Detection of MET mRNA in gastric cancer in situ. Comparison with immunohistochemistry and sandwich immunoassays

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Abstract

Determination of predictive biomarkers by immunohistochemistry (IHC) relies on antibodies with high selectivity. RNA in situ hybridization (RNA ISH) may be used to confirm IHC and may potentially replace it if suitable antibodies are not available or are insufficiently selective to discriminate closely related protein isoforms. We validated RNA ISH as specificity control for IHC and as a potential alternative method for selecting patients for treatment with MET inhibitors. MET, the HGF receptor, is encoded by the MET proto-oncogene that may be activated by mutation or amplification. MET expression and activity were tested in a panel of control cell lines. MET could be detected in formalin fixed paraffin, embedded (FFPE) samples by IHC and RNA ISH, and this was confirmed by sandwich immunoassays of fresh frozen samples. Gastric cancer cell lines with high MET expression and phosphorylation of tyrosine-1349 respond to the MET inhibitor, BAY-853474. High expression and phosphorylation of MET is a predictive biomarker for response to MET inhibitors. We then analyzed MET expression and activity in a matched set of FFPE vs. fresh frozen tumor samples consisting of 20 cases of gastric cancer. Two of 20 clinical samples investigated exhibited high MET expression with RNA ISH and IHC. Both cases were shown by sandwich immunoassays to exhibits strong functional activity. Expression levels and functional activity in these two cases were in a range that predicted response to treatment. Our findings indicate that owing to its high selectivity, RNA ISH can be used to confirm findings obtained by IHC and potentially may replace IHC for certain targets if no suitable antibodies are available. RNA ISH is a valid platform for testing predictive biomarkers for patient selection.

Key words: immunohistochemistry, MET, RNA ISH, sandwich immunoassay

The development of new cancer therapies increasingly depends on the use of predictive biomarkers for patient selection, because targeted therapies address decreasing subpopulations. Because biomarkers are becoming increasingly embedded into the early phases of drug development, understanding the target biology, and robust and validated assays are essential for identifying the appropriate patients for particular treatments.

The use of predictive biomarkers for patient selection faces three major challenges: availability of relevant tumor material, intratumor heterogeneity and availability of validated assays. Also important, however, is the development of a

responder hypothesis, which defines a quantitative threshold that is necessary for response to treatment during preclinical studies. From a series of preclinical models, the properties necessary for response to treatment, e.g., the extent of target expression, must be deduced. The biomarker assay must be sensitive and robust, especially near the response threshold, to discriminate potential responders from non-responders.

Gastric cancer is the third most frequent cause of death from cancer among men and fifth among women worldwide. More than 700,000 deaths worldwide and 10,700 deaths in the US are caused by gastric cancer each year (Torre et al. 2015, Siegel et al. 2016). Although the prognosis is very good for cases of asymptomatic early stage disease detected by endoscopic screening (Ono et al. 2001, Yada et al. 2013), the prognosis is poor for advanced cancer stages with less than one year median survival with conventional chemotherapies (Bilici 2014).

Trastuzumab was approved in 2010 as the first targeted agent for advanced gastric cancer. Her2 amplifications or overexpression have been reported in 7–34% of investigated tumors (Gravalos and Jimeno 2008) and trastuzumab combined with chemotherapy improved overall survival significantly compared to chemotherapy alone (Bang et al. 2010). Consequently, trastuzumab has been approved for treatment of Her2 positive advanced gastric cancer.

The vascular endothelial growth factor (VEGF) receptor antibody, ramucirumab, was the second targeted agent approved for treatment of gastric cancer, although not in a selected patient population (Wilke et al. 2014).

