

Detection of *MET* amplification in gastroesophageal tumor specimens using IQFISH

Jan Trøst Jørgensen¹, Karsten Bork Nielsen², Jens Møllerup², Anna Jepsen², Ning Go³

¹Department of Companion Diagnostic Research, Dx-Rx Institute, Fredensborg, Denmark; ²R&D, Diagnostics and Genomics Group, Agilent Technologies Denmark Aps, Glostrup, Denmark; ³Medical Sciences, Amgen Inc., Thousand Oaks, CA, USA

Contributions: (I) Conception and design: JT Jørgensen, KB Nielsen, N Go; (II) Administrative support: All authors; (III) Provision of study materials or patients: JT Jørgensen, KB Nielsen, A Jepsen, N Go; (IV) Collection and assembly of data: JT Jørgensen, KB Nielsen, N Go; (V) Data analysis and interpretation: JT Jørgensen, KB Nielsen, J Møllerup, N Go; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Jan Trøst Jørgensen. Dx-Rx Institute, Baunevaenget 76, DK-3480 Fredensborg, Denmark. Email: jan.trost@dx-rx.dk.

Background: The gene mesenchymal epithelial transition factor (*MET*) is a proto-oncogene that encodes a transmembrane receptor with intrinsic tyrosine kinase activity known as Met or cMet. *MET* is found to be amplified in several human cancers including gastroesophageal cancer.

Methods: Here we report the *MET* amplification prevalence data from 159 consecutive tumor specimens from patients with gastric (G), gastroesophageal junction (GEJ) and esophageal (E) adenocarcinoma, using a novel fluorescence in situ hybridization (FISH) assay, *MET/CEN-7* IQFISH Probe Mix [an investigational use only (IUO) assay]. *MET* amplification was defined as a *MET/CEN-7* ratio ≥ 2.0 . Furthermore, the link between the *MET* signal distribution and amplification status was investigated.

Results: The prevalence of *MET* amplification was found to be 6.9%. The FISH assay demonstrated a high inter-observer reproducibility. The inter-observer results showed a 100% overall agreement with respect to the *MET* status (amplified/non-amplified). The inter-observer CV was estimated to 11.8% (95% CI: 10.2–13.4). For the signal distribution, the inter-observer agreement was reported to be 98.7%. We also report an association of *MET* amplification and a unique signal distribution pattern in the G/GEJ/E tumor specimens. We found that the prevalence of *MET* amplification was markedly higher in tumors specimens with a heterogeneous (66.7%) versus homogeneous (2.0%) signal distribution. Furthermore, specimens with a heterogeneous signal distribution had a statically significantly higher median *MET/CEN-7* ratio (2.35 versus 1.04; $P < 0.0001$).

Conclusions: The novel FISH assay showed a high inter-observer reproducibility both with respect to amplification status and signal distribution. Based on the finding in the study it is suggested that *MET* amplification mainly is associated with tumor cells that is represented by a heterogenous growth pattern.

Keywords: Mesenchymal epithelial transition factor (*MET*); fluorescence in situ hybridization (FISH); gastroesophageal cancer; amplification; reproducibility; signal distribution

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Introduction

The gene mesenchymal epithelial transition factor (*MET*) is located on chromosome 7, which is a proto-oncogene that encodes a transmembrane receptor with intrinsic tyrosine kinase activity known as Met (or cellular-MET, c-Met). This receptor is also called the hepatocyte growth factor receptor (HGFR) after its ligand; hepatocyte growth factor (HGF). Under normal circumstances HGF activation of Met is tightly controlled by mechanisms such as paracrine ligand delivery and ligand activated receptor internalization and degradation. Despite these controls, the HGF/Met signaling contributes to oncogenesis and tumor progression in several human cancers, including gastroesophageal, colorectal, lung, breast, renal and more (1,2).

In gastroesophageal cancer, *MET* amplification has been reported with a prevalence of 1.5% to 30.5% (2-8), depending on the method used for the detection and the assay cut-off. *MET* amplification has been shown to be a factor of poor prognosis in gastroesophageal cancer (4,6,8,9). It has been suggested that *MET* amplification is associated with higher grade and higher stage tumors, and with a shorter median overall survival (3). The association between *MET* amplification and a poor prognosis in gastroesophageal cancer has also been confirmed in a recent meta-analysis (4,10). Beside the prognostic characteristics it has been suggested that *MET* amplification in gastroesophageal cancer potentially possess predictive properties in relation to Met targeted therapy and, thereby, could act as a companion diagnostic for some of the new therapies under development (3,11). Furthermore, in non-small cell lung cancer it has been suggested that *MET* amplification is involved with acquired resistance to EGFR tyrosine kinase inhibitors (12,13).

