MET amplification status in therapy-naïve adeno- and squamous cell carcinomas of the lung

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Translational relevance

Met is one of the most promising new therapeutic targets in lung cancer. An important activating mechanism is *met* amplification. There are, however, conflicting data on the prevalence of *met* amplification and the criteria for measurement of *met* changes in pulmonary carcinomas. Therefore, *in situ* hybridization has not yet been fully evaluated as a potential biomarker. In this work we describe our *met* FISH findings on a cohort of nearly 700 well characterized therapy-naïve lung cancers. Based on this to date largest series, we describe prevalence and patterns of *met* amplification and propose clear-cut evaluation criteria. Furthermore, we provide evidence that *met* amplification occurs in adeno- and squamous cell carcinomas at the same frequency and can be found even in EGFR and KRAS mutated tumors. These findings are relevant for the treatment of lung cancer with both EGFR and met inhibitors.
ABSTRACT

Purpose: MET is a potential therapeutic target in lung cancer and both MET tyrosine kinase inhibitors and monoclonal antibodies have entered clinical trials. MET signaling can be activated by various mechanisms including gene amplification. In this study we aimed to investigate MET amplification status in adeno- and squamous cell carcinomas of the lung. We propose clearly defined amplification scores and provide epidemiologic data on MET amplification in lung cancer.

Experimental design: We evaluated the prevalence of increased MET gene copy numbers in 693 treatment-naïve cancers by fluorescence in situ hybridization, defined clear cut-off criteria, and correlated FISH results to MET immunohistochemistry.

Results: Two thirds (67%) of lung cancers do not have gains in MET gene copy numbers whereas 3% show a clear-cut high-level amplification (MET/centromer7 ratio ≥2.0 or average gene copy number per nucleus ≥6.0 or ≥10% of tumor cells containing ≥15 MET copies). The remaining cases can be subdivided into intermediate (6%) and low level gains (24%). Importantly, MET amplifications occur at equal frequencies in squamous and adenocarcinomas without or with EGFR or KRAS mutations.

Conclusion: MET amplification is not a mutually exclusive genetic event in therapy-naïve NSCLC. Our data suggest that it might be useful to determine MET amplification a) before EGFR inhibitor treatment to identify possible primary resistance to anti-EGFR treatment, and b) to select cases that harbor KRAS mutations additionally to MET amplification and, thus, may not benefit from MET inhibition. Furthermore, our study provides comprehensive epidemiologic data for upcoming trials with various MET inhibitors.
INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the Western world, and an estimated number of 230,000 newly diagnosed cases will occur in the year 2013 in the US with approximately 160,000 deaths (1). Better understanding of the molecular pathophysiology of non-small cell lung cancer (NSCLC) has led to the development of more selective and targeted treatment options with improved outcome rates (2, 3). With the discovery of Epidermal growth factor receptor (EGFR) mutations, EML4-ALK (Echinoderm microtubule-associated protein like 4-anaplastic lymphoma kinase) translocations and the ROS1 translocation, lung cancer treatment has been fundamentally changed and shown to be much more effective than ancient chemo-radiation therapy regimens (4-7).

More recently, the MET receptor tyrosine kinase and its ligand the hepatocyte growth factor (HGF), also known as scatter factor (SF), were identified as further therapeutically relevant targets in lung cancer (3). MET is a heterodimeric transmembrane receptor tyrosine kinase, composed of an extracellular α-chain and a transmembrane spanning β-chain linked via disulfide bonds (2) and its gene is localized to chromosome 7q (8). Binding of HGF to MET triggers receptor dimerization and transphosphorylation, which lead to conformational changes of the receptor with subsequent activation of the tyrosine kinase (TK). Activation of the TK domain in turn mediates downstream signaling via the phosphoinositide 3-kinase (PI3K)/AKT, RAS-RAC/RHO, mitogen–activated protein kinase (MAPK) and phospholipase C (PLC) pathways (8, 9). During embryogenesis, MET receptors are expressed on epithelial cells as well as on muscle precursor cells to mediate epithelial to mesenchymal transition which is essential for limb bud development (10). MET signaling is maintained throughout adult life where it plays an important physiologic role for cell migration, cell growth, cell differentiation, angiogenesis and cell survival (11). Dysregulation of the MET-HGF signaling pathway has been
reported in many different cancer types (3) and results in cancer cell proliferation, survival, invasion, motility and the formation of metastasis (12-14). MET overexpression was shown in various cancer tissues and correlated with disease progression (15-17). However, MET expression in non-small cell lung cancer is reported controversially and ranges from 5%-75% with most of the studies indicating a negative prognostic impact (18).

