Non-BAC Component but not Epidermal Growth Factor Receptor Gene Mutation is Associated with Poor Outcomes in Small Adenocarcinoma of the Lung

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Objective: The purpose of this study was to identify risk factors for poor clinical outcome after surgical resection of small lung adenocarcinoma.

Materials and Methods: Clinical records of 127 patients who had pathologic stage IA lung adenocarcinoma 20 mm or less and who had undergone a lobectomy with mediastinal lymph node dissection were reviewed. The percentage of non-bronchioloalveolar carcinoma (non-BAC) components quantified objectively, and epidermal growth factor receptor gene (*EGFR*) mutation determined by polymerase chain reaction-based assay were retrospectively linked with clinical data.

Results: Based on the percentage of non-BAC component, 127 patients were classified as follows: 26 in group I, BAC, 46 in group II mixed subtype with ≥ 50% BAC, 18 in group III, mixed subtype with under 50% BAC, and 37 in group IV, mixed subtype with all non-BAC components or a pure pattern of one of the non-BAC components. Groups I and II were considered to be a "low non-BAC component type" and groups III and IV were considered to be a "high non-BAC component type." *EGFR* mutations in exon19 and exon21 were observed in 64 patients (50.4%). In terms of recurrence, the high non-BAC component type was the only independent factor for recurrence ($p = 0.029$). Regarding survival, the high age ($p = 0.028$) and high non-BAC component type ($p = 0.046$) were independent risk factors for poor overall survival. They were also independent risk factors for poor disease-free survival ($p = 0.025$ and $p = 0.027$, respectively).

Conclusion: The high non-BAC component but not *EGFR* mutation status, is an independent risk factor for both recurrence and poor prognosis in patients with stage IA lung adenocarcinoma ≤20 mm.

Key Words: Lung cancer, Adenocarcinoma, Stage IA, Bronchioloalveolar carcinoma, Non-BAC component, *EGFR*.

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Lung cancer is the leading cause of death from cancer in Japan and many countries.[1,2] Survival among patients with non-small-cell lung cancer (NSCLC) remains unsatisfactory because many cases present with advanced disease and are unresectable.[3] Even patients with early stages of the disease who undergo complete resection sometimes undergo a recurrence, resulting in a poor prognosis.[4,5] Approximately 30% of patients with stage IA NSCLC die within 5 years of surgery.[6]

Recent studies have demonstrated the usefulness of postoperative adjuvant chemotherapy among patients with stage IB to IIIA NSCLC who have undergone complete resections.[7–9] Kato et al. reported that adjuvant chemotherapy with uracil-tegaful prolonged survival among patients with stage I adenocarcinoma and a tumor diameter of greater than 20 mm. They also concluded that patients with tumor ≤20 mm in diameter should be excluded from adjuvant therapy unless a subgroup with a poor prognosis were identified.[10] This proposal also implies that adjuvant chemotherapy for patients with high risk for poor outcome with stage IA NSCLC may be useful for improving survival in this population.

The major subtypes of adenocarcinoma identified by the world health organization (WHO) consist of bronchioloalveolar carcinoma (BAC), and acinar, papillary or solid tumors with mucin. BAC is defined as a noninvasive tumor that has lepidic spread—i.e., replacement of the bronchiolar or alveolar epithelium by tumor cells without stromal, vascular or pleural invasion and typically has a good prognosis.[11,12] However, in practice, the majority of tumors consist of mixtures of two or more subtypes. Tumors with BAC components are classified as mixed type adenocarcinomas with a BAC component, the prognosis for which reportedly worsens along with the degree of non-BAC components.[13,14]

