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Prevalence and clinical association of *MET* gene overexpression and amplification in patients with NSCLC: Results from the European Thoracic Oncology Platform (ETOP) Lungscape project

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ABSTRACT

Introduction: In a well-defined NSCLC cohort of the ETOP Lungscape program, we explored the epidemiology of IHC MET overexpression and amplification, their inter-correlation, and their association to outcome.
Methods: Resected NSCLC were assessed for *MET* gene copy number (GCN) and expression using silver in-situ hybridization (SISH) and immunohistochemistry (IHC) on TMAs in a multicenter setting. *MET* amplification was defined as MET/centromere ratio ≥ 2 (with average *MET* GCN ≥ 4), high *MET* GCN as CGN ≥ 5 and MET IHC+ as ≥2+ intensity in ≥50% of tumor cells. A total of 182 MET IHC+ and EGFR/KRAS WT tumors were analyzed for *MET*ex14 skipping mutation.
Results: MET IHC+ was found in 23.8% of 2432 patients, significantly associated with female gender, small tumor size, and adenocarcinoma histology. We observed a high inter-laboratory variability in IHC and SISH analysis. *MET* amplification prevailed in 4.6% and *MET* GCN ≥ 5 in 4.1% of 1572 patients. *MET* amplification and *MET* GCN ≥ 5 were not significantly associated with any tumor characteristics or stage. Both were

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significantly associated with IHC MET positivity ($p < 0.001$). *MET*ex14 skipping mutation prevailed in 5 of 182 (2.7%) MET IHC+ WT EGFR/KRAS NSCLC, 4 of which within the 88 adenocarcinomas (4.5%). No association of IHC MET overexpression, SISH *MET* amplification or high *MET* GCN was found with OS, RFS or TTR.
Conclusion: MET overexpression is found in 23.8% of surgically resected NSCLC. *MET* amplification prevails in 4.6% and is associated with MET overexpression. Both have no influence on prognosis. The large inter-laboratory variability in IHC highlights the challenge of MET IHC analysis in routine practice.

1. Introduction

Non-small cell lung cancer (NSCLC) is known to be molecularly heterogeneous [1] and its growth and dissemination has been associated with various oncogenic drivers [2–4]. The c-Mesenchymal-Epithelial Transition factor (c-MET) receptor tyrosine kinase (RTK) pathway is frequently activated in many human cancers, and has been shown to have a critical role in tumor formation, progression, metastasis and angiogenesis, as well as resistance to other cancer treatments. Inappropriate activation of *MET* can be induced by specific genetic mutations, transcriptional upregulation or ligand-dependent autocrine or paracrine mechanisms. The most frequent cause of constitutive *MET* activation in human tumors is increased protein expression as a consequence of transcriptional upregulation, in the absence of gene amplification or mutation [5–7]. Amplification of the *MET* gene, with consequent protein overexpression and constitutive kinase activation, has been reported in a number of human primary tumors, including NSCLC, notably in the context of acquired resistance to epidermal growth factor receptor (EGFR) inhibitors [8,9]. Both MET overexpression and amplification have been related to a poor prognosis in NSCLC patients [6–10], while amplification is hypothesized to possess a transforming and driver capability. *MET* exon 14 (*MET*ex14) skipping mutations have recently been discovered as another driver mechanism that prevails in approximately 3% of non-squamous NSCLC and is associated with *MET* genomic amplification and overexpression [7,11].

The true prevalence of MET overexpression and amplification, in newly diagnosed NSCLC, is not yet known accurately, and clinical correlations are emerging slowly. In addition, a standard definition for c-*MET* amplification is still lacking. In this study, we aim at investigating the epidemiology and the natural history of MET positivity in stage I–III NSCLC and increasing our understanding of patient groups likely to benefit from MET-directed targeted treatments [12].

2. Methods

2.1. Study design

This is a cohort study of surgically resected, stage I–III NSCLC cases from the Lungscape cohort [13]. Comprehensive clinical annotations and molecular data are available in the iBiobank database (https://etopdata.etop-eu.org). Tissue microarrays (TMAs) constructed in the context of the ETOP Lungscape program have been analysed at their site of origin, using the same methods and procedures.