The receptor tyrosine kinase, MET, was discovered in 1984 as part of an oncogenic fusion protein, TPR-MET, in a carcinogen-treated cell line. The name MET was chosen after the carcinogen (n-methyl-N ʟnitro-N-nitrosoguanidine) used in the study that caused the appearance of the TPR-MET fusion protein (Cooper et al. 1984). Its causative role in cancer development was confirmed by the discovery of activating mutations in its kinase domain causing constitutive kinase activity in hereditary renal papillary carcinomas (Schmidt et al. 1997). The role of MET in development, tissue regeneration and cancer has been reviewed extensively (Trusolino et al. 2010, Gherardi et al. 2012, Peters and Adjei 2012, Ghiso and Giordano 2013). MET overexpression has been reported in many cancer types and this is associated with poor prognosis. Strong overexpression caused by genomic amplification of the MET gene has been described in gastric cancer (Giordano et al. 1989, Kiyose et al. 2012) and in response to treatment with epidermal growth factor receptor (EGFR) targeting drugs such as gefitinib for non-small cell lung cancer (Engelman et al. 2007) and with cetuximab for colorectal cancer (Bardelli et al. 2013). These findings led to the development of 23 small molecule inhibitors and antibodies that target MET and its ligand, hepatocyte growth factor (HGF); more than 240 clinical trials were conducted in 2014 (Furlan et al. 2014). The first phase three trial on a tyrosine kinase inhibitor, tivantinib, or non-small cell lung carcinoma (NSCLC), however, was terminated owing to lack of efficacy. Further studies currently are underway using a population with high MET expression. Also, the development of the single-armed MET antibody, onartuzumab, was stopped at phase three, although phase two had produced promising results (Spigel et al. 2013). Both studies failed to reach the primary end point of improved overall survival, although the drugs exhibited significant efficacy in preclinical studies.

The history described above emphasizes the need for an appropriate patient selection strategy (Garber 2014, Hirsch et al. 2014). In our experience, many anti-MET antibodies are only weakly selective. Typical immunohistochemistry (IHC) assays demonstrate MET over-expression in ≤ 50% of NSCLC cases depending on the antibodies used; however, in only 5% of cases the pathway is actively generating signals, as shown by receptor phosphorylation (Tsuta et al. 2012). The findings described above suggest that the commonly used IHC approach has selectivity problems. This was corroborated by a recent report that > 50% of the MET positive cases by IHC could not be confirmed by mass spectrometry. A perfect match, however, was reported between genomic amplification and mass spectrometry (Catenacci et al. 2014).

Clinical findings also support the use of FISH analysis of gene amplification as a predictive biomarker. Crizotinib achieved an objective response in two thirds of FISH positive cases (Sholl et al. 2013). This finding supports the use of FISH analysis for patient selection; however, this method is expensive and laborious compared to IHC, and it is not available in every clinical laboratory. Moreover, gene amplifications can be non-functional, as described for 5–10% of the Her2 FISH positive breast cancer cases without Her2 mRNA or protein expression (Luoh et al. 2013). For fibroblast growth factor receptor 1, mRNA and protein expression, but not gene copy numbers, were found to predict sensitivity toward FGFR inhibitors (Wynes et al. 2014).

RNA ISH is closer to the most relevant biomarker, which would be protein expression if not functional activity. By contrast to detection of proteins by IHC, detection of the coding mRNA is independent of antibodies and is highly sensitive and selective according to the design of the oligo-probes (Wang et al. 2012). This technique recently has been used to detect MET RNA in FFPE tissue samples and produced a good correlation with both IHC and clinical relevance by correlation with prognosis (Choi et al. 2014).

In addition to highly specific and validated assays, a clear and quantitative responder hypothesis is required to develop a targeted therapy. To obtain statistically significant findings, only patients who have the potential to respond to treatment should be recruited for the study. Traditionally, the expression of a target, which was selected based on reports of its overexpression compared to normal tissue was sufficient for participation in a clinical study. We propose, however, that a quantitative responder hypothesis should be generated and that patients be recruited accordingly.

Because successful clinical development of targeted therapies depends on carefully selected patients, we set out to confirm the feasibility of RNA ISH for detection of functionally relevant expression levels of MET that are predictive of oncogenic addiction. Two among 20 cases of gastric cancer with high expression and activity levels in sandwich immunoassays, by IHC and confirmed by mass spectrometry were clearly identified by RNA ISH. Therefore, we consider that RNA ISH is a valuable additional control for IHC, and potentially an alternative method for patient selection.

Materials and methods

Anonymized tumor samples were purchased from Indivumed (www.indivumed.com) (Hamburg, Germany). Sample collection was performed according to the principles of good clinical practice stated in the Declaration of Helsinki (1964). We used double-blinded historical samples from donors who had given informed consent for research on the samples for commercial purposes. The need for ethics committee approval was waived by the local institutional review board (Hamburger Ärztekammer), because samples were obtained from patients undergoing surgical removal of their primary tumors with curative intent and no additional procedures were necessary. All samples were obtained from the resected

tumor material post-surgery. Small blood samples were obtained from the venous access required for the surgery. Informed consent was obtained and use of the samples after double-blinding was approved by the local data protection officer, the "Hamburgische Beauftragte fuer Datenschutz und Informationssicherheit" in Hamburg, Germany.

