characteristics. A multivariate analysis was performed for the Met biomarker status (amplification by FISH, expression by IHC, and expression by Met SRM) and outcomes after adjustments for baseline covariates, including age, sex, race, histology, biopsy, diagnosis, stage, lesion status, curative-intent surgery, and perioperative therapy.

TABLE 1. Number of Gastroesophageal Adenocarcinoma Tumor Samples Evaluated for MET/MET by the Method of Assessment

Method	Samples	Samples With Outcome Data
FISH	344	338
FISH + IHC	304	
FISH + SRM	255	
IHC	332	324
IHC + SRM	229	
SRM	282	281

Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; SRM, selected reaction monitoring. Samples were analyzed for MET amplification by FISH and/or for Met protein expression by IHC or SRM; the quantities for each individual subgroup as well as paired evaluation subgroups are presented in the middle column. The quantity of each individual subgroup with corresponding outcome data is presented in the far right column.

RESULTS

There were 447 samples available: 344 (77.0%) were evaluable by MET FISH, 332 (74.3%) were evaluable by Met IHC, and 282 (63.1%) were evaluable by Met SRM (Table 1). Intermethod correlations were performed with tumors evaluable by more than 1 approach (discussed in further detail later). A comparison of single-method subsets by patient demographic and clinical characteristics indicated a balanced representation of each subset (Supporting Tables 1 and 2 [see online supporting information]).

When MET gene amplification was defined as a MET/CEP7 ratio ≥ 2 per cell,¹¹ 16 of 344 tumors (4.7%) were MET-amplified (Supporting Table 3 [see online supporting information]). When it was scored as the percentage of tumor cells with a mean MET GCN ≥ 5,¹² 18 tumors (5.2%) were amplified. When it was defined as ≥10% of tumor cells having at least 15 copies of MET, 9 tumors (2.6%) were amplified. These results were similar to previously published rates of MET gene amplification in GEC.^{9-11,13,26}

Regardless of the definition of amplification, MET FISH was an indicator of a poor prognosis in univariate analyses. Amplification-positive patients exhibited an increased risk of death (Fig. 1A), and the median OS of unamplified patients was much longer than the median OS