Detection of *MET* amplification can be performed by several techniques such as Southern blot, polymerase chain reaction (PCR), and fluorescence in situ hybridization (FISH). Here we report data on *MET* amplification in tumor specimens from patients with gastric (G), gastroesophageal junction (GEJ) and esophageal (E) adenocarcinomas with a *MET* FISH assay using the formamide-free fast IQFISH hybridization buffer (14). The purpose of this study was to investigate the inter-observer reproducibility, and to evaluate the *MET* signal distribution and the prevalence of *MET* amplification in tissue specimens from patients with G/GEJ/E adenocarcinomas.

Methods

Specimens

The study included 159 formalin-fixed and paraffin-embedded G/GEJ/E adenocarcinoma specimens. These specimens were collected consecutively from the screen population related to a phase II study with the investigational Met tyrosine kinase inhibitor AMG337 (Amgen, Thousand Oaks, CA, USA) (11). All specimens were anonymized with respect to the identity of the patients and no data on patient demographic was available. The study was conducted according to the Helsinki Declaration, and informed consent was obtained from the patients before testing was conducted. Before study initiation the protocol was reviewed and approved by the Chesapeake Institutional Review Board, Columbia, MD, USA. Evaluation of the stained slides was performed independently by two different technologists and subsequently reviewed independently by two pathologists.

MET IQFISH testing

The *MET* FISH staining was performed using the *MET*/CEN-7 IQFISH Probe Mix [for investigational use only (IUO), Agilent Technologies, Glostrup, Denmark] in combination with the Histology FISH Accessory Kit (Agilent Technologies) reagents and IQFISH staining procedure. Briefly, the FFPE specimens mounted on positively charged glass slides were exposed to heat pre-treatment using microwave oven followed by pepsin digestion at 37 °C to prepare the tissue for probe hybridization. Co-denaturation of target DNA in the specimens and the probe was done for 10 min at 66 °C followed by hybridization at 45 °C for 90 min using a Hybridizer (Agilent Technologies). The hybridization was performed simultaneously for the Texas Red-labeled DNA probe (*MET*) and the fluorescein-labeled PNA probe (CEN-7). The tissue specimens were subjected to stringent wash at 63 °C for 10 min before dehydration and drying. The dried slides were subsequently mounted using Fluorescence Mounting Medium containing DAPI.

The *MET* FISH stained slides were evaluated using a fluorescence microscope with 20× and 40× objectives. The enumeration was performed with a 100× objective. The *MET*/CEN-7 ratio was calculated by enumeration of 20 nuclei from the invasive tumor area. Based on the ratio, specimens were categorized into amplified (*MET*/CEN-7 ≥ 2.0) or non-amplified (*MET*/CEN-7 < 2.0) categories.

