Title

Validity of Using Immunohistochemistry to Predict Treatment Outcome in Patients with Non-Small-Cell Lung Cancer Not Otherwise Specified

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22 **Abstract**

23 *Purpose*: Histology samples are important for the appropriate administration of tumor type-specific
24 cytotoxic and molecular-targeted therapies for the treatment of non-small-cell lung cancer (NSCLC). When
25 biopsy samples lack a definite morphology, a diagnosis can be selected from three subtypes based on
26 immunohistochemistry (IHC) results, as follows: favor adenocarcinoma (ADC), favor squamous cell
27 carcinoma (SQC), or not otherwise specified (NOS)-null. In terms of patient outcome, however, the validity
28 of IHC-based classifications remains unknown.

29 *Methods*: A large series of 152 patients with advanced NSCLC whose diagnoses had been made based on
30 morphological findings and who had been homogeneously treated were enrolled. We used IHC staining
31 (TTF-1, SP-A, p40, and CK5/6) to examine tumor samples and refined the diagnoses. We then analyzed
32 the pathological subgroups according to the IHC staining results.

33 *Results*: IHC profiling resulted in 50% of the cases being classified as favor ADC, 31% being classified as
34 favor SQC, and 19% being classified as NOS-null. Compared with the favor ADC and favor SQC groups,
35 the NOS-null group had a significantly poorer outcome. Pemetrexed-containing platinum regimens
36 produced a response rate similar to that of other platinum doublet regimens in the favor ADC group (44%

vs. 46%), whereas it produced a poorer response in the favor SQC group (0% vs. 52%) and the NOS-null group (0% vs. 24%). The favor ADC group tended to have a higher percentage of EGFR-positivity and ALK-positivity than the favor SQC group (25% vs. 11% and 7% vs. 0%, respectively).

Conclusions: These findings support the use of immunohistological subtyping of NSCLC biopsy specimens to select patient-appropriate treatments.

Keywords

Immunohistochemistry; Non-small-cell lung cancer; Not otherwise specified; Pemetrexed; Outcome

Background

Recent clinical studies indicate that histology is an important factor in individualizing treatment for patients with non-small-cell lung cancer (NSCLC), based on the safety and efficacy outcomes of the administration of tumor type-specific cytotoxic chemotherapy and molecular-targeted agents (Einhorn 2008; Hirsch et al. 2008; Scagliotti et al. 2008; Stinchcombe et al. 2010). The majority of NSCLCs are detected at an advanced stage, and a histological diagnosis must generally be performed using a small amount of tumor tissue obtained from the primary or metastatic site via a transbronchial biopsy, transbronchial needle aspiration, percutaneous needle lung biopsy, or other core needle biopsy. Although correct NSCLC subtyping is extremely important, these samples can be too small for a definite assessment of the precise tumor

morphology because of insufficient viable cells or poor tumor differentiation. Previously, such cases in which morphological diagnostic criteria were unavailable were classified as NSCLC, not otherwise specified (NOS). Nowadays, American Thoracic Society (ATS)/European Respiratory Society (ERS)/International Association for the Study of Lung Cancer (IASLC) guidelines and the World Health Organization (WHO) Classification of Tumors recommend the use of immunohistochemistry (IHC) when biopsy samples lack a definite morphology, with a diagnosis being made immunohistochemically according to three subtypes: favor adenocarcinoma (ADC), favor squamous cell carcinoma (SQC), and NOS-null (Travis et al. 2015; Travis et al. 2013). IHC markers are useful for identifying specific cell lineages and for distinguishing ADC from SQC. Several previous studies have proposed different panels of IHC markers, with thyroid transcription factor-1 (TTF-1) generally being used as an ADC marker and p40 or p63 being used as an SQC marker (Rekhtman et al. 2011; Whithaus et al. 2012). In addition, some institutes use other IHC markers, such as surfactant apoprotein A (SP-A) as an ADC marker and cytokeratin 5/6 (CK5/6) as an SQC marker. Molecularly targeted therapy is a major treatment strategy for cancer and is most successful for subgroups of tumors, highlighting the need for the exceptional classification of clinically related molecular tumor phenotypes based on a better understanding of the mutations in relevant genes, especially oncogenic driver mutations. EGFR mutation (Paez et al. 2004; Pao et al. 2004) and ALK rearrangement (Soda et al. 2007) are associated with an ADC histology; however, there are no definite opinions as to how EGFR or ALK is expressed in subgroups classified according to IHC or the effects of molecular-targeted

drugs. Chemotherapy is conducted based on IHC findings in patients with initially diagnosed as NSCLC-NOS on Hematoxylin-Eosin staining; however, in terms of the patients' treatment outcomes, the validity of the three IHC-based subtypes remains uncertain. In the current study, we retrospectively examined a consecutive series of patients with advanced or recurrent NSCLC-NOS after chemoradiotherapy who had been diagnosed morphologically and had received palliative chemotherapy and in whom adequate tissue samples for performing IHC were available. The patients' diagnoses were refined based on the presence of four IHC markers (TTF-1, p40, SP-A, and CK5/6), and the patients' responses to treatment and outcomes were then examined after classifying the patients into three groups: favor ADC, favor SQC, and NOS-null.

MATERIALS AND METHODS

Case Selection

Two hundred and ninety-four NSCLC-NOS patients whose diagnoses were based on morphology findings obtained using a tissue biopsy performed using bronchoscopy, transbronchial needle aspiration, or a core needle biopsy from a primary or metastatic site at the National Cancer Center Hospital East between 2009 and 2015 were retrospectively selected. Among them, 197 consecutive patients with morphologically diagnosed advanced or recurrent NSCLC-NOS (IIIB and IV stages, UICC TNM 7th edition) subsequently underwent chemotherapy. Thirty-five cases with tumor samples that were inadequate for immunohistochemistry (IHC) and 9 cases whose diagnoses were confirmed by re-biopsy were subsequently excluded. Thus, a total of 152 patients were finally included in this analysis (Fig. 1). All the samples were