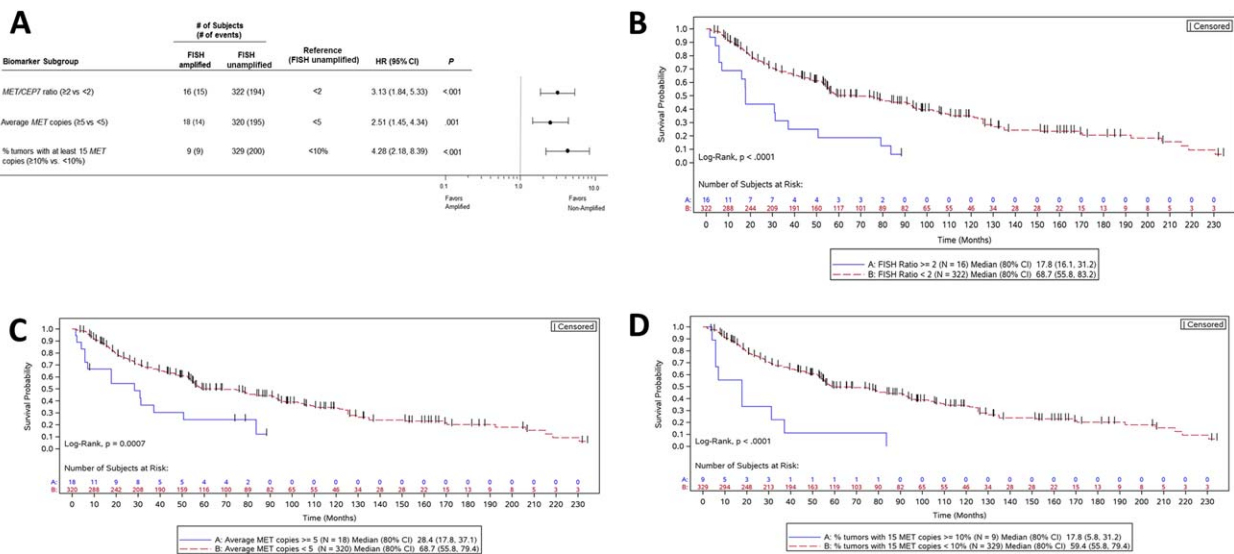


Figure 1. Patient risk and overall survival as assessed by the MET FISH status. (A) Cox proportional hazards model evaluation of FISH classification with respect to overall survival. HRs along with 95% CIs are depicted for MET-amplified tumors versus unamplified tumors as characterized by 3 separate criteria. (B-D) Kaplan-Meier curves depicting the overall survival of subjects with MET-amplified tumors versus unamplified tumors as characterized by (B) the MET/CEP7 ratio, (C) the average gene copy number, and (D) the percentage of tumor cells containing at least 15 copies of MET. CEP7 indicates centromere enumeration probe for chromosome 7; CI, confidence interval; FISH, fluorescence in situ hybridization; HR, hazard ratio.

Biomarker Subgroup	Comparison group	# of Subjects (# of events)		Reference group	HR (95% CI)	P
		Comparison group	Reference group			
MET FISH Ratio	≥2	16 (15)	322 (194)	<2	2.55 (1.27, 5.09)	.008
SRM HER2	Equivocal	19 (14)	215 (136)	Unamplified	1.45 (0.77, 2.70)	.246
	Amplified	20 (14)	215 (136)	Unamplified	1.70 (0.90, 3.19)	.102
Age	≥65	212 (133)	126 (76)	<65	1.59 (1.05, 2.39)	.027
Sex	Male	187 (114)	151 (95)	Female	0.85 (0.59, 1.21)	.361
Race	African American/Asian	26 (18)	64 (40)	Caucasian	0.98 (0.39, 2.49)	.973
	Caucasian-Italian	240 (145)	64 (40)	Caucasian	0.33 (0.15, 0.73)	.006
	Multiple Races/Unknown	8 (6)	64 (40)	Caucasian	1.96 (0.60, 6.46)	.268
Histology	Diffuse	71 (47)	192 (117)	Intestinal	1.02 (0.62, 1.68)	.942
	Other	75 (45)	192 (117)	Intestinal	1.13 (0.74, 1.72)	.571
Biopsy	Biopsy	37 (22)	301 (187)	Resection	0.59 (0.25, 1.40)	.233
Diagnosis	Gastric	298 (184)	40 (25)	EGJ/esophageal	0.93 (0.42, 2.07)	.868
Stage	III	144 (103)	163 (80)	I and II	2.35 (1.63, 3.39)	<.001
	IV	15 (10)	163 (80)	I and II	1.83 (0.49, 6.77)	.366
Lesion Status	Primary	325 (199)	13 (10)	Metastatic	0.54 (0.09, 3.22)	.498
Surgery	Yes	318 (196)	20 (13)	No	0.52 (0.09, 3.03)	.469
Perioperative Therapy	Yes	122 (71)	216 (138)	No	0.67 (0.43, 1.05)	.083

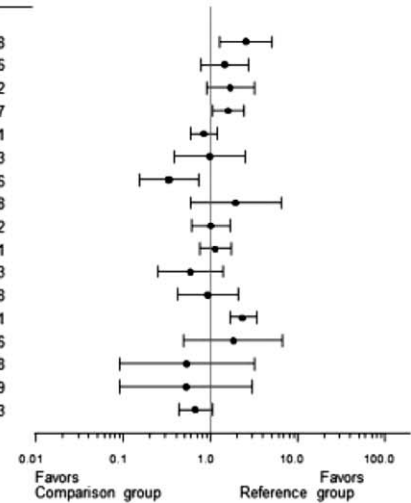


Figure 2. *MET* amplification is an independent indicator of overall survival: Cox proportional hazards model evaluation of the FISH classification with respect to overall survival. A multivariate analysis was performed, and the *MET/CEP7* ratio was used as the approach to FISH characterization. HRs along with 95% CIs for each baseline covariate are depicted. CEP7 indicates centromere enumeration probe for chromosome 7; CI, confidence interval; EGJ, esophagogastric junction; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; SRM, selected reaction monitoring.