2.2. Training and external quality assessment (EQA)

Representatives of all participating centers took part in a central 1.5-day immunohistochemistry (IHC)-scoring workshop and in a silver in-situ hybridization (SISH)-training webinar held by the first author (L.B.). All centers successfully completed the EQA of MET IHC/SISH analysis on EQA TMAs and full tissue sections.

2.3. Definitions of MET overexpression/amplification

C-MET protein expression has been measured using the CONFIRM anti-total c-MET (SP44) rabbit monoclonal antibody (Ventana) on the Ventana Benchmark XT platform using established scoring criteria [14]. A dual-color SISH has been used to analyze C-*MET* gene copy number (GCN) [15].

MET positivity (overexpression) corresponds to maximum IHC MET score 2+ or 3+ with ≥50% of stained tumor tissue ≥2+. The TMA spot with the highest IHC MET staining was selected for scoring. Similarly, the spot with the highest number of *MET* signals was selected, and 50 non-overlapping tumor cells were analyzed for number of *MET* and centromere signals. For ***MET* amplification** or copy number gains, there is no global consensus definition. In the literature several alternative definitions have been applied [15–19]. In the current context, the primary definition for SISH *MET* amplification is: *MET*/Centromere ratio (*MET*-ratio) ≥ 2 with average *MET* GCN ≥ 4. An alternative categorization was explored, with 3 levels of amplification intensity, also using the GCN ≥ 4 restriction: No amplification: *MET*-Ratio < 1.8; Low amplification: 1.8 ≤ *MET*-Ratio ≤ 2.2; Intermediate: 2.2 < *MET*-Ratio < 5; High: *MET*-Ratio ≥ 5. Dropping the GCN ≥ 4 restriction leads to the Camidge et al. [18] definition, investigated in a sensitivity analysis.

Increased *MET* GCN was explored using two different thresholds: the overall median GCN and the (high) value 5.

2.4. METex14 mutation analysis

*MET*ex14 splice site mutations have been reported to be enriched in *MET* strongly IHC positive and highly amplified NSCLC, and particularly frequently in the sarcomatoid subtype [20]. Therefore, *MET*ex14 investigation was performed on a "*MET*+ enriched cohort", of 182 patients with *MET* amplification or overexpression or sarcomatoid histology, without EGFR/KRAS mutations. Mutation analysis was done by direct Sanger sequencing. Valid point mutation had to be detectable in at least two independent PCR reactions with one of the primer pairs (Table A1). Identified deletions were confirmed in an independent PCR reaction. The used DNA amount ranged between 4 ng and 20 ng for most samples. Analysis was done manually with "SeqScape v2.5" software and validated with automated variant calling by the "Sequencher v4.10.1" software (More details in the Appendix).

2.5. Statistical analysis

Prevalence of MET overexpression, amplification or increased/high GCN is expressed as a percentage with corresponding 95% exact binomial confidence interval (CI).

Clinico-pathological characteristics are compared between groups of patients (by their MET status), using the Fisher's exact, the Mantel-Haenszel, the Mann-Whitney or the Kruskal-Wallis test, depending on type of data.

The main endpoint is overall survival (OS; time from surgery to death from any cause). Secondary endpoints are recurrence-free survival (RFS) and time-to-relapse (TTR) [13,16]. Kaplan-Meier curves by MET status and log-rank tests are used for evaluating differences in outcome. Cox proportional hazards models (unadjusted/adjusted) assess the effect of MET status on the outcome.

All tests are performed at the 5% significance level. Statistical analysis is carried out in SAS version 9.3. Additional statistical methodology is provided in the Appendix.

3. Results

3.1. Study cohorts

A total of 2709 retrospective cases from 17 centers have been captured in the ETOP iBiobank as of *7th July 2015* (clinico-pathological details in Table A2): 2432 (90%) have available IHC MET (cohort-1), 1572 (58%) SISH *MET* GCN (cohort-2), with 1540 cases in common.