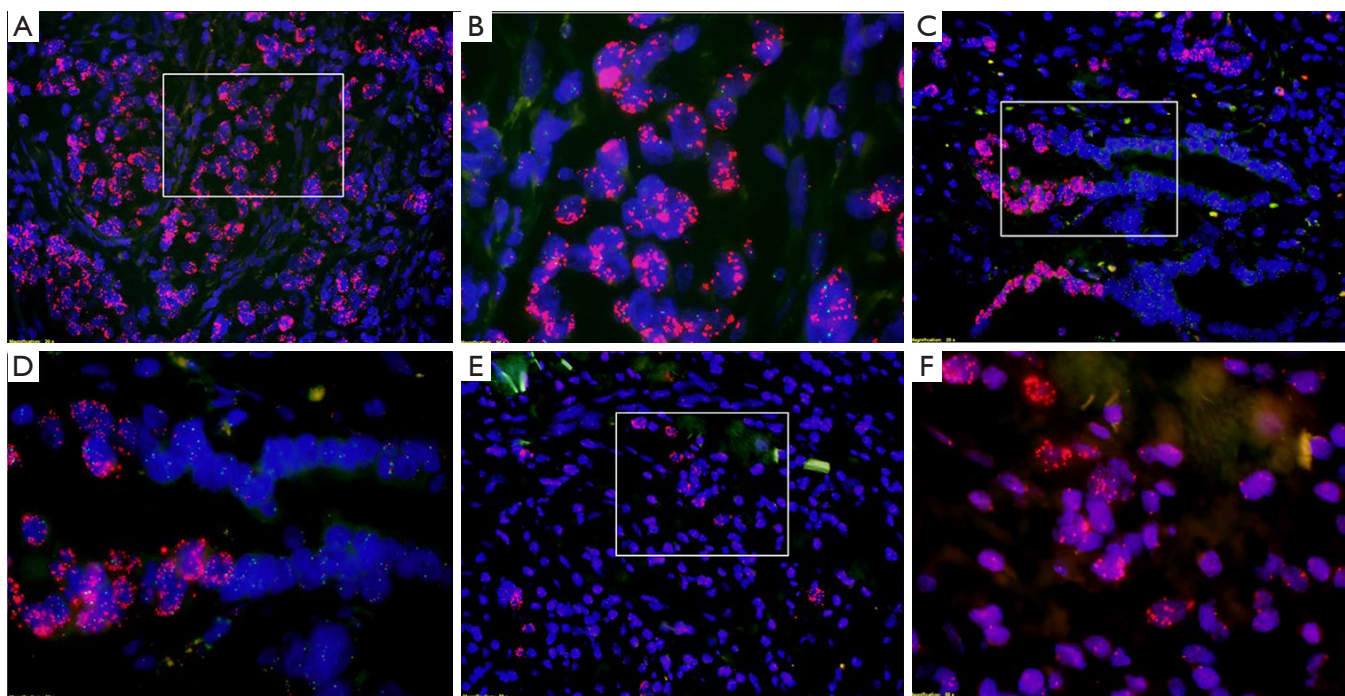


Figure 1 Images of the different type of signal distributions in gastroesophageal cancers the left column (20× objective) and right column (50× objective), *MET* IQFISH testing. (A,B) Illustrate homogeneous signal distribution [G tumor specimen with a *MET*/CEN-7 ratio of 9.11]; (C,D) illustrate heterogeneous focal signal distribution (GEJ tumor with a *MET*/CEN-7 ratio of 7.00); (E,F) illustrates heterogeneous mosaic signal distribution (G tumor with a *MET*/CEN-7 ratio of 6.97). *MET*, mesenchymal epithelial transition factor; G, gastric; GEJ, gastroesophageal junction.

Specimens with a ratio between 1.8 and 2.2 (borderline cases) were subjected to enumeration of additional 20 nuclei and the ratio was then recalculated for the 40 nuclei to determine if amplification was present or not. Normal cells within the specimen served as an internal control for successful staining. Normal cells should exhibit the ratio expected for normal diploid cells with a one-to-one relationship of *MET* gene and centromere-7 signals.

MET signal distribution

Beside the evaluation of *MET* FISH status (amplified/non-amplified) the *MET* signal distribution was also evaluated. In the gastroesophageal cancer specimens, the *MET* signal can be classified as having a homogeneous or heterogeneous distribution. A homogeneous signal distribution is when the majority of the tumor cells in a specimen are equally amplified or equally non-amplified. For the heterogeneous signal distribution, different levels of amplification are

observed in the cells in the specimen. Tumor specimens with a heterogeneous signal distribution can be sub-divided into two subcategories. If the *MET* amplified tumor cells are grouped together, the signal distribution is categorized as focal, whereas, if the *MET* amplified tumor cells in the specimen are interspersed with cells with a lower or normal *MET*/CEN-7 ratio, the signal distribution is categorized as mosaic. *Figure 1* shows examples of the different type of signal distributions. A similar phenotypical signal distribution pattern has been described for *HER2*, another gene known to be amplified in a subset of patients with gastroesophageal cancer (15).

Statistical analyses

Mainly descriptive statistical methods were used. For the inter-observer reproducibility, the coefficient of variation (CV) was calculated after Log transformation of data. The Wilcoxon ranks sum test was used to test the difference in

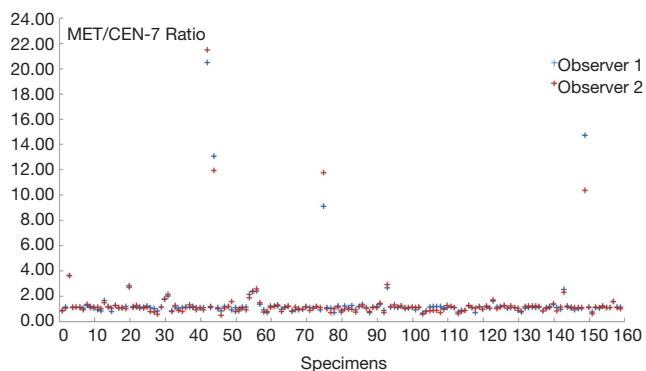
Table 1 Characteristics of the study specimens

