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ORIGINAL ARTICLE – CANCER RESEARCH

Validity of using immunohistochemistry to predict treatment outcome in patients with non-small cell lung cancer not otherwise specified

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Abstract

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

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

Results IHC profiling resulted in 50% of the cases being classified as favor ADC, 31% being classified as favor SQC, and 19% being classified as NOS-null groups. Compared with the favor ADC and favor SQC groups, the NOS-null group had a significantly poorer outcome. Pemetrexed-containing platinum regimens 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

Introduction

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

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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. 2013, 2015). 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 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 after definitive chemoradiotherapy NSCLC-NOS (III and IV stages, UICC TNM 7th edition) subsequently underwent chemotherapy. Postoperative recurrence cases were excluded, as a definitive pathological diagnosis had been made using the surgical specimen. 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 reviewed and confirmed to lack a definite ADC or SQC morphology by two separate pathologists (TO and GI).

Immunohistochemistry and mutation analyses

All the examined specimens were collected before treatment. A block containing the most extensive tumor component was selected from each specimen. Four-micrometer sections were then cut from the paraffin blocks and mounted on slides. The sections were deparaffinized with xylene and rehydrated in a graded ethanol series. After the slides were placed in a high buffer, antigen retrieval was performed in a microwave oven and the slides were allowed to cool for 1 h at room temperature. Next, the slides were washed three times in phosphate-buffered saline and reacted with the nuclear markers TTF-1 (SPRING clone SP141, 1/200) and p40 (abcam clone BC28, 1/200) in a first run and then with the cytoplasmic markers SP-A (ANTIBODY SHOP clone6F10, 1/200) and CK5/6 (abcam clone D5/16, 1/100) and incubated overnight at 4 °C. The slides were subsequently incubated with EnVision™ (Dako, Glostrup, Denmark) for 30 min at room temperature and then subjected to a color reaction by developing in 2% 3,3′-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxidase. Finally, the sections were counterstained with Meyer's hematoxylin, dehydrated, and mounted. A surgically resected specimen of squamous cell carcinoma with normal bronchial and alveolar epithelium was used as an internal control. All IHC-stained tumor biopsy slides were reviewed by two separate pathologists (TO and GI) who were blinded to the patients' clinical outcomes. Discrepancies in the assignment of the staining pattern between the two pathologists were later resolved by consensus using a multi-headed microscope. A mutation analysis was conducted by PCR-based hypersensitive EGFR mutation testing, by the immunohistochemical detection of ALK protein or by fluorescence in situ hybridization for detecting ALK fusions in local laboratories, according to standard testing