Disturbed MET expression can occur by multiple mechanisms, including overexpression, kinase activation, paracrine and autocrine activation via HGF, MET mutation, epigenetic changes as well as MET gene amplification (2, 19-22). In NSCLC patients, amplification of the MET gene was shown to correlate with poor clinical prognosis (2) and increased MET gene copy numbers in general were shown to be an independent prognostic factor in surgically resected non-small cell lung cancer (16). Furthermore, the activation of the MET-HGF signaling pathway has been described as one of the most critical events responsible for acquired anti-EGFR therapy resistance, and patients with increased MET gene copy numbers showed poor prognosis and resistance to EGFR tyrosine kinase inhibitors (TKI) (15, 16, 23). Some data indicate that about 20% of patients with acquired resistance to EGFR inhibition therapy and about 3% of treatment-naïve patients harbor MET amplification (24). In preclinical models, lung cancer cell lines with MET amplification were shown to be dependent on MET signaling for growth and survival (25). As a result, many new MET inhibition compounds (including antibody inhibition, small molecule inhibitors and others) have entered clinical trials (9). However, to date the frequency of MET amplification in NSCLC remains controversial and it ranges from 3% to 10%, depending on the detection technique, cut-off criteria and selection of samples (15-18, 26). The aim of this study was to evaluate the prevalence of increased MET gene copy numbers in treatment-naïve non-small cell lung cancer patients via fluorescence in situ hybridization technique (FISH), to compare the findings to existing criteria for amplification as well as to potentially define clear cut-off criteria for subgroups of
MET amplification. Therefore, a cohort of nearly 700 therapy-naïve non-small lung cancer cases was examined including different molecular subtypes such as wild-type adenocarcinomas and KRAS- or EGFR- mutated adenocarcinomas, as well as a group of squamous cell carcinomas.
MATERIALS AND METHODS

Patients

The study was carried out with a total of 693 non-small cell lung cancer tumor samples with sufficient material for molecular diagnostics. 277 (40%) female patients and 416 male patients (60%) were included into the study with a mean age of 65.8 years (range 30-92 years). About two third of the samples consisted of biopsy specimens, the remaining cases were surgical resection samples. Unselected consecutive lung cancer patients who did not undergo prior non-surgical treatment were screened for MET amplification as part of the routine molecular diagnostics program (27) of the Network Genomic Medicine Cologne (NGM) between January 2011 and July 2013. The study was conducted in concordance with the local ethical guidelines and was reviewed by the institutional ethics committee.

Samples and subgroups

The entire cohort of 693 non-small cell lung cancer tumor samples consisted of 651 primary tumors, 16 lymph node metastases and 26 samples of distant metastases from non-small cell lung cancer.

A total of 519 adenocarcinomas and 174 squamous carcinomas (SCC) were included into the study. The group of adenocarcinomas consisted of three genetically characterized subgroups: i) KRAS mutated carcinomas, ii) EGFR mutated adenocarcinomas, and iii) adenocarcinomas lacking KRAS and EGFR mutations (in the following referred to as wild-type adenocarcinomas). It is noteworthy that among the EGFR mutated tumors, there were two lesions that showed clear histological and immunohistological criteria for squamous cell carcinoma. However these tumors were not included into the group of squamous cell carcinomas but were kept in the group of EGFR mutated carcinomas. Beside these two incidentally found tumors, squamous cell carcinomas were not systematically examined for EGFR mutations. A
subset of squamous cell carcinomas was checked for KRAS mutations, but all were negative.

According to these data a total of four subgroups was defined: 1) wild-type adenocarcinomas (n=212, 30%), 2) KRAS-mutated adenocarcinomas (n=171, 25%), 3) EGFR-mutated carcinomas (n=138, 20%, including the two squamous cell carcinomas) and 4) squamous cell carcinomas (SCC: n=172, 25%).