Characteristics	n	Percent (%)
Type of tumor		
Gastric	106	66.7
Gastroesophageal junction	24	15.1
Esophageal	29	18.2
Tumor site		
Primary tumor	155	97.5
Recurrence at primary site	3	1.9
Not provided	1	0.6
Type of sample		
Resection	58	36.5
Biopsy	101	63.5

Table 2 Inter-observer agreement

Factors	Observer 1	Observer 2
<i>MET</i> non-amplification	148	148
<i>MET</i> amplification*	11	11
Total	159	159
<i>MET</i> /CEN-7 ratio		
Median (range)	1.05 (0.62–20.41)	1.03 (0.44–21.43)
Mean (SD)	1.43 (2.20)	1.40 (2.16)

*, *MET* amplification: *MET*/CEN-7 ratio ≥ 2.0 . *MET*, mesenchymal epithelial transition factor.

**Figure 2** The individual *MET*/CEN-7 ratios obtained by the two observers for the 159 tumor specimens.

median *MET*/CEN-7 ratios between tumor specimens with

a homogeneous and a heterogeneous signal distribution. Excel and SAS JMP PRO v11.0.0 was used for the statistical analyses.

Results

MET status and inter-observer reproducibility

A total of 159 consecutive G/GEJ/E adenocarcinoma specimens were included in the study. An overview of the characteristics of the specimens with respect to type of tumor, sites and type of sample are given in *Table 1*. Two thirds (66.7%) of the specimens tested originates from the stomach and the remaining one third from esophagus or the GEJ region. Most of the specimens were biopsies (63.5%) which nearly all (97.5%) were taken from the primary tumor site. Both observers found that the same 11 out of 159 samples were *MET* amplified giving a prevalence of 6.9% and hence an inter-observer agreement of 100%, as shown in *Table 2*. Using Log data transformation, the inter-observer CV for the *MET*/CEN-7 ratio was estimated at 11.8% (95% CI: 10.2–13.4). The individual *MET*/CEN-7 ratios obtained by the two observers for the 159 tumor specimens are plotted in *Figure 2*. Observers agreed on signal distribution in 98.7% of specimens (157 out of 159), i.e., for one specimen observer disagreed on focal *vs.* mosaic heterogeneous signal distribution and for one specimen homogeneous *vs.* heterogeneous mosaic signal distribution.

Signal distribution

Beside the calculation of the *MET*/CEN-7 ratios and the evaluation of the amplification status, a qualitative assessment of the *MET* signal distribution pattern was performed. This assessment showed that 12 out of the 159 tumor specimens exhibit a heterogeneous signal distribution, either focal or mosaic. *Table 3* gives the prevalence of *MET* amplification as well as the median and the mean *MET*/CEN-7 ratios for the specimens according to their signal distribution. Eight out of the 12 (66.7%) specimens that had a heterogeneous signal distribution were amplified, whereas this only applied to 3 out of 147 (2.0%) for the specimens with a homogeneous signal distribution. Both the mean and the median *MET*/CEN-7 ratios were markedly higher for the specimens with a heterogeneous signal distribution. Comparing the median *MET*/CEN-7 ratios for the two groups showed a statistically significantly higher value for the specimens with a heterogeneous

Table 3 Signal distribution and *MET* amplification

Factors	Heterogeneous signal distribution	Homogeneous signal distribution
Specimens tested	12	147
Specimens with <i>MET</i> amplification*	8	3
Percent of specimens with <i>MET</i> amplification* (%)	66.7	2.0
<i>MET</i> /CEN-7 ratio		
Median (range)	2.35 (1.52–16.68)	1.04 (0.62–20.41)
Mean (SD)	4.06 (4.60)	1.52 (1.75)

*, *MET* amplification: *MET*/CEN-7 ratio ≥ 2.0 . *MET*, mesenchymal epithelial transition factor.

signal distribution compared to the specimens with a homogeneous signal distribution ($P < 0.0001$, Wilcoxon rank sum test).

Discussion

In the current study, *MET* amplification was detected in 6.9% of the 159 consecutive specimens originating from individual patients with gastroesophageal cancer. The vast majority of the tested specimens were primary tumors and mainly taken as biopsies from the stomach. Comparing the prevalence of *MET* amplification in this study with the relatively few data available in literature, the prevalence seems to be in the lower end of the reported range. For gastroesophageal cancer, the prevalence of *MET* amplification has been reported in the range of 1.5% to 30.5% (2-8). However, when such data is compared, it is important also to take the analysis method used and the assay cut-off into consideration. The highest prevalence reported originate from Southern blot analyses and PCR based assays as these methods are unable to distinguish between true gene amplification and polysomy, which then result in higher estimates for the prevalence of *MET* gene amplification (5). The *MET* amplification prevalence of 6.9% observed in this study is within the range 1.5% to 8.3% found in other studies by means of FISH (2,5,6).

