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Detection of MET and SOX2 amplification by quantitative real-time PCR in non-small cell lung carcinoma

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Abstract. Non-small cell lung carcinoma is a leading cause of cancer-related death. Amplification of the two oncogenes *MET* and *SOX2* is frequently encountered in non-small-cell lung carcinoma. This study aimed to use real-time quantitative PCR to assess the correlation of *MET* and *SOX2* amplification with clinicopathological factors. This study was conducted using 115 tissue samples including 57 squamous cell carcinomas (SCCs), 50 adenocarcinomas (ADCs) and 8 adenosquamous carcinomas (ADSCs). A total of 67 patients (58.3%) had a history of smoking. Our results showed that the frequency of *MET* amplification in SCCs was significantly higher compared to ADCs (χ^2=8.0, P=0.005). *SOX2* showed a markedly preferential amplification in SCCs compared to ADCs in the smoking group cases (P=0.014). Lymph node invasion correlated with *MET* amplification in SCCs marginally more significantly compared to ADCs (P=0.02). The amplified *MET* occurred more frequently in SCCs compared to ADCs correlated to tumor dimension at a small scale (<5 cm) (P=0.01). No significant difference in *SOX2* amplification was found with regards to lymph node metastasis or tumor dimension. *SOX2* and *MET* amplifications were not associated with gender or age. However, *MET* amplification in SCCs among patients younger than 64 years of age was higher compared to ADCs and ADSCs (P=0.03). Among ADSCs, *MET* was not amplified among patients who had never been smokers or were younger than 64 years of age. Neither *MET* nor *SOX2* were amplified in tumors with dimensions <5 cm and without lymph node invasion. Findings of this study showed that *MET* and *SOX2* amplifications are more common in the SCCs of smokers. Moreover, *MET* amplification is intrinsic in SCCs particularly among smokers, with regards to tumor growth, lymph node invasion and negative correlation to *SOX2* amplification. The incidence of discrepancy in the amplifications of *MET* and *SOX2* in SCCs and ADCs suggests that the *MET* and *SOX2* genes play different roles in SCC and ADC tumorigenesis, respectively, particularly among smokers.

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Introduction

Histological subtypes of non-small-cell lung carcinomas (NSCLC) include adenocarcinomas (ADC), squamous cell carcinomas (SCC) and adenosquamous carcinomas (ADSC). NSCLCs represent 80% of lung cancers and are further classified into ADC, including bronchioloalveolar carcinoma (BAC), and SCC (1). Receptor tyrosine kinases (RTKs) regulate various key processes in mammalian development, cell function and tissue homeostasis. Alterations at the level of the receptor and its ligand lead to the activation of a number of signaling pathways, each of which may contribute to cancer progression. Deregulation of RTKs by mutation, gene rearrangement, gene amplification and overexpression of both receptor and ligand play a role as causative factors in the development and progression of various types of human cancer (2-4). The tyrosine-kinase epidermal growth factor receptor (EGFR) pathway has been shown to play a crucial role in the pathogenesis of NSCLC, leading to the development of targeted therapeutic agents using small molecule EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib or erlotinib (5-7).

Recent clinical evidence of EGFR-TKIs in refractory and advanced NSCLCs potentially indicate that EGFR-TKIs target other deregulated growth factor signaling pathways, such as the hepatocyte growth factor (HGF)/MET pathway. A recent study showed that *MET* amplification leads to gefinitib secondary resistance and may also explain the resistance noted in certain patients (8). The *MET* gene is located on band 7q31, and encodes a transmembrane tyrosine kinase receptor for HGF/scatter factor (SF), located on band 7q25. The *MET* gene is the prototypic member of a subfamily of RTKs. The *MET* RTK family is structurally distinct from other RTK families and is the only known high-affinity receptor for HGF, also known as SF (9,10). In addition to the proliferative and antiapoptotic activities that are common to various growth factors, *MET* elicits unique motogenic and morphogenic effects by stimulating cell-cell detachment, migration, invasion,

tubule formation and branching (11,12). These activities of the *MET* signaling pathway provided examples of the mechanisms by which this pathway is involved in tumor development and progression. *MET* is usually considered to be an oncogene (13) and appears to play a role in ADCs. Cigarette smoking induces overexpression of HGF in type II alveolar pneumocytes and lung cancer cells (14). Overexpression of HGF in lung cancer cells induces alveolar differentiation/proliferation, and *MET* activation may play a crucial role in well-differentiated lung ADCs (15-17). *MET* amplification has already been described in gastric and esophageal cancers (18,19).