3.2. Prevalence of MET overexpression and amplification – overall, by histology

Positive IHC MET staining (MET+) is present in 579 cases (23.8%; 95% CI [22.1%, 25.6%]): 332 IHC 2+, 247 IHC 3+. *MET*+ is significantly higher in adenocarcinomas compared to squamous cell carcinomas (33.2%; 95% CI [30.6%, 35.9%] vs 12.7%; 95% CI [10.8%, 14.9%], p-value < 0.001). MET H-score [16] distribution is presented in Figure A1 .

The overall prevalence of SISH *MET* amplification, is 4.6% (95% CI [3.6%, 5.7%]), not significantly different between adenocarcinomas and squamous cell carcinomas (5%; 95% CI [3.6%, 6.7%] vs 3.6%; 95% CI [2.3%, 5.4%], p-value = 0.25). For the alternative 3-level SISH *MET* amplification, the prevalence is: Low 1.8% (95% CI [1.2%, 2.6%]), Intermediate 2.5% (95% CI [1.8%, 3.4%]), High 1% (95% CI [0.5%, 1.6%]), also not significantly different between adenocarcinomas and squamous cell carcinomas (p-value = 0.26).

Based on the median value of average *MET* GCN per cell (2.28), we classify patients in 'increased *MET* GCN" (≥2.28, n = 787) and "not increased *MET* GCN" (< 2.28, n = 785). The overall prevalence of high (≥5) *MET* GCN is 4.1% (95% CI [3.2%, 5.2%]). In both cases, no significant difference between adenocarcinomas and squamous cell carcinomas is detected (p-values: 0.22, 0.053).

3.3. Exploration of MET-ratio and GCN distributions

The distribution of GCN, overall and by MET-ratio level (< 2 or ≥2) is displayed in Figure A2 and the distribution of *MET*-ratio, overall and by GCN level, in Figure A3 . Both figures reveal skewed distributions, while a positive correlation between the two measures emerges (Fig. 1).

3.4. Association IHC-SISH for assessing MET

IHC MET positivity is significantly associated with SISH *MET* amplification and increased/high *MET* GCN (p-value < 0.001 in all cases, Table 1). *MET* amplification is present in 9.7% of MET+ and 3.0% of MET- patients and high *MET* GCN (≥5) in 8.5% of MET+ vs. 2.8% of MET- patients. *MET* amplification and increased/high GCN is significantly more common for IHC3+ compared to IHC2+ (p-values 0.02, 0.005, < 0.001, respectively; Table 1).

MET GCN is significantly higher for MET+ patients (median GCN 2.7 vs 2.2 for MET-, p-value < 0.001). *MET*-ratio does not differ significantly between MET+ and MET- (data not shown).

3.5. Association of MET overexpression/amplification with clinico-pathological characteristics

IHC MET positivity is more frequent in women (30.9% vs 20.1% in male), in never smokers (30% vs 23.0%/21.9% in former/current smokers), in adenocarcinomas (33.2% vs 12.7% in squamous cell