of patients who had a *MET/CEP7* ratio ≥ 2 (17.8 vs 68.7 months; $P < .0001$; Fig. 1B), patients who had a mean *MET* GCN ≥ 5 (28.4 vs 68.7 months; $P < .0007$; Fig. 1C), or patients for whom $\geq 10\%$ of tumor cells had ≥ 15 copies of *MET* (17.8 vs 59.4 months; $P < .0001$; Fig. 1D). Using the *MET/CEP7* ratio and adjusting for numerous baseline covariates (age, sex, race, histology, biopsy, diagnosis, stage, lesion status, curative-intent surgery, and perioperative therapy), we found that *MET* amplification (FISH ratio ≥ 2) was independently prognostic of OS (hazard ratio [HR], 2.55; 95% CI, 1.27-5.09; $P < .008$) in comparison with a ratio < 2 (Fig. 2).

According to IHC, 273 of 332 samples (82.2%) exhibited Met expression at any intensity (intensity score $\geq 1+$) and any extensity level (percentage of tumor cells positive; Supporting Table 4 [see online supporting information]). When Met IHC positivity (combined percentages of cells that were positive for Met expression at 1+, 2+, and 3+) was slightly more restrictively defined as any staining intensity $\geq 1+$ in either $\geq 25\%$ or $\geq 50\%$ of tumor cells,^{11,21,26} 117 samples (35.2%) and 43 samples (13.0%), respectively, were scored as positive.

Increased expression of Met protein according to IHC was also indicative of a poor prognosis for GEC in a univariate analysis. Met IHC-positive patients exhibited a worse prognosis, and there was a general upward trend in the HR with increasing IHC extensity staining (Fig. 3A).

Patients whose tumors had $\geq 25\%$ positive membrane staining at an intensity $\geq 1+$ (predefined cutoffs) experienced shorter OS than patients with lower staining levels (54.2 vs 78.4 months; HR, 1.39; 95% CI, 1.04-1.86, $P = .025$). The survival difference between patients at the $\geq 50\%$ positive membrane staining extensity criterion was slightly greater (43.2 vs 78.2 months; HR, 1.62; 95% CI, 1.10-2.28, $P = .013$; Fig. 3B,C). However, a multivariate analysis adjusted for known prognostic covariates indicated that Met IHC, according to these 2 definitions of positivity, was not an independent marker of prognosis ($P = .36$; Fig. 3D).

According to mass spectrometry-based SRM, 231 of 282 samples (81.9%) had Met levels below the lower limit of quantitation of 200 amol/ μg (Supporting Table 5 [see online supporting information]). Protein concentrations in the remaining 51 samples (18.1%) ranged from 200 to 3245.5 amol/ μg .^{11,27} Using expression levels previously established in an independent cohort¹¹ and minimum P value and hazard modeling, we tested and established 400 amol/ μg as the cutoff for Met-positive expression because this value was determined to have the most significant effect on OS (Fig. 4A). As such, 22 tumors (7.8%) were classified as Met-positive in this study. The median OS for patients above this cutoff was significantly shorter than the median OS for their Met-negative counterparts (22.6 vs 59.4 months; HR 1.76