As already mentioned, we included unselected consecutive patients from our screening program. The given frequencies of the four groups, however, do not reflect the prevalence of these genetic findings in our entire screening cohort, since the selection of the patients was based on their molecular characteristics to adjust the frequencies of patients in the four groups. The same range (from 20% to 30%) was important for further statistical analysis. Despite the fact that the analyzed cohort was numerically adjusted to the same size of the included molecular subgroups, the entire cohort consists of unselected representative cases which were consecutively included (solely based on the four molecular subtypes which were collected prospectively in parallel). The subgroups are, therefore, representative and suitable for the evaluation of prevalence and the comparison between subgroups.

No cytology specimens were included. All tumor tissues were fixed in buffered formalin and embedded in paraffin blocks. Mutational analyses for EGFR and KRAS were carried out as previously described (28).

All primary diagnoses were reviewed by two experienced pathologists, according to current recommendations and tumor diagnoses were made in accordance to the current WHO classification system (29).

**FISH assay**

Fluorescence *in situ* hybridization was carried out as previously described (30, 31). Briefly, three to four µm tissue sections were hybridized overnight with the ZytoLight SPEC MET/CEN7 Dual Color Probe (ZytoVision, Bremerhaven, Germany). Normal
tissue including vessels, fibroblasts, lymphocytes or non-neoplastic lung tissue served as internal controls. Tumor tissue was entirely scanned for amplification hot spots by using a 63x objective and appropriate filter sets (DM5500 fluorescent microscope; Leica). If MET signals showed a homogenous distribution, random areas were used for reading the slides. Twenty contiguous tumor cell nuclei from three areas – either hot spots or from randomly selected regions -, resulting in a total of 60 nuclei, were individually evaluated with the 100x or 63x objectives by counting green MET and orange centromere 7 (CEN7) signals.

MET/CEN7 ratio, the percentage of tumor cells with ≥4, ≥5 and ≥15 MET signals, and the average MET copy number per cell were calculated. All FISH-assays were evaluated by one reader (HUS).

Patients were classified into the following four groups of MET amplification status:

i) **high-level amplification** was defined in tumors with
   a) a MET/CEN7 ratio ≥2.0 or
   b) an average MET gene copy number per cell of ≥6.0 or
   c) ≥10% of tumor cells containing ≥15 MET signals.

ii) **intermediate-level of gene copy number gain** being defined as
   a) ≥50% of cells containing ≥5 MET signals and
   b) criteria for high-level amplification are not fulfilled

iii) **low-level of gene copy number gain** was defined as
   a) ≥ 40 % of tumor cells showing ≥4 MET signals and
   b) criteria for high-level amplification or intermediate-level of gene copy number gain are not fulfilled

iv) all other tumors were classified as **negative**.

The evaluation and interpretation criteria were derived from our observation on several hundred tumors (see below) and influenced by earlier published data in
which a modified Colorado score was recommended for the evaluation of MET amplification (15, 32, 33).

**MET immunohistochemistry**

We selected 65 cases from all subgroups (EGFR mutated carcinomas, KRAS mutated adenocarcinomas, wild-type adenocarcinomas and squamous cell carcinomas) with sufficient tumor material for further immunohistochemical staining. Cases were selected on the basis of previous FISH results, i.e. five tumors with high level MET amplification, five low/intermediate cases and 55 negative tumors. Stainings were carried out by using the CONFIRM SP44 anti-MET monoclonal antibody (Ventana Medical Systems, Tucson, AZ; cat No. 790-4430). Staining was done on an automated stainer system (Bench Mark ULTRA) where slides had been dewaxed, pretreated by cell conditioner No 1 at 95°C for 8 min, treated with CC1 (4 times at 20, 36, 52 and 64 minutes) and thereafter incubated by SP44 for 16 min. Finally, slides were counterstained by hematoxylin II. Immunoscoring was done according to the criteria recently proposed for non-small cell lung cancer (34, 35) by evaluating both staining intensity (negative, weak, moderate, or strong) and the percentage of these intensities. Four diagnostic subgroups (“immunoscores”) have been delineated: 3+ (≥50% of tumor cells stained exhibiting strong staining intensity); 2+ (≥50% of tumor cells with moderate or higher staining intensity but <50% strong intensity); 1+ (≥50% of tumor cells with weak or higher staining intensity but <50% with moderate or higher intensity); or 0 (no staining or <50% of tumor cells with any intensity). MET expression status was determined in a blinded fashion (by JR) in particular without any knowledge of FISH data.