The transcription factor *SOX2*, a member of the SRY-high mobility box transcription factor family, is expressed in epithelial cells of the foregut, including the pharynx, esophagus, trachea, bronchi and bronchioles, but is excluded from the peripheral and alveolar regions of the lung (20). *SOX2* is also expressed in developing respiratory epithelium, but is restricted to the conducting airways of the mature lung (21). *SOX2* is induced in the bronchiolar epithelium during repair following toxicant-induced injury (22). Overexpression of *SOX2* in lung epithelium during early development disrupted branching morphogenesis, resulting in cystic lungs and neonatal death (21). It has been suggested that *SOX2*, which belongs to group B of the *SOX* family, plays a critical role in cell fate determination, differentiation and proliferation (23,24). A recent study showed *SOX2* amplification on band 3q26.3 and intense *SOX2* immunostaining in lung SCC, indicating potential active transcriptional regulation by *SOX2*. *SOX2*-overexpressing lung epithelial cells and embryonic stem cells (ESCs) reveal that *SOX2* contributes to the activation of ESC-like phenotypes and provides clues pertaining to the de-regulated genes involved in the malignant phenotype (25). Limited data are available with regards to the *MET* and *SOX2* copy number in NSCLC. Our study aimed to simultaneously analyze the *MET* and *SOX2* status in a NSCLC cohort to elucidate the potential role of the *MET* and *SOX2* signaling pathway and to demonstrate *MET* and *SOX2* gene amplification to its clinicopathological features.

Materials and methods

Samples. A total of 115 primary lung cancers were analyzed from Chinese patients whose surgeries were performed in Beijing Chest Hospital, China, between 2007 and 2009. Resected tumors were formalin-fixed and paraffin-embedded until the DNA was extracted. Corresponding non-malignant peripheral lung tissues were also collected. The specimens were reviewed by two reference pathologists (Hai-Qing Zhang and Yi-Ran Cai) to confirm the diagnosis and predominance (>70%) of cancer tissue in the tumor specimens. BAC was defined as previously described (26). The two observers were blind to the patient outcomes. Clinicopathological characteristics such as age, gender, histological subtype and feature of tumors were obtained.

DNA extraction. Genomic DNA was derived from formalin-fixed paraffin-embedded tumors in blocks. The tissues (50-100 mg) were scraped off the block, de-paraffinized twice in xylene, rinsed twice with absolute ethanol and washed with pure water. The tissues were suspended in 500 μl of glutaraldehyde containing 100-200 μg of proteinase K (Promega, Madison, WI, USA) and incubated overnight at 55˚C. Finally, the DNA was purified through columns (DNeasy Tissue Kit, Tiangen, Beijing, China) following the manufacturer's instructions. The lysis mixture was centrifuged for 1 min to remove undigested tissue and then the eluant (DNA) was combined with 200 μl of GB buffer, vortexed and incubated at 70˚C for 10 min. Following the addition of 210 μl of 100% ethyl alcohol, the sample was vortexed, added to a spin column and centrifuged at 9000 rpm for 1 min. The filtrate was discarded, 500 μ1 of GD buffer was added, and the column was centrifuged at 8000 rpm for 1 min. The filtrate was discarded, and the preceding step was repeated twice, with the column spin being rinsed by PW buffer. The DNA was then suspended in 100 μl of TE [10 mM Tris (pH 8) and 1 mM EDTA (pH 8)]. The DNA concentration was measured by UV absorbance at 260 nm, and the samples were stored at 4˚C until further use.

Real-time quantitative PCR. The Taq Man PCR reaction was used to evaluate the amplification of *MET* and *SOX2* genes in lung cancer. This PCR reaction included a dual-labeled fluorogenic probe (5_ reporter and 3_ quencher dyes) and the amount of fluorescence detected was directly proportional to the amount of DNA synthesized (27-29). The Taq Man probes and primers were designed using Primer Express software (Applied Biosystems, Warrington, UK) and were optimized according to the manufacturer's guidelines. Target gene probes (MET and SOX2) and reference gene probes (GAPDH) contained a TAMRA dye at the 3_ end and FAM as a reporter dye at the 5_ end. The primer and probe sequences were: GAPDH primer (forward, 5'-TCG ACA GTC AGC CGC ATC TTC TTT-3'; reverse, 5'-ACC AAA TCC GTT GAC TCC GAC CTT-3'; and probe, 5'-6FAM-AGC CAC ATC GCT CAG ACA CCA TGG G-TAMRA-3'); MET primer (forward, 5'-TGC AGC GCG TTG ACT TAT TCA TGG-3'; reverse 5'-GAA ACC ACA ACC TGC ATG AAG CGA-3'; and 5'-6FAM-AGG AGA CCT CAC CAT AGC TAA TCT TGG G-TAMRA-3'); SOX2 primer (forward, 5'-CAC ATG AAG GAG CAC CCG GAT TAT-3'; reverse 5'-GTT CAT GTG CGC GTA ACT GTC CAT-3'; and 5'-6FAM-TGA AGA AGG ATA AGT ACA CGC TGC CC-TAMRA-3'). Monoplex real-time quantitative PCR was performed using a 50 ng template extracted from paraffin-embedded tissues. The reactions were carried out in a total volume of 25 μl containing 1X SsoFast probes supermix (BioRad, USA). The primer and probe concentrations were optimized for each target according to the manufacturer's instructions. The PCR program consisted of 50˚C for 2 min and 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 56˚C for 1 min. In each run, the templates were assayed in triplicate and for the majority of samples each run was repeated at least twice.