93 reviewed and were confirmed to lack a definite ADC or SQC morphology by two separate pathologists
94 (T.O. and G.I.).
95
96 **Immunohistochemistry and mutation analysis**
97 All the examined specimens were collected before treatment. A block containing the most extensive tumor
98 component was selected from each specimen. Four-micrometer sections were then cut from the paraffin
99 blocks and mounted on slides. The sections were deparaffinized with xylene and rehydrated in a graded
100 ethanol series. After the slides were placed in a high buffer, antigen retrieval was performed in a microwave
101 oven and the slides were allowed to cool for 1 h at room temperature. Next, the slides were washed three
102 times in phosphate-buffered saline and reacted with the nuclear markers TTF-1 (SPRING clone SP141,
103 1/200) and p40 (abcam clone BC28, 1/200) in a first run and then with the cytoplasmic markers SP-A
104 (ANTIBODY SHOP clone6F10, 1/200) and CK5/6 (abcam clone D5/16, 1/100) and incubated overnight at
105 4°C. The slides were subsequently incubated with EnVision™ (Dako, Glostrup, Denmark) for 30 min at
106 room temperature and then subjected to a color reaction by developing in 2% 3,3′-diaminobenzidine in 50
107 mM Tris-buffer (pH7.6) containing 0.3% hydrogen peroxidase. Finally, the sections were counterstained
108 with Meyer’s hematoxylin, dehydrated, and mounted. A surgically resected specimen of squamous cell
109 carcinoma with normal bronchial and alveolar epithelium was used as an internal control. All IHC-stained
110 tumor biopsy slides were reviewed by two separate pathologists (T.O and G.I) who were blinded to the

111 patients' clinical outcomes. Discrepancies in the assignment of the staining pattern between the two
112 pathologists were later resolved by consensus using a multi-headed microscope. A mutation analysis was
113 conducted using next-generation sequencing and/or a commercial companion diagnostic kit.

114 **Statistical analyses**

115 Qualitative data were compared using the Chi-squared test and the Fisher exact test. Overall survival (OS)
116 was defined as the time between the date of the start of first-line chemotherapy or chemotherapy after
117 disease relapse and death, and progression-free survival (PFS) was calculated from the date of the start of
118 chemotherapy until the date of clinical and/or radiological progression or any cause of death. The best
119 response to therapy was recorded as a complete response (CR), partial response (PR), stable disease (SD),
120 progressive disease (PD), or not evaluable (NE) according to the Response Evaluation Criteria in Solid
121 Tumors (RECIST version 1.1) criteria. If a patient had SD at the first assessment performed within 8 weeks
122 after the start of treatment, the patient's best response depended on the results of subsequent assessments.
123 The disease control rate (DCR) and the response rate (RR) percentages were calculated based on the best
124 responses. The chi-square test was used to test the association between the IHC profile and the efficacy of
125 chemotherapy. Survival estimates were calculated using the Kaplan-Meier's method. Statistical analyses
126 were conducted using JMP software (ver. 12.2). P <0.05 was considered significant.

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Results

Immunohistochemical subtyping and clinical characteristics

The immunohistochemical findings and clinical characteristics are summarized in Table 1. After the specimen review and the application of IHC for four markers (TTF-1, p40, SP-A, and CK5/6), the NSCLCs were distributed as follows. Seventy-six of the 152 cases (50%) were TTF-1 and/or SP-A positive (in specimens with TTF-1 positivity, the p40 and CK5/6 results did not matter; in specimens with SP-A positivity, both p40 and CK5/6 were negative) and were subtyped as “NSCLC favor ADC” based on a glandular immunophenotype. Meanwhile, 47 of the 152 cases (31%) were p40 and/or CK5/6 positive (TTF-1 was negative, and SP-A was positive or negative) and were subtyped as “NSCLC favor SQC” according to a squamous phenotype (Fig. 2). None of the cases were TTF-1 and p40 negative and SP-A and CK5/6 positive. Finally, 29 of the 152 cases (19%) did not express any specific immunoprofile and were consequently classified as “NSCLC NOS-null”.

The number of men and the SCC antigen levels were significantly smaller in the favor ADC group than in the favor SQC group (sex, $p = 0.038$; SCC, $p < 0.001$). The favor ADC group had a significantly higher carcinoembryonic antigen (CEA) level than the favor SQC group ($p = 0.012$). More patients received molecularly targeted drugs in the favor ADC group than in the favor SQC group ($p < 0.001$). There were no statistical differences among the favor ADC, favor SQC, and NOS-null subgroups in terms of smoking status, performance status, cytokeratin 19 fragment (CYFRA), or the number of patients who received

147 cytotoxic chemotherapy as a first-line treatment (Table 1).

148 **Mutational analysis**

149 The frequencies of driver mutations in the different morphological NSCLC-NOS subgroups are shown in
150 Table 2. Overall, an EGFR mutation examination was performed in 120 of the 152 patients: 70 of the 76
151 patients (92%) in the favor ADC group, 28 of the 47 patients (60%) in the favor SQC group, and 22 of the
152 29 patients (76%) in the NOS-null group. EML4-ALK fusion examinations were performed in 90 of the
153 152 cases: 56 of the 76 patients (74%) in the favor ADC group, 22 of the 47 patients (47%) in the favor
154 SQC group, and 12 of the 29 patients (41%) in the NOS-null group. Examinations of the RET fusion gene
155 and the ROS1 fusion gene were only performed in 8 cases each.

156 The patients in the favor ADC group tended to have more EGFR-activating mutations and ALK fusions
157 than those in the favor SQC group (26% vs. 11%, $p = 0.10$; and 7% vs. 0%, $p = 0.098$, respectively). In
158 addition, significantly more EGFR-activating mutations were detected in the favor ADC group than in the
159 NOS-null group (26% vs. 5%, $p = 0.03$), although a significant difference in the incidence of ALK fusion
160 was not observed between the favor ADC and NOS-null groups (7% vs. 17%; $p = 0.56$). Among the 8
161 patients who underwent examinations for RET and ROS1 fusions, one patient who had been diagnosed as
162 favor ADC tested positive for a RET fusion.

163 **Treatment and tumor response**

164 Overall, 100 patients received a platinum-containing regimen as their first-line treatment, and the rate of

objective response rate (RR) and the disease control rate (DCR) were 40% and 70%, respectively. The RR and DCR percentages were assessed for each group and were further divided according to the use of pemetrexed (PEM)-containing platinum regimens and other platinum doublet regimens (Table 3). In terms of the response to PEM-containing platinum regimens, no statistical difference in DCR was seen between the favor ADC group and the favor SQC group (70% vs. 71%, p = 0.92). However, the RR was significantly higher in the favor ADC group than in the favor SQC group (44% vs. 0%, p = 0.0096). In terms of the response to other platinum doublet regimens, no statistical difference in DCR (67% vs. 74%, p = 0.59) or RR (46% vs. 52%, p = 0.66) was seen between the favor ADC group and the favor SQC group. The NOS-null cohort had the worst response to PEM-containing platinum regimens (RR = 0% and DCR = 33%) and to other platinum doublet regimens (RR = 24% and DCR = 53%) of the three subgroups. The RR for PEM-containing platinum regimens was significantly different from that for other platinum doublet regimens in the favor SQC group (0% vs. 52%, p = 0.0035) but not in the favor ADC (44% vs. 46%, p =0.87) or NOS-null group (0% vs. 24, p = 0.10). A total of 18 patients received an EGFR/ALK-tyrosine kinase inhibitor (TKI) as a first-line treatment. Among these patients, the 15 patients in the favor ADC group had an RR of 86% and DCR of 93% in the EGFR-TKI, while the response of one patient in the favor SQC cohort was not assessed. Of the 2 people who received ALK-TKI in the favor ADC group, one had PR, and the other was not assessed.