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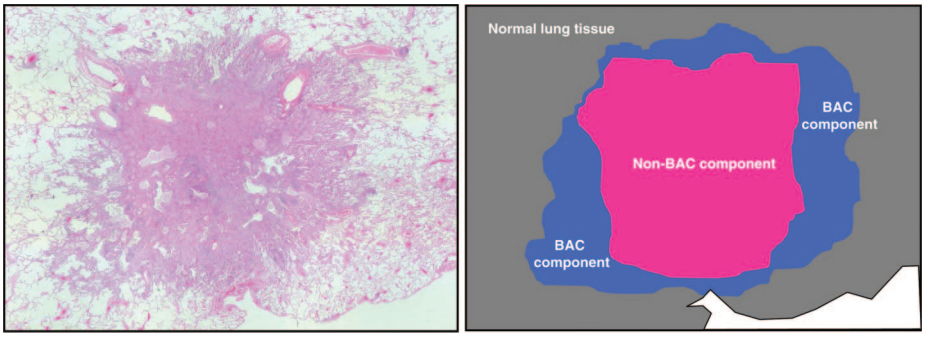

FIGURE 1. Histology of mixed adenocarcinoma with BAC component (Table 3, patient No. 6). *A*, Hematoxylin and eosin staining (×10 magnification). *B*, Schema of the tumor components. Non-BAC component percentage calculated by NIH image J software was 58.0%.

TABLE 1. Characteristics of Patients According to the Non-BAC Component Percentage

Subsets	Group I (n = 26)	Group II (n = 46)	Group III (n = 18)	Group IV (n = 37)
Non-BAC component (%)	0	>0, ≤50	>50, <100	100
Age	63.5 ± 9.1	61.9 ± 10.0	65.6 ± 12.8	64.3 ± 8.9
Sex (male/female)	8/18 (30.8%)	17/29 (37.0%)	11/7 (61.1%)	22/15 (59.5%)
Smoking (never/ever)	18/8 (69.2%)	33/13 (71.7%)	7/11 (38.9%)	14/23 (37.8%)
EGFR mutation (±)	13/13 (50.0%)	28/18 (60.9%)	9/9 (50.0%)	14/23 (37.8%)

EGFR, epidermal growth factor receptor.

Mutations within the tyrosine kinase domain of the epidermal growth factor receptor (*EGFR*) gene are characteristic of adenocarcinomas of the lung. *EGFR* mutations have been reported to be more frequent in adenocarcinomas having BAC components.[15,16] Furthermore, the *EGFR* mutation has been reported to be a good prognostic factor for lung adenocarcinoma compared with the *EGFR* wild-type, although this is still controversial.[17–19] Taken together, for the purpose of focusing on candidates for adjuvant chemotherapy, it is important to know the degree of non-BAC components, the *EGFR* mutation status, or preferably, both, as potential risk factors for an unfavorable clinical outcome in stage IA adenocarcinoma of the lung equal to or less than 20 mm in diameter.

In this study, we reviewed the clinical data of 127 patients with stage IA adenocarcinoma of the lung ≤20 mm in diameter and performed a quantitative estimation of the non-BAC component in the tumor and an examination *EGFR* mutation. Subsequently, we retrospectively analyzed the risk factors for recurrence and poor prognosis after surgery in this population.

PATIENTS AND METHODS

Patients

Between January 1995 and December 2002, 734 consecutive patients with NSCLC underwent pulmonary resections at the Department of Cancer and Thoracic Surgery, Okayama University Hospital. Among them, 127 patients with pathologic stage IA adenocarcinoma of the lung ≤20 mm in diameter who had undergone a lobectomy with mediastinal lymph node dissection as a complete resection were included in the current study. Fifty-eight were male and 69 were female with a median age of 65 years (range 38–84 years). The primary treatment in this group was pulmonary resection without chemotherapy or radiotherapy before surgery. Never-smokers were defined as a lifetime exposure of 100 cigarettes or less, ever-smokers were those with lifetime exposure of more than 100 cigarettes. Institutional review

TABLE 2. Relationship between *EGFR* Mutation and Clinicopathological Variables in Patients With Stage IA Lung Adenocarcinoma <20 mm in Diameter