Standard curve. Real-time PCR technology is often used for the relative quantification of nucleic acids (30). The slightest impurities in the sample and uneven template fragmentation due to improper fixation severely distort absolute quantification. Consequently, a relative quantification algorithm is strongly recommended. In performing relative calculations, the distortions of the amplification efficiency due to fixation artifacts or sample impurities were eliminated. Whether PCR

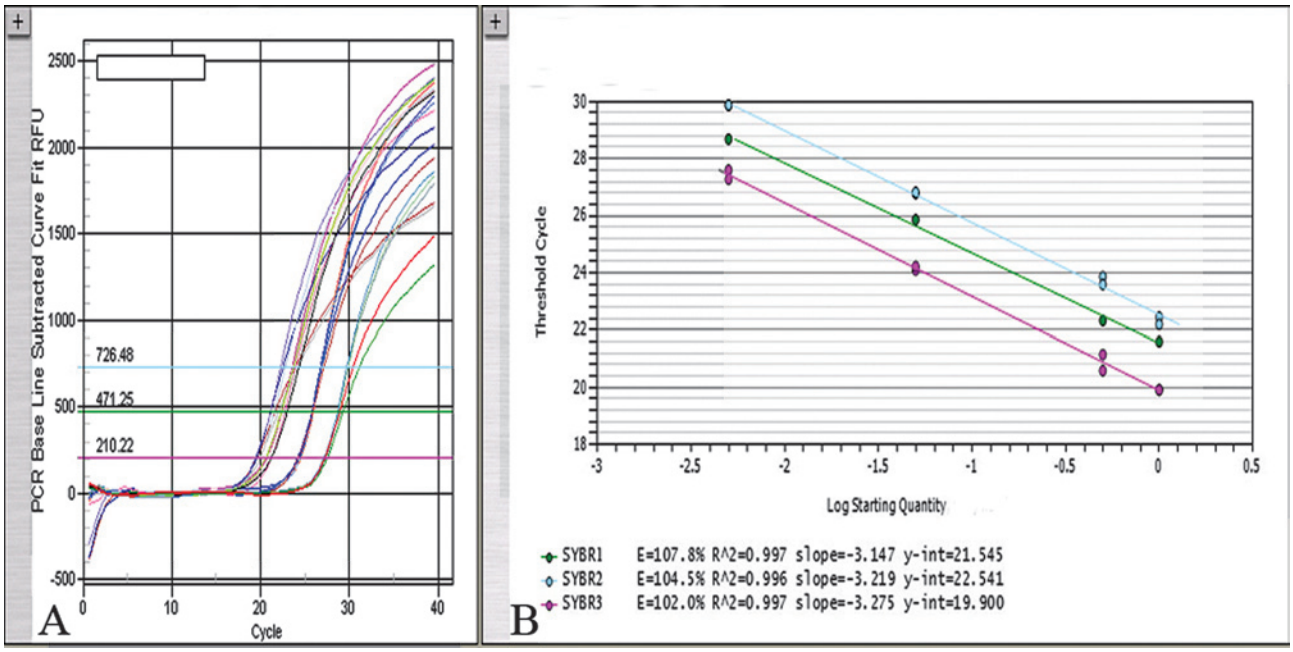

Figure 1. Taq Man GAPDH, MET and SOX2 standard curves obtained by real-time PCR applied on DNA from FFPE tissue. (A) Amplification plots for four serial dilutions of a normal DNA sample. DNA dilution increases from left to right from 0, 1:2, 1:20 to 1:200 per triplicate. (B) Standard curves plotting the log dilution (x) against the threshold cycle numbers (Ct). The magenta line represents the standard curve obtained for GAPDH, green line for MET and sky-blue line for *SOX2*. Regression parameters of the standard curves are displayed: E, efficiency of amplification; R^2, coefficient of correlation; y-int, intercept.