**Statistics**
For statistical analysis, the SPSS software, version 21.0, (IBM Germany) was used. Chi-square, Fisher’s exact and $t$-tests were used, if appropriate. All tests were two-sided, with a 95% confidence interval.
RESULTS

Detection of MET amplification by FISH

We obtained valid and easily evaluable FISH data for all 693 samples. Based on our findings in this representative cohort of pulmonary adeno- and squamous cell carcinomas we have noticed, that nearly two thirds of these tumors do not harbor any copy number gains of the MET gene. Thus, the majority of non-small cell lung cancers is disomic for chromosome 7 with a regular number of MET gene copies, indicated by one to two MET and CEN7 signals in the fluorescence in situ hybridization analysis (Figure 1). However, one third of the tumors showed an increase in either MET or CEN7 signals or in both of them. It is noteworthy, that most of those cases are characterized by an uneven, i.e. heterogeneous or focal distribution of signals. We have observed focally amplified tumor clones in a background of tumor tissue with only slight increase in copy numbers as well as cancers in which isolated amplified cells occurred patchily distributed throughout the entire tumor tissue. Based on these experiences, we were able to establish evaluation criteria for MET FISH (see Materials and Methods section for details) in which we also included previously published data. By applying these criteria we categorized lung carcinomas into four amplification patterns (Figure 2). The average MET gene copy number per tumor cell showed a great variability throughout the entire cohort (range: 1.3 to 31.0; mean: 3.2). Mean copy numbers per nucleus were 2.5, 3.7, 4.9 and 9.3 for non-amplified, low-level, intermediate-level and high-level tumors, respectively. Another unique finding in MET FISH on non-small cell lung cancer is the occurrence of amplicons, which include parts of or the entire centromeric region (Figure 1H) without a numerically balanced MET copy number gain. This phenomenon may result in a MET/CEN7 ratio below 1.0. Thus we noticed ratios from only 0.2 up to 14.1 (mean: 1.2, 1.3, 1.5, 2.9 and 1.3 for non amplified, low level, intermediate level, high level cases and the entire cohort, respectively). Tumor cells containing large clusters with 15 or more MET gene copies were nearly
restricted to the group of high-level cases and occurred only rarely in all other subgroups.

**Distribution of amplification patterns**

A total of 227 tumors (33%) showed criteria for any type of *MET* copy number gains (high-level amplification, intermediate-level or low-level copy number gain). 22 carcinomas fulfilled the criteria for high-level amplification (3%; Table 1, Figure 2), 43 (6%) tumors reached the criteria for intermediate-level and 162 tumors (24%) for low-level gene copy number gain (Figure 2).

The different subgroups, i.e. *EGFR*-mutated carcinomas, *KRAS*-mutated adenocarcinomas, wild-type adenocarcinomas and squamous cell carcinomas did not show any statistically significant difference in the frequency of low, intermediate or high level *MET* amplification (Figure 2). Furthermore, *MET*/CEN7 ratio, gene copy number per cell, percentage of tumor cells containing ≥4, ≥5 or ≥15 signals were not significantly different between the different tumor groups (Figure 3). We found three high level amplified cases in the group of *EGFR*-mutated carcinomas, four high-level amplified tumors among *KRAS*-mutated adenocarcinomas, and six highly amplified cases in the squamous cell carcinoma cohort. The highest number of nine high level amplified cases was found among wild-type adenocarcinomas. This distribution, however, was not statistically significant. To note, one of the *EGFR*-mutated tumors with histologic and immunohistologic characteristics for squamous cell carcinoma showed low-level *MET* copy number gain.

According to sex, the group of high-level amplified tumors was distributed as follows: 14 (64%) male patients and 8 (36%) female patients without significant enrichment for any sex when comparing the amplification negative and positive patients (p = 0.87). Furthermore, there was no difference in the mean age at diagnosis. The mean age of the entire cohort was 65.8 years (range 30-92 years) with a mean age of all high-level amplified cases of 63.7 years (range 45-91, p=0.35). However, the group
of EGFR-mutated patients (irrespective of the MET status) showed a higher age at diagnosis (mean: 68.0 years) when compared to KRAS-mutated tumors (mean age: 63.7 years) or wild-type adenocarcinomas (mean age: 64.5 years; t-Test: p=0.001 and p=0.008, respectively).

Of all high-level amplified cases (n=22), 18 tumors showed an average number of MET signals ≥ 6 (mean: 4.5), 11 cases showed a MET/CEN7 ratio ≥ 2 (mean 1.5) and 11 of these cases exhibited ≥10% of tumor cells containing ≥15 MET signals or large clusters. Only five cases fulfilled all three criteria (Table 1).