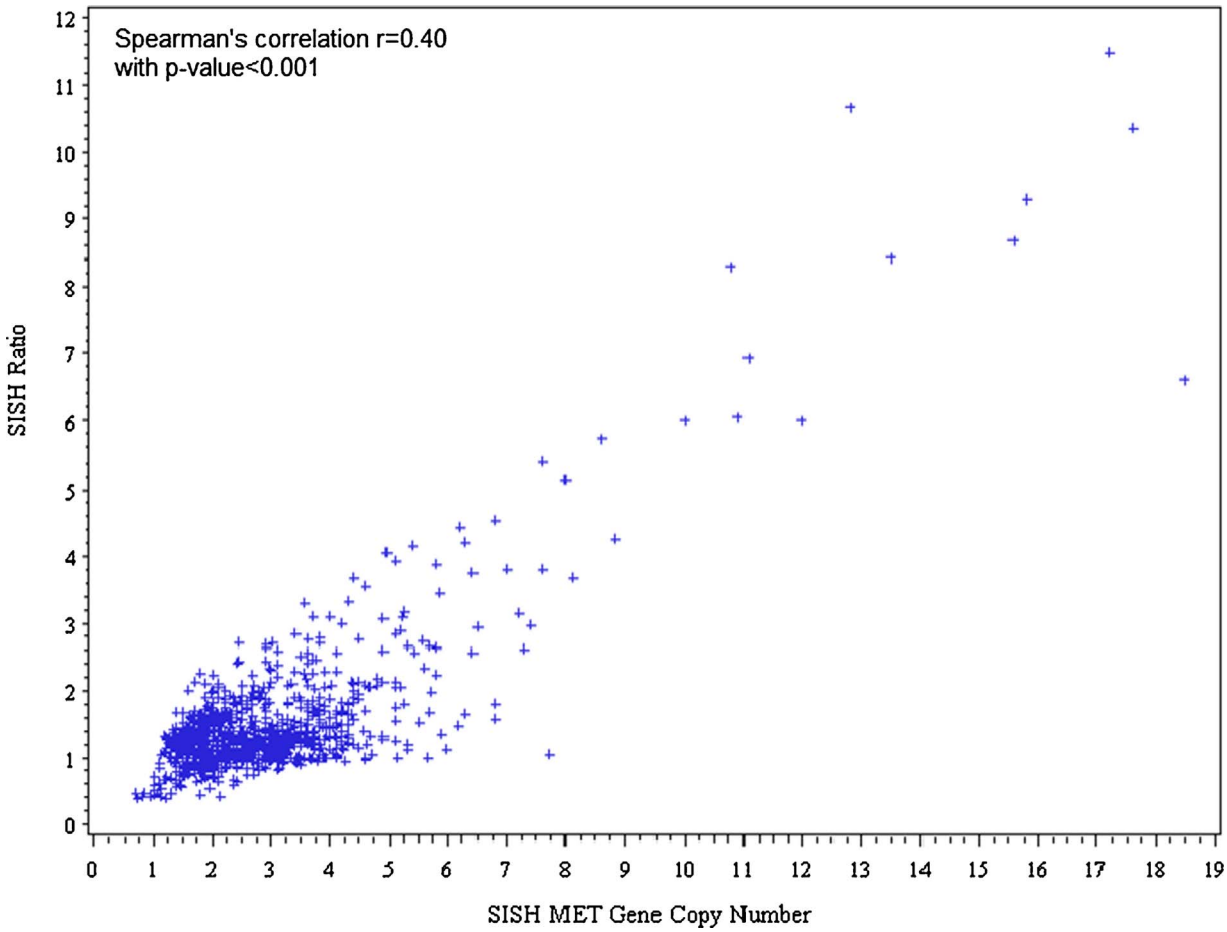

Fig. 1. Scatterplot of SISH GCN with SISH Ratio.

Table 1
Association of IHC MET positivity with SISH MET positivity (N = 1540).

SISH method	Overall IHC MET + (n = 351)	*IHC 2 + (n = 181)*	*IHC 3 + (n = 170)*	*p-value* (2 + vs 3 +)	IHC MET− (n = 1189)	p-value MET + vs MET−
SISH *MET* Amplification						
Yes	34 (9.7)	*11 (6.1)*	*23 (13.5)*	*0.02**	36 (3.0)	< 0.001*
No	317 (90.3)	*170 (93.9)*	*147 (86.5)*		1153 (97.0)	
SISH *MET* GCN (by median value 2.28)						
High	234 (66.7)	*108 (59.7)*	*126 (74.1)*	*0.005**	544 (45.8)	< 0.001*
Low	117 (33.3)	*73 (40.3)*	*44 (25.9)*		645 (54.2)	
SISH *MET* GCN (by threshold 5)						
High	30 (8.5)	*6 (3.3)*	*24 (14.1)*	*< 0.001**	33 (2.8)	< 0.001*
Low	321 (91.5)	*175 (96.7)*	*146 (85.9)*		1156 (97.2)	

Note: *Fisher's exact test.

carcinomas), in smaller tumors (26.9% for size ≤4 cm vs 18.6% for size > 4 cm), in patients with surgery after 2006 (26.6% vs 20.9% for surgeries up to 2006) (all p-values ≤ 0.001, except for smoking where p-value = 0.006).