would be quantitative when the DNA had been extracted from fixed tissues under our experimental conditions was defined. For fixed tissues, the 260 nm DNA concentration is not an exact reflection of the DNA quantity to be amplified, since it is altered by the presence of PCR inhibitory factors, as well as by degradation or chemical modification of the DNA due to the fixative. Consequently, a standard curve was constructed by amplifying serial dilutions (0, 1:2, 1:20, 1:200) of normal DNA from 500 to 2.5 ng (measured at 260 nm) (Fig. 1). A constant numerical ratio of target and reference genes was measured in this concentration range. The PCR efficiency in all of the cases was controlled (range 100-110%). The standard deviation (SD) of the values was in the range of 0.5-1 cycle. Data analysis was carried out using iCycler IQ5 optical system software (version 2.0, BioRad) which calculates the threshold cycle numbers (Ct). The N cut-off value for gene amplification was determined for the *MET* and *SOX2* genes in paraffin wax-embedded tissues using cumulative frequencies of the values. Individual measurements that fell outside the 97.5 percentile (the cut-off point) were considered to be outside the normal values. Only standard curves with correlation coefficients of ≥0.98 were used. The copy number change of the *MET* and *SOX2* genes in correlation to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was determined using the formula $(T_{target\ gene}/T_{GAPDH})/(N_{target\ gene}/N_{GAPDH})$, where $T_{target\ gene}$ and T_{GAPDH} were determined from sample DNA using *MET*, *SOX2* and *GAPDH*; and the normalized ratio of *MET* and *SOX2* was determined from a normal sample of 20 tuberculosis patients selected at random. The results of samples for increased *MET* and *SOX2* relative copy numbers were confirmed by repeating the experiments ≥3 times. An interval for the normalized ratio values was calculated corresponding to the mean (M) ±2 SD. A lung tumor sample was considered to be amplified if its ratio was >M +2 SD, or as deleted if its ratio was <M -2 SD (31).

Statistical analysis. The associations between the categorical variables were examined using the χ^2 or Fisher's exact tests. Statistical tests were two-sided. The significant level α=0.05 and P<0.05 were considered to indicate statistical significance for the cross table (Table I), but when referred to co-comparison within the cross table, the significant level was specified at α/n (n = combinations of comparison). The correlation between *MET* and *SOX2* amplifications was evaluated by calculating the r value. The statistical analysis was performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) for Windows.

Results

Patient characteristics and histopathological features. The clinicopathological characteristics of 115 non-small cell carcinomas are listed in Table I. The patients were primarily male (70.4%, n=81). A total of 67 patients (58.3%) had a former or current history of smoking. The median age was 58 years (range 27-77). The patients were diagnosed with 57 SCCs (49.5%) and 50 ADCs (43.5%), including 8 BACs and 8 ADSCs (7%). ADSCs, as a special substance in histological types, which exhibit ADC and SCC, were used as connectors to assess the relationship between ADC and SCC. A total of 54 patients (47%) presented with lymph node metastasis. Of the tumors, 10 (8.7%) were well differentiated, whereas 58 (50.4%) exhibited moderate and 47 (40.9%) exhibited poor differentiation. From a total of 57 cases in the SCC subgroup, 28 cases (49.1%) had tumor dimensions of <5 cm, 49 cases (86%) had a history of smoking and all 57 cases were moderately or poorly

Table I. Correlation of *SOX2* and *MET* gene amplifications with their pathological features.

	SOX2				*MET*			
	Amplification		Non-amplification		Amplification		Non-amplification	
Variable	Patient no.	%	Patient no.	P	Patient no.	%	Patient no.	P-value
All 57 cases	30	26.1	85		13	11.3	102	
Gender								
Male	24	29.6	57	0.18	10	12.4	71	0.75
Female	6	17.7	28		3	8.8	31	
Age, years								
<64	20	27	54	0.75	9	12.2	65	0.77
≥64	10	24.4	31		4	9.8	37	
Histological type								
Adenocarcinoma	10	20	40	0.39	1	2	49	0.02
Squamous cell carcinoma	18	31.6	39		11	19.3	46	
Adenosquamous carcinoma	2	25	6		1	12.5	7	
Differentiation of tumor								
Well	2	20	8	0.94	0	0	10	0.6
Moderate and poor	28	26.7	77		13	12.4	92	
Tumor size (cm)								
<5	18	23.7	58	0.5	8	10.5	68	0.76
≥5	12	30.8	27		5	12.8	34	
Lymph node metastasis								
Positive	17	31.5	37	0.29	6	11.1	48	0.95
Negative	13	21.3	48		7	11.5	54	
Smoking status								
No history	12	25	36	0.82	1	2.1	47	0.008
Former or current	18	26.9	49		12	17.9	55	

differentiated. Additionally, in 24 cases (42.1%) a local lymph node invasion was detected. Of 50 cases in the ADC subgroup, 38 (76%) had no history of smoking, 43 (86%) had tumor dimensions of <5 cm, 40 (80%) were moderately or poorly differentiated, 28 (56%) were female and 35 (70%) were <64 years of age. The ADSCs histologically comprised ADC and SCC patterns. In this subgroup, 6 cases (75%) were smokers, 5 cases had tumor dimensions of <5 cm, 4 cases were <64 years of age, and 4 cases exhibited lymph node metastasis.