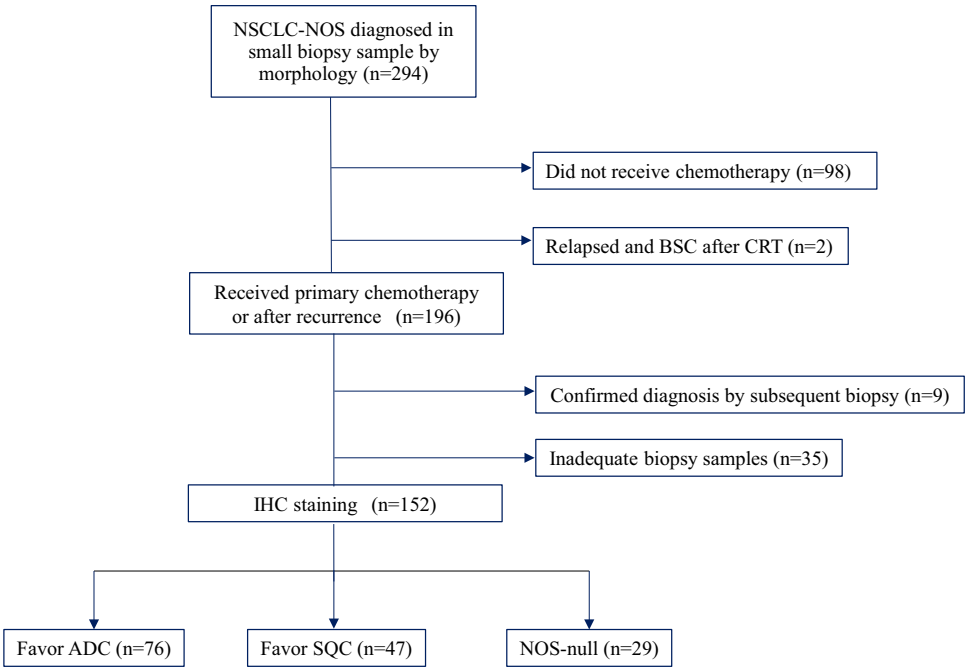


Fig. 1 Flow diagram of the study selection process. *NSCLC-NOS* non-small cell lung cancer not otherwise specified, *BSC* best supportive care, *CRT* chemoradiotherapy, *IHC* immunohistochemistry, *ADC* adenocarcinoma, *SQC* squamous cell carcinoma

practices, and/or next-generation sequencing by LC-SCRUM (Lung Cancer Genomic Screening Project for Individualized Medicine in Japan) (Bando 2017).

Statistical analyses

Qualitative data were compared using the Chi squared test and Fisher's exact test, and continuous variables were compared using the Mann–Whitney *U* test. The overall survival (OS) was defined as the time between the date of the start of first-line chemotherapy or chemotherapy after disease relapse and death, and the progression-free survival (PFS) was calculated from the date of the start of chemotherapy until the date of clinical and/or radiological progression or any cause of death. The best response to therapy was recorded as a complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), or not evaluable (NE) according to the Response Evaluation Criteria in Solid Tumors (RECIST version 1.1) criteria. If a patient had SD at the first assessment performed within 8 weeks after the start of treatment, the patient's best response depended on the results of subsequent assessments. The disease control rate (DCR) and the response rate (RR) percentages were calculated based on the best responses. The Chi square test was used to test the association between the IHC profile and the efficacy of chemotherapy. Survival estimates were calculated using the Kaplan–Meier method, and the significance of the differences between the prognosis and clinicopathological factors was evaluated by a multivariate survival analysis using Cox's regression model. Statistical analyses were conducted using the JMP software program (ver. 12.2). $P < 0.05$ was considered significant.

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

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
First-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

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

Mutational analyses

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

The patients in the favor ADC group tended to have more EGFR-activating mutations and ALK fusions than those in the favor SQC group (26% vs. 11%, $P = 0.10$; and 7% vs. 0%, $P = 0.098$, respectively). In addition, significantly more EGFR-activating mutations were detected in the favor ADC group than in the NOS-null group (26% vs. 5%, $P = 0.03$), although a significant difference in the incidence of ALK fusion was not observed between the favor ADC and NOS-null groups (7% vs. 17%; $P = 0.56$). Among the eight patients who underwent examinations for RET and ROS1 fusions, one patient who had been diagnosed as favor ADC tested positive for a RET fusion. Although there were no correlations between the serum CEA level and the EGFR mutation rate ($P = 0.73$) or the ALK fusion analysis rate ($P = 0.46$), the rate of EGFR gene mutations

