Clinical Features Reflect Exon Sites of EGFR Mutations in Patients with Resected Non-Small-Cell Lung Cancer

The aim of the current study was to determine the clinical significance according to the subtypes of epidermal growth factor receptor (EGFR) mutations and presence of *KRAS* mutations in operable non-small-cell lung cancer (NSCLC). We sequenced exons 18-21 of the EGFR tyrosine kinase domain and examined mutations in codons 12 and 13 of *KRAS* in tissues of patients with NSCLC who had undergone surgical resection. EGFR mutations were more frequent in never-smokers than smokers (33% vs. 14%, respectively; p=0.009) and in females than in males (31% vs. 16%, respectively; p=0.036). Mutations in exon 18-19 and 20-21 were found in 10 and 22 patients, respectively. Never-smokers and broncho-alveolar cell carcinoma features were positively associated with a mutation in exon 18-19 (p=0.027 and 0.016, respectively). The five-year survival rate in patients with a mutation in exons 18-19 (100%) was higher than that in patients without such mutation (47%; p=0.021). *KRAS* mutations were found in 16 patients (12%) and were not related to the overall survival (p=0.742). Patients with an EGFR mutation in exons 18-19 had better survival than patients without such mutation. Subtypes of EGFR mutations may be prognostic factors in patients undergoing curative resection.

Key Words : *Carcinoma, Non-small-cell Lung; Receptor, Epidermal Growth Factor; Genes, Ras; Mutation*

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INTRODUCTION

Lung cancer is the leading cause of cancer mortality worldwide (1). The overall 5-yr survival for lung cancer is approximately 15% and has improved only marginally over the past 30 yr despite the progress of modern chemotherapy (2). Therefore, new treatment strategies are needed to improve the prognosis of this dismal disease.

Aberrant signaling from the epidermal growth factor receptor (EGFR) is known to be important in the development and progress of non-small-cell lung cancer (NSCLC) (3). Several agents designed to inhibit EGFR tyrosine kinase such as gefitinib and erlotinib showed good tolerability and antitumor activity in NSCLC (4, 5). Somatic mutations in the region of EGFR encoding the tyrosine kinase domain have been identified to be an important factor determining the sensitivity to these drugs (6, 7). The tyrosine kinase domain of EGFR consists of N and C lobes. Until now, mutations had been found in EGFR exons 18-21, which encode the N lobe and part of the C lobe. Mutations in the different exons target structures around the ATP-binding cleft of EGFR, including the phosphate-binding loop (P-loop), the aC-helix, and the activation loop (A-loop) (8). In theory, all mutations could result in conformational changes and increased activity of the protein, as well as changes in its sensitivity to tyrosine kinase inhibitors (TKI) (6, 7, 9).

Recent studies have reported inconsistent results for EGFR mutations as a prognostic factor in cancer. The Canadian BR.21 trial reported that the presence of EGFR mutations confers no survival benefit on patients treated with erlotinib, whereas the results of the TRIBUTE trial found that EGFR mutations are a good prognostic factor (10-14). We surmised that there might be subgroups of EGFR mutations showing different survival rates.

KRAS mutations, which could result in resistance to EGFR inhibitors, are frequently reported changes in the EGFR signaling pathway in NSCLC (15, 16). These mutations are detected in 15-20% of NSCLC patients (16). A recent meta-analysis suggested that *KRAS* mutations might be another prognostic factor for overall survival in NSCLC (17).

We undertook this study to identify a subgroup of EGFR mutations and to define the prognostic role of *KRAS* mutations involved in the overall survival of patients with resected NSCLC.

PATIENTS AND METHODS

Patients

Tumor tissues were procured from patients who had undergone surgical resection at the Korea Cancer Center Hospital

from May 1995 to May 2004. One hundred and thirty three formalin-fixed paraffin-embedded tissues were available for the analysis. All patients had pathologically proven localized NSCLC. All pathology was reviewed using the WHO classification (18). Pure broncho-alveolar cell carcinoma (BAC), BAC with focal invasion, and adenocarcinoma with BAC features were considered as one entity (adenocarcinoma with BAC features) (19, 20). The study protocol was reviewed and approved by the institutional review board of the Korea Cancer Center Hospital.