Progression-free survival and overall survival

The median follow-up time was 11.8 months. The patients in the favor ADC group tended to have a longer PFS (median: 5.5 months, 95% CI = 4.6-6.3 months) than those in the favor SQC group (median: 4.4 months, 95% CI = 3.4-5.7 months) after the first-line treatment, representing a 46% lower risk of disease progression in the favor ADC group (HR = 0.54, 95% CI = 0.21–1.42, p = 0.20), and a significantly longer PFS than those in the NOS-null group (median: 3.0 months, 95% CI = 1.7-3.4 months), representing a 56% lower risk of disease progression in the favor ADC group (HR = 0.46, 95% CI = 0.29–0.76, p = 0.003) (Fig. 3A). Regarding outcome, the OS was significantly longer in the favor ADC group (median: 19.5 months, 95% CI = 14.8-27.9 months) than in the favor SQC group (median: 15.0 months, 95% CI = 7.3-19.1 months, HR = 0.59, 95% CI = 0.38–0.92, p = 0.02) and the NOS-null group (median: 6.9 months, 95% CI =4.3-10.7 months, HR=0.39, 95% CI = 0.25–0.63, p < 0.001). The overall survival rates at 1 year were 67% (95% CI = 55%-77%) in the favor ADC group, 51% (95% CI = 37%-65%) in the favor SQC group, and 20% (95% CI = 10%-39%) in the NOS-null group (Fig. 3B).

Regarding the effect of EGFR-TKIs, the median PFS was 12.3 months (95% CI =8.2-25.0 months) and the median OS was 33.5 months (95% CI = 14.8-42.7 months) in EGFR-sensitive mutated NSCLC patients. When patients who had been treated with EGFR/ALK-TKIs were excluded, no significant difference in OS was seen between the favor ADC group and the favor SQC group (median: 15.2 months vs. 15.0 months, HR = 0.78, 95% CI = 0.49–1.24, p = 0.29).

In the ADC group, the 23 patients who received a PEM-containing platinum regimen tended to have a

better PFS and OS than the 24 patients who received other platinum doublet regimens (median PFS: 5.8 vs. 4.8 months, HR = 0.56, 95% CI = 0.28–1.06, p = 0.075; median OS: 21.1 vs. 15.9 months, HR = 0.74, 95% CI = 0.38–1.45, p = 0.38), but the differences were not statistically significant (Fig. 3C, 3D). In the favor SQC group, no differences in PFS and OS were seen between the 7 patients who received PEM-containing platinum regimens and the 23 patients who received other platinum doublet regimens (median PFS: 5.3 vs. 5.3 months, HR = 1.25, 95% CI = 0.48–2.91, p = 0.63; median OS: N.R. vs. 9.6 months, HR = 0.43, 95% CI = 0.10–1.3, p = 0.14). In the NOS group as well, no differences in PFS and OS were seen between the 6 patients who received PEM-containing platinum regimens and the 17 patients who received other platinum doublet regimens (median PFS: 3.0 vs. 3.3 months, HR = 0.95, 95% CI = 0.30-2.6, p = 0.92; median OS: 6.1 vs. 6.0 months, HR = 0.91, 95% CI = 0.32-2.2, p = 0.84).

Discussion

A precise histological diagnosis is critical for the administration of tumor type-specific cytotoxic chemotherapy and molecular-targeted agents; however, little evidence is available regarding the use of IHC subtyping for histology-based treatment decisions. The results of this study show that IHC subtyping is a promising tool for making histology-driven treatment decisions. The present study investigated the validity of an IHC-based, three-tiered classification (favor ADC, favor SQC and NOS-null) in terms of patient outcome using small biopsy samples of advanced or recurrent NSCLC-NOS that had been diagnosed morphologically and subsequently treated with chemotherapy. The results showed that the favor ADC,

219 favor SQC, and NOS-null classifications were substantially different from each other in terms of patient
220 characteristics and clinical outcomes. For example, the favor ADC group had a higher CEA level than the
221 favor SQC group, while the favor SQC group had a higher SCC level than the favor ADC group. In addition,
222 PEM-containing platinum regimens enabled a better RR in the favor ADC group than in the favor SQC or
223 NOS-null group. The median OS was significantly longer in the favor ADC group than in the favor SQC
224 group, but the significant difference between the favor ADC and the favor SQC groups disappeared when
225 patients who were treated with TKIs were excluded. In previous phase III trials, the median OS of patients
226 with genetic mutation-negative advanced non-squamous NSCLC who were treated with cytotoxic
227 chemotherapy was about 13.4-13.9 months (Barlesi et al. 2014; Patel et al. 2013; Paz-Ares et al. 2013),
228 whereas patients with advanced squamous NSCLC had a median OS of about 12.4-13.6 months (Govindan
229 et al. 2017; Shukuya et al. 2015). Although the median OS of this study tended to be slightly longer than
230 the results of previous trials, it was similar for patients with advanced favor ADC and those with favor
231 SQC; the latter observation agrees with previous reports describing no significant difference in median OS
232 between patients with genetic mutation-negative advanced non-squamous NSCLC and those with
233 squamous NSCLC. Based on the above results, favor ADC seems to have the same properties as
234 morphological ADC, and PEM and TKI are likely to be effective in these patients. The NOS-null group had
235 the worst prognosis, possibly because of the poorly differentiated nature of this subgroup of tumors. This
236 finding is in agreement with another study reporting that histopathological marker-null large cell carcinoma

may have a more aggressive course, compared with solid predominant ADC and non-keratinizing SQC that would previously have been classified as large cell carcinoma (Rekhtman et al. 2013). In addition, pulmonary pleomorphic carcinoma has a more aggressive clinical course than other types of NSCLC that may have been included in this study (Fishback et al. 1994; Lin et al. 2016; Venissac et al. 2007).