We used 20 cases of gastric cancers at different stages that were removed during surgery. We obtained matched samples from the same excised tumor, either fresh frozen or FFPE. All cell lines were obtained from ATCC (Manassas, VA) and grown in RPMI1640 (F1215; Biochrom, Berlin, Germany), Iscove Basal Medium (F0465; Biochrom) or DMEM (4166-029; ThermoFisher Scientific, Waltham, MA), supplemented with 10% fetal calf serum and 2 mM glutamine.

BAY 853474 is a reversible, ATP-competitive inhibitor of MET. It inhibits MET with an IC 50 of 1 nM in the biochemical assay and 2.9 nM in a cell based mechanistic assay. BAY 853474 is highly selective; it inhibits only Rsk2 with IC 50 of 906 nM and causes < 50% inhibition of Rsk1, Rsk3, Flt1, MKK6, PKGα and PKGβ at 1 μM.

To generate cell lysates, cells were grown to 70% confluence in a T 75 flask, washed with phosphate buffered saline (PBS) and frozen in liquid nitrogen for later preparation of lysates using 500–900 μl MSD lysis buffer supplemented with phosphatase inhibitor cocktail (P0044 and P-5726, respectively (Sigma, Munich, Germany) and Halt protease inhibitor cocktail (87785; Pierce/ ThermoFisher Scientific). Cell lysates were aliquoted, then stored at −80° C before use for sandwich immunoassays or western blotting.

Cell pellets were prepared from washed cells in an Eppendorf tube by 10 min centrifugation at 2,000 × g followed by fixation in 10% neutral buffered formalin for 8–18 h at room temperature. The pellets were stored in 70% ethanol until embedment in paraffin using a vacuum infiltrator (ASP200S; Leica, Nussloch, Germany).

RNA ISH analysis of MET expression was performed using RNAscope 2.0 reagents from Advanced Cell Diagnostics, (ACD; Hayward, CA) according to the manufacturer's instructions. mRNA specific probes specific for MET and ubiquitin were designed by ACD. Pretreatment protocol 2 was used for 15 min. For better discrimination of moderate and strong expression, we adjusted the scoring according to the following system: 0, no staining or <1 dot/ cell; 1, 1–3 dots/ cell; 2, 4–10 dots/ cell with no or few dot clusters; score 3, >10 dots/ cell with fewer than 10% positive cells with dot clusters; 4, >10 dots/ cell and >10% positive

cells with dot clusters; 5, ≥10 dots/ cell with >50% positive cells with dot clusters.

The antibody clone, SP44 (Spring Biosciences, Pleasanton, CA), diluted 1:100 in antibody diluent (S2022; Dako, Glostrup, Denmark) was used for IHC. Antigen retrieval was performed in pH 9 target retrieval solution (S2367; Dako) for 17 min in a pressure cooker. Staining was performed using the Dako Envision system. Evaluation and scoring of ISH and IHC were performed by a board-certified pathologist (AF).

MET expression and phosphorylation of tyrosine-1349 were analyzed by electrochemilumines-cence-based sandwich immunoassays (Mesoscale Discovery, Rockville, MD) in lysates made from fresh frozen tumor material or exponentially growing cell cultures. Cells were harvested by trypsinization, washed twice with PBS and lysed in MSD lysis buffer supplemented with Halt protease inhibitor cocktail (87785; Pierce/ ThermoFisher Scientific) and phosphatase inhibitors (P0044 and P-5726, respectively; Sigma). Protein concentration in the lysates was determined using the Bradford assay (Bradford 1976). A protein content of 20 µg/ well was used and the results are reported as MSD counts/ µg.

MET expression levels in different cell lines also were analyzed by western blot. A 10 µg protein extract of exponentially growing cells was loaded on a NuPAGE Novex 4–12% Bis–Tris Mini Gel, blotted onto a nitrocellulose membrane and detected using IRDye conjugated secondary antibodies. The monoclonal antibody, SP44 (Spring Biosciences), diluted 1:500 in tris-buffered saline containing Tween 20 (TBST) was used as primary antibody.