No patient or tumor characteristic is significantly different between patients with or without SISH *MET* amplification (primary definition). However, the low prevalence of *MET* amplification makes the acquisition of significant results difficult. This is also true for the GCN categorization based on the value 5, where only one significant result appears: patients with high GCN are younger than those with low GCN (median ages 60.6 and 66.4 respectively, p-value = 0.003).

Increased *MET* GCN is more frequent in never smokers (61.1% vs. 53.5%/38.7% in former/current smokers), in patients without previous cancer history (57.6% vs 45.7% in patients with previous cancer history), in younger patients (median age 65.4 years for patients in High group vs 66.7 in Low group), and in patients with surgery after 2006 (57.4% vs 43.4% for surgeries up to 2006) (all p-values ≤ 0.001).

The 3-level *MET* amplification displays a similar time-effect (p-value < 0.001), while intermediate-high level is more common in male (4.2% vs 1.8% in female, p-value = 0.013) and former/current smokers (4.1%/3.9% vs 0.9% in never smokers, p-value = 0.018).

MET positivity (overexpression/amplification, increased/high GCN) is not significantly associated with ALK positivity (IHC/FISH) (all p-values non-significant), evaluated on adenocarcinomas with available ALK results (IHC ALK for 1092/771 patients from cohort-1/cohort-2; FISH ALK for 226/168 patients from cohort-1/cohort-2).

The significant associations between year of surgery with MET IHC positivity and increased *MET GCN*, respectively, were further explored.

