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MET-FISH Evaluation Algorithm: Proposal of a Simplified Method

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Abstract

MET amplifications (METamp) occur in 5% of NSCLC and represent in most case mechanisms of resistance to ALK and/or EGFR-targeted therapies. METamp detection can be performed using different techniques, although Fluorescence In-Situ Hybridization (FISH) remains the gold-standard, especially in the context of subclonality. To date current evaluation algorithms of MET amplifications are time consuming. Aim of the study was to identify a faster, equally reliable diagnostic algorithm for the detection of METamp, which is currently classified in negativity and low/intermediate/high-level amplification. N=497 NSCLC cases with available MET-FISH data had been selected. The results based on the first evaluated 20 cells had been re-calculated and compared with the definitive results based on 60 cells. For n=464 (93.4%) identical results had been obtained when counting 20 cells instead of 60 cells. Thirty-three cases (5.6%) showed a discrepancy, leading to an incorrect upgrade to a higher diagnostic category (n=25) and to an incorrect downgrade (n=8). We propose a simplified, yet equally reliable MET FISH-algorithm: after accurate screening of the whole tumor slide, twenty tumor cells have to be evaluated and results calculated: If the result is negative, or if all criteria of high-level METamp are fulfilled, the case can be signed out as such. All other cases should be considered as equivocal and additional 40 cells have to be counted. Given that, reliable results can be obtained by counting 20 cells only and an “equivocal” category for cases that need further investigation have been clearly defined.

Keywords

Evaluation Algorithm; Fish; MET-Amplification; Non-Small-Cell-Lung Cancer

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Conflict of Interest

RB provided lectures and was part of Advisory Boards for AbbVie, Amgen, AstraZeneca, Bayer, BMS, Boehringer-Ingelheim, Illumina, Lilly, Merck-Serono, MSD, Novartis, Qiagen, Pfizer, Roche, Targos MP Inc. RB is Co-Founder and Scientific Advisor for Targos Mol. Pathology Inc. RB is Testifying Advisor for MSD in GBA-Assessment for Pembrolizumab. RB has received funding from the Deutsche Krebshilfe for the Network Genomic Medicine. SMB has received speaker honoraria and personal fees from Pfizer, Novartis, Roche, Bayer, AstraZeneca, Molecular Health, GSK, MSD and Targos; speaker honoraria and non-financial support from BMS; non-financial support from Janssen. The authors declare no further conflict of interest. RC was supported by the Else Kröner-Fresenius Stiftung (2016-Kolleg.19). AMS, CA and BH were supported by Roche Pharma AG. The authors have no further conflict of interest to disclaim.

Introduction

Lung cancer remains the main cause of cancer related deaths worldwide [1]. New therapies targeting specific genetic alterations fundamentally changed the treatment of patients with advanced stage disease [2]. As consequence, the identification of actionable genetic alterations is part of the routine diagnostic setting [3]. One of the most recent targets are aberrations of the *Mesenchymal epithelial transition factor (MET)* [4, 5]. According to current data [4], *MET* is altered in approximately 5% of non-small cell lung cancers (NSCLC) and is considered as adverse prognostic factor [6-11] and a potential predictive marker for response to anti *MET* therapeutics [12]. *MET* pathway activation occurs by several mechanisms that affect cancer cell survival, growth, and invasiveness [4, 12]; among those, *MET* amplifications. Preclinical and clinical evidence suggests that *MET* amplifications may occur as primary oncogenic driver in subsets of treatment-naive lung cancers as well as a secondary driver of acquired resistance after treatment with targeted therapies [12]. *MET* copy number gains arise from two distinct processes: polysomy and amplification [13]. While polysomy occurs in cases of chromosomal duplication, true amplification occurs in the setting of focal gene duplication and represents a real oncogenic driver [14, 15]. *MET* inhibition is now confirmed to lead to clinically meaningful antitumor activity with rapid and durable response and low side effects, especially in the context of *MET* exon 14 skipping mutations or higher level of amplifications [10, 16, 17].

***MET* Amplification as Resistance Mechanism in *EGFR*- or *ALK*-altered NSCLC**

MET activation has been implicated as an oncogenic driver in *epidermal growth factor receptor (EGFR)*- or *anaplastic lymphoma kinase (ALK)*-positive NSCLC and can mediate primary and secondary resistance to *ALK* and *EGFR* tyrosine kinase inhibitors (TKI) [18, 19]. *MET* inhibition may overcome *ALK* resistance combining *MET* and *ALK* inhibition [19, 20] or analogously, combining *MET* and anti-*EGFR* inhibition (erlotinib) [21] to overcome *MET* amplification mediated resistance to *EGFR* inhibition [22].