A previous study showed that NSCLC-NOS tends to be more aggressive than morphologically diagnosed tumors; however, no statistically significant differences were observed among the IHC subtypes. In the present study, only 5 favor SQC patients were included; this limited number of cases prevented a reliable statistical analysis of the survival data from being performed (Pelosi et al. 2014). Another previous study reported that patients with favor ADC had an OS that was comparable to that of patients with morphological ADC, compared with patients with NOS-null. This previous study restricted patients to those with advanced NSCLC who had undergone chemotherapy during a specific time period (2005–2010) prior to the approval of PEM as a first-line treatment for advanced non-squamous NSCLC, and patients with favor SQC were not included in the study (Righi et al., 2014). As expected, the favor ADC group had a significantly better outcome than the NOS-null group in terms of best response and OS. At present, PEM-containing platinum regimens are indicated for the treatment of patients with advanced non-squamous NSCLC, and our study included patients (even those with favor SQC) who had received PEM-containing platinum regimens. To the best of our knowledge, this is the first study to compare the patient responses and outcomes of specific chemotherapies including PEM between patients with favor ADC and those with favor SQC subtypes. A

255 previous phase III study showed OS was statistically superior for cisplatin plus PEM versus cisplatin plus
256 gemcitabine (GEM) in patient with non-squamous NSCLC; in contrast, a significant improvement in
257 survival was seen for cisplatin plus GEM versus cisplatin plus PEM in patients with SQC (Scagliotti et al.
258 2008). In the current study comparing the favor ADC, favor SQC, and NOS-null subtypes, no effect was
259 seen in terms of the response to PEM-containing platinum regimens. In contrast, no significant differences
260 in PFS and OS were observed between the PEM-containing platinum regimens and other platinum doublet
261 regimens in each of the three subgroups. These apparently contradictory results suggest that the small
262 sample sizes or the use of maintenance therapy may have affected the results, since when the RR was taken
263 into consideration, the validity of actively using PEM-containing platinum regimens for patients with favor
264 SQC or NOS-null subtypes was questionable. Some previous studies have demonstrated that for cases not
265 containing ADC components, the frequency of positivity for EGFR-activating mutations and ALK
266 rearrangements is very low (Comprehensive genomic characterization of squamous cell lung cancers
267 2012; Inamura et al. 2008; Marchetti et al. 2005; Miyamae et al. 2011; Salido et al. 2011; Sugio et al. 2006;
268 Takeuchi et al. 2008; Tsao et al. 2011). In our study, EGFR-positivity tended to be more common in the
269 favor ADC group than in the other two subtypes, whereas ALK positivity was only confirmed in the NOS-
270 null group. A large-scale meta-analysis performed in 2015 reported that the frequency of EGFR mutation
271 in Japanese ADC was 45% (21%-68%), which was not significantly different from the result of this study
272 (Midha et al. 2015). One previous study using surgical specimens showed a correlation between ALK-

positive lung cancers and TTF-1 immunoreactivity (Inamura et al. 2009), while ALK was not detected in the favor SQC group in our study. Taken together, these results suggest that a TTF-1-positive ADC component might have been included in the NOS-null group, which was ALK positive, since our study used small samples and the entirety of the tumors could not be evaluated. Since the statistical difference in OS between the favor ADC and favor SQC groups disappeared when patients treated with TKIs were excluded, the difference was considered to be attributable to the difference in the frequency of driver mutations and the effect of TKIs.

Several limitations of this study should be acknowledged. First, the study had a retrospective design and was performed at a single center. Therefore, the possibility of an unintentional selection bias cannot be fully excluded. Second, some of the treatments might have been selected based on cytology results, although the present study only examined tissue samples. Therefore, the present findings need to be confirmed in a prospective study.

In conclusion, we demonstrated that the favor ADC subtype has the same properties as morphological ADC, and the efficacy of PEM and TKI can be expected for patients with this subtype. In addition, the favor SQC subtype exhibited different properties from the favor ADC subtype, since PEM was not effective and the frequency of driver mutations was relatively small; however, the prognosis was still better than that of the NOS-null subtype. Among the three subgroups classified according to IHC, the frequency of driver mutations, the chemo-effectiveness, and the patient prognosis were all different, supporting the significance

291 of adding IHC to the discrimination of NSCLC-NOS subtypes.

292

293 **Figure Legends**

294 Figure 1. Flow diagram of the study selection process. NSCLC-NOS, non-small cell lung cancer-not
295 otherwise specified; BSC, best supportive care; CRT, chemoradiotherapy; IHC, immunohistochemistry;
296 ADC, adenocarcinoma; SQC, squamous cell carcinoma.

297 Figure 2. Immunohistochemistry (IHC) staining in a representative case. Positive TTF-1 and SP-A IHC
298 staining indicates favor adenocarcinoma. Positive p40 and CK 5/6 IHC staining and negative TTF-1 IHC
299 staining indicates favor squamous cell carcinoma. A: TTF-1 (SPRING clone SP141, 1/200), B: p40 (abcam
300 clone BC28, 1/200), C: SP-A (ANTIBODY SHOP clone6F10, 1/200), D: CK5/6 (abcam clone D5/16,
301 1/100).

302 Figure 3. Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS). (A) PFS and
303 (B) OS in the favor adenocarcinoma (ADC) group, favor squamous cell carcinoma (SQC) group, and not
304 otherwise specified (NOS)-null group. (C) PFS and (B) OS in the favor adenocarcinoma (ADC) group for
305 patients receiving a pemetrexed (PEM)-containing regimen or other platinum regimen. ADC,
306 adenocarcinoma; SQC, squamous cell carcinoma; NOS, not otherwise specified.

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Compliance with ethical standards

Conflict of interest

Dr. Kirita reports personal fees from AstraZeneca , personal fees from Boehringer Ingelheim, personal fees from Chugai phamaceutical, personal fees from MSD, personal fees from Novartis Pharma, personal fees from Pfizer, personal fees from Roche, personal fees from Boston, outside the submitted work. Dr. Udagawa reports personal fees from AstraZeneca , grants and personal fees from MSD, personal fees from Taiho , personal fees from Amco, grants and personal fees from Abbvie, personal fees from Chugai phamaceutical, personal fees from Bristol-Myers Squibb, personal fees from Ono Pharmaceutical , grants from Daiichi Sankyo, grants from AMGEN, outside the submitted work. Dr. Matsumoto reports grants from Chugai Pharmaceutical Co., Ltd., grants from Novartis Pharma K.K., grants from Merck Serono Co., Ltd., outside the submitted work. Dr. Yoh reports grants and personal fees from Ono Pharmaceutical , grants and personal fees from Taiho Pharmaceutical, grants and personal fees from Chugai Pharma , grants and personal fees from AstraZeneca , grants and personal fees from Lilly Japan , personal fees from Boehringer Ingelheim , grants and personal fees from Novartis , grants from Pfizer , grants and personal fees from MSD, grants from Bristol-Myers Squibb , grants from Bayer , outside the submitted work. Dr.

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345 **Ethical approval**

346 The study was conducted with the approval of the Institutional Review Boards of the National Cancer

347 Center. The IRB approval number for this study was 2016-125. All the methods were performed in

348 accordance with the approved guidelines.