Variables and Subsets (n)	No.	No. of *EGFR* Mutation Cases (%)	p
Age (yrs)			
<64	71	36 (12.7%)	0.94
≥64	56	28 (50.0%)	
Sex			
Male	58	21 (36.2%)	0.0034
Female	69	43 (62.3%)	
Smoking status			
Ever smoker	55	18 (32.7%)	0.0005
Never smoker	72	46 (63.9%)	
Serum CEA level (ng/ml)*			
≥5.0	14	5 (43.9%)	0.28
<5.0	112	58 (51.8%)	
Tumor size (mm)			
≥15	56	29 (51.8%)	0.92
<15	71	35 (49.3%)	
Presence of BAC component			
Yes	37	14 (37.8%)	0.070
No	90	50 (55.6%)	

EGFR, epidermal growth factor receptor; CEA, carcinoembryonic antigen; BAC, bronchioloalveolar carcinoma.
*The serum CEA level was not evaluated in one patient.

TABLE 3. Information of Patients Who Died

No.	Recurrence	Age (yrs)	Sex	Smoking	CEA (ng/ml)	T Size (mm)	Non-BAC Component (%)	*EGFR*
1	Yes	68	Male	Ever	5.9	9	100	WT
2	Yes	76	Male	Ever	18.2	14	100	WT
3	Yes	56	Male	Ever	1.7	20	100	Exon19 del
4	Yes	71	Female	Ever	15.2	18	100	Exon19 del
5	Yes	65	Female	Never	1.1	15	100	Exon21 L858R
6	Yes	70	Male	Ever	1.4	15	58.0	WT
7	Yes	71	Male	Ever	2.1	20	85.5	WT
8	Yes	69	Male	Ever	6.1	16	54.2	Exon19 del
9	No	65	Male	Ever	3.5	18	100	WT
10	No	72	Male	Ever	4.5	15	100	WT
11	No	84	Male	Ever	6.2	16	55.2	WT
12	No	81	Male	Ever	8.9	18	62.7	Exon19 del
13	No	84	Male	Never	3.2	20	0	WT
14	No	66	Female	Never	1.7	17	0	WT

CEA, carcinoembryonic antigen; *EGFR*, epidermal growth factor receptor; WT, wild-type; del, deletion.

board permission and informed consent were obtained for all patients.

Clinical Data

The following clinicopathologic variables were evaluated: age, sex, smoking status, preoperative serum carcinoembryonic antigen (CEA) level, pathologic tumor size, the non-BAC component percentage, and *EGFR* mutation status. The cutoff CEA level was set at 5.0 ng/ml between the normal and elevated groups. Clinicopathologic staging was determined according to the tumor-node-metastasis classification of malignant tumors of the International Union against Cancer.[20] Pathologic finding including BAC were determined based on WHO classification as mentioned.[11]

Patients were examined at the outpatient clinic at least every 6 months for 2 years after surgery and annually thereafter. All patients underwent a complete blood count, blood chemistry analysis, plain chest radiograph, measurement of serum CEA level, and a computed tomography (CT) scan of the chest and abdomen to screen for recurrent disease when appropriate. Biopsies of new lesions suspected to be recurrences were performed, if possible, and the attending physician made the final diagnosis regarding relapse. The overall survival (OS) and the disease-free survival (DFS) periods were calculated from the date of surgery until the date of death or the last follow-up for OS, and the date of recurrence and death or the last follow-up for DFS.

Analysis of Area of Non-BAC Component with National Institutes of Health (NIH) Image J Software

Maximally cut surface specimens of tumor tissue samples were formalin-fixed and paraffin-embedded. These samples were observed under BIOZERO BZ-8000 microscopy (Keyence, Osaka) at a 10× magnification and the percentage of BAC and non-BAC components was analyzed with NIH image J software (×1.38), which is freely available from the NIH Website (http://rsb.info.nih.gov/ij/).