SOX2 and MET gene amplifications in non-small-cell lung carcinoma. The amplifications of the *MET* and *SOX2* oncogenes were detected by relative quantitative real-time PCR analysis in 115 NSCLC samples with paraffin-embedded tissues. The normalized ratios of *MET* and *SOX2* calculated from non-cancer tissues were 0.942±0.09 and 0.96±0.08, respectively. Of this cohort, 11.3% (13/115) and 26.1% (30/115) exhibited MET and SOX2 amplifications, respectively. No deletion was found in either of the genes. The patients were classified into amplified and unamplified groups and stratified by other factors (Table I, Figs. 2 and 3).

In ADCs, the 38 cases with no history of smoking were unamplified in the *MET* gene vs. 10 cases amplified in *SOX2*. *MET* amplification indicated a low level in this group despite clinicopathological characteristics such as tumor dimension, lymph node invasion and age. Of the 12 smokers, 1 case was found to be amplified in *MET* vs. no cases in *SOX2*. Among 43 cases exhibiting a tumor dimension of <5 cm, 2.3% of the cases were found to be amplified in *MET* and 20.9% in *SOX2*. Of the ADCs, 26 cases were positive for lymph node invasion and 6 cases (23.1%) indicated *SOX2* amplification vs. no cases detected in *MET*.

In the SCC subpopulation, all 57 cases were moderately or poorly differentiated, 18 cases (31.6%) had *SOX2* amplification and 11 cases (19.3%) had *MET* amplification, including 10 cases (91%) with a history of smoking. When subdivided by the smoking status of SCCs, the amplification level of the *SOX2* gene was higher than that in ADCs among smokers (χ^2=6.25, P=0.014; below the significant level of 0.017). Of the 35 cases that were younger than 64 years of age showed 1 case (2.86%) amplified in *MET* and 8 (22.9%) in *SOX2* gene. From the 35 cases, *MET* amplification was noted in 7 cases (25%) with a tumor dimension <5 cm each, and 4 (13.8%) with a tumor dimension ≥5 cm. *MET* and *SOX2* gene amplification were not significantly associated with tumor dimension or lymph node invasion. In this study, we demonstrated that *MET*

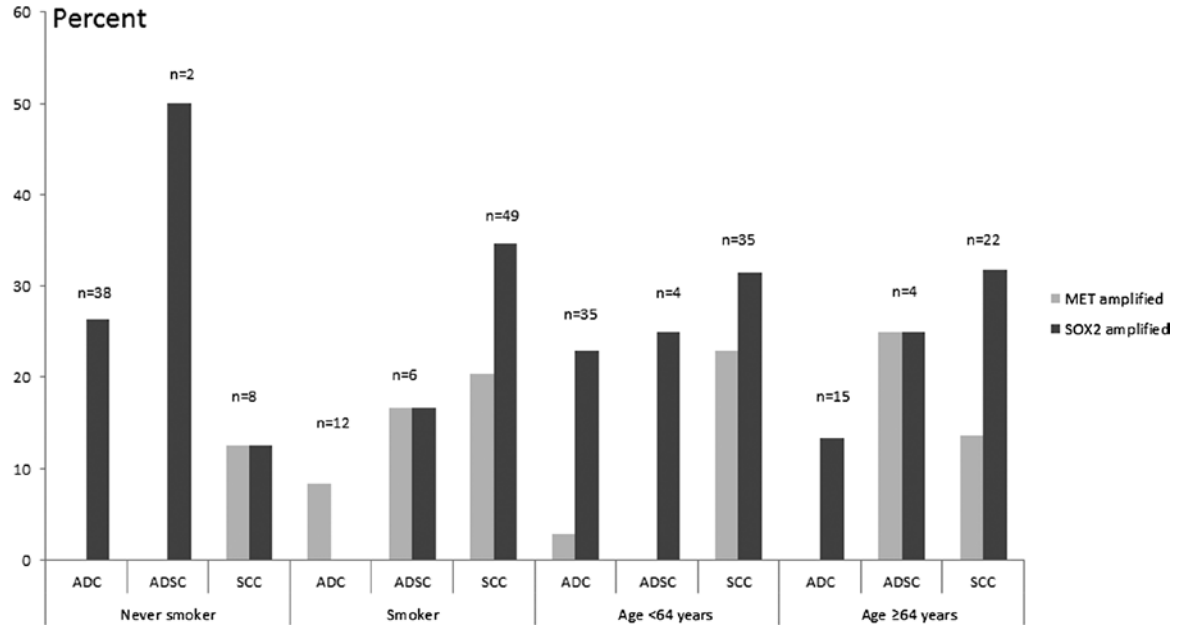

Figure 2. *MET* and *SOX2* gene amplifications in various histological types stratified by smoking history and age. *MET* amplification in squamous cell carcinomas (SCCs) among patients <64 years of age was higher compared to adenocarcinomas (ADCs) (P=0.03). *SOX2* showed preferential amplification in SCCs from smokers as compared to ADCs (P=0.014).