The overall inter-observer agreement with respect to the *MET* status (amplified/non-amplified) was 100%. The two observers detected the same 11 tissues specimens as being *MET* amplified. Furthermore, the median and mean *MET*/CEN-7 ratios obtained by the two observers were almost identical as can be seen from *Table 2*. The largest differences between the two observers were found for the specimens that had the highest *MET*/CEN-7 ratios, as shown in *Figure 2*, where enumeration of the *MET* hybridization

signals could be challenging. However, these differences have no influence on the *MET* status determination, with regards to amplified or non-amplified, as they lie far above the assay cut-off at a *MET*/CEN-7 ratio of 2.0. Discrepancy between observers for the higher gene/centromere ratios has also been reported for *HER2* using the FISH method (16,17), and is due to the nature of enumerating signals in the nuclei. When high amplification levels are present, signals may lie in clusters, where it is difficult or impossible to enumerate the precise signal numbers. In such situations, a qualified estimate of every cluster in the relevant nuclei is performed, which will introduce variation between observers. The inter-observer CV was estimated at 11.8%, which is in the lower end of previous reported CVs for this type of assay (17).

As part of the study a qualitatively assessment of the *MET* signal distribution pattern at the tissue level was performed. When the 159 tumor specimens were divided into homogeneous and heterogeneous signal distribution patterns, as shown in *Table 3*, the prevalence of *MET* amplification was much higher in the group of patients who harbored tumors with a heterogeneous signal distribution compared to the group with a homogeneous signal distribution. In the group with a heterogeneous signal distribution 8 out of the 12 (66.7%) tumor specimens were amplified, whereas this only was the situation for 3 out of 147 (2.0%) for the tumor specimens with a homogeneous signal distribution. We also found that the median *MET*/CEN-7 ratio for the tumor specimens with a heterogeneous signal distribution was significantly higher than for the tumor specimens with a homogeneous signal distribution.

Intra tumor heterogeneity and tumor aneuploidy are important mechanisms for development of drug resistance, which possess major challenges in relation to anti-cancer therapy (18). The different *in situ* hybridization techniques

offers the possibility of combining genetic/chromosomal information with spatial phenotypic characteristics of the tumor. The FISH data from the current study indicate that a specific phenotypic characteristic is linked to *MET* amplification, which could suggest that the *MET* positive tumor cells are dominated by a heterogeneous signal distribution pattern in gastroesophageal cancer. Recent published data on the *HER2* gene has likewise shown a similar link between heterogeneous signal distribution and gene amplification and hence the associated tumor growth pattern (15).

To the best of our knowledge this relationship between *MET* amplification and a heterogeneous signal distribution has not previously been described for gastroesophageal cancer. As this specific relationship, also has been shown for *HER2* this might be a common feature linked to gene amplification in gastroesophageal cancer (15). However, further studies will be needed to confirm these findings and show if this relationship will have prognostic and/or predictive implications for patients with *MET* amplified gastroesophageal cancer. Furthermore, we underline that the hypothesis raised about the link between *MET* amplification and the heterogeneous signal distribution in gastroesophageal cancer is generated on a relatively small number of tumor specimens and therefore must be validated in a larger study.

Conclusions

The novel FISH assay showed a high inter-observer reproducibility both with respect to *MET* status and the signal distribution pattern. Based on the finding in the study it is suggested that *MET* amplification mainly is associated with tumor cells that is represented by a heterogenous growth pattern. However, due to the relative small sample size in the study the signal distribution pattern findings require further validation.

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None.

Footnote

Conflicts of Interest: N Go is an employee of Amgen. KB Nielsen, J Mollerup, and A Jepsen are employees of Agilent Technologies. JT Jørgensen has worked as a consultant for Agilent Technologies, Euro Diagnostica, and Oncology

Ventures and has given lectures at meetings sponsored by AstraZeneca, Merck Sharp & Dohme, and Roche.

Ethical Statement: The study protocol was approved by the Chesapeake Institutional Review Board (No. Pro00009139), approval date December 19, 2013. Written informed consent was obtained from the patients before testing was conducted.

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