For proliferation assays, cells were seeded at 2,000 cells/ well in 96 well plates, grown for 24 h, then BAY 853474 was added. Cell culture was continued for 72 h, then viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany). For selected reaction monitoring (SRM) analysis, 10 µm tissue sections were cut using a Thermo S2055 microtome (Thermo Fisher Scientific, San Jose, CA), then microdissected to maximize tumor content. Samples were processed for SRM analysis using a TSQ Vantage triple quadrupole mass spectrometer as described earlier (Catenacci et al. 2014).

Results

To test the performance of the different assay techniques and to define a relevant level of MET activity for gastric cancer, we used a panel of different gastric cancer cell lines. Three of the cell lines (MKN45, HS746T, SNU-5), which are known to exhibit a genomic MET gene amplification, were used as positive controls. The MET gene amplification causes MET overexpression, its ligand-independent autophosphorylation and activation of downstream intracellular signaling pathways (MKN45, HS746T and SNU-5 cells) have 12, 22 and 20 gene copies, respectively (Wang et al. 2016). For comparison, we used three other gastric cancer cell lines without MET amplification. The lung cancer cell lines, H520 and H661, served as MET negative controls as described by Tang et al. (2008). The gold standard for specificity is the use of sandwich immunoassays using two antibodies.

We first tested the expression and phosphorylation of MET in lysates prepared from exponentially growing cells using commercially available sandwich immunoassays. Figure 1a shows the expression levels of MET and Fig. 1b shows the phosphorylation of tyrosine-1349, which is
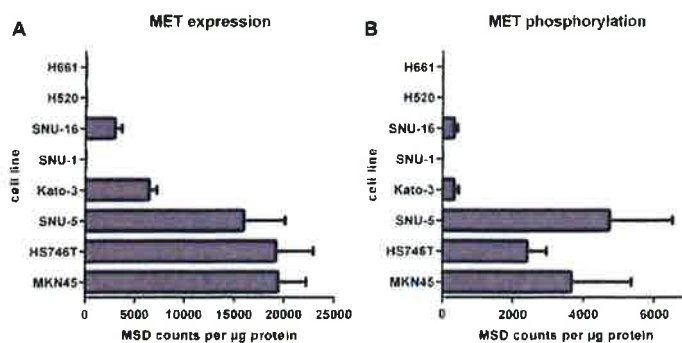

Fig. 1. Expression and autophosphorylation levels of MET in various cell lines. A) Expression of MET in different cell lines tested by a sandwich immunoassay (MSD). B) Phosphorylation of tyrosine-1349 in different cell lines as shown by MSD.

phosphorylated after receptor dimerization and serves as a docking site for the intracellular adaptor protein, Gab1 (Lock et al. 2003). The cell lines with MET amplification show very strong expression; two other gastric cancer cell lines, Kato-3 and SNU-16, exhibit moderate MET expression, while SNU-1 cells are MET-negative (Wang et al. 2016). The MET negative lung cancer cell lines, H520 and H661, also confirmed the specificity of the assay. The MET expression levels were confirmed by the functional activity as shown by MET autophosphorylation (Fig. 1b). The SP44 antibody used for IHC here and elsewhere was first tested by western blotting and the MET expression levels were confirmed (data not shown).

We established a staining protocol using the SP44 monoclonal antibody and tested its performance first on formalin fixed cell pellets of the control cell lines. The staining was highly selective and the relative intensities matched the results of the western blot and the sandwich immunoassays (Fig. 2a). The performance of the RNA ISH assay on the same cell pellets (Fig. 2b) matched the IHC data.

To define therapeutically relevant expression levels, we compared the response of the cell lines to the MET inhibitor, BAY-853474 (Fig. 3a), a highly selective ATP-competitive inhibitor with 1 nM affinity in biochemical assays. In a three day proliferation assay, BAY-853474 efficiently blocked the growth of MKN45, HS746T and SNU-5 cell lines with double-digit nM potency and 100% efficacy, which verified the oncogenic addiction of these cell lines to MET (Table 1). Three other gastric cancer cell lines and the MET negative control cell lines failed to exhibit a response to MET inhibition. These results are consistent with the report by Wang et al. (2016), who found that HS746T, SNU-5 and MKN45 were responsive to both ABT-700, an anti-MET antibody and the MET-kinase inhibitor, PF-4217903, whereas SNU-16, Kato-3
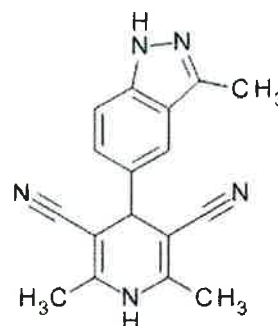