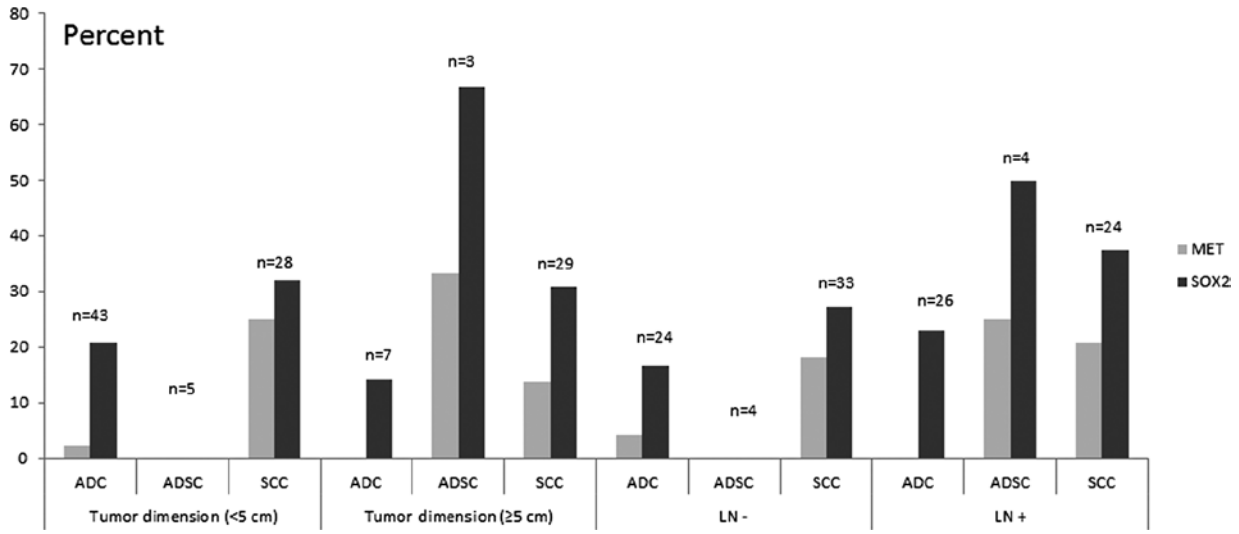

Figure 3. *MET* and *SOX2* gene amplifications in various histological types stratified by tumor dimension and local lymph node metastasis status. Within the lymph node metastasis subgroup, *MET* amplification in squamous cell carcinomas (SCCs) was marginally higher than in adenocarcinomas (ADCs) (P=0.02). SCCs were significantly amplified in *MET* compared to ADCs correlated to the tumor dimension at a smaller scale (<5cm) (P=0.01), whereas no significant correlation was noted when tumors were larger (≥5 cm). No significant difference in *SOX2* amplification was found related to lymph node metastasis or tumor dimension. LN^-: negative lymph node invasion; LN^+: positive lymph node invasion.

amplification was negatively correlated with SOX2 amplification although the difference was not statistically significant (r=-0.19, P>0.05).

In the ADSC group, the 8 cases were moderately or poorly differentiated. Of these cases, 1 (12.5%) and 2 (25%) exhibited *MET* and *SOX2* amplification, respectively. Only 1 of 2 cases among non-smokers exhibited *SOX2* amplification, whereas no cases exhibited *MET* amplification. ADSC patients younger than 64 years of age were not amplified in *MET* gene, and only 1 case (25%) was amplified in *SOX2* gene. Of the cases with lymph node invasion, 1 (25%) and 2 (50%) showed in *MET* and *SOX2* amplification, respectively. No amplification was noted in either *MET* and *SOX2* without lymph node metastasis (Fig. 2).

A significant difference was found in the *MET* amplification level among former or current smokers vs. non-smokers (χ^2=6.99, P<0.01). *SOX2* amplification occurred in 20% of ADCs, 19.3% of SCCs and 25% of ADSCs (P=0.39). *MET* amplifications were detected in ADCs (2%), SCCs (19.3%) and ADSCs (12.5%) (χ^2=7.96, P=0.02). Further analysis showed that the *MET* level was higher in SCCs than in ADCs (χ^2=8.0, P=0.005; compared under a significant level of 0.017). *SOX2*