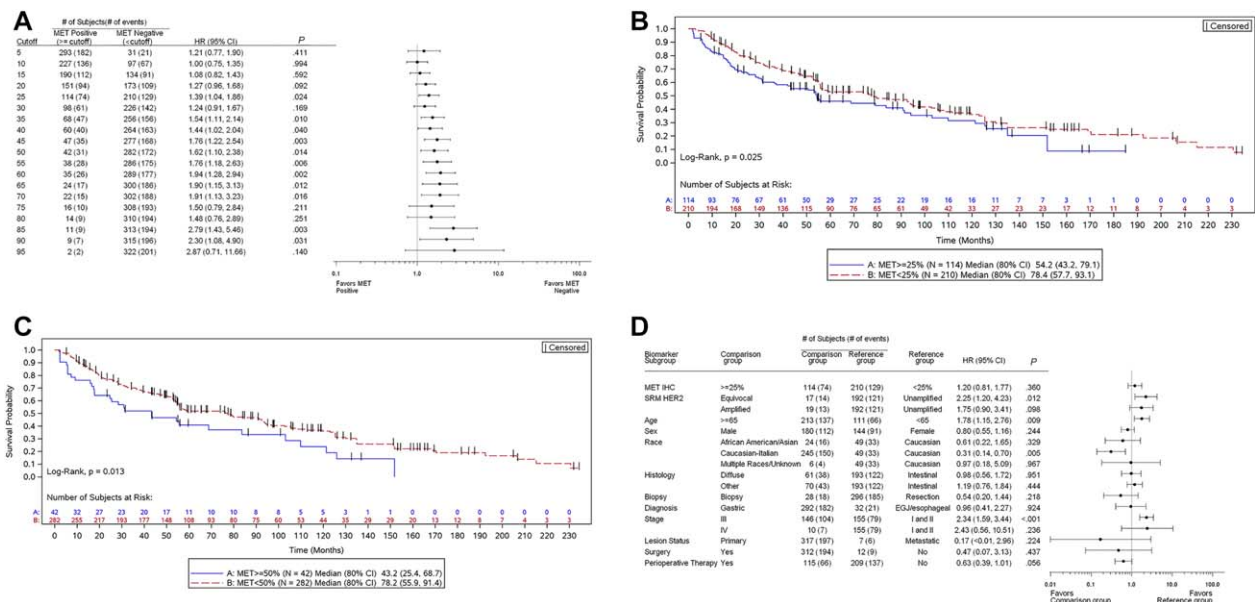


Figure 3. Patient risk and overall survival as assessed by Met IHC expression. (A) Cox proportional hazards model evaluation of IHC classification with respect to overall survival. HRs along with 95% CIs comparing Met-positive and Met-negative patients at each cutoff value are depicted. (B,C) Kaplan-Meier curves depicting the overall survival of the subjects by the Met IHC status with cutoffs of (B) 25% and (C) 50%. (D) Cox proportional hazards model evaluation of the IHC status with respect to overall survival. A multivariate analysis was performed, and 25% staining was used as the Met-positive cutoff. HRs along with 95% CIs for each baseline covariate are depicted. CI indicates confidence interval; EGJ, esophagogastric junction; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; IHC, immunohistochemistry; SRM, selected reaction monitoring.

(95% CI 1.06-2.9); $p = 0.0258$; $P = .0258$; Fig. 4B). After adjustments for covariates, patients with Met expression levels ≥ 400 amol/ μg exhibited a significantly higher risk of death (HR, 1.85; 95% CI, 1.04-3.30; Fig. 4C).

FISH and SRM results were assessed for correlations with the clinicopathological variables listed in Supporting Table 1 (see online supporting information). FISH amplification was associated with an esophageal primary location (16.3%) versus a distal gastric location (3.7%), and a Met SRM level ≥ 400 amol/ μg was associated with a higher tumor stage (III/IV; see online supporting information). There were no other statistically significant correlations observed.

FISH, IHC, and SRM results were also assessed for cross-method sensitivity and specificity; we used subsets of tumor samples that were analyzed by multiple methods (Table 1). With a $MET/CEP7$ ratio ≥ 2 as the reference, Met IHC at the $\geq 25\%$ staining cutoff was 80% sensitive (12 of 15 FISH-positive samples were identified) and 69.2% specific (200 of 289 negative samples were identified) for MET amplification by FISH (Fig. 5A), and there was a significant correlation ($P < .0001$). With SRM at ≥ 400 amol/ μg as the reference, Met IHC exhibited 68.8% sensitivity (11 of 16 Met SRM-positive samples

were identified) with 68.5% specificity (146 of 213 Met SRM-negative samples; $P = .002$; Fig. 5B). Finally, in comparison with a $MET/CEP7$ ratio ≥ 2 , SRM (≥ 400 amol/ μg) identified 5 of 13 MET -amplified tumors (38.5% sensitivity) and 230 of 242 unamplified tumors (95.0% specificity) with a significant correlation between the 2 methods ($P < .001$; Fig. 5C). Thus, although both Met IHC and Met SRM correlated with MET FISH amplification status in this study. In addition, although a very limited subset was used, applying a previously defined cutoff of ≥ 1500 amol/ μg ¹¹ resulted in a Met SRM specificity of 100% for identifying MET amplification (no negative FISH samples were identified within this high Met-positive SRM cutoff).