DNA Extraction and Mutation Analyses of the *EGFR* Gene

The DNAs of 20 frozen lesions were isolated by digestion with proteinase K followed by phenol-chloroform (1:1) extraction and ethanol precipitation from frozen specimen.[21]

TABLE 4. Uni- and Multi-variate Analysis for Recurrence and Survival

	Recurrence			
Variables	Univariate HR (95% CI)	*p*	Multivariate HR (95% CI)	*p*
Age (≥64 vs. <64)	3.82 (0.83–1.75)	0.086	2.38 (0.48–11.8)	0.29
Sex (male vs. female)	2.11 (0.62–7.21)	0.23	2.52 (0.35–18.2)	0.36
Smoking status (ever vs. never)	3.64 (0.97–13.7)	0.057	3.15 (0.34–29.1)	0.31
CEA level (≥5.0 vs. <5.0)	5.28 (1.54–18.1)	0.008	3.00 (0.65–13.8)	0.16
Tumor size (≥15 vs. <15)	1.15 (0.35–3.76)	0.82	1.64 (0.45–5.99)	0.46
Non-BAC component (>50% vs. ≤50%)	14.4 (1.85–112.8)	0.011	10.6 (1.27–88.2)	0.029
EGFR mutation status (yes vs. no)	1.19 (0.36–3.90)	0.77	1.42 (0.38–5.29)	0.60

HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; *EGFR*, epidermal growth factor receptor.

For the 107 lesions that were formalin fixed and paraffin embedded, DNAs were isolated by DEXPAT (TaKaRa, Shiga, Japan) following the manufacturer's instructions. *EGFR* mutations were examined limited to exon19 deletions and exon21 L858R point mutations by mutant-enriched polymerase chain reaction (PCR) assays as described previously.[22] Mutant-enriched PCR is a two-step PCR with intermittent restriction digestion to eliminate wild-type genes selectively, thus enriching the mutated genes at a high sensitivity. The product of the amplification by both the methods was analyzed with 12% polyacrylamide gel electrophoresis (PAGE) via ethidium bromide staining. The common deletions of exon19 were distinguished from the wild-type based on PCR product length polymorphisms using 12% PAGE. For exon21, Sau96I digestion which can specifically digest the mutant type was performed before 12% PAGE. These methods are predicted to detect approximately 85% of *EGFR* tyrosine kinase domain mutations.[23–25]

Statistical Analysis

Differences in significance among the categorized groups were compared using Fisher exact test or χ^2 tests when appropriate. Uni- and multi-variate analyses for relationships between survival and clinicopathologic variables were performed using a Cox proportional hazards model. Survival curves were drawn using the Kaplan–Meier method which was analyzed with the log-rank test. All data were analyzed using StatView® 5.0 Program for Windows (SAS Institute Inc., Cary, NC). All statistical tests were two-sided, and probability values <0.05 were regarded as statistically significant.

RESULTS

The Status of Non-BAC Component and *EGFR* Mutation

The proportion of BAC and non-BAC components was determined using NIH image J software (Figure 1). On the basis of the non-BAC component percentage, all the tumors of the 127 patients were classified into four categories: those with BAC (group I, 26 patients), mixed subtype with $\geq 50\%$ BAC (group II, 46 patients), mixed subtype with $<50\%$ BAC (group III, 18 patients), and mixed subtype with all non-BAC components or a pure pattern of one of the non-BAC patterns (group IV, 37 patients). The patient characteristics of each group are shown in Table 1. *EGFR* mutations were observed in 64 patients (50.4%), 38 as exon19 deletions and 26 as an L858R point mutation. The relationship between *EGFR* mutation and clinicopathologic factors is shown in Table 2. *EGFR* mutations were more frequently found in the female-sex ($p = 0.0034$) and never-smokers ($p = 0.0005$) and tended to be frequently found in cases with BAC components (group I, II, and III) ($p = 0.070$).