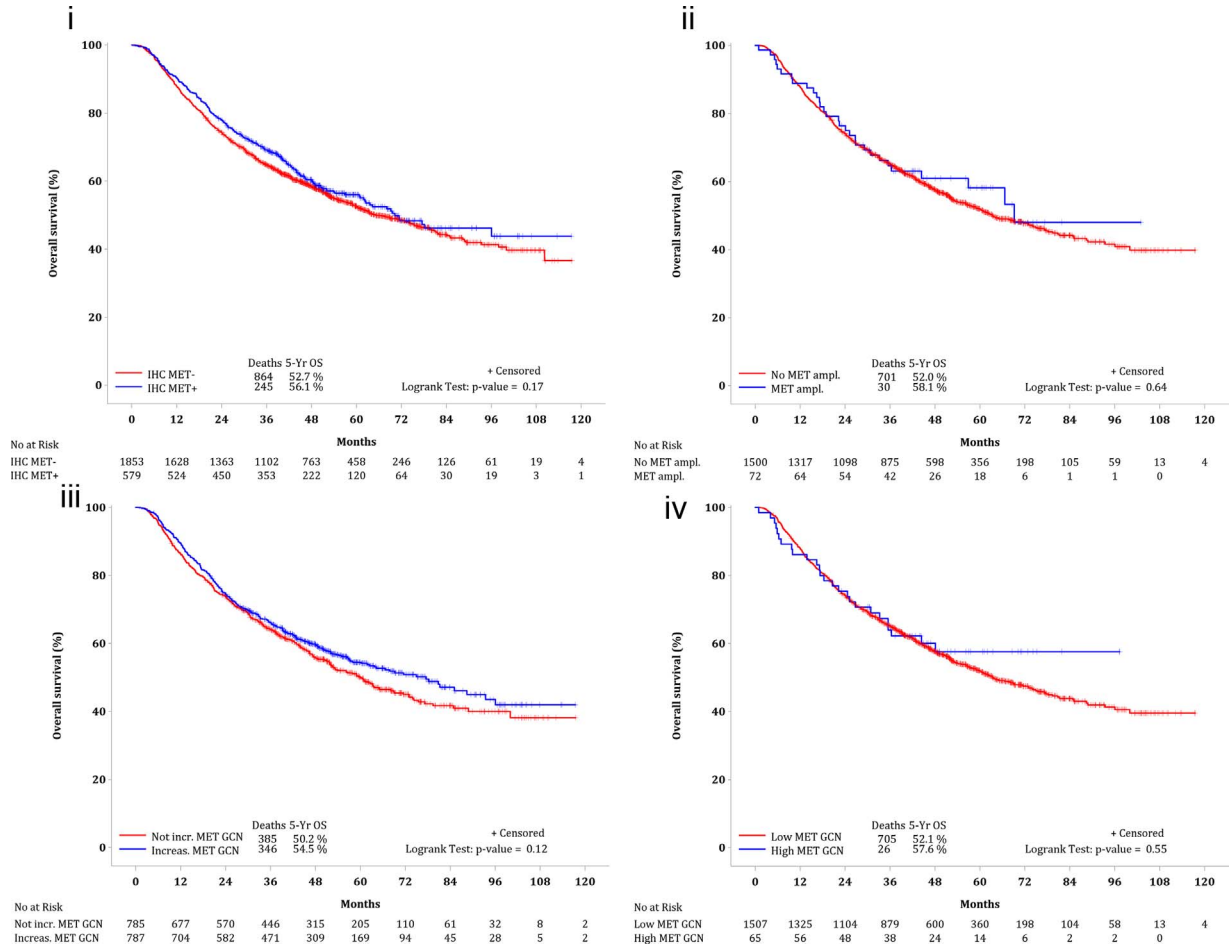

Fig. 2. (i) Overall Survival (OS) by IHC MET positivity. (ii) Overall Survival (OS) by SISH MET amplification. (iii) Overall Survival (OS) by SISH MET GCN (threshold of median). (iv) Overall Survival (OS) by SISH MET GCN (threshold of 5).

In the cohort of patients with available IHC, for surgeries up to 2006, the percentage of former/current smokers is higher than for those after 2006 (89.2% vs. 84.8%; p-value = 0.0017). This is also the case in the smaller cohort of patients with SISH *GCN* information (88.6% vs. 83.7%, p-value = 0.0060). Together with the association between never smoking and IHC MET positivity and increased *MET GCN* these findings can partly explain the observed correlations between MET IHC and increased *GCN* with year of surgery. Patients with increased *MET GCN* might be further enriched for after 2006 since the percentage of patients with a previous cancer history is significantly lower after 2006 than up to 2006 (15.9% vs. 24.8%, $p < 0.001$) and at the same time negatively associated with increased MET *GCN*, as described above.

3.6. Clinical significance of MET overexpression/amplification: association with outcome

The median follow-up time is 57.3 months (Interquartile Range: 45.2–73.9). Overall, 47.7% of patients are alive with no evidence of disease while 32.6% have died with disease.

The 5-year OS of the Lungscape cohort is 53.7% (95% CI: 51.6%, 55.7%) with median OS 69.1 months (95% CI: 62.8, 76.3); the 5-year RFS is 47.2% (95% CI: 45.1%, 49.2%), with median RFS 52.5 months (95% CI: 47.4, 57.9); and the 5-year TTR is 57.5% (95% CI: 55.4%, 59.5%), with median TTR 103 months (95% CI: 97.0, Not Estimable).

None of these outcome variables (overall and by histology) differs significantly by MET overexpression/amplification, increased/high *MET* GCN (Kaplan-Meier for OS: Fig. 2 (all patients), Appendix Figure A4 (by histology type); RFS and TTR: Appendix Figures A5 –A8).

Cox models for OS, RFS and TTR show no statistical significant effect of MET status to the outcome.

3.7. Inter-laboratory variability of MET overexpression and GCN assessment

The MET IHC positivity rate between the centers varied highly from 1.6% to 41.6% (Table A3), while increased levels of GCN by SISH ranged from 12.0% to 98.9% (Table A5). Variability of MET amplification and high GCN levels (> 5) was not evaluable due to the low prevalence. Blinded IHC re-scoring of the TMA sections of two outlier centers by the first author (L.B.) revealed statistically significant over-rating and under-rating, respectively, by the two centers. Excluding the data from these two centers did not change the conclusion of all statistical analyses in this study (data not shown).