DISCUSSION

This study evaluated Met as a prognostic biomarker for GEC by contrasting the technical methods of FISH, IHC, and SRM and by assessing various criteria for defining genomic or proteomic positivity. Moreover, an association of each of these methods/definitions with clinical outcomes was evaluated via univariate and covariate adjusted analyses. The findings are consistent with some

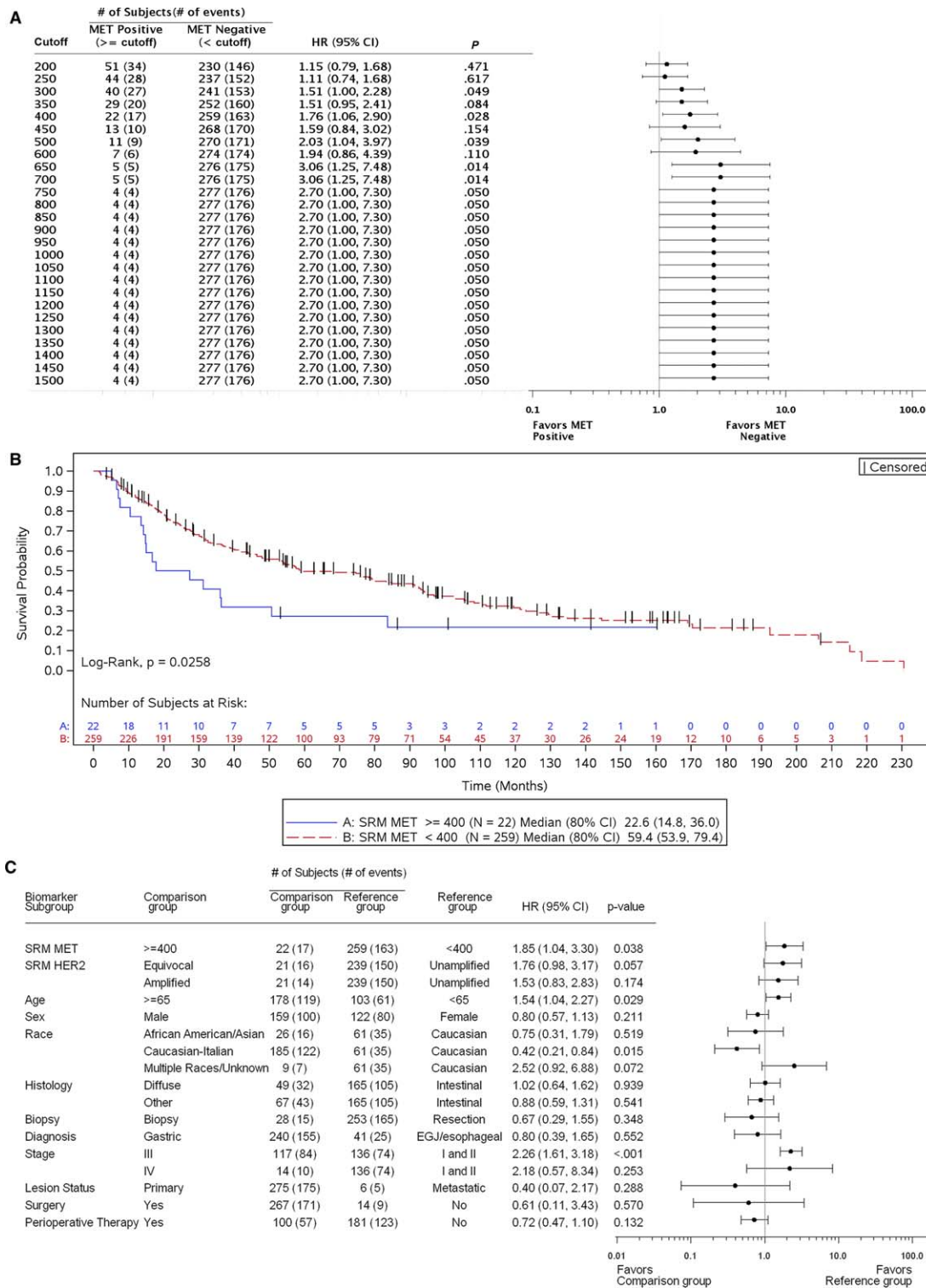


Figure 4. Patient risk and overall survival as assessed by Met SRM expression. (A) Cox proportional hazards model evaluation of Met SRM quantitation with respect to overall survival. HRs along with 95% CIs comparing Met-positive and Met-negative patients at each cutoff value are depicted. The number of *MET*-amplified tumors present within each Met-positive cutoff group is presented. (B) Kaplan-Meier curves depicting the overall survival of subjects by Met SRM expression with 400 amol/μg as the Met-positive cutoff. (C) Cox proportional hazards model evaluation of Met SRM expression with respect to overall survival. A multivariate analysis was performed, and 400 amol/μg was used as the Met-positive cutoff. HRs along with 95% CIs for each baseline covariate are depicted. CI indicates confidence interval; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; SRM, selected reaction monitoring

		MET FISH Status			P
		Amplified	Not amplified	Total	
Met IHC (≥25%)	Positive	12	89	101 (33.2%)	<.0001
	Negative	3	200	203 (66.8%)	
Total		15 (4.9%)	289 (95.1%)	304 (100%)	

		Met SRM (≥400 amol/μg)			P
		Positive	Negative	Total	
Met IHC (≥25%)	Positive	11	67	78 (34.1%)	.002
	Negative	5	146	151 (65.9%)	
Total		16 (7.0%)	213 (93.0%)	229 (100%)	

		MET FISH Status			P
		Amplified	Not amplified	Total	
Met SRM (≥400 amol/μg)	Positive	5	12	17 (6.7%)	<.001
	Negative	8	230	238 (93.3%)	
Total		13 (5.1%)	242 (94.9%)	255 (100%)	

Figure 5. Correlation of *MET*/MET analytical approaches. *MET* gene amplification results were compared with (A) Met IHC and (C) Met SRM cutoff-derived protein expression; (B) the correlation between Met IHC and Met SRM was also examined. FISH indicates fluorescence in situ hybridization; IHC, immunohistochemistry; SRM, selected reaction monitoring.