Risk Factors for Recurrence and Poor Prognosis

By March 2007, 14 patients (11.0%) died in clinical course and 11 patients (8.7%) developed recurrences after surgical resection. The median follow-up time among the surviving patients was 67 months (range, 12–134 months). The details of the patients who died are shown in Table 3. Of the total study population, the 5-year OS and DFS rates were 92.9% and 91.3%, respectively. In this study, the status of the non-BAC component was divided into two groups: high non-BAC component type ($>50\%$) and low non-BAC component type ($\leq 50\%$), following the classification of previous studies.[13,26] Based on univariate analyses, high CEA level (hazard ratio [HR] = 5.28, 95% confidence interval [CI]: 1.54–18.1, $p = 0.008$) and high non-BAC component type (HR = 14.4, 95% CI: 1.85–112.8, $p = 0.011$) are risk factors significantly associated with recurrence. No significant differences in disease recurrence were observed for age, sex, smoking status, pathologic tumor size, or *EGFR* mutations status. Multivariate analysis indicated that high non-BAC component type was the only independent factor associated with recurrence (HR = 10.6, 95%CI: 1.27–88.2, $p = 0.029$). With regard to survival, univariate analyses indicated that high age (OS; HR = 11.4, 95% CI: 1.49–90.9, $p = 0.019$ and DFS; HR = 6.41, 95% CI: 1.47–27.8, $p = 0.014$), male sex (OS; HR = 4.76, 95% CI: 1.33–17.1, $p = 0.017$ and DFS; HR = 3.08, 95% CI: 1.08–8.76, $p = 0.035$), ever smoking status (OS; HR = 5.42, 95% CI: 1.51–19.5, $p = 0.0096$ and DFS; HR = 3.50, 95% CI: 1.23–10.0, $p = 0.019$), high CEA level (OS; HR = 6.98, 95% CI: 2.42–20.1, $p = 0.0003$ and DFS; HR = 5.06, 95% CI: 1.87–13.7, $p = 0.0014$), and high non-BAC

TABLE 4. Continued

Overall Survival				Disease-Free Survival			
Univariate HR (95% CI)	*p*	Multivariate HR (95% CI)	*p*	Univariate HR (95% CI)	*p*	Multivariate HR (95% CI)	*p*
11.4 (1.49–90.9)	0.019	10.2 (1.29–83.3)	0.028	6.41 (1.47–27.8)	0.014	5.62 (1.24–25.6)	0.025
4.76 (1.33–17.1)	0.017	2.34 (0.14–39.7)	0.56	3.08 (1.08–8.76)	0.035	1.77 (0.19–16.2)	0.61
5.42 (1.51–19.5)	0.0096	1.45 (0.037–13.1)	0.80	3.50 (1.23–10.0)	0.019	1.41 (0.14–1.45)	0.77
6.98 (2.42–20.1)	0.0003	3.17 (0.88–11.4)	0.077	5.06 (1.87–13.7)	0.0014	2.37 (0.72–7.74)	0.15
2.55 (0.85–7.62)	0.094	1.45 (0.47–4.70)	0.49	1.99 (0.76–5.22)	0.16	1.15 (0.42–3.15)	0.78
8.48 (1.90–37.9)	0.0051	5.00 (1.03–24.2)	0.046	6.72 (1.93–23.4)	0.0028	4.42 (1.18–16.6)	0.027
1.73 (0.58–5.20)	0.33	1.60 (0.49–5.23)	0.44	1.86 (0.69–5.04)	0.22	1.55 (0.54–4.49)	0.42

component type (OS; HR = 8.48, 95% CI: 1.90–37.9, p = 0.0051 and DFS; HR = 6.72, 95% CI: 1.93–23.4, p = 0.0028) were significantly related to the poor OS and DFS. No significant differences in survival were observed according to pathologic tumor size, or *EGFR* mutations. Multivariate analysis indicated that high age (OS; HR = 10.2, 95% CI: 1.29–83.3, p = 0.028, and DFS; HR = 5.62, 95% CI: 1.24–25.6, p = 0.025) and high non-BAC component type (OS; HR = 5.00, 95% CI: 1.03–24.2 p = 0.046 and DFS; HR = 4.42, 95% CI: 1.18–16.6, p = 0.027) were independent factors associated with poor OS and DFS (Table 4).