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350 **Informed consent**

351 All the specimens were collected after obtaining written comprehensive informed consent from the

352 patients.

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Fig. 1

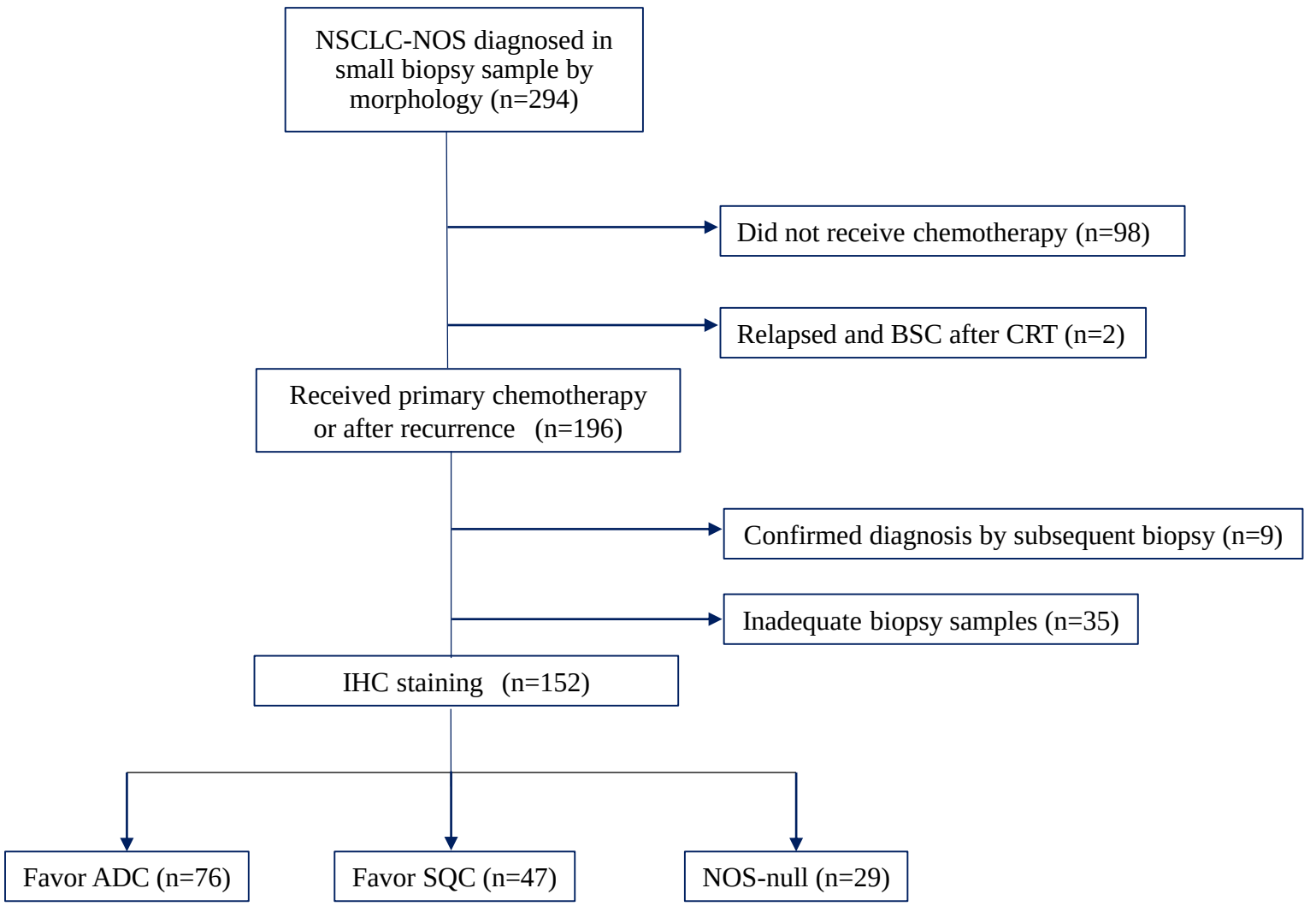
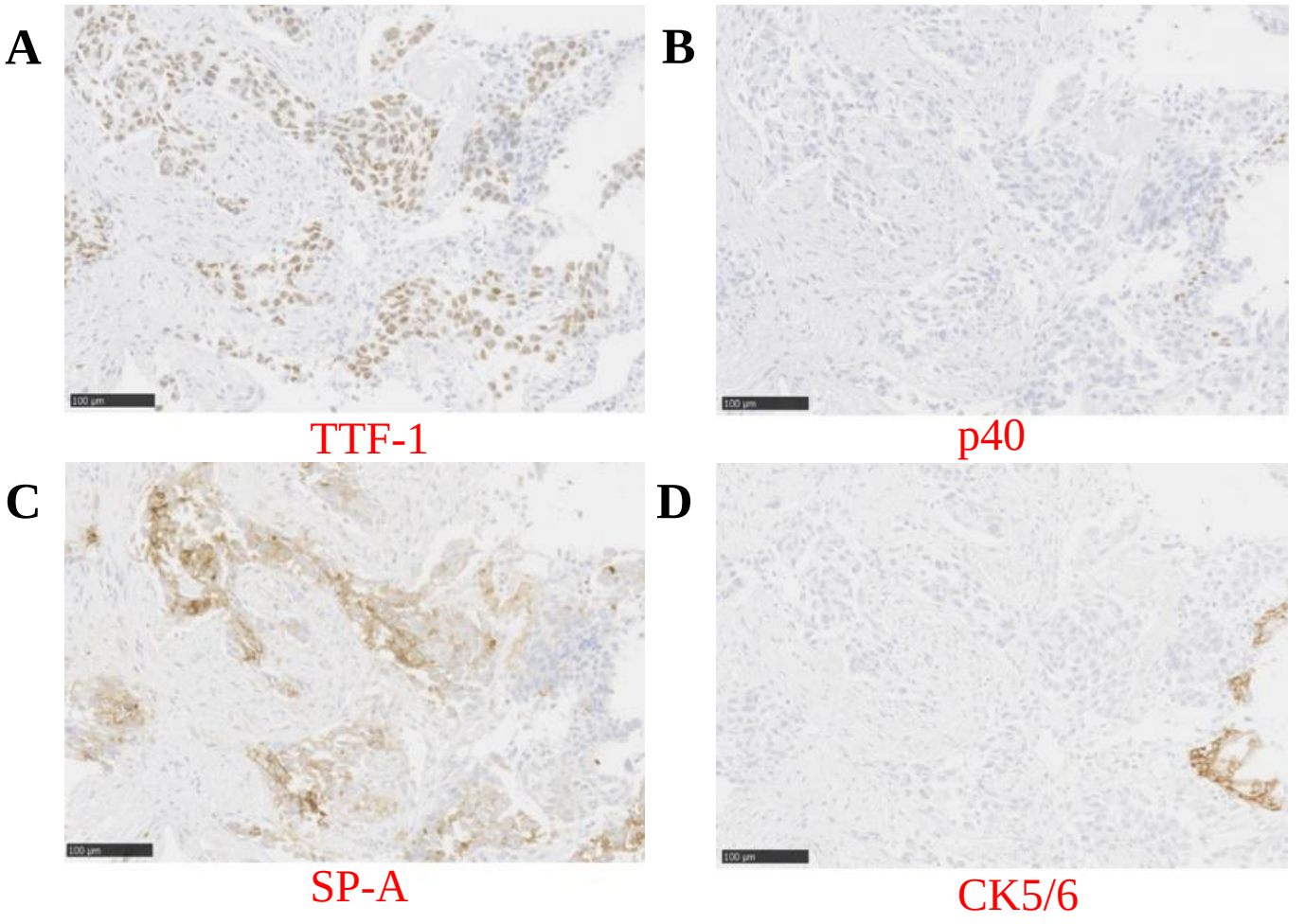