DNA Sequencing for EGFR and *KRAS* mutations

DNA was extracted from formalin-fixed paraffin-embedded tissue sections of 10 μm thickness. DNA (100 ng) was amplified in 20 μL of reaction solution containing 2 μL of 10$\times$ buffer (Roche, Mannheim, Germany), 1.7-2.5 mM/L MgCl$_2$, 250 μM deoxynucleoside triphosphate, 2.5 units of DNA polymerase (Roche), and 0.3 μM each primer pair for EGFR (exon 18: forward, 5′-TCCAAATGAGCTGGCAA-GTG-3′ and reverse, 5′-TCCCAAACACTCAGTGAAAC-AAA-3′; exon 19: forward, 5′-ATGTGGCACCATCTCAC-AATTGCC-3′ and reverse, 5′-CCACACAGCAAAGCAG-AAACTCAC-3′; exon 20: forward, 5′-CATTCATGCGTC-TTCACCTG-3′ and reverse, 5′-CATATCCCCATGGCAA-ACTC-3′; exon 21: forward, 5′-GCTCAGAGCCTGGCA-TGAA-3′ and reverse, 5′-CATCCTCCCCTGCATGTGT-3′) or *KRAS* (codons 12-13: forward, 5′-TTATGTGTGAC-ATGTTCTAAT-3′ and reverse, 5′-AGAATGGTCCTGC-ACCAGTAA-3′; codon 61: forward, 5′-TCAAGTCCTTT-GCCCATTTT-3′ and reverse, 5′-TGCATGGCATTAGCA-AAGAC-3′). Fragments were amplified with a 5 min initial denaturation at 94℃, followed by 30 cycles of 1 min at 94℃, 1 min at 57℃ (EGFR mutation) or 55℃ (*KRAS* mutation), and 1 min at 72℃, with a final 10 min extension at 72℃. Purification and sequencing were performed as previously described (13).

Statistical analysis

The primary endpoint was overall survival, the definition of which was the time from diagnosis to death from any cause. The secondary endpoint was disease-free survival, which was defined as the time from diagnosis to the earliest occurrence of relapse or death from any cause. Overall and disease-free survivals were calculated by the Kaplan-Meier method (21). The Pearson χ^2 or Fisher's exact test was used for categorical variables. Mutation type (wild type, mutations in exons 18-19 or 20-21) was correlated with sex, smoking history, and pathology using multinomial logistic regression with the mutation type as the categorical dependent variable. A log-rank test was performed for the univariate analysis of survival.

Recursive partitioning using RPART, with exponential scaling for survival data, was applied to classify patients according to their death risk (22, 23). The splitting process stopped when the number of splits was two to avoid overfitting.

S-PLUS® was used for statistical analyses. All *p* values reported are the results of two-sided tests, and values less than 0.05 were considered significant.

RESULTS

Patient characteristics

The baseline characteristics of the patients are shown in Table 1. Sixty-three (47%) patients were male. The median age was 63 yr. The predominant pathology was adenocarcinoma (99 patients, 74%). Never-smokers comprised 52% of patients. The proportions of stage I, II, and III cancers were 36%, 17%, and 47%, respectively. EGFR mutations were detected in 24% of patients. The mutation rate was higher in never-smokers than in smokers (33% vs. 14%, respectively; *p*=0.009) and in females than in males (31% vs. 16%, respectively; *p*=0.036). The frequency of EGFR mutations was not significantly greater in younger (<50 yr) than in older ($\geq$50 yr) patients (33% vs. 23%, respectively; *p*=0.322) or in patients with adenocarcinomas than in those with other histologies (27% vs. 5%, respectively; *p*=0.139). *KRAS* mutations were detected in 16 of 133 (12%) patients, and were detected more frequently in males than in females (19% vs. 6%, respectively; *p*=0.018), more often in smokers than in never-smokers (19% vs. 6%, respectively; *p*=0.022). Neither pathology nor age was a significant factor predicting the presence of *KRAS* mutations.

Table 1. Patient characteristics

Characteristic	N (%)
Age (yr)	
Median	63
Range	38-89
Sex	
Male	63 (47)
Female	70 (53)
Smoking history	
Ever-smoker	64 (48)
Never-smoker	69 (52)
Histology	
Adenocarcinoma	99 (74)
Other	34 (26)
Histology according to BAC features	
Adenocarcinoma with BAC features	17 (13)
Other	116 (87)
Stage	
I	48 (36)
II	22 (17)
III	63 (47)

Types of EGFR and *KRAS* mutations

Mutations in codons 12 and 13 of *KRAS* were found in 16 patients and one patient, respectively (one patient had a double mutation). G12D (nine patients) was the most common mutation (one patient also carried G13D). G12C (two patients) and G12V (two patients) were detected. One patient carried G12S, and G12A was observed in two patients. There was no mutation in codon 61. No patient had a tumor in which both EGFR and KRAS were mutated.