smaller studies demonstrating similar concordance patterns between Met SRM and IHC and/or FISH¹¹ and comparable adverse prognoses in univariate analyses when the Met biomarker (FISH, IHC, or SRM) was positive.^{10,12,15,21} Notably, the findings are unique in demonstrating an independently worse prognosis for amplification as determined by the FISH ratio and overexpression by SRM according to an adjusted multivariate analysis; in contrast, IHC with the currently applied positivity criteria was not an independent prognostic biomarker.²⁶

Numerous complexities contribute to determining the strength of Met as a predictive and/or prognostic biomarker; these can often be associated with a lack of consensus on a diagnostic assay (FISH, next-generation sequencing, IHC, and SRM) and varying reported incidence rates of (and thresholds for) Met positivity (Supporting Table 6 [see online supporting information]). In

this study, we assessed the prognostic value of *MET* amplification by FISH and Met expression by IHC and SRM without consideration of their predictive value for the benefit of anti-Met therapy or of other emerging prognostic/predictive Met biomarkers, including *MET* mutations and/or exon 14 skipping, HGF aberrations, and Met aberrations within circulating tumor cells or circulating tumor DNA.^{5,6,12,28-33,41-44}

Generally, *MET* amplification denotes an increased GCN, is considered a genomic driver of the tumor, and results in extreme overexpression of the Met protein.^{11,14} However, with respect to amplification and consequent overexpression, the degree of amplification is important to consider: low-level amplification (eg, *MET/CEP7* ratio = 3:1.5 = 2) generally does not correlate with extreme overexpression of the protein (Supporting Table 6 [see online supporting information]). This was previously demonstrated with the FISH ratio and SRM expression