Fig. 3. BAY-853474, a potent selective MET inhibitor.

and SNU-1 did not respond to these compounds. The same findings also had been reported earlier for the MET inhibitor PHA-665752 (Smolen et al. 2006), which demonstrated the same result independent of the inhibitors used. These cells grow without requiring MET signaling; the MET inhibitor, BAY-853474, has no measurable effect on these cells. This was surprising because Kato-3 and SNU-16 cells express MET and exhibit active phosphorylation of tyrosine-1349. This finding suggests that careful determination of a response threshold is required for clinical studies. The preclinical values obtained here generate a first responder hypothesis. An IHC score >2 is required; a score of 3 appears to be sufficient. An RNA ISH score >2 is required, a RNA ISH score of 4 appears to be sufficient. More than 6,000 counts/µg MET and 500 counts/µg phosphotyrosine-1349 would be necessary for a response to treatment. After creating the IHC and RNA ISH assays, we used a set of 20 clinical cases of gastric cancer to test the performance of the assays on clinical FFPE samples. In IHC, two of 20 gastric cancer samples stained strongly for MET, while two other cases showed areas of moderate intensity. Most of the samples stained weakly or did not stain. Figure 4 shows
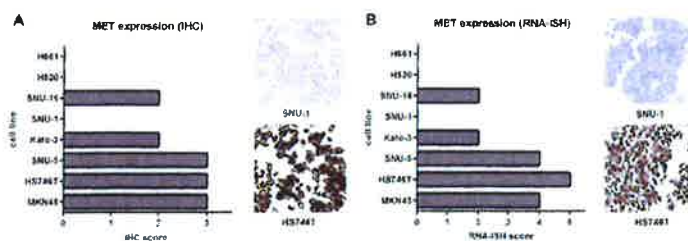

Fig. 2. Expression of MET in various cell lines assessed by IHC vs. RNA in situ hybridization. A) Expression of MET in different cell lines tested by IHC, with examples for negative and score of 3 staining. B) Expression of MET in different cell lines tested by RNA ISH, with examples for negative and score of 5 staining. Scale bars = 50 µm.

Table 1. IC50 and efficacy of BAY-853474 in 72 h proliferation assay on different gastric and lung cancer cell lines, and correlated with MET expression levels

Cell line	MKN45	HS746T	SNU-5	Kato-3	SNU-1	SNU-16	H520	H661
MET expression (IHC)	+++	+++	+++	++		++		
Bay 853474 IC50	44 nM	17 nM	17 nM					
Bay 853474 efficacy	98%	96%	100%					

representative images of the outcomes including all moderately and strongly stained samples.

High expression of MET was confirmed by strong signals for MET RNA using in situ hybridization (Fig. 5). The two cases (A1362 and A1516) that displayed strong signals with IHC were confirmed by high RNA levels. These findings are consistent with the literature concerning the prevalence of MET amplification in gastric cancer. A recent review identified 8–21% amplifications (Peng et al. 2014). The prevalence of MET amplification may be lower in Japanese patients (Kiyose et al. 2012). Two more cases (A1525 and A1550) showed moderate IHC signals. These moderate expressers could not be confirmed by elevated RNA signals, because the RNA signals for these samples were within the commonly observed weak signal intensity range. The comparison

between IHC and ISH results is summarized in Fig. 5a,b.

Because a potential drawback of RNA ISH is its sensitivity to RNA degradation, we controlled the RNA levels by testing for the presence of ubiquitin RNA. Fig. 6 shows high levels of ubiquitin C RNA in sample A1525, whereas. MET RNA levels are very low. We can rule out issues of RNA quality as the reason for low MET RNA ISH in this case. On the other hand, sample A1550 exhibited relatively low levels of ubiquitin RNA, so in this case, RNA degradation cannot be ruled out.