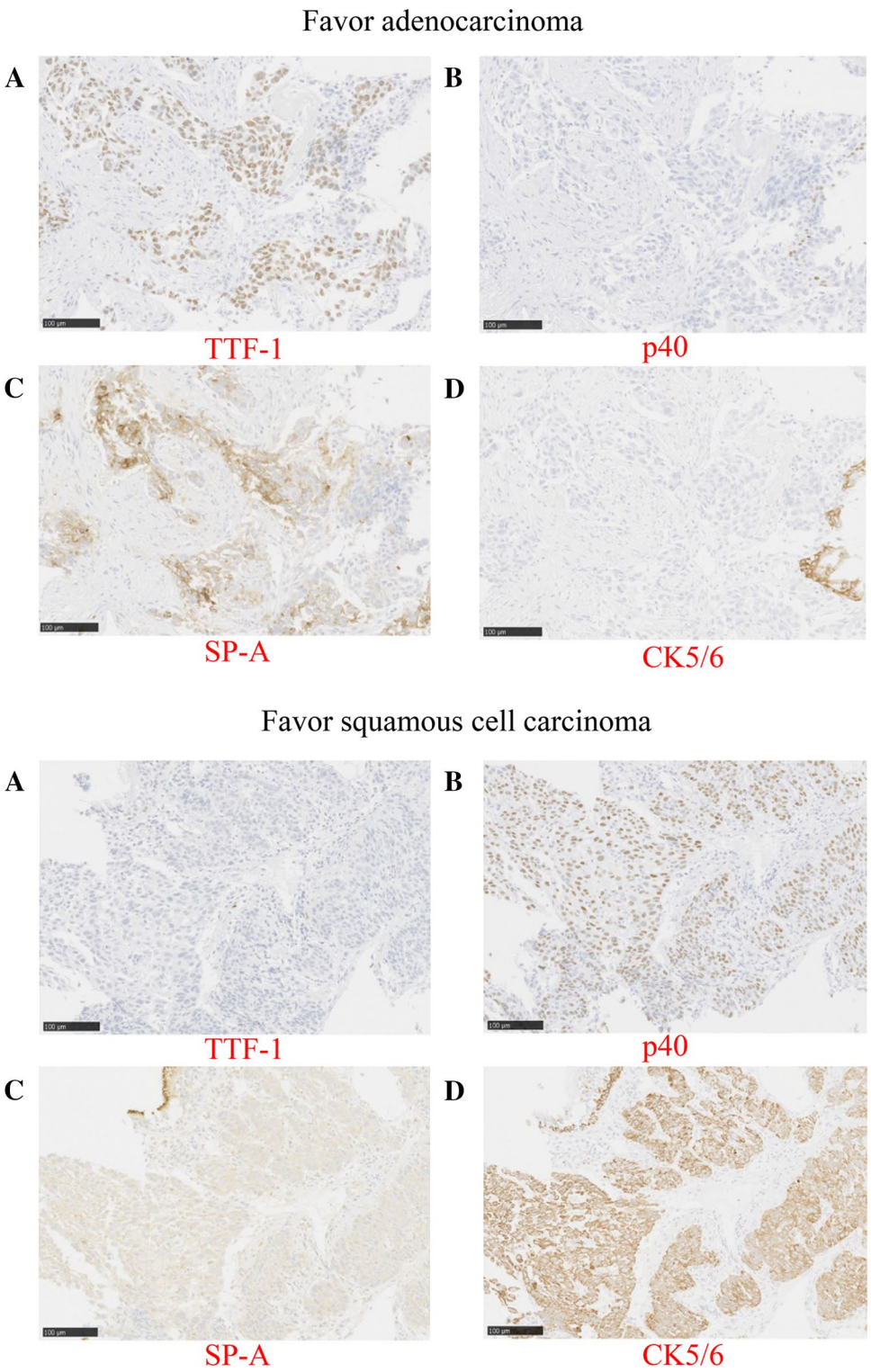


Fig. 2 Immunohistochemistry (IHC) staining in a representative case. Positive TTF-1 and SP-A IHC staining indicates favor adenocarcinoma. Positive p40 and CK 5/6 IHC staining and negative TTF-1 IHC staining indicate favor squamous cell carcinoma. **a** TTF-1 (SPRING clone SP141, 1/200), **b** p40 (abcam clone BC28, 1/200), **c** SP-A (ANTIBODY SHOP clone6F10, 1/200), **d** CK5/6 (abcam clone D5/16, 1/100)

was significantly associated with a high serum CEA level (median CEA: 15.2 vs. 5.5, $P = 0.018$). The rate of ALK fusion was not significantly associated with the serum CEA level (median CEA: 2.0 vs. 6.3, $P = 0.061$).