EGFR mutations were detected in 32 patients (Table 2). Mutations in exon 20 were most frequent (16 patients). P772_H773insPR in exon 20 was detected in 11 patients. All mutations present in exon 19 were deletion types; del K745_E749

Table 2. Types of EGFR mutations. Numbers in parentheses indicate the numbers of patients with the mutations. (*) one patient carried two different mutations

Exon 18	Exon 19	Exon 20	Exon 21
G719D(1)*	del K745_E749(4)	L799R(1)	L861Q(1)*
G719C(1)	del E746_E750(1)	D807N(1)	L858R(6)
K713F(1)	del L747_P753(1)	T805I(1)	
	del A750_K757(1)	P772_H773insPR(11)	
		H773_V774insH(1)	
		S768_V769insAWT(1)	

Table 3. Clinical characteristics according to exonal sites of EGFR mutations

	Exon 18-19 mutation (N=10) OR (*p* value)	Exon 20-21 mutation (N=22) OR (*p* value)
Female	4.41 (0.068)	1.93 (0.175)
Young age (<50 yr)	1.85 (0.467)	1.65 (0.430)
Never-smoker	10.7 (0.027)	2.09 (0.129)
BAC features	5.93 (0.016)	0.34 (0.309)

(four patients), del A750_K757 (one patient), del E746_A750 (one patient), and del L747_P753 (one patient). L858R in exon 21 was found in six patients. One patient carried two mutations, in exon 18 and exon 21 (G719D and L861Q, respectively).

Multinomial logistic regression was used to compare two subgroups of EGFR mutations in the different exons with those of the wild type (the double mutation in exons 18 and 21 was considered to be a mutation in exon 18). The odds ratio was calculated with the wild type as the reference group. Non-small-cell lung cancer with a mutation in exon 18 or 19 was positively associated with never-smokers (OR, 10.7; *p*= 0.027) and BAC features (OR, 5.93; *p*=0.016). All cases with a mutation in exon 18 or 19 were adenocarcinoma. Females also showed a positive relationship with mutations in exons 18 or 19, but the association was not significant (OR, 4.41; *p*=0.068). Compared with mutations in exons 18-19, muta-
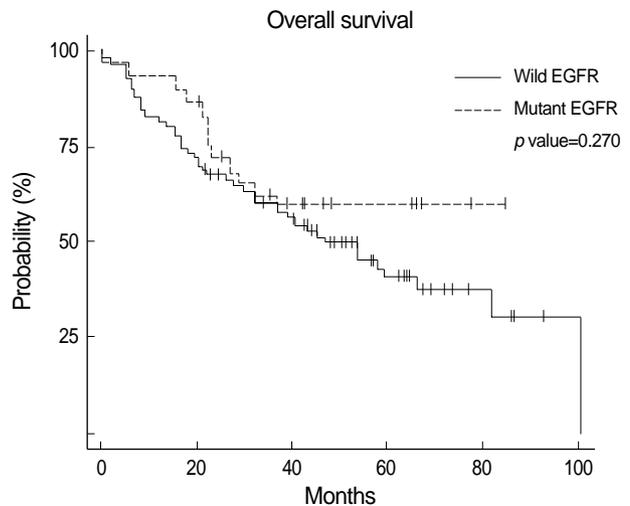
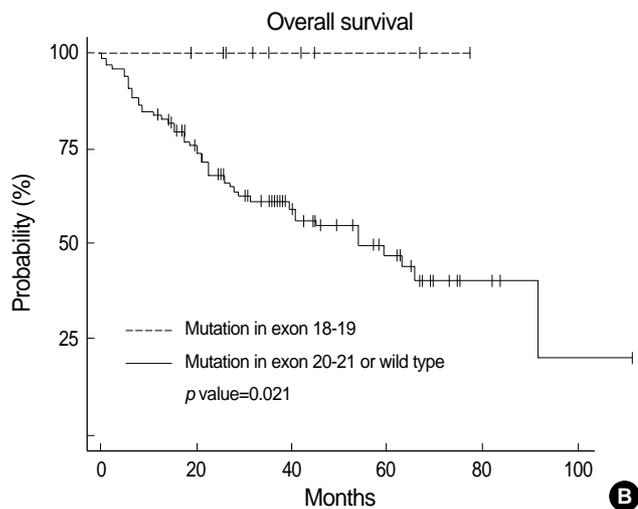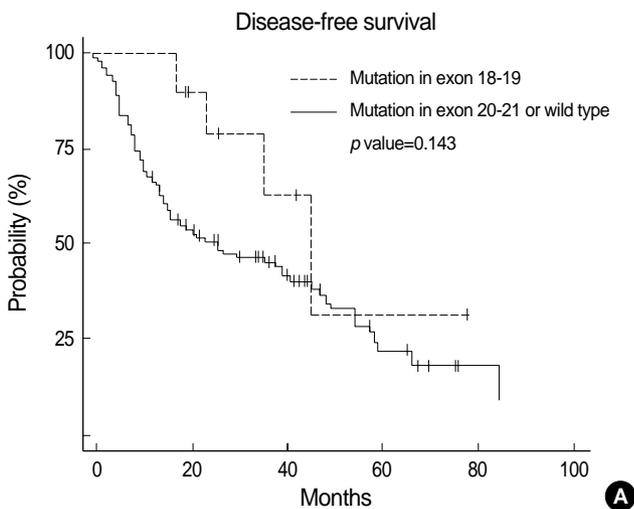Fig. 1. Kaplan-Meier plots of overall survival according to EGFR mutations.