**Correlation between MET amplification and protein expression**

In order to evaluate whether our FISH evaluation score is related to and can be confirmed by c-MET receptor protein expression, 55 tumor cases were selected comprising the different amplification levels. Over all, immunohistochemistry and FISH results were statistically significantly correlated (Table 2; Chi-Square: p<0.0001). Even if one subdivides IHC stainings into “positive” (scores 2+ and 3+) and “negative” (scores 0 and 1+), those categories were significantly correlated with FISH results (high level vs. negative/low level/intermediate level; Fisher’s Exact test: p=0.001). Most interestingly, immunoscore 3+ was strongly correlated with high-level amplification. Four out of five cases (80%) with high-level amplification were intensely stained resulting in immunoscore 3+ (Table 2, Figure 4). One highly amplified tumor reaching immunoscore 2+ showed markedly lower MET gene copy numbers compared with the 3+ cases, but yet fulfilled the criteria of high level amplified cases. Moreover, immunoscore 3+ was exclusively noticed in tumors with high level amplification. On the other hand, negative or weak immunostainings (“IHC negative” cases; immunoscores 0 or 1+) were associated with FISH negativity. Tumors with low level/intermediate level MET gain showed mostly a 2+ immunoscore (three out of five low/intermediate level cases). However, 2+ staining was also seen in FISH negative tumors as well as in one highly amplified carcinoma. Therefore, we
conclude i) that our FISH findings could be confirmed by immunohistochemistry as an independent method, ii) that immunohistochemistry and FISH are basically correlated to each other, and iii) that this FISH-IHC correlation is best in clearly positive and negative categories (FISH: negative and high level amplification, IHC: scores 0/1+ and score 3+) whereas IHC and FISH are only weakly correlated in borderline categories (FISH: low/intermediate level, IHC score 2+) which, however, account for a significant proportion of lung cancers (FISH: 205/693 (30%), IHC: 14/65 (22%) in our cohort).
DISCUSSION

Activated MET is a potential therapeutic target in lung cancer and several drugs with anti-MET activity have entered early or even late clinical trials (3). Most of them are small molecule tyrosine kinase inhibitors, but also monoclonal antibodies and anti-HGF compounds have been developed and introduced to clinical application. It is important to keep in mind that MET signaling can be the result of various activated mechanisms in tumor cells. Gene amplification is a major pro-oncogenic event leading to MET receptor activation. Other known mechanisms such as gain-of-function-mutations, protein overexpression, paracrine and autocrine activation via HGF, as well as epigenetic changes have been reported and need to be taken into account (15, 16, 18, 36). Furthermore, some of these mechanisms may be related to each other. To date, two major issues are still unsatisfactorily addressed: i) which type of MET activation is relevant for which tumor entity and its initiation and propagation, and ii) in terms of biomarkers, which type of activation is predictive for which anti-MET compound.

In this study, we aimed to investigate MET amplification as one relevant mechanism of MET activation in non-small cell lung cancer. By analyzing a large cohort of squamous cell carcinomas and adenocarcinomas, to the best of our knowledge, we provide the currently most comprehensive data set on the prevalence of MET amplifications in therapy-naïve non-small cell lung cancer. Secondly, we are able to define amplification patterns of the MET gene and thirdly, we correlate our findings with common genetically defined subgroups of pulmonary carcinomas. Our findings may be useful in the context of screening programs for patients in upcoming clinical trials with c-MET inhibitors. Furthermore, they may also have impact on the future application of EGFR TKI therapy administration since our data provide evidence that MET amplification occurs also in EGFR mutated carcinomas prior to TKI treatment and hence may represent a mechanism of primary resistance to TKIs in these tumors.
One major finding of our study is the fact that MET amplification can occur in both adenocarcinomas and squamous cell carcinomas of the lung and that MET amplifications appear even in the background of EGFR and KRAS mutations. Thus MET amplification does not fulfill the criteria of a genetically epistatic oncogenic driver lesion at least in these cases (37). However, here we show unambiguously that MET amplification is not a mutually exclusive event to KRAS and/or EGFR mutation in adenocarcinomas. Our data reveal that both, high level amplification as well as low/intermediate level copy number gains occur in treatment-naïve carcinomas of different genetic background and that there is no statistically significant difference in the frequency amongst the subgroups.