Treatment and tumor response

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)

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

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 1 patient in the favor SQC cohort was not assessed. Of the two people who received ALK-TKI in the favor ADC group, one had PR, and the other was not assessed.

The progression-free 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). According to the findings of multivariate analyses, the pathological subtype and TKI therapy were independent prognostic factors for the PFS, while the smoking history, ECOG PS, clinical staging, pathological subtype, and TKI therapy were independent prognostic factors for the OS (Table 4).

Regarding the effect of EGFR-TKIs, the median PFS was 12.3 months (95% CI 8.2–25.0 months) and the

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)

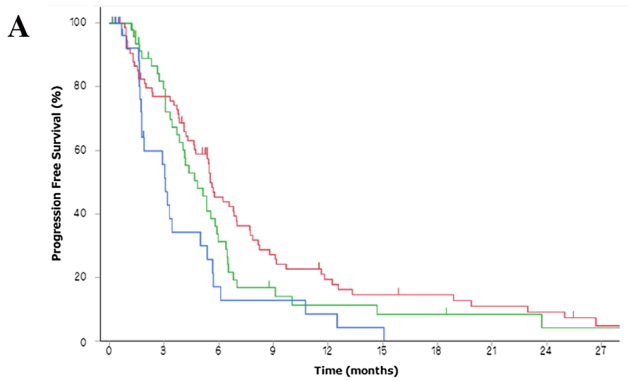
ADC adenocarcinoma, *SQC* squamous cell carcinoma, *NOS* not otherwise specified, *PEM* pemetrexed, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *NE* not evaluable

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, $P = 0.29$).

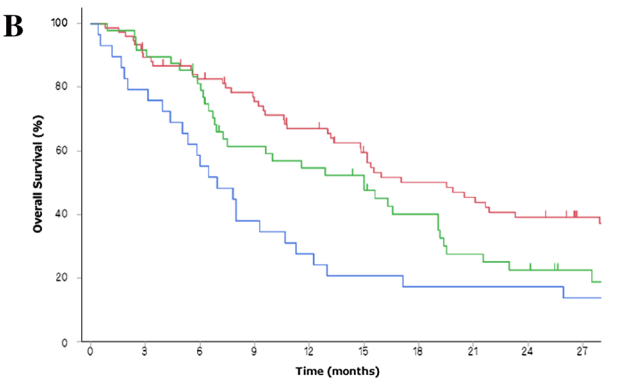
In the ADC group, the 23 patients who received a PEM-containing platinum regimen tended to have a better PFS and OS than the 24 patients who received other platinum doublet regimens (median PFS: 5.8 vs. 4.8 months, HR = 0.56, 95% CI 0.28–1.06, $P = 0.075$; median OS: 21.1 vs. 15.9 months, HR = 0.74, 95% CI 0.38–1.45, $P = 0.38$), but the differences were not statistically significant (Fig. 3c, d). 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

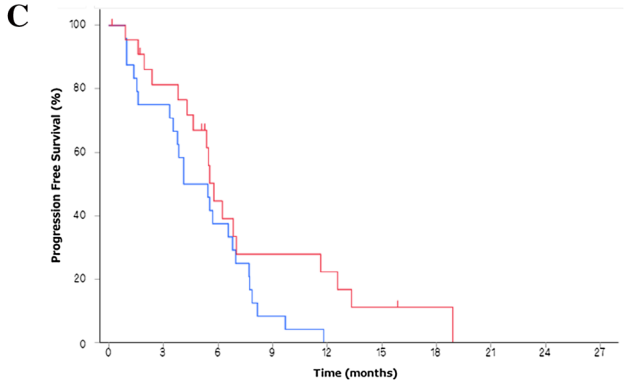
A precise histological diagnosis is critical for the administration of tumor type-specific cytotoxic chemotherapy and molecular-targeted agents; however, little evidence is available regarding the use of IHC subtyping for histology-based treatment decisions. The results of this study show that IHC subtyping 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, favor SQC, and NOS-null classifications were substantially different from each other in terms of patient characteristics and clinical outcomes. For example, the favor ADC group had a higher CEA level than the favor SQC group, while the favor SQC group had a higher SCC level than the favor ADC group. In addition, PEM-containing platinum regimens enabled a better RR in the favor ADC group than in the favor SQC or NOS-null group. The median OS was significantly longer in the favor ADC group than in the favor SQC group, but the significant difference between the favor ADC and the favor SQC groups disappeared when patients who were treated with TKIs were excluded. Our data also showed that the IHC-based subtype