Identification of *MET* Amplification

The definition of clinically relevant *MET* amplification however has been a long-debated topic and several criteria have been proposed. *MET* amplification can be identified through different molecular techniques, however, the most widely used method in the clinical setting remains Fluorescence *In-Situ* Hybridization (FISH), also allowing the detection of the exact number of gene copies per cell. Numerous ongoing trials enrolling *MET* amplified NSCLC, show that cut-offs for the definition of *MET* amplification vary [22]. Recently, early data from clinical studies point out [12, 16] that a high-level amplification is necessary to obtain a significant clinical response [16]. As the level of amplification seems to be of prognostic [4, 23] and predictive value [5], FISH remains the most reliable, fast and cost-effective method for the identification of *MET* amplification in the clinical setting [4]. However a unique definition of high-level amplification is still debated. Some authors propose to use the *MET*/centromere 7 (CEP7) copy number ratio [16, 24, 25]. As described by Schildhaus et al [23], *MET* amplification can also co-occur in the context of

amplification of the centromere, leading to a ratio < 2 , so that the author has proposed a more complex approach including both *MET* gene copy number amplification and *MET*/centromere 7 copy number ratio. This approach has also been preferred in numerous large clinical trials (such as [NCT02414139](#) [NCT01610336](#), [NCT01982955](#) (INSIGH study), and [NCT02143466](#) (TATONN study)) [22]. In addition, in a very recent study [4] Overbeck et al propose a new category of *MET* high-level amplification identifying the higher unequivocal *MET*-amplification level, defined as average *MET* gene copy number of 10 independently from the *MET*/centromere 7 copy number ratio. With a steadily increasing number of cases, currently applied FISH-criteria can be time-consuming in the daily clinical setting and sometimes may not even be possible in the case of very small biopsies, not reaching the required number of tumor cells. Therefore, the aim of the current work was to develop a faster and equally reliable *MET*-FISH evaluation algorithm that allows the identification of all potentially relevant *MET*-amplification levels in basically all patient samples.

Materials and Methods

Case Collection

The archives of the Institute of Pathology at the University Hospital of Cologne, Cologne, Germany and of the Institute of Pathology at *Klinikum Stuttgart*, Stuttgart, Germany have been searched retrospectively for NSCLC cases at every stages, for which *MET*-FISH analysis has been performed as part of the routine diagnostic work-up according to the internal protocol of the Network Genomic Medicine established in Cologne. For the purpose of this study, only biopsy or resection specimens have been included in the study resulting in n=400 consecutive cases derived from the Institute of Pathology of the University Hospital of Cologne and n=97 consecutive cases from the Institute of Pathology at *Klinikum Stuttgart*. Cytology samples were excluded as specific cut-off criteria were established on formalin-fixed paraffin-embedded (FFPE) tissues samples. Of n=497 cases, n=400 (80.5%) were classified as negative, n=60 (12.1%) as low-level amplification, n=11 (2.2%) as intermediate-level amplification, n=26 (5.2%) as high-level amplification.

Histomorphological Analysis

Histology was reviewed by 3 experienced pathologists (AMS, RC and RB). The diagnosis of NSCLC was confirmed in all cases. Prior to the study, approval by the local ethics committee had been granted and all patients had signed written informed consent.

Fluorescence *In-Situ* Hybridization (FISH)

MET-FISH was performed as part of the routine diagnostic setting using a commercially available FISH probe Zytolight® SPEC *MET*/CN7 Dual color Probe (product nr. Z-2087-200) and its implementation kit (product nr. Z-2028-5/-20) provided by ZytoVision (ZytoVision GmbH, Bremerhaven, Germany) according to manufacturer's instructions. The probe is composed by ZyGreen-marked (excitation 503 nm/emission 528 nm) gene-sequence including the *MET*-region and by ZyOrange-marked (excitation 547 nm, emission 572 nm) gene-sequence including the centromeric alphasatellite-region D7Z1 of chromosome 7. Slides were reviewed at high magnification power ($\times 63$) and scored according to previous published guidelines [23]: the entire tumor area was initially screened

and the spots with most signals were selected for further analysis. A total number of n=60 cells derived from 3 areas of 20 continuous cells each were counted. As previously described by our group, green *MET* signals and orange *CEP7* were counted during routine diagnostic defining 4 groups of amplification status [23]:

1. High-level amplification defined as tumors with *MET/CEP7* ratio ≥ 2.0 or an average *MET* gene copy number per cell of ≥ 6.0 or $\geq 10\%$ of tumor cells containing ≥ 15 *MET* signals.
2. Intermediate-level of gene copy number (GCN) gain defined as $\geq 50\%$ of cells containing ≥ 5 *MET* signals and criteria for high-level amplification not being fulfilled.
3. Low-level of gene copy number gain defined as $\geq 40\%$ of tumor cells showing ≥ 4 *MET* signals and criteria for high-level amplification or intermediate-level of gene copy number gain not being fulfilled.
4. All other tumors were classified as negative.

An example of each category is depicted in Figure 1. In order to verify, whether a more effective evaluation algorithm was possible, the results of the original reports were pulled and re-calculated first using only the first counted 20 cells and then compared to the final result based on 60 counted cells. In both cases the same endpoints (*MET* mean GCN, *CEP7* mean GCN, *MET/CEP7* GCN-ratio and number of cells with ≥ 4 / ≥ 5 / ≥ 15 *MET* signals) were used and the cases were classified according to the following categories:

1. Identical results when counting 20 and when counting 60 cells.
2. Discrepant results leading to a potential upgrade to a higher category when counting only 20 cells.
3. Discrepant results leading to a potential downgrade to a lower category when counting only 20 cells.

Data Analysis

Automated evaluation of the number of signals of *MET*, *CEP7* as well as calculation of *MET/CEP7* ratio and identification of low- /intermediate- / high-level amplification were performed using the FileMaker® software and Microsoft Office Excel.

Results

The reported frequency of *MET* positivity was similar to the main literature data [4, 23]. The dataset of n=497 cases was distributed as follow: (i.) n=26 cases showed high-level amplification. Five of them would have been now classified as Top-level according to the new classification proposed by Overbeck and colleagues; (ii.) n=11 intermediate-level amplification. (iii.) n=60 low-level amplification; (iv.) n=400 negative result. Comparing the results after counting 20 or 60 cells, high concordance was observed. In details, for n=464 (93.4%) cases identical results were obtained when counting only 20 cells instead of 60 cells were obtained. N=25 cases (5.0%) cases would be upgraded to higher category

when counting only 20 cells. N=8 cases (1.6%) would be downgraded counting only 20 cells (Figure 2 and Supplementary Table 1). The results obtained from our re-analysis lead to the proposal of the following diagnostic algorithm, which allows a more rapid, but equally reliable and precise evaluation of *MET* gene copy numbers (Figure 3). For all cases classified as negative, the analysis is completed after counting 20 representative cells and the case can be signed out as such. Cases classified as high-level amplification by fulfilling all three criteria ($MET-GCN \geq 6$, at least 10% of tumor cells containing ≥ 15 *MET* signals and $MET/CEP7$ ratio ≥ 2) can be signed out as high-level amplification, otherwise (i.e. if only one or two of the criteria for high-level amplification are fulfilled) additional 40 cells have to be evaluated. For all cases that reach the criteria for low-level or intermediate-level amplification, the evaluation of additional 40 cells is mandatory. Given the current evidence of a significant prognostic value of top-level amplifications, defined by gene copy number higher than 10, we suggest including this new category as proposed by Schildhaus and colleagues [5].