Kaplan–Meier survival curves stratified by non-BAC component level and *EGFR* mutation are shown in Figure 2. As consistent with the results with the Cox proportional hazards model, OS and DFS were significantly shorter in patients with high non-BAC component type than those with low non-BAC component type (OS, p = 0. 0.0008 and DFS, p = 0.0005). On the other hand, there was no difference in OS and DFS periods between patients with *EGFR* mutant and wild-type.

DISCUSSION

Recent randomized phase III trials have shown that patients with stage IB–IIIA NSCLCs are candidates for adjuvant chemotherapy after complete surgical resection.[7–9] The indications for adjuvant chemotherapy among patients with stage IA NSCLC, on the other hand, are still under debate, despite the performance of subset analyses in certain randomized trials.[10] To select candidates for adjuvant chemo-

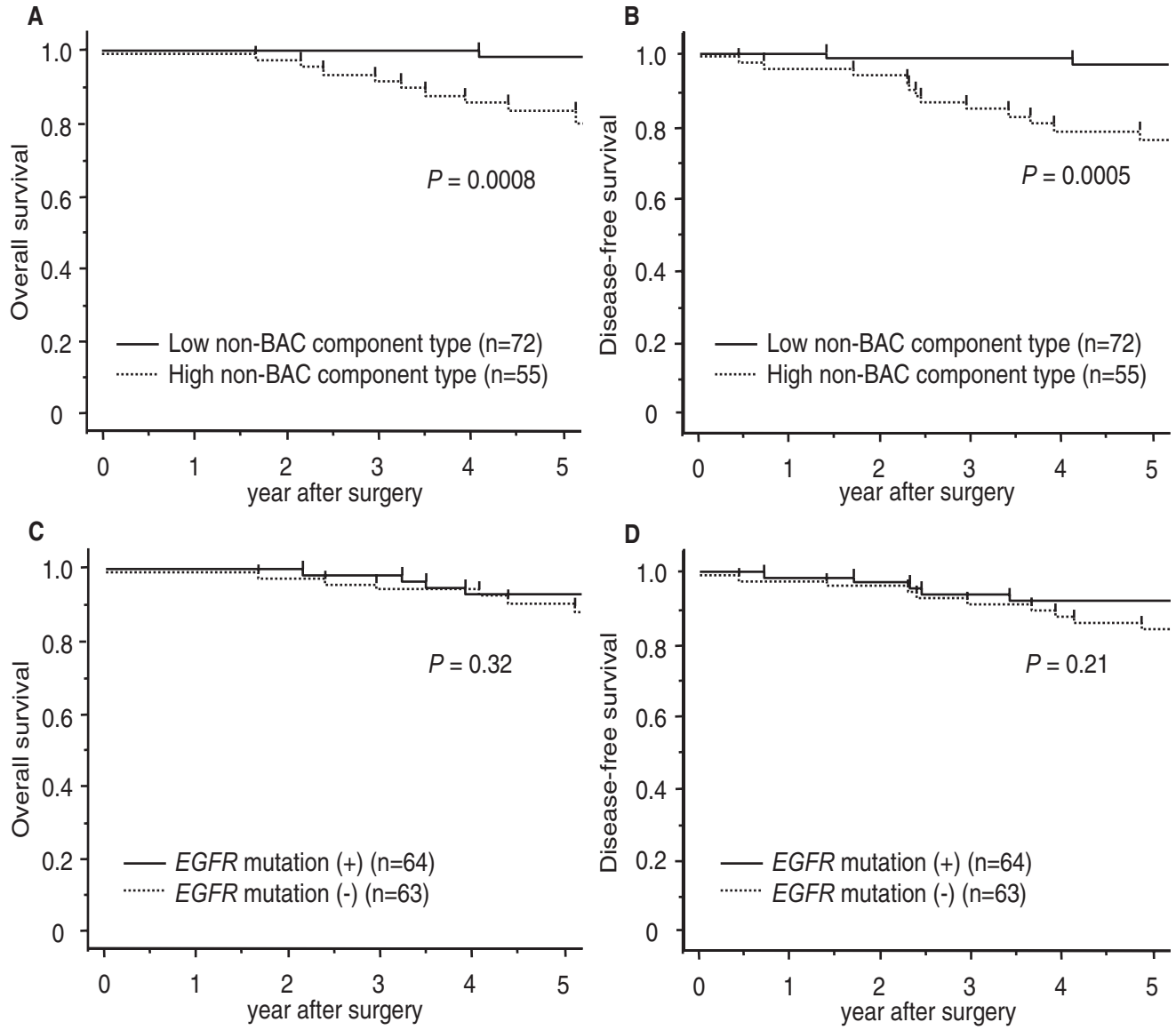

FIGURE 2. Kaplan–Meier survival curves. *A*, Overall survival stratified by non-BAC component level. *B*, Disease-free survival stratified by non-BAC component level. *C*, Overall survival stratified by *EGFR* mutation. *D*, Disease-free survival stratified by *EGFR* mutation.

therapy among stage IA patients with equal to or less than 2 cm tumors whose prognosis is supposed to be excellent, two issues should be identified: (1) the risk factors for poor outcome and (2) the predictors of efficacy of the therapeutic agents used. This study was conducted to clarify the former issue.

We previously reported the poor differentiation to be a risk factor for recurrence and poor prognosis in patients with stage IA NSCLC ≤20 mm in diameter.[27] However, the grade of tumor differentiation is based on subjective criteria and is not officially defined. Thus, the assessment of risk factors based on more objective parameters is necessary for precise assessment. For this study, we limited the subjects to adenocarcinomas because this subtype is the most common among the lung cancers with well defined characteristics in the WHO criteria.