To extend our understanding of quantitative expression levels, we analyzed matching fresh frozen material from the same tumors using sandwich immunoassay (MSD). Figure 7 shows that the two cases with very high MET expression detected by IHC (Fig. 7a) and RNA ISH (Fig. 7b) were confirmed by sandwich immunoassay (Fig. 7c). Both
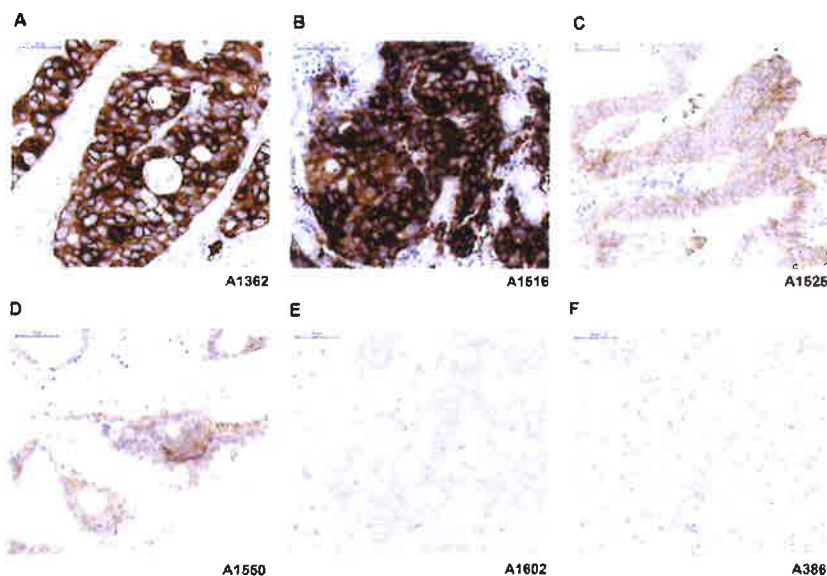

Fig. 4. Examples of different MET expression levels shown by IHC on FFPE gastric cancer samples. A) Sample A1362, IHC score of 3. B) Sample A1516, IHC score of 3. C) Sample A1525, IHC score of 2. D) Sample A1550, IHC score of 2. E) Sample A1602, IHC score of 1. F) Sample A386, IHC score of 0. Scale bars = 50 μm.
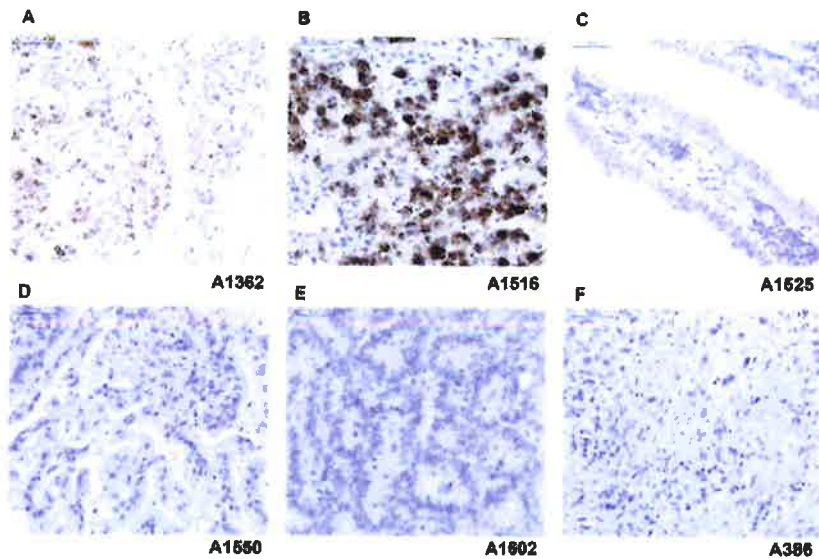
Fig. 5. Examples for different MET RNA levels shown by RNA ISH on FFPE gastric cancer samples. A) Sample A1362, RNA ISH score of 3. B) Sample A1516, RNA ISH score of 4. C) Sample A1525, RNA ISH score of 1. D) Sample A1550, RNA ISH score of 1. E) Sample A1602, RNA ISH score of 1. F) Sample A386, RNA ISH score of 0. Scale bars = 50 μm.
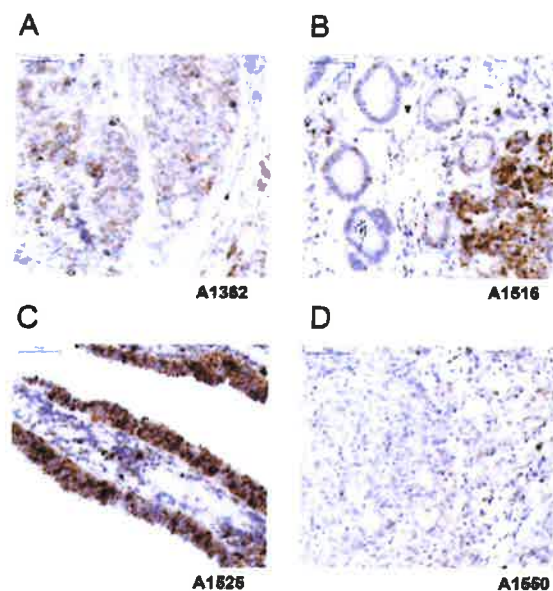