amplification was correlated with patient characteristics and tumor pathology. With the exception of a higher frequency observed in SCCs with smoking status as compared to ADCs, *SOX2* amplification was not significantly associated with any other clinical or pathological variable, including gender, age, histological subtype, grade and lymph node invasion. *MET* amplification did not correlate with gender. Only 2 (3.51%) SCCs with a history of smoking presented co-amplification of *MET* and *SOX2*. No co-amplification of the two genes was detected in ADCs or ADSCs. A total of 8 (22.9%) SCCs younger than 64 years of age (n=35) were correlated with amplified *MET* gene. However, no correlation was noted with amplified *MET* in ADSCs, and only 1 (2.9%) in ADCs (P=0.03) (Fig. 2). *MET* amplification was also detected preferentially in patients with a former or current history of smoking (17.9%), compared with non-smokers (2.1%). Among the patients with no history of smoking, no cases of ADCs and ADSCs had amplified *MET* with the exception of 1 SCC. No significant difference was noted among smokers with *MET* amplification in ADCs (8.3%, n=12), ADSCs (16.7%, n=6) or SCCs (20.4%, n=49) (Fig. 2). In addition, when stratified into subgroups by age and differentiation, the frequency of *MET* amplification in SCCs younger than 64 years of age and moderate differentiation was marginally higher than those in ADCs and ADSCs (P=0.06, n=66). *MET* amplification in SCCs were more frequent than in ADCs combined with clinicopathological characteristics such as younger than 64 years of age, lymph node metastasis and a tumor dimension of <5 cm (Figs. 2 and 3). A total of 2 cases (20%) of well-differentiated ADCs were detected with SOX2 amplification. In contrast, 8 cases (20%) of ADCs, 2 cases (25%) of ADSCs and 18 cases (31.6%) of SCCs amplified in *SOX2* with moderate and poor differentiation were detected (P=0.44).

MET and *SOX2* amplifications were not significantly correlated whether or not they were distinguished by histological type. In ADCs discriminated by smoking status, however, it was observed that *MET* amplification occurred preferentially in smokers, whereas *SOX2* amplification was noted in non-smokers (Fig. 2).

Discussion

The tyrosine kinase receptor *EGFR* pathway has been extensively studied in NSCLC since gefitinib and erlotinib are used in NSCLC, with a clinical response more commonly observed in ADC/BAC histology arising in non-smokers, females and patients of East Asian ethnicity (5). Recent findings have shown that *MET* amplification is associated with gefitinib resistance during therapy (8). On the other hand, it has been demonstrated that, using high-throughput analysis both in cell lines and in patients with lung cancer, subpopulations of cells with *MET* amplification existed prior to drug exposure (32). The *MET* gene comprises 21 exons and 20 introns (33,34). The role of *MET* in human tumors is enhanced by mutation or amplification, leading to oncogenic changes such as cell proliferation, reduced apoptosis, angiogenesis, altered cytoskeletal function and metastasis. We found that in the subgroup with local lymph node invasion, the *MET* copy level in SCCs was marginally higher than in ADCs. Studies have shown that *MET* and its ligand HGF are mis- and over-expressed in head and neck SCC (HNSCC), resulting in the constitutive activation of the RTK system. This aberrant activity probably induces the mechanism of invasive growth by conferring an invasive potential to these tumors. *HGF* and *MET* orchestrate invasive growth during the progression of HNSCC by integrating a number of independent biological responses using a specific set of signaling pathways. Specifically, *HGF*/*MET* may facilitate the detachment of neoplastic cells from primary HNSCCs through *MAPK* signaling, resulting in the Snail-mediated transcriptional down-regulation of E-cadherin (35). *MET* was also found to be deleted in ADC/BACs and SCCs; *MET* deletion is not a prognostic factor. However, *MET* appeared to be less frequently deleted compared with *MET* amplification (5). We identified a population with *MET* amplification (11.3%, 13/115) in our cohort including 11 cases of SCCs, with 10 cases being smokers. A previous study showed that the *MET* gene copy status was not associated with gender, smoking history, histology or stage. However, true MET amplification occurred more frequently in patients with SCC than in those with ADC. The incidence of *MET* amplification between SCC and ADC was significantly different (36). The clinicopathologic factors of *MET* amplification in NSCLC are conflicting in a number of studies. Okuda *et al* (37) reported that an increased *MET* gene copy number (GCN) was observed in 5.6% of patients with NSCLC, who were male and smokers, whereas no difference in the *MET* GCN status was noted with regards to histological type. In contrast, other studies observed that *MET* amplification (or high GCN) was not significantly associated with gender, smoking history or histology (38,39). However, Go *et al* (36) showed that the majority of patients with true *MET* amplification were male smokers with SCC. Partially consistent with this study, we found that *MET* amplifications are not only associated with smoking status, but are more prevalent in SCCs as compared to ADCs, suggesting that *MET* amplification may be more involved in the oncogenesis of SCCs resulting from smoking than ADCs and may play a key role in lymph node invasion in SCC compared to ADC.