NSCLC-NOS diagnosed in small biopsy sample by morphology (n=294)
Did not receive chemotherapy (n=98)
Relapsed and BSC after CRT (n=2)
Received primary chemotherapy or after recurrence (n=196)
Confirmed diagnosis by subsequent biopsy (n=9)
Inadequate biopsy samples (n=35)
IHC staining (n=152)
Favor ADC (n=76)
Favor SQC (n=47)
NOS-null (n=29)

Fig. 2

Favor adenocarcinoma



Favor squamous cell carcinoma

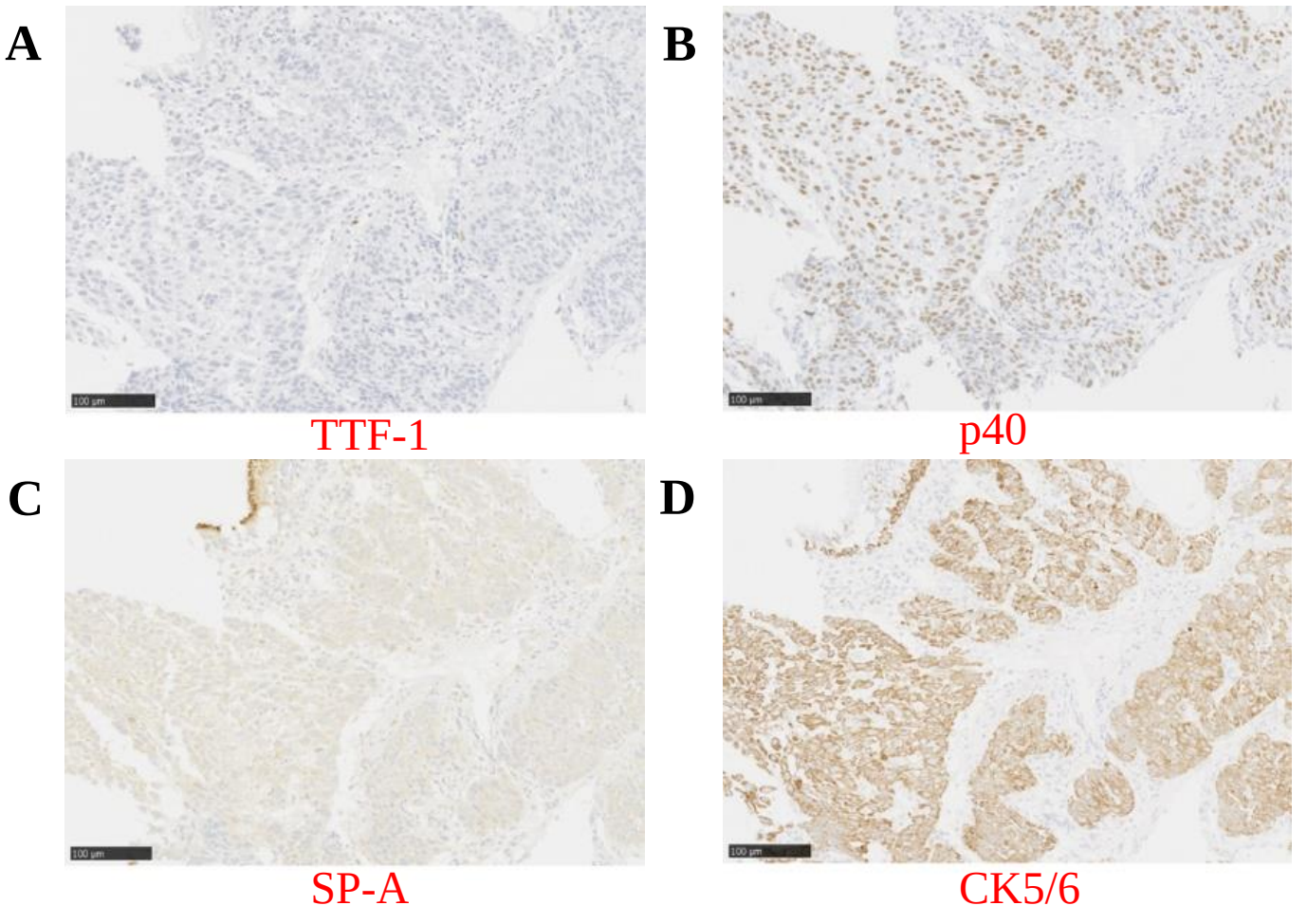
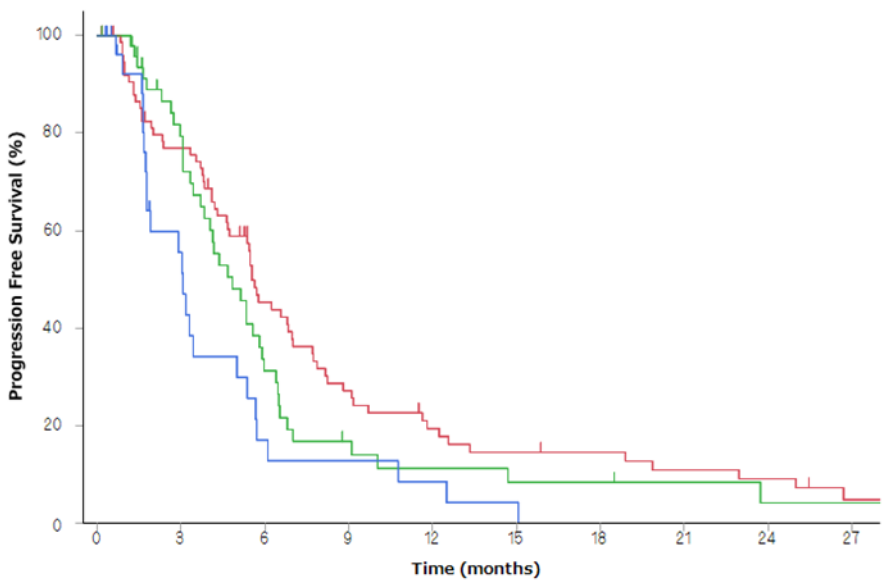