3.8. Prevalence of METex14

The overall prevalence, on the "MET+ enriched" cohort, of *MET*ex14 is 2.75% (5/182), (95% CI: 0.9%, 6.3%). In adenocarcinomas, the prevalence increases to 4.5% (4/88; 95% CI: 1.3%, 11.2%). One *MET*ex14 was found among the 80 squamous cell carcinomas, corresponding to a prevalence of 1.3% (95% CI: 0.03%, 6.8%). The characteristics of the 5 *MET*ex14 cases are listed in Table A7.

4. Discussion

There is growing interest in *MET* as a potential oncogenic driver in NSCLC. Despite recent failures of clinical trials in patients with MET protein overexpression, MET remains to be an interesting candidate therapeutic target in NSCLC, in light of ongoing trials investigating the activity of MET-targeting agents in patients with *MET* amplifications and a recent report on their activity on *MET*ex14 [11,21]. Data on the prevalence and clinicopathological associations of *MET* alterations have been controversial, so far. In this largest cohort of therapy-naïve, resected NSCLC analysed so far, neither MET expression nor GCN status including amplification and polysomy were significantly associated with tumor stage and they had no impact on the measured outcome.

Our overall prevalence of 23.8% MET IHC overexpression in NSCLC is within the previously reported range (10–81%), and the higher MET positivity rate in adenocarcinomas as compared to squamous cell carcinomas concurs with previous reports [10,20,22]. We simulated a diagnostic real-life situation by using small TMAs, as well as a standardized protocol on an automated immunostainer, a well-defined scoring system combined with scoring training and an EQA by a ring trial. The between-centers variability of MET overexpression prevalence demonstrates that harmonization of MET IHC analysis remains a challenge despite all targeted efforts. This is not surprising in light of the history of HER2/neu IHC testing in breast and gastric cancer, where similar problems have been encountered [23–25]. Possible reasons for such variability include differences in pre-analytical conditions that are often difficult to track down across different centers, and individual shifting of the threshold for negative/positive findings [26]. Variation in fixation can affect the performance of certain antibodies [27]. This factor might also account for the significant association found between MET IHC positivity and small tumor size (< 4 cm) in our study, since the positivity rate might be lower in larger tumors due to relative underfixation in the central part of the tumor. Similarly, long storage of the paraffin blocks might also negatively affect the epitopes of the MET protein, which would explain the lower positivity rate of specimens obtained before 2006 than the more recent years. In general, however, long-term storage of paraffin blocks does not seem to significantly impact IHC staining [26]. Part of this finding could also be explained by the higher proportion of former/current smokers in the patients treated up to 2006 as opposed to those treated after 2006, given the significant negative association between a smoking history and MET positivity.

The problem of individual IHC threshold shift (despite central training and passed EQA) is illustrated by the two study centers with the lowest (1.6%) and highest (41.6%) prevalence of MET expression. Review of the MET IHC TMA sections of these centers revealed remarkable over- and underrating, respectively. Major inter-center variability is also present in the case of the assessment of increased GCN (≥ the median value of 2.28). This could be due difficulties in accurately scoring gene copy numbers around a low threshold and by variability of technical hybridization efficiency between the centers.

MetMab trial criteria for scoring MET IHC in this study had been defined in a stringent manner. MET IHC positive is either IHC 2+ (strong) or IHC 3+ (very strong), in ≥50% of the tumor cells, while less intensive or no staining (0/1+) is considered as negative. Notably, 2+ MET IHC score has an intensity that would traditionally be assigned to strong staining in IHC diagnostic routine across different markers, while the 1+ IHC MET score would often fall into the category of moderately strong staining if the specific MetMab criteria were not considered and trained for. This shift towards strong staining in the MetMab criteria might explain different prevalence of MET overexpression in the literature. For example, the representative figure of a 2+ MET IHC shown in a recent paper [20] does not meet the stringent criteria of MetMab IHC Score 2+ and should rather be rated as 1+. This threshold shift could explain the higher prevalence of MET positivity as compared to our study (33.5% vs. 23.8%).