SOX2 is a key transcription factor involved in the stabilization of embryonic stem cells in a pluripotent state (40). Advances of stem cell biology have proven that both embryonic and cancer stem cells exist. Functions of such cancer stem cells include self-renewal which drives tumorigenesis, and aberrant differentiation that contributes to cellular heterogeneity. It was suggested that tumors contain a cellular population that retains key stem cell properties (41) which, in turn, have gene expression signatures closely related to embryonic stem cells (42). A high *SOX2* expression was found in breast cancer (43), testicular germ cell tumors (44) and gastric adenocarcinoma (23). Expression of *SOX2* protein has not been extensively studied in lung cancer. However, a recent study showed that *SOX2* is strongly and diffusely expressed in approximately 90% of pulmonary SCC and 20% of ADC (45). To the best of our knowledge, this is the first report concerning high *SOX2* amplification in a cohort of NSCLC. We showed that the amplification of *SOX2* in SCCs and ADCs was 31.6 and 20%, respectively. *SOX2* is considered to be a master pluripotency controller that was recently identified as a major novel oncogene, recurrently amplified and activated in SCC (46,47). These studies used a similar strategy of chromosomal aberrations screening to identify the *SOX2* locus as one of the

most frequently amplified sites over the SCC genome. They have further highlighted the recurrent *SOX2* activation and its indispensable role for squamous cell survival. The studies showed that *SOX2* is involved in the early steps of lung SCC since it participates in transforming human bronchial epithelial cells. Furthermore, *SOX2* overexpression induces the expression of the squamous markers p63 and keratin 6, indicating that *SOX2* plays a role in SCC differentiation (47). However, neither study assessed the impact of the recurrent activation of *SOX2* in advanced primary tumors nor how *SOX2* may mechanistically be involved in tumor progression and aggressiveness. The above-mentioned studies therefore elucidate and offer novel perspectives on the multiple roles that *SOX2* exerts on SCC carcinogenesis.

In our study population, 89.1% (49/55) of SCC and 24% (12/50) of ADC had a history of smoking, and 34.7% (17/49) of SCCs had *SOX2* amplification, whereas no amplification was found in ADCs. In contrast, 10 of 38 (26.3%) cases involving patients with no history of smoking and with ADC presented *SOX2* amplification, indicating that *SOX2* amplification may be an activating pathway to ADC. Additionally, SCCs presented a higher proportion of *SOX2* amplification than in ADCs and ADSCs among smokers (Fig. 2). The evidence points to the discrepancy in the oncogenesis of SCC and ADC. Comparative genomic hybridization studies have demonstrated that more than 90% of SCCs and approximately 20% of ADCs have copy number gain involving 3q26,4, i.e., the same proportions that have been shown to have a high level of *SOX2* expression at the protein level (45). Notably, these studies have found a high level of amplification only in SCC (48). These observations warrant additional studies to determine the molecular mechanisms involved in *SOX2* expression in ADC. In this study, the *SOX2* gene was more likely to be amplified in the subgroup of patients with no history of smoking and with ADCs. This pilot study was biased to include surgically resectable, early stage tumors. Consequently, sufficient higher-stage tumors in order to perform a well-powered statistical analysis were not available. However, we hypothesize that the *SOX2* and *MET* genes play different roles in the carcinogenesis of SCCs and ADCs, respectively. Previous studies suggest that overexpression of SOX2 is key in the activation of the Nanog/Oct4/SOX2 pathway, which promotes tumor cell proliferation and is associated with the short survival time of patients in stage I ADC (49). *SOX2* is also activated in more advanced SCC tumors, as previously reported (25,45). We conclude that SOX2 is activated not only by overexpression but also by amplification as previously mentioned (25,50). *MET* and *SOX2* gene amplifications are more common in the SCCs of smokers. Moreover, *MET* amplification is intrinsic to SCCs, particularly among smokers, with regards to tumor growth, local lymph node metastasis and negative correlation with *SOX2* amplification. The incidence of amplifications of *MET* and *SOX2* is in the early stage of tumorigenesis in NSCLC. We speculate that the *SOX2* gene is not only activated by amplification but is also affected by other regulators that promote its transcription, affecting its downstream genes.

ADSCs are morphologically mixed tumors comprising the two cell components ADC and SCC. To determine whether these types of tumors are a 'simple' mix of ADC and SCC or whether they present molecular specificities, as compared with the molecular characterization of the two components, Bastide *et al* found that genes were differentially expressed when comparing ADCs SCCs, ADSCs SCCs and ADCs ADSCs (51). Partially consistent with their findings, we observed that, when classifying the three histological subtypes, using *MET* and *SOX2* gene amplifications that distinguished ADCs and SCCs, all ADSCs were identified as intermediate between ADCs and SCCs, with some being similar to ADCs, but others to SCCs. The results indicate that ADSCs are considered a mix of ADCs and SCCs, in various proportions. Moreover, molecular specificities were observed since we found *MET* and *SOX2* gene amplifications among smokers in the three histological types. In conclusion, the ADSC mixed lung tumors are more complex than a 'simple' mix of ADC and SCC components. A recent study showed that neuroendocrine differentiation and ERK proliferation pathways appeared to be preferentially deregulated in ADSCs compared to ADCs and SCCs, which warrants further investigation since these pathways may partially explain the high clinical aggressiveness of ADSCs (51). Amplification of *SOX2* was also found to be more preferential in ADSCs than the *MET* gene (Figs. 2 and 3). One limitation of our study is the insufficient number of cases. Thus, more studies should be conducted to determine the molecular specificities of ADSCs.

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