It is well known, that MET gene amplification shows higher frequencies in patients who received and relapsed after therapies with EGFR inhibitors (23). However it remained unclear whether MET activation occurs prior to treatment (primary resistance) or after the exposure to EGFR inhibitors (secondary resistance). To date, only very few data about the MET amplification status in treatment-naïve non-small cell lung cancer patients are available. Data from our study indicate that 2% (3/138) of EGFR mutated lung cancers and 2% (4/171) of KRAS mutated lung adenocarcinomas harbor simultaneous high-level MET amplification, a percentage which even increases, if one includes intermediate and low level cases. These findings might be an explanation for at least a part of the non-responders to EGFR TKI therapies. Therefore, it might be necessary, to determine the MET status in all EGFR mutated non-small cell lung cancer cases prior to treatment, particularly when administrating EGFR inhibitors. As a possible result, the addition of MET inhibitors to EGFR TKIs might be necessary in amplified cases.

Furthermore, one has to keep possible KRAS mutations in mind when administrating unselected MET inhibition therapy and being confronted with treatment resistance. Based on our data it might be wise to determine the KRAS status of patients who are candidates for an anti-MET therapy.
In a recent report by the TCGA network (38) the frequency of MET amplifications was 2.2% which is basically in the range of our findings. Data from this particular publication, however, suggest that high level MET amplification is mutually exclusive with EGFR and KRAS mutations since MET amplification could not be detected in these molecular subgroups. This finding is in contrast to our aforementioned observation. One explanation for this discrepancy might be the number of analyzed samples. The TCGA cohort consisted of 230 pulmonary adenocarcinomas among them 32% KRAS (n=75) and 11% EGFR (n=26) mutants and a total of six MET amplified cases. On the other hand 171 KRAS and 138 EGFR mutated cases were included in our study, and we could detect a total of 22 samples with high level MET amplification. We found that EGFR/MET and KRAS/MET co-alterations are rare with 0.6% (3 out of 519 cases) and 0.4% (2/519) of all adenocarcinomas, respectively. Therefore, this phenomenon might not be perceptible in smaller cohorts. As mentioned before we are, however, convinced that co-alterations of MET and canonical oncogenic drivers might have direct clinical implications given the high incidence of lung cancer.

In summary, we here show that two thirds of non-small cell lung cancers do not reveal any changes in MET gene copy numbers whereas 3% of these tumors harbor high level amplification. Both of these categories can be considered as certain, to some extent independently from classification and scoring systems (see below).

Interestingly, nearly 30% of pulmonary non-small cell carcinomas in our study showed a moderate increase in MET gene copy numbers. Since this cohort was more heterogeneous and showed larger variations in gene copy numbers and MET/CEN7 ratio than negative or high level amplified cases, we defined the groups of intermediate level copy number gain (tumors showing ≥ 50% of cells with ≥ 5 MET gene copies per cell) and low level copy number gain (≥ 40% of cells with ≥ 4 MET copies). Intermediate level cases account for 6% and low level tumors for 24% of all
investigated non-small cell lung cancers. To date, it is unclear whether patients with these tumors will also benefit from anti-MET treatment.

Our aim was to propose an easily applicable MET amplification classification system, which might serve as a reliable basis and as a potential biomarker for consecutive studies of MET inhibitors. The defined thresholds are suggested on the basis from the experiences in our large cohort of nearly 700 consecutive lung cancer cases and previous attempts (15, 18, 32, 33). Our proposal allows two define a clear-cut high level amplification category, which is defined by certain and easily reproducible criteria (i.e., \( \text{MET/CEN7 ratio} \geq 2.0 \) or average gene copy number \( \geq 6.0 \) per tumor cell or \( \geq 10\% \) of tumor cells containing \( \geq 15 \) MET signals). These parameters are widely accepted as criteria for true amplification of many genes, and – based on our findings - are suitable to select cases with the highest level of MET amplification for ongoing clinical trials (the top 3%). On the other hand, our scoring system clearly defines a large cohort (67% of all squamous cell and adenocarcinomas), which does not show any significant MET gene copy alteration. Application of our proposed low and intermediate level categories is to date somewhat arbitrary since – as already mentioned – the clinical relevance is still unclear. We decided to introduce those categories only i) to facilitate the definitions of negative and high level amplified categories and ii) to allow a better comparability with previous attempts of MET scoring. Some of those publications have used our criteria for low and/or high level amplification as parameters for “MET positivity”. These attempts resulted in a high proportion of “MET positive” cases whereas most of these authors did not discriminate between polysomic tumors and true amplifications.