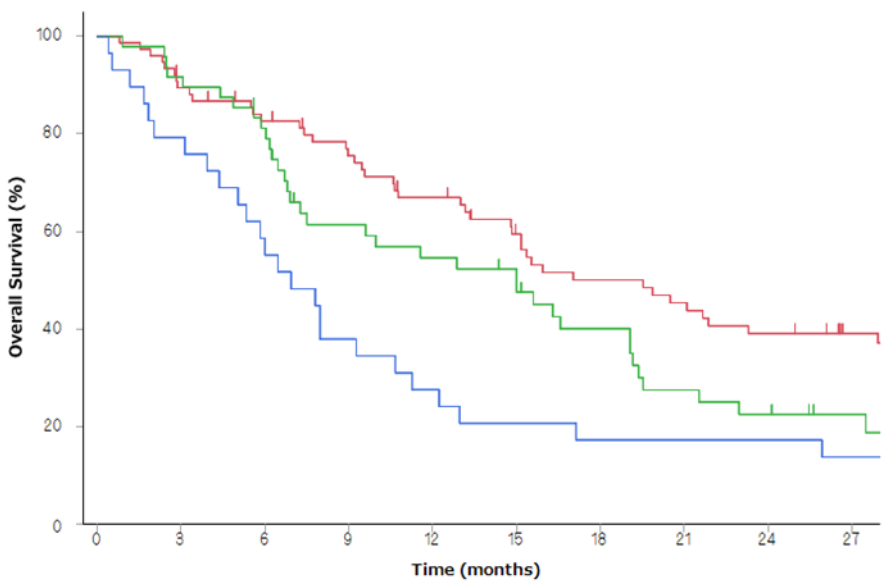
Fig. 3

A



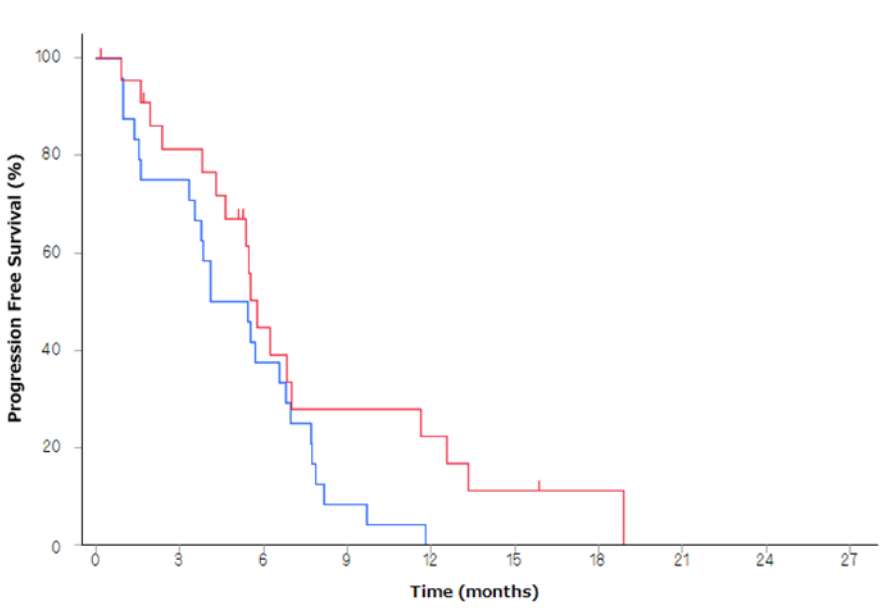
Group	Median PFS months (range)	6-month PFS, % (95% CI)
Favor ADC	5.5 (4.6-6.3)	6.1 (4.6 to 7.9)
Favor SQC	4.4 (3.4-5.7)	3.9 (2.1 to 6.0)
NOS-null	3.0 (1.7-3.4)	1.9 (0.6 to 4.7)

B



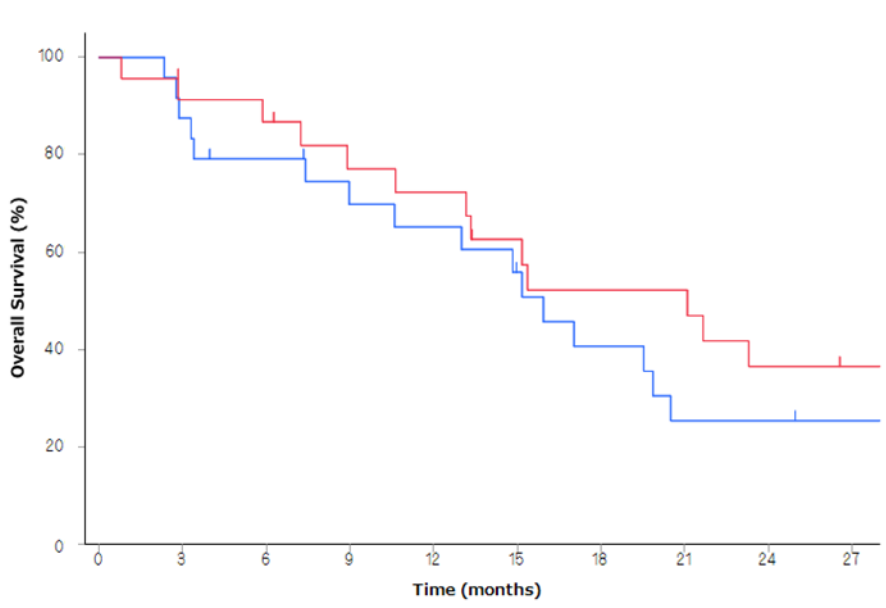
Group	Median OS months (range)	1-year OS, % (95% CI)	2-year OS, % (95% CI)
Favor ADC	19.5 (14.8-27.9)	67 (55 to 77)	39 (28 to 51)
Favor SQC	15.0 (7.3-19.1)	51 (37 to 65)	20 (10 to 35)
NOS-null	6.9 (4.3-10.7)	20 (10 to 39)	7 (2 to 24)

C



Group	Median PFS months (range)	6-month PFS, % (95% CI)
PEM-containing	5.8 (4.3-7.0)	5.6 (2.9 to 8.9)
other platinum	4.8 (3.3-6.8)	5.4 (3.0 to 8.3)

D



Group	Median OS months (range)	1-year OS, % (95% CI)	2-year OS, % (95% CI)
PEM-containing	21.1 (10.6-33.5)	67 (46 to 84)	37 (19 to 59)
other platinum	15.9 (9.0-20.5)	61 (40 to 78)	25 (11 to 48)

Table 1. Baseline Demographics.