Discussion and Conclusions

The identification of *MET* genetic alterations represents a crucial point in the molecular assessment of non-small cell lung cancer in advance stage of disease since *MET* may act both as primary driver and as mechanism of resistance to ALK-/EGFR- delivering therapies [12, 18, 19]. Over the last two years, the importance of *MET* alterations has further grown since two drugs targeting *MET*, capmatinib [5] and tepotinib [26], obtained FDA approval. With a steadily increasing number of cases of lung cancer needing a molecular characterization and the fact that FISH currently represents the gold-standard for detection of *MET* amplifications, *MET* FISH analyses can be time-consuming when using currently applied evaluation criteria. Clear cut-off criteria for *MET* amplification remain debated, but the most recent studies on *MET* amplifications agree that higher levels of amplification better respond to *MET* targeted therapies in clinical studies [5, 26] and are associated with poorer prognosis [4]. The use of next generation sequencing in the assessment of *MET* amplification, although time-sparing is not suggested since the sensibility is not superior and it provides no description concerning any subclonal variation [6]. The use of immunohistochemistry is as well not recommended for the identification of *MET* amplification, since the specificity is too low, as already published by our group [27]. In this work an updated, shorter, yet equally reliable algorithm for the evaluation of *MET* amplification has been presented, improving the current diagnostic algorithm [23]. According to the results based on n=497 unselected, consecutive *MET* FISH analyses of NSCLC, the same results are obtained in 93.4% (n=464) of cases when counting 20 cells instead of 60 cells. Of thirty-three (6.6%) discordant cases, n=25 (5.0%) would have been upgraded and n=8 (1.6%) would have been downgraded when counting only 20 cells, as listed in the results section and in Figure 2. An upgrade from negative result to low-level amplification (n=9, 1.8%) as well as an upgrade from low-level to intermediate-level amplification (n=9, 1.8%) can be avoided as cases are added to the “equivocal” category and need counting of additional 40 cells as described by the algorithm. Seven (n=7, 1.4%) cases would have been falsely classified as high-level when only counting 20 cells. However, this relevant misclassification can be avoided, if only cases that fulfil all three possible

criteria for high-level amplification (MET/CEP7 ratio ≥ 2.0 and average MET gene copy number per cell of ≥ 6.0 and $\geq 10\%$ of tumor cells containing ≥ 15 MET signals) are directly classified as high-level after counting 20 cells. All other cases are classified as “equivocal” and need the counting of additional 40 cells. Of note, this may lead to missing cases with very focal high-level amplification when thorough screening of a case is not performed. If a very focal high-level amplification in therapy-naïve patients however is of clinical relevance needs to be evaluated in further studies. In addition, we believe that the downgrade to a negative result instead of the identification of a clinically not relevant low-level amplification (n=5, 1.0%) can be prevented by thorough slide screening but is rather of no clinical relevance. All cases classifying as low-level (n=2, 0.4%) or intermediate-level (n=1, 0.2%) when using 20 cells will automatically be considered “equivocal” and will be reanalyzed, resulting in counting a total of 60 cells. In contrast to next-generation sequencing based methods, even if in our algorithm just 20 cells are counted, the clonal variations of amplifications are still not missed out, since before starting the count the whole tumor surface has to be screened looking for clonal events. In conclusion, here we propose an updated *MET*-FISH algorithm that provides a much more efficient yet equally reliable way to identify clinically relevant *MET* amplifications in NSCLC in the routine clinical setting, even in the case of very small biopsies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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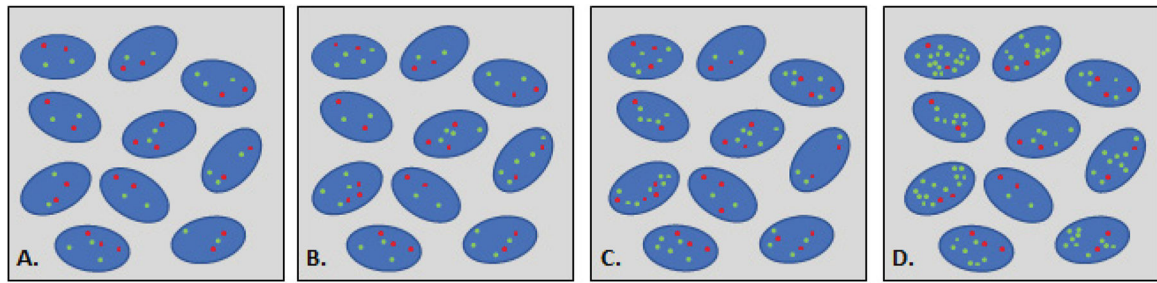
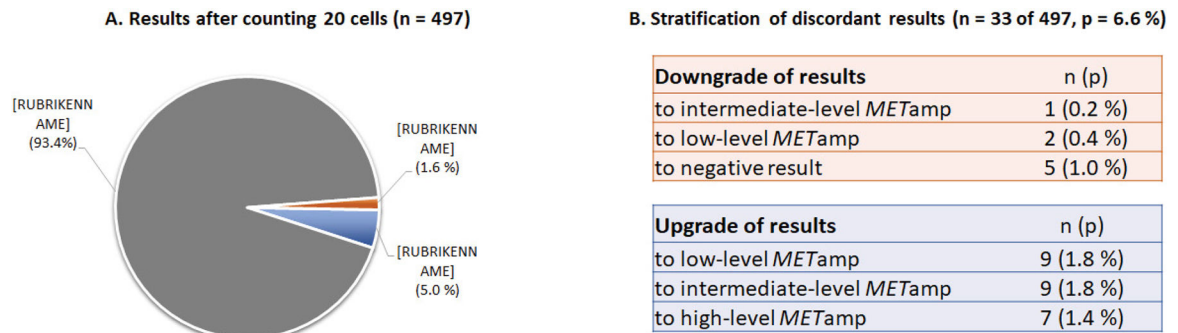