In a subgroup of 65 cases, we could finally verify our FISH findings by immunohistochemistry in which MET protein overexpression was highly associated with high level amplification, indicating that our FISH findings basically have a biologic relevance. Larger studies, however, are needed to clarify whether MET amplification status and MET protein expression (measured by
immunohistochemistry) are correlated and which biomarker assay has the best predictive value. Our findings indicate that the IHC 2+ cases represent a genetically diverse group of tumors. Therefore, IHC might have limited diagnostic value especially in these tumors and met in situ hybridization could be superior to IHC – especially in characterizing genetically diverse borderline cases. Therefore, we propose to include the genomics-based information, i.e. the amplification status, obtained by FISH, in the panel of potential biomarkers.
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References:


33. Hirsch FR, Varella-Garcia M, Bunn PA, Jr., Di Maria MV, Veve R, Bremmes RM, et al. Epidermal growth factor receptor in non-small-cell lung carcinomas:


TABLES
Table 1: Characteristics of 22 pulmonary carcinomas with high level MET amplification.

<table>
<thead>
<tr>
<th>Age</th>
<th>Gender</th>
<th>Subgroup</th>
<th>MET/CEN7 Ratio (threshold: ≥2.0)</th>
<th>Average MET gene copy number per nucleus (threshold: ≥6.0)</th>
<th>Percentage of tumor cells containing ≥15 MET signals (threshold: ≥10%)</th>
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mean(range)  
64(45-91)  
mean(range)  
2.9 (1.0-14.1)  
mean(range)  
9.2 (3.8-31)  
mean(range)  
18.9 (0-95)
EGFR; *EGFR* mutated carcinoma; KRAS, *KRAS* mutated adenocarcinomas, wt, wildtype adenocarcinoma; SCC, squamous cell carcinoma. Grey boxes indicate fulfilled criteria. Note that all criteria for high level amplification are fulfilled in only five cases. High level amplified tumors represent 3.2% of all pulmonary carcinomas and can occur in the background of *EGFR* and *KRAS* mutations of adenocarcinomas as well as in squamous cell carcinomas.
Table 2: Correlation of *MET* amplification status with MET protein expression

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For definition of immunohistochemistry scores and FISH categories see Materials and Methods section.
FIGURE LEGEND

Figure 1: MET amplification patterns.
Amplification patterns are detected by using a dual color FISH assay with green (MET) and orange probes (centromere 7). A) Negative case with one to two MET copies per tumor cell nucleus. B) Low level of copy number gain, defined by ≥4 MET copies in ≥40% of tumor cells. C) Intermediate level of copy number gain, defined by ≥5 MET copies in ≥50% of tumor cells. D) High level amplification with very high gene copy number and homogenous signal distribution. E) High level amplification with heterogeneously signal distributed MET copies. F) High-level amplification with focal, so called “hot-spot” amplification areas. G) Heterogeneous signal distribution in a non-amplified case. H) Frequently occurring clusters of CEN7 signal without increased MET gene copy number leading to ratio below 1.

Figure 2: Frequency of the different amplification levels in NSCLC subgroups.
A) all cases combined, B) EGFR-mutated carcinomas, C) KRAS-mutated adenocarcinomas, D) wild-type adenocarcinomas and E) squamous cell carcinomas.

Figure 3: Frequencies of applied amplification criteria in the context of the different subgroups.
a) MET/CEN7 ratio (threshold: ≥2.0), b) average MET gene copy number per tumor cell nucleus (threshold: ≥6.0), c) percentage of tumor cells containing ≥15 MET signals, d) percentage of cells containing ≥5 MET signals and e) percentage of cells containing ≥4 MET signals are indicated for lung cancer subgroups. Note that a), b) and c) are criteria for high level amplification; d) defines the intermediate level and e) the low level group. Thresholds are indicated by horizontal lines.
Figure 4: MET Immunohistochemistry.

A) weak, B) moderate and C) strong staining intensity. Intensities and the proportion of immunostained cells result in the final immunoscore (0 to 3+; see Materials and Method section for details).
Figure 1
Figure 2
Figure 3
Figure 4
Clinical Cancer Research

MET amplification status in therapy-naïve adeno- and squamous cell carcinomas of the lung


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