both evaluated as linear variables in comparison with a binary FISH ratio ≥ 2 .¹¹ This is also quite analogous to human epidermal growth factor receptor 2 (*HER2*) amplification, as recently described.²⁷ Therefore, the ease of categorizing into binary subsets (ratio \geq or < 2) should be weighed against the weaker observed correlation with expression when higher and lower FISH ratios are combined within 1 category. This may have implications for the strength of FISH as a prognostic and predictive biomarker. Ultimately, targeted therapies inhibit proteins, so excluding patients with gene amplification (low-level) who lack resulting overexpression may enrich the cohort most likely to benefit. Regardless, the rates of *MET* amplification in this study with a FISH ratio ≥ 2 (4.7%), a FISH GCN ≥ 5 (5.2%), and a FISH GCN ≥ 15 in $> 10\%$ of cells (2.6%) were consistent with rates previously reported,^{9-11,13,15} and they were the poorest prognostic factors among the *MET* biomarkers evaluated. This is intuitive because when it is amplified, *MET* is the driver oncogene portending significant metastatic and aggressive tumor behavior. In this setting, Met is exceptionally overexpressed (several-fold higher than Met-expressing tumors in the absence of gene amplification).¹¹ Although a lower SRM cutoff of ≥ 400 amol/ μg (set to define overexpression and not amplification) had low accuracy in discerning *MET* amplification by FISH, the predefined level of ≥ 1500 amol/ μg did show 100% specificity (in a small cohort analyzed with both FISH and SRM analysis). This level of ≥ 1500 amol/ μg has consistently demonstrated excellent accuracy in identifying truly cluster *MET*-amplified tumors.¹¹ However, when heterogeneous *MET* amplification was present within a sample, FISH identified the amplified areas, whereas SRM represented the aggregate Met expression level of the entire tumor; this resulted in lower than expected expression levels of a homogeneously amplified tumor (Supporting Fig. 1 [see online supporting information]).¹¹

In contrast to amplification, Met protein overexpression determined by IHC or SRM includes the small subset with overexpression due to gene amplification but consists mainly of expression without amplification. The incidence of Met overexpression depends on the lower limit set for positivity. Indeed, the rate reportedly varies widely from 24% to 70%, in part because of the variability of this lower limit along with other analytic variables associated with IHC, the assay typically used for Met expression. As previously described, Met SRM is more objective than IHC and correlates better with FISH ratio and GCN values.¹¹ This was confirmed in this study: Met overexpression, as determined by SRM, accounted for

18.1% of the samples at any level above the lower limit of detection (≥ 200 amol/ μg) and for 7.8% of the samples at the predefined threshold of ≥ 400 amol/ μg . In contrast, 82.2% of the samples in this study demonstrated any positivity by IHC, and 35.2% and 13% demonstrated any positivity by any staining intensity (intensity ≥ 1 + in $\geq 25\%$ and $\geq 50\%$ of tumor cells, respectively). Interestingly, a recent phase 3 trial, RILOMET-1, reported a Met positivity rate of 81% versus a phase 2 rate of 64% with the Dako IHC criteria (intensity ≥ 1 + with extensity $\geq 25\%$); this suggests interstudy variability even with the same IHC antibody and scoring.^{21,26} In addition to observed variability between the 2 rilotumumab trials using the Dako antibody, it is also possible that the rate of Met positivity differs between locally advanced disease and metastatic disease and between primary tumor and metastatic biopsies.

In this study, although IHC was associated with a poor prognosis in the univariate analysis, this did not persist after adjustments for other prognostic covariates. The low-level expression of Met may be associated with these poor prognostic covariates yet not be independently associated with outcomes upon adjustments. In contrast, extreme overexpression as a result of *MET* amplification or by SRM (determined here to be ≥ 400 amol/ μg) in the absence of *MET* amplification remained prognostic after adjustments for other variables.