The challenge of MET IHC testing despite major efforts for harmonization, coupled with the heterogeneous nature of MET expression [28], might explain why a recent phase III randomized trial failed to demonstrate benefit of onartuzumab, an anti-MET monoclonal antibody, in patients with advanced, MET IHC positive NSCLC [29].

In contrast to MET expression, *MET* amplification is emerging as a clinically more relevant biomarker to predict response to treatment with *MET* inhibitors, as illustrated by very promising data regarding crizotinib activity in that context and the fact that *MET* amplification is used as an inclusion criterion in clinical trials [18]. The prevalence of SISH *MET* amplification in our cohort (4.6%), and its significant association with MET overexpression, is in line with previous studies (reviewed in [30]). Still, only a small fraction of MET IHC positive tumors were *MET* amplified (9.7%), including those with high-level

amplification (ratio ≥ 5; 1.4%). Although there is no consensus definition of *MET* amplification, it appears that amplifications with a MET/centromere ratio ≥2.0 are relevant, high-level amplifications being most selective for response to treatment [18,31,32]. This is supported by a previous study showing that high-level amplification but not low-level amplification or polysomy (i.e. high GCN without amplification) were mutually exclusive for EGFR or KRAS driver mutations [20], although this has not been confirmed by others [19]. Interestingly, a lower GCN (< median) but not amplification status was associated with longer archival age in our study, suggesting impaired hybridization efficiency over time, as previously demonstrated for ALK FISH [33]. Amplification seems to be more robust and technically less affected by this potential technical limitation.

*MET*ex14 is emerging as a comparatively rare but important predictive marker, since excellent response rates to crizotinib have been reported [7,34–36]. The *MET*ex14 prevalence of 2.75% in our small series is identical to the 2.7% in a recent series of 11,205 patients [34]. We also found *MET*ex14 in a squamous cell carcinoma, emphasizing that *MET*ex14 analysis also provides a therapeutic opportunity in rare patients with squamous cell carcinoma. Although the low number of cases does not allow for final conclusions, the relatively high rate (4.6%) of *MET*ex14 positive adenocarcinomas in our study indicates that selection for *MET*ex14 by high MET expression and absence of other driver mutations might be a valid strategy for the time being where systematic *MET*ex14 testing is not yet feasible. This would be in line with previous studies showing a very high sensitivity of MET IHC to identify *MET*ex14, particularly in stage IV NSCLC [7,37]. The rare occurrence of MET IHC negative stage I–III NSCLC despite *MET*ex14 might be due to pre-analytical factors such as fixation problems of individual resection specimens that could negatively affect the MET immunoreactivity [7]. Like *ALK* rearrangement, both *MET* amplification and *MET*ex14 are predictors of response to the MET/ALK/ROS1 inhibitor crizotinib.

The lack of any significant association between MET expression or amplification with prognosis is in keeping with previous studies [15,38,39]. In case of *MET* amplification, however, these data are challenged by other reports where *MET* amplification appeared as independent marker of poor outcome in surgically resected NSCLC [17,20,40,41]. This discrepancy remains difficult to explain by technical factors or amplification criteria alone, but might be explained by use of different variables in multivariate analyses, differences in sample size, histology and inclusion of stage IV patients in some studies.

We think that our study draws a realistic picture of the challenges of MET testing in clinical practice across many different laboratories in different countries. Nevertheless, there are some limitations to be considered. Although we used several TMA spots per specimen, the TMA format is not fully equivalent to whole tissue specimens and might not fully control for the often heterogeneous expression seen in case of MET.

In conclusion, our data show that MET expression and increased GCN or amplification have no prognostic impact in a large multicenter cohort of mostly European patients, analyzed in a manner similar to clinical practice. The challenge of inter-laboratory discordance of MET IHC and SISH testing emphasizes the need for training and systematic quality control.

Conflict of interests

David Shames and Katja Schulze are employees of Genentech Inc., Ashis Das-Gupta is employee of F. Hoffmann-La Roche Ltd. All other authors declare no competing interests.

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Appendix A

Lungscape Consortium

Appendix L ungscape 002 MET

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Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2017.07.021.

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