Figure 1:

Different level of amplification. Red dot: copy of centromere 7, green dot: copy of *MET* gene. A. Negativity; B. Low-level amplification; C. intermediate-level amplification; D. high-level amplification.

**Figure 2:**

Comparison of results after counting 20 or 60 cells. A. Concordant results have obtained in n=464 (93.4%) of cases, discordant results in n=33 (6.6%) cases. B. Analysis of the n=33 discrepant cases after counting 20 or 60 cells. 8 cases (1,6%) would be downgraded in a lower diagnostic category after counting only 20 cells as following: n=5 (1.0%) downgraded to negative result instead of low-level amplification; n=2 (0.4%) downgraded to low-level instead of intermediate-level amplification; n=1 (0.2%) downgraded to intermediate-level instead of high-level amplification. 25 cases (5.0%) would be upgraded to higher category when counting only 20 cells: n=9 (1.8%) upgraded to low-level result instead on negative; n=9 (1.8%) upgraded to intermediate-level instead of low-level amplification; n=5 (0.4%) upgraded to high-level instead of low- or intermediate-level amplification.

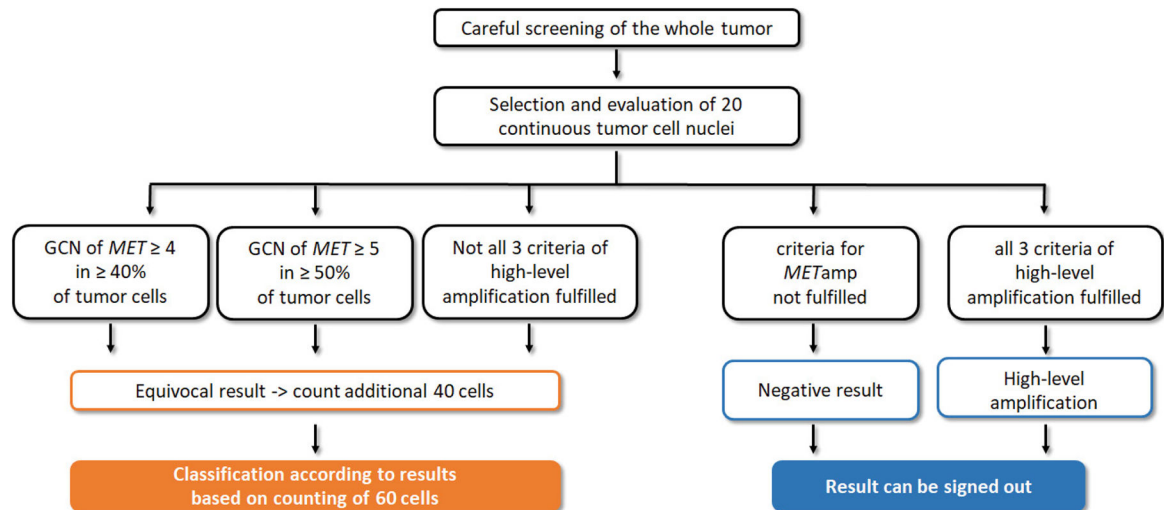


Figure 3:

Diagnostic algorithm of MET amplification. After screening the whole tumor slide, the area with greater number of signals per cell is evaluated counting 20 cells. In case of preliminary low-level or intermediate-level amplification, or just one/two criteria for high-level amplification are fulfilled, 40 more cells need to be counted. The final result is based on the evaluation of 60 cells according to previous described criteria. In case of negative result as well as if all three criteria for high-level amplification are fulfilled, the analysis can be signed down.