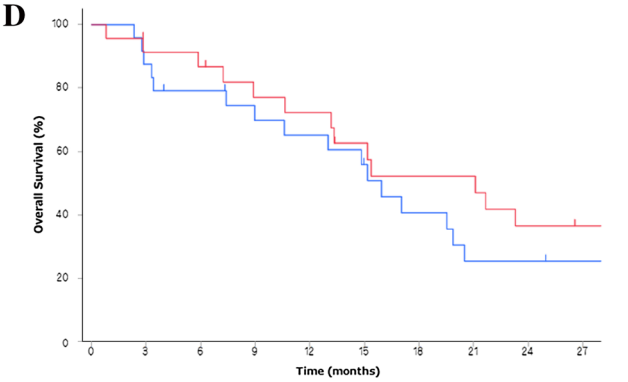
Group	Median PFS months (range)	6-month PFS, % (95% CI)
Favor ADC	5.5 (4.6-6.3)	6.1 (4.6 to 7.9)
Favor SQC	4.4 (3.4-5.7)	3.9 (2.1 to 6.0)
NOS-null	3.0 (1.7-3.4)	1.9 (0.6 to 4.7)



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)



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)



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)

Fig. 3 Kaplan–Meier curves for progression-free survival (PFS) and overall survival (OS). **a** PFS and **b** OS in the favor adenocarcinoma (ADC) group, favor squamous cell carcinoma (SQC) group, and not otherwise specified (NOS)-null group. **c** PFS and **d** OS in the favor adenocarcinoma (ADC) group for patients receiving a pemetrexed (PEM)-containing regimen or other platinum regimen. *ADC* adenocarcinoma, *SQC* squamous cell carcinoma, *NOS* not otherwise specified

and TKI therapy were independent prognostic factors for both the PFS and OS in multivariate analyses.

In previous phase III trials, the median OS of patients with genetic mutation-negative advanced non-squamous NSCLC who were treated with cytotoxic chemotherapy was about 13.4–13.9 months (Barlesi et al. 2014; Patel et al. 2013; Paz-Ares et al. 2013), whereas patients with advanced squamous NSCLC had a median OS of about 12.4–13.6 months (Govindan et al. 2017; Shukuya et al. 2015). Although the median OS of this study tended to be slightly longer than the results of previous trials, it was similar for patients with advanced favor ADC and those with favor SQC; the latter observation agrees with previous reports describing no significant difference in median OS between patients with genetic mutation-negative advanced non-squamous NSCLC and those with squamous NSCLC. Based on the above results, favor ADC seems to have the same properties as morphological ADC, and PEM and TKI are likely to be effective in these patients. The NOS-null group had the worst prognosis, possibly because of the poorly differentiated nature of this subgroup of tumors. This finding is in agreement with another study reporting that

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

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 five 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 previous phase III study showed OS was statistically superior for cisplatin plus PEM versus cisplatin plus gemcitabine (GEM) in patient with non-squamous NSCLC; in contrast, a significant improvement in survival was seen for cisplatin plus GEM versus cisplatin plus PEM in patients with SQC (Scagliotti et al. 2008). In the current study comparing the favor ADC, favor SQC, and NOS-null subtypes, no effect was seen in terms of the response to PEM-containing platinum regimens. In contrast, no significant differences in PFS and OS were observed between the PEM-containing platinum regimens and other platinum doublet regimens in each of the three subgroups. These apparently contradictory results suggest that the small