Fig. 2. Kaplan-Meier plots of (A) disease-free survival and (B) overall survival according to the presence of EGFR mutations in exons 18-19.

tions in exons 20-21 did not show a significant association with never-smokers or females (Table 3).

Types of EGFR mutations were associated with disease-free and overall survival

The median follow-up time was 29 months. By May 2005, the numbers of observed relapses and deaths were 83 and 54, respectively. The presence of EGFR mutations was not significantly associated with overall survival ($p=0.27$) (Fig. 1). Interestingly, we found that the five-year survival rate (5YSR) for mutations in exons 18-19 (5YSR, 100%) was higher than that for mutations in exons 20-21 (5YSR, 57%) or the wild type (5YSR, 44%; $p=0.056$). For the analysis of different demographic features and statistical power, the types of mutations in the different exons were collapsed to mutations in

exons 18-19 and others. There was no significant difference in disease-free survival between these two groups; the median disease-free survival was 45 months for patients with mutant exons 18-19 and 26 months for the others ($p=0.143$) (Fig. 2A). The presence of a mutation in exons 18-19 was a significant prognostic factor for overall survival (5YSR, 100% vs. 47% in patients without such mutations; $p=0.021$) (Fig. 2B). The *KRAS* mutation was not a prognostic factor for disease-free survival or overall survival ($p=0.371$ and 0.742, respectively) (Fig. 3).