Patient characteristics	Total	Favor ADC	Favor SQC	NOS-null	Favor ADC vs Favor SQC	Favor ADC vs NOS-null	Favor SQC vs NOS-null
	N=152 (%)	N=76 (%)	N=47 (%)	N=29 (%)	*P*-value	*P*-value	*P*-value
Gender							
Male	117 (76)	52 (68)	40 (83)	25 (86)	0.033	0.053	0.89
Age, year median (range)	65（23-84)	64 (23-81)	67 (35-84)	65 (40-77)	0.027	0.64	0.16
Smoking							
Current	59 (39)	29 (38)	17 (35)	13 (45)	0.75	0.76	0.74
ex-smoker	74 (48)	36 (47)	25 (52)	13 (45)			
never smoker	19 (13)	11 (15)	5 (13)	3 (10)			
ECOG PS							
0-1	126 (83)	62 (82)	42 (89)	22 (76)	0.24	0.52	0.12
2-4	26 (17)	14 (18)	5 (11)	7 (24)			
Stage							
IIIA or IIIB	33 (22)	10 (13)	16 (34)	7 (24)	0.007	0.19	0.36
IV	119 (78)	66 (87)	31 (66)	22 (76)			
Tumor marker							
CEA, ng/ml median(range)	6.2 (0.3-1325)	6.3 (0.3-1325)	6.2 (0.6-40.3)	7.5 (0.8-774)	0.012	0.24	0.49
CYFRA, ng/ml median(range)	4.9 (0.9-359.7)	4.9 (0.9-51.1)	4.9 (1.2-295.8)	4.9 (1.2-359.7)	0.14	0.49	0.62
SCC, ng/ml median(range)	1.0 (0.2-182.6)	1.0 (0.3-5.8)	1.0 (0.2-182.6)	0.9 (0.4-7.9)	<0.001	0.72	0.001
1st line treatment							
Platinum doublet	100 (66)	47 (62)	30 (65)	23 (79)	0.82	0.081	0.15
non-platinum	34 (22)	12 (16)	16 (33)	6 (21)			
TKI	18 (12)	17 (22)	1 (2)	0	<0.001	<0.001	0.32
Survival							
alive or lost of follow-up	32 (22)	19 (25)	12 (27)	1 (3)	0.84	0.005	0.01
Dead	120 (78)	57 (75)	35 (73)	28 (97)			

ADC, adenocarcinoma; SQC, squamous cell carcinoma; NOS, not otherwise specified; EGOG PS, Eastern Cooperative Oncology Group performance status; TKI, tyrosine kinase inhibitor.

Table 2. Frequency of driver mutations in different morphological NSCLC-NOS groups.

n(%)	Total	Favor ADC	Favor SQC	NOS-null	Favor ADC vs Favor SQC *P*-value	Favor ADC vs NOS -null *P*-value
Analyzed EGFR mutation	120	70	28	22		
positive	22 (18)	18 (25)	3 (11)	1 (5)	0.10	0.03
Del 19	11 (9)	10 (14)	0	1 (5)		
L858R	10 (8)	7 (10)	3 (11)	0		
G719C	1 (1)	1 (1)	0	0		
Wild type	98 (82)	52 (75)	25 (88)	21 (95)		
Analyzed ALK fusion gene	90	56	22	12		
positive	6 (7)	4 (7)	0	2 (17)	0.20	0.33
negative	84 (93)	52 (93)	22	10 (83)		
Analyzed RET fusion gene	8	6	0	2		
positive	1 (13)	1 (20)	0	0		
negative	7 (87)	5 (80)	0	2		
Analyzed ROS1 fusion gene	8	6	0	2		
positive	0	0	0	0		
negative	8	6	0	2		

NSCLC-NOS, non-small cell lung cancer-not otherwise specified; ADC, adenocarcinoma; SQC, squamous cell carcinoma.

Table 3. Distribution of best responses to first-line platinum based treatment in the different groups.

Response N(%)	total	Favor ADC	Favor SQC	NOS-null
PEM contained				
Total	36	23	7	6
CR	0	0	0	0
PR	10	10	0	0
SD	13	6	5	2
PD	12	6	2	4
NE	1	1	0	0
Objective response				
No. of patients with response	10 (28)	10 (44)	0	0
No. of patients with disease control	23 (64)	16 (70)	5 (71)	2 (33)
PEM not contained				
Total	64	24	23	17
CR	0	0	0	0
PR	27	11	12	4
SD	15	5	5	5
PD	16	8	4	4
NE	6	0	2	4
Objective response				
No. of patients with response	27 (42)	11 (46)	12 (52)	4 (24)
No. of patients with disease control	42 (66)	16 (67)	17 (74)	9 (53)

ADC, adenocarcinoma; SQC, squamous cell carcinoma; NOS, not otherwise specified; PEM, pemetrexed; CR, complete response; PR, partial response; SD, stable disease; PD progressive disease; NE, not evaluable.

Table 4. Results of the multivariable analysis of the survival

Variable	PFS			OS		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Gender						
Female (reference)	1			1		
Male	0.72	0.45-1.17	0.18	0.97	0.56-1.78	0.92
Age, years						
<65 (reference)	1			1		
≥65	0.90	0.59-1.35	0.60	0.90	0.60-1.34	0.59
Smoking						
ever (reference)	1			1		
never	0.70	0.35-1.32	0.27	0.42	0.17-0.99	0.048
ECOG PS						
0-1 (reference)	1			1		
2-4	1.1	0.58-1.92	0.78	5.1	2.98-8.42	<0.001
Stage						
IIIA or IIIB (reference)	1			1		
IV or recurrence after CRT	0.99	0.62-1.62	0.60	1.79	1.08-3.13	0.024
Subtype						
Favor ADC (reference)	1			1		
Favor SQC	1.26	0.77-2.03	0.35	1.69	1.04-2.75	0.035
NOS-null	1.87	1.11-3.09	0.019	2.41	1.44-3.99	<0.001
TKI						
untreated (reference)	1			1		
treated	0.47	0.26-0.80	0.004	0.38	0.19-0.70	0.002

PFS, progression-free survival; OS, overall survival; ECOG PS, Eastern Cooperative Oncology Group performance status; CRT, chemoradiotherapy; ADC, adenocarcinoma; SQC, squamous cell carcinoma; NOS, not otherwise specified; TKI, tyrosine kinase inhibitor.