sample sizes or the use of maintenance therapy may have affected the results, since when the RR was taken into consideration, the validity of actively using PEM-containing platinum regimens for patients with favor SQC or NOS-null subtypes was questionable. Some previous studies have demonstrated that for cases not containing ADC components, the frequency of positivity for EGFR-activating mutations and ALK rearrangements is very low (Comprehensive genomic characterization of squamous cell lung cancers 2012; Inamura et al. 2008; Marchetti et al. 2005; Miyamae et al. 2011; Salido et al. 2011; Sugio et al. 2006; Takeuchi et al. 2008; Tsao et al. 2011). In our study, EGFR positivity tended to be more common in the favor ADC group than in the other two subtypes, whereas ALK positivity was only confirmed in the NOS-null group. A large-scale meta-analysis performed in 2015 reported that the frequency of EGFR mutation in Japanese ADC was 45% (21–68%), which was not significantly different from the result of this study (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. In the present study, the CEA levels in patients with EGFR mutations were higher than in wild-type cases. Some previous studies have shown that the serum CEA level is associated with EGFR mutations in patients with advanced NSCLC (Facchinetti et al. 2015; Shoji et al. 2007). Our results are in line with these previous studies describing an association between EGFR mutations and the serum CEA level. This information might have affected the treatment strategy, such as decisions concerning genetic testing, for patients with morphologically diagnosed advanced NSCLC-NOS.

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 of adding IHC to the discrimination of NSCLC-NOS subtypes. Our findings suggest that, based on IHC results, favor ADC groups should undergo further genetic testing. However, for favor SQC and NOS-null groups, the usefulness of genetic testing remains unclear, and further studies will be required to clarify this point.

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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 Pharmaceutical, personal fees from MSD, personal fees from Novartis Pharma, personal fees from Pfizer, personal fees from Roche, and 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 Pharmaceutical, personal fees from Bristol-Myers Squibb, personal fees from Ono Pharmaceutical, grants from Daiichi Sankyo, and grants from AMGEN, outside the submitted work. Dr. Matsumoto reports grants from Chugai Pharmaceutical Co., Ltd., grants from Novartis Pharma K.K., and 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, and grants from Bayer, outside the submitted work. Dr. Niho reports personal fees from Bristol-Myers Squibb, personal fees from MSD, personal fees from Chugai, grants and personal fees from AstraZeneca, grants from Merck Serono, and grants and personal fees from Pfizer, outside the submitted work. Dr. Goto reports grants and personal fees from Eli Lilly, during the conduct of the study; grants and personal fees from AbbVie Stemcentrx, grants from Ignyta, personal fees from F. Hoffmann-La Roche, grants and personal fees from Life Technologies Japan, grants from Oxonc, personal fees from Otsuka Pharmaceutical, grants and personal fees from RIKEN GENESIS, personal fees from SRL, grants and personal fees from Sumitomo Dainippon Pharma, grants and personal fees from AstraZeneca, grants from Astellas Pharma, grants from Amgen Astellas BioPharma, grants and personal fees from Boehringer Ingelheim, grants and personal fees from Bristol-Myers Squibb, grants and personal fees from Chugai Pharmaceutical, grants and personal fees from Daiichi Sankyo, grants from Eisai, grants and personal fees from Kyowa Hakko Kirin, grants and personal fees from MSD, grants and personal fees from Merck Serono, grants and personal fees from Novartis Pharma, grants and personal fees from ONO Pharmaceutical, grants and personal fees from Pfizer, grants and personal fees from Taiho, grants and personal fees from Takeda Pharmaceutical, and grants from Janssen Pharmaceutical, outside the submitted work. None of the other authors has any conflict of interest to declare.

Ethical approval The study was conducted with the approval of the Institutional Review Boards of the National Cancer Center. The IRB approval number for this study was 2016-125. All the methods were performed in accordance with the approved guidelines.

Informed consent All the specimens were collected after obtaining written comprehensive informed consent from the patients.

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