We applied recursive partitioning to construct a classification model for survival (Fig. 4A). Age (<50 vs. ≥50 yr), stage, pathology, *KRAS* mutation, and exon site of EGFR mutation (designated Ex 18, Ex 19, Ex 20, Ex 21, and wild type) were used in the model. The standardized event rate, which is a special case of the event rate in the Poisson model for censored data with exponential scaling, was set to one for the entire sample at the first node. The classification tree started at the top where 54 of the 133 total patients developed events. The model chose the exon site of the mutation for the first split and cancer stage for the second. The resulting terminal nodes (node 1, node 2, and node 3) were groups with mutations in Ex 18-19, stage I-II, and stage III according to increasing standardized event rates (0.17, 0.89, and 1.41, respectively). The observed five-year survival rates were 100%, 53%, and 46%, respectively. Fig. 4B shows the Kaplan-Meier estimates of overall survival according to each node. The survival rates of the three groups were significantly different ($p=0.026$).
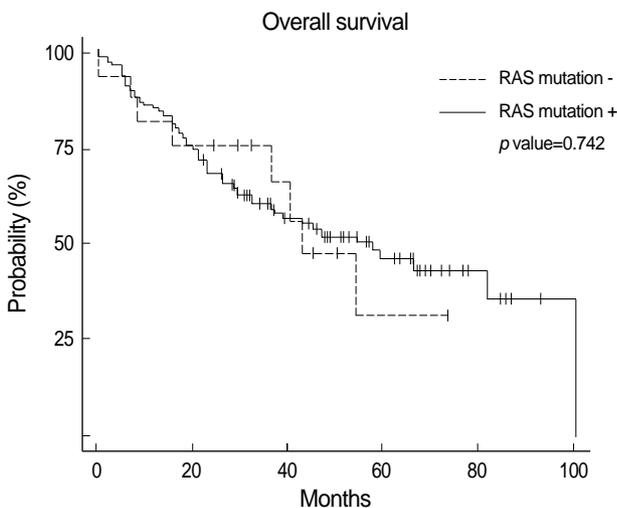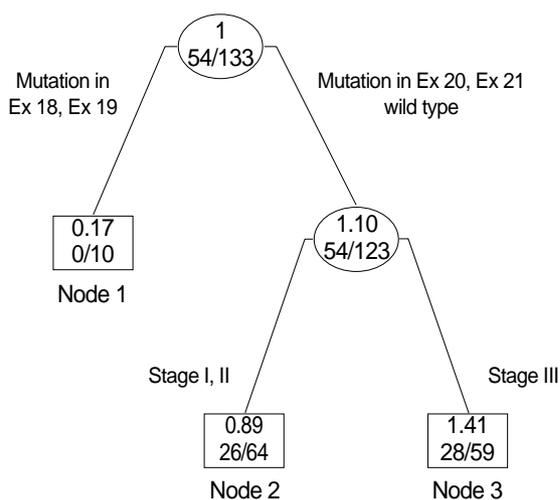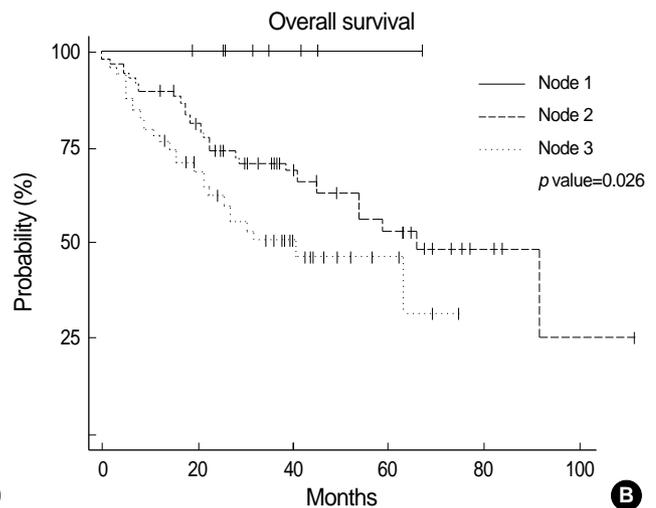

Fig. 3. Overall survival according to *KRAS* mutations by Kaplan-Meier method.

DISCUSSION

Recently, the BR.21 trial reported the survival benefits of erlotinib as a second-line therapy, in which the subgroup of

Fig. 4. (A) The final tree fitted by recursive partitioning using an exponential scaling method. The standardized event rate and events/total number of observations per subgroup are given. (B) Kaplan-Meier plots of overall survival according to three different nodes.

never-smokers displayed better survival (11). The results of a subset analysis in the TRIBUTE trial showed a survival gain for never-smokers when treated with chemotherapy and erlotinib (24). It seems clear that some subgroups might respond to TKI or show a survival benefit.

Most studies have based their analyses on the assumption that all types of EGFR mutations have the same prognostic significance in terms of overall survival. Mutations in exon 18 are associated with the P-loop. Deletion of exon 19 occurs just downstream from a lysine residue at a critical position for ATP binding, and L858R mutations occur adjacent to the DFG motif, which stabilizes the A-loop. All types of mutations, including these mutations, could lead to conformational changes that might theoretically result in a response to TKI (9, 25). However, in most studies, no structural modeling or biochemical analysis has been performed for novel mutations. Furthermore, not all investigators analyzed exons 18-21 of EGFR (13, 14).

Shigematsu et al. reported a mutation in exon 20 that is found in 9% of patients with EGFR mutations (26). In our study, 16 of 32 patients carried a mutation in exon 20, which is a relatively high proportion compared with those reported previously. It should be noted that there was a preponderance of early-stage cancers and adenocarcinomas in our study population. Our results indicate that mutations in exon 20 were more common in females (10 patients), never-smokers (10 patients), and those with adenocarcinomas (13 patients), which is consistent with the known demographic features of EGFR mutations. Racial differences could be a possible cause.