Fig. 6. Control for integrity of the RNA in FFPE cell pellets by in situ hybridization of ubiquitin as housekeeping gene control. A) Sample A1362, RNA ISH score of 4. B) Sample A1516, RNA ISH score of 4. C) Sample A1525, RNA ISH score of 5. D) Sample A1550, RNA ISH score of 2. Scale bar = 50 μm.

cases also exhibit active signaling as demonstrated by high phosphorylation of tyrosine-1349 (Fig. 7d). The phosphorylation levels are within the range for cell lines with oncogenic addiction to MET activity as shown in Fig. 1. This result shows that functionally relevant MET expression levels can be detected by RNA ISH and confirmed by ELISA-like approaches. Only one of the two moderate
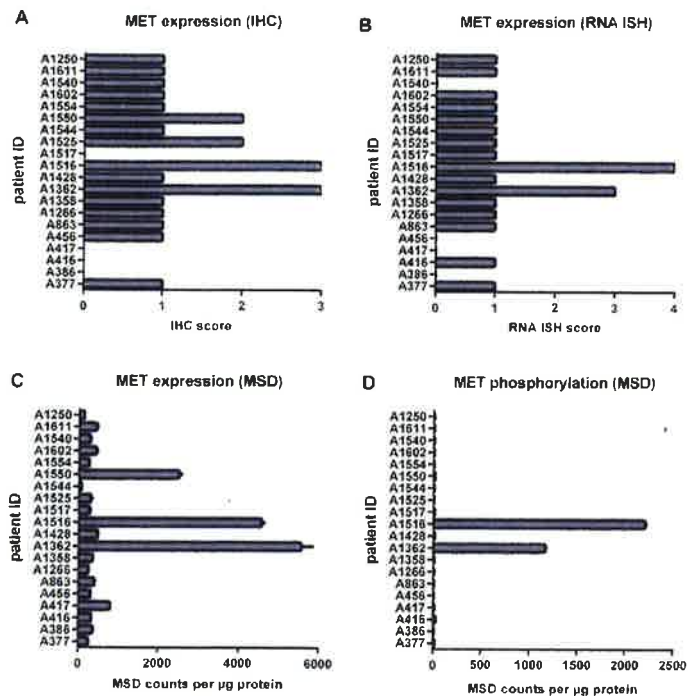
Fig. 7. MET expression tested by IHC compared to the presence of MET RNA detected by RNA ISH (A) and FFPE samples (B). The results were confirmed by testing matched fresh frozen samples for MET expression (C) and phosphorylation of tyrosine-1349 (D) as indicators of molecular activation by Mesoscale Discovery (MSD) assays.

expressers (A1550) could be confirmed by sandwich immunoassay, but it did not exhibit increased phosphorylation. Case A1525 could not be confirmed in any way. A possible explanation for this may be local heterogeneity in the tumor as even the matched samples were derived from different parts of the tumor. Potentially, the matched fresh frozen sample contained a very limited number of tumor cells. An alternative explanation would be false positive staining by IHC, although the clear membrane staining restricted to the epithelial layer appeared to be specific. As another independent control, we analyzed FFPE material by mass spectrometry The two high expressers were confirmed (A1362 with 3.23 fmol/ μg and A1516 with 4.60 fmol/ μg) using this method, but the questionable cases with moderate IHC staining were below the detection limit of mass spectrometry as were all other cases except A1250 with 0.39 fmol/ μg).

Taken together, our findings demonstrate that RNA in situ hybridization on FFPE material is feasible and sufficiently sensitive to detect high levels of MET expression with high specificity.

Using the FFPE material for IHC and RNA ISH is a good complement to stringent specificity control.