Finally, Met may be a negative predictive biomarker for standard cytotoxic therapies^{15,34} and a positive predictive biomarker for Met-directed therapies, particularly when one is selecting for *MET* amplification.^{13-15,35,36} However, all trials to date selecting for *MET* amplification have been open-label, single-arm trials, undoubtedly because of the low incidence of *MET* amplification, aggressive tumors with a quickly progressing clinical course, and difficulty in accruing cases for proper randomization. Because of the putative negative prognosis rendered by *MET* amplification, single-arm trials may underestimate the benefit of anti-Met therapies if this overall poor baseline prognosis is not considered. On the other hand, despite early evidence for the predictive value of anti-Met therapy for tumors that overexpress Met (regardless of the GCN/amplification status),^{21,23} 2 recent phase 3 trials, RILOMET-1 (with the anti-HGF antibody rilotumumab) and METGastric (with the anti-Met antibody onartuzumab), both failed to meet their primary endpoints in Met-positive patients as determined by 2 different IHC methods.^{26,37} Although it is quite plausible that Met inhibition, in the setting of Met overexpression lacking amplification, is not sufficient to improve outcomes (ie, a

negative prognostic biomarker is not by default also a positive predictive biomarker for a targeted therapy), it is also possible that the positivity criteria were too lenient. The phase 1 patient who responded to onartuzumab monotherapy²³ was later found to have an expression level of 526.93 amol/ μg .¹¹ Indeed, the positivity rate in RILOMET-1 ($\geq 1 +$ intensity, $\geq 25\%$ extensity) was 81%, with only 21% of the patients (71 patients on rilotumumab vs 57 patients on a placebo) having higher level expression ($\geq 2 +$ intensity, $\geq 50\%$ extensity).²⁶ In fact, in the METGastric trial, a predefined subset analysis (IHC: $\geq 2 +$ intensity, $\geq 50\%$ extensity) suggested improved OS with an HR of 0.64 ($P = .06$) with 105 and 109 patients in the onartuzumab and control arms, respectively, or 38% of all enrolled patients. Because the trial closed early with only 70% of the intended accrual (562 of 800), the intended power to detect an HR of 0.49 in the higher expressing subset was compromised; moreover, if the more likely true HR is 0.6 to 0.8, the trial was significantly underpowered to detect this true benefit. Unfortunately, because METGastric and even more so RILOMET-1 did not have sufficient power to evaluate the benefit in those samples/patients with higher expression cutoffs, they do not provide guidance for this select patient population. Coupling these results with the inherent limitations associated with IHC (antigen dependency, variability in tissue staining, lack of temporal reproducibility, and subjective scoring of positive staining) suggests that the technique may not be a definitive method for identifying patients likely to respond to Met-targeted therapy. Regardless, it is clear that low-level Met expression or, worse, treatment of all-comers with no selection does not predict a benefit from anti-Met agents.^{26,37,38}

In summary, *MET* FISH and Met SRM were independently associated with a poor prognosis in this large cohort of GEC patients, whereas Met IHC at the currently applied positivity cutoffs was not independently associated with outcomes. SRM is a novel technology demonstrating clinical utility with Met and Her2^{11,27,39,45} and additionally has the advantage of multiplex peptide analysis,²² which is analogous to next-generation sequencing for genomic aberrations. The results of this study suggest that amplification by FISH and/or overexpression by SRM should be incorporated into multivariate models when one is assessing the prognostic significance of other novel covariates. Moreover, amplification and overexpression, as determined by FISH and SRM, may better direct treatment and, in particular, Met-targeted therapies for GEC principally in the setting of next-generation clinical trial designs.⁴⁰

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CONFLICT OF INTEREST DISCLOSURES

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AUTHOR CONTRIBUTIONS

Daniel V. T. Catenacci: Study conception and design, clinical trial and translational statistical design, sample collection, assay performance, data management, data interpretation, and manuscript writing and editing. **Agnes Ang:** Study conception and design, sample collection, assay performance, data management, data interpretation, and manuscript writing and editing. **Wei-Li Liao:** Sample collection, assay performance, data management, and manuscript writing and editing. **Jing Shen:** Clinical trial and translational statistical design and manuscript writing and editing. **Emily O'Day:** Sample collection, assay performance, data management, and manuscript writing and editing. **Robert D. Loberg:** Data interpretation and manuscript writing and editing. **Fabiola Cecchi:** Manuscript writing and editing. **Todd Hembrough:** Study conception and design, data interpretation, and manuscript writing and editing. **Annamaria Ruzzo:** sample collection, assay performance, data management, and manuscript writing and editing. **Francesco Graziano:** Study conception and design, data interpretation, and manuscript writing and editing.

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