The proportions of mutations in exons 18-19 among EGFR mutants have varied in previous studies. Cappuzzo et al. reported a frequency of 47% (seven of 15 mutations), and found that gene amplification is a more important factor in survival than is mutation (14). The BR.21 trial showed that 40% of mutations occurred in exons 18-19 (16 of 40 mutations) (10). Of the other studies that showed a survival benefit for TKI, Han et al. reported that 65% of mutations occurred in exons 18-19 (11 of 17 mutations) and Eberhard et al. reported that 69% of mutations occurred in exons 18-19 (20 of 29 mutations) (12, 13). In another study, which reported a better response in patients with deletion mutations (16 patients with deletions in exons 18-19 mutation) and better survival in patients with mutant EGFR, exon 18-19 mutations comprised 61% of all mutations (27). The proportion of exon 18-19 mutations appears to be higher in studies that show a survival benefit for TKI in patients with EGFR mutations (10, 12-14, 27). Therefore, the inconsistent results of previous studies regarding EGFR mutations might be due to the varying proportions of exon 18-19 mutations among the mutant EGFRs examined. This could lead to different analyses of the putative 'EGFR mutation' as a prognostic factor. The current study suggests that the exonal site of EGFR muations and stage were more important prognostic factors than pathology. Our results also show a positive association between never-

smokers and exon 18-19 mutations, compared with exon 20-21 mutations. The TRIBUTE molecular study found that EGFR mutations were a prognostic factor in NSCLC, but the BR.21 study did not (10). Both studies reported better survival for never-smokers. These studies did not undertake molecular analysis of all patients. Therefore, the discrepancy for EGFR mutations but the consistency for never-smokers could be explained if never-smokers have a greater tendency to carry exon 18-19 mutations. In addition, different demographic features according to BAC feature and smoking history may explain the results of another study to observe better response in the BAC subtype and never-smoker (20).

Most previous studies for EGFR mutations examined patients with advanced-stage cancers, whereas we performed this study on localized tumors. The subgroup analysis of TRIBUTE showed that EGFR mutations did not confer a survival benefit in combination with chemotherapy, but did confer better survival with no relationship to the type of treatment (12). This suggests that EGFR mutations themselves are a prognostic factor for survival. We postulated that survival differences caused by EGFR mutations could be observed in resected NSCLC. Shigematsu et al. found no survival difference according to the presence of EGFR mutations, which was consistent with our study (26). They evaluated the survival outcome between the L858R mutation and exon 19 mutation. Contrasting with our results, patients with the L858R mutation appeared to achieve better survival although the difference was not statistically significant (p=0.05). It should be considered that this study involved a heterogeneous group including advanced-stage cancers and presented the results of univariate analysis. In addition, its study population consisted of various ethnicities and the proportion of adenocarcinoma was lower than our study. These differences might have led to different results for overall survival according to mutational types of EGFR. In the current study, some patients (n=15) received gefitinib in relapse. There was no difference in terms of response to gefitinib according to mutational sites (data not shown). Even when these patients were excluded in survival analysis, patients with mutations in exon 18-19 showed better overall survival (p=0.045).

It is not clear whether the various types of mutant EGFRs affect the survival of patients with tumors by different signal transduction pathways. Although both deletion of exon 19 and L858R mutation activated EGFR by a common pathway (AKT and STAT), EGF-induced phosphorylation of Y845, one of autophosphorylation sites in EGFR, was observed only in cell lines with the L858R mutation (28). Some investigators have reported that phosphorylated Y845 regulates cell survival by another downstream pathway (29). We hypothesize that mutations on either side of the αC-helix of EGFR (mutations in exons 18-19 and exons 20-21) cause different conformational changes in EGFR that lead to the activation of various downstream pathways. A definitive explanation of the variations in survival according to the exon site of the

EGFR mutation requires further studies.

In conclusion, the present study demonstrated better survival for NSCLC patients with mutations in exons 18-19. This suggests that survival may differ according to the exon sites of the EGFR mutation. Our results, together with those of previous studies, should be interpreted with caution because the numbers of patients with mutations were small and the studies were performed retrospectively. Further laboratory studies of the downstream pathways involved and analysis of the actual crystal structures of the EGFR mutants are anticipated. A large prospective study is required to confirm these findings.

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