Discussion

Immunohistochemistry on FFPE tissue currently is the standard method for patient selection based on target expression. The small dynamic range and the high potential for cross reactivity, however, are major issues with this method. The recent failure of Genentech's phase 3 study of onartuzumab raised concerns about a patient selection strategy based on MET IHC (Garber 2014, Hirsch et al. 2014).

We experienced false positive staining on tumor samples using several other commercially available MET antibodies (data not shown). We made similar experiences for phospho-site specific antibodies. An antibody directed against the activation loop phospho-sites exhibited strong cross reaction with activated EGFR, and also an antibody against the intracellular docking site, phosphotyrosine-1349, stained nearly all tested clinical samples despite appearing to be specific for a small

panel of cell line controls. The limitations of IHC for patient selection for anti-MET therapy have been summarized by Lee et al. (2016). These investigators reported that MET positivity could not be confirmed by DNA-FISH in many studies. These reports are consistent with that of Watermann et al. (2015), who described lack of correlation between MET gene amplification, protein expression and phosphorylation using the same antibodies on NSCLC samples. Kawakami and Okamoto (2016) proposed to recruit patients based only on DNA-FISH positivity. This would ensure high specificity, but unfortunately, DNA-FISH is compromised by inter-operator variability, false negative results and the potential lack of expression of amplified sequences.

We established a new MET IHC method using the SP44 antibody plus an RNA ISH assay as an antibody-independent specificity control suitable for use with FFPE material. Using high quality samples, we have shown here that RNA ISH is a valuable tool for confirming target expression in tumor samples, at least for high expression levels. Our approach appears valid for oncogenic drivers, such as Her2 and MET, that are highly overexpressed owing to genomic amplification of their coding sequences.

The suitability of RNA ISH for detecting HER2 mRNA in breast cancer has been demonstrated and RNA ISH sensitivity has been shown to be higher compared to DNA FISH for gene amplification (Bernet et al. 2012). This suggests that an additional 7% of breast cancer patients may be eligible for herceptin therapy (Bernet et al. 2012). Her2 detection by RNA ISH correlates well with IHC and DNA-FISH (Vassilakopoulou et al. 2014). HER2 expression also could be detected reliably by RNA ISH in gastric cancer (Kim et al. 2013). We confirm here the applicability of RNA ISH for analyzing MET expression in FFPE samples. We have shown for MET that alternative methods, such as RNA ISH, can be used to identify biologically relevant overexpression levels associated with response to treatment at least in cell culture.

We believe that generating a quantitative responder hypothesis before establishing thresholds in a clinical study is critical for successful clinical studies. Often, the fact that expression of a protein is associated with bad prognosis is used as evidence for its functional importance, but we propose that experimental proof of its functional importance as a tumor driver is necessary. Of course, a preclinical responder hypothesis can only indicate which level of biomarker expression is required; the actual threshold for response can

be determined only in a well-designed clinical trial with retrospective biomarker analysis.

Because active molecular signaling is driving the cancer, it is advisable to analyze the MET phosphorylation levels rather than MET protein expression alone. The sandwich immunoassay for MET phosphorylation was the most selective and relevant assay among the assays characterized here; it was quantitative and produced the best signal to background level of the assays tested. In principle, the sandwich immunoassay is the best assay for selecting patients for MET inhibitor therapy. Because fresh biopsies are not common for screening purposes, however, analyses still must be based on historical FFPE material for the foreseeable future.

A combination of IHC and RNA ISH for specificity control is a practical approach, because the results correlate with MET phosphorylation. Tumor heterogeneity is a potential issue for biopsy based treatment decisions. The fact that the investigator can judge the heterogeneity of the sample and evaluate the tumor cells remains an advantage of histology based methods. In this respect, the possibility of controlling the selectivity of IHC by using RNA ISH constitutes a big step forward. Of course, preservation of RNA by rapid fixation and good sample handling is a prerequisite. Larger studies are required to determine whether the RNA ISH method is sufficiently robust for routine use in clinical settings. In addition, RNA integrity must be controlled by testing the expression of housekeeping genes such as ubiquitin or cyclophilin.

We demonstrated the feasibility of RNA ISH for detecting functionally relevant expression levels of MET in FFPE gastric cancer samples. Our findings demonstrate the potential of the RNA ISH method as a validation tool to verify the specificity of new antibodies. This highly selective method also may be useful for studies for which no suitable antibodies are available or when several homologous protein isoforms must be discriminated.

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