Previous studies examined the impact of non-BAC or BAC component on the clinical outcome of patients with lung adenocarcinomas. Sakao and his colleagues reported that a high non-BAC component was strongly associated with poor prognosis in resected adenocarcinoma,[14] which suggested that tumors with both BAC and non-BAC components can be subdivided into two groups based on the degree of non-BAC component. Higashiyama and his colleagues reported that a ground-glass opacity (GGO) of less than 50% was a risk factor for lymph node metastasis, advanced stage and poor clinical outcome in adenocarcinoma of tumor <20 mm in diameter, although the GGO ratio was determined in a subjective manner (investigators' impression). In addition, previous reports have suggested a GGO of greater than 50% on high-resolution CT images to be a preoperative indicator of favorable outcome using NIH image J software[26]. Therefore, we set the threshold of the percentage of non-BAC component at 50.0% and used NIH image J software in this study for objective quantification.

The main result reported here is that the high non-BAC component type is the only independent risk factor for both recurrence and a poor prognosis. On the other hand, *EGFR* mutation had no correlation with recurrence or survival on either univariate or multivariate analysis. *EGFR* mutation tended to be more frequent in tumors with a BAC component, as shown in previous studies.[28,29] These results suggest that the *EGFR* mutation is a surrogate factor of the BAC component not directly related to prognosis. Taken together, the degree of non-BAC component seems to be a useful indicator of a need for postoperative adjuvant chemotherapy in patients with small lung adenocarcinoma.

In conclusion, we found that tumors with high percentage of non-BAC component, but not *EGFR* mutation, are a risk factor for both recurrence and poor prognosis in patients with stage IA lung adenocarcinoma ≤20 mm in diameter. Whereas randomized prospective studies are mandatory, the status of non-BAC component can be used as a suggestive indictor for adjuvant chemotherapy in small adenocarcinoma of the lung.

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