Analysis of *ERBB4* Mutations and Expression in Japanese Patients with Lung Cancer

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Abstract: Only the kinase domain of *ERBB4* has been analyzed in East Asian populations, but a recent large-scale mutation analysis has indicated a higher incidence of mutations in the extracellular domain. Mutations in the extracellular and kinase domains of *ERBB4* were examined by direct sequencing in 72 patients with primary lung cancer and 8 cell lines. In addition, *ERBB4* expression was determined in 60 patients by quantitative real-time polymerase chain reaction. We investigated the relationship between *ERBB4* expression and clinicopathologic characteristics including prognosis. One patient possessed Q793Q polymorphism in the kinase domain. However, we detected no mutations in extracellular or kinase domains of *ERBB4*. There was no significant difference in the clinicopathologic characteristics including prognosis of patients with high or low expression of *ERBB4*. The clinical significance of *ERBB4* in lung cancers is negligible.

Key Words: ERBB4 mutation, Extracellular domain, Lung cancer, Expression, Prognosis.

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The ERBB family of tyrosine kinase receptors consists of four members: epidermal growth factor receptor (*EGFR*), *ERBB2*, *ERBB3*, and *ERBB4*.[1] According to the NCBI database, the sequences of the kinase domains of *EGFR* and *ERBB4* are 79% identical, and *EGFR* and *ERBB4* have common ligands: heparin-binding epidermal growth factor, betacellulin, and epiregulin.[1] *EGFR* mutation has been revealed to play an important role in non-small cell lung cancer (NSCLC).

Two mutation analyses of the *ERBB4* kinase domain in East Asian patients with NSCLC have been reported: 5 of 217 (2.3%) in Korean patients[2] and none of 105 in Japanese patients.[3] Conversely, a large-scale mutation analysis in 188 patients with lung adenocarcinoma detected nine *ERBB4* mutations: two in the kinase domain, one in the transmembrane domain, and six in the extracellular domains (Figure 1*A*).[4]

The extracellular domains of the *ERBB* family are consisted of four distinct protein domains. There are two homologous large (L) domains and two cysteine-rich (CR) domains, which occur in the order L1–CR1–L2–CR2 (Figure 1*A*).[5] The L1 and L2 domains form the ligand binding pocket and the CR1 and CR2 domains are deeply involved in receptor dimerization.[5] Mutations in the *ERBB4* gene are more frequently present in the extracellular domain, especially the CR1 domain, than in the kinase domain.[4] However, only the kinase domain has been analyzed in East Asian populations.[2,3] We considered that analysis of mutations in the extracellular domains and the kinase domain of *ERBB4* would be of value.

In this study, we searched for mutations in the CR1 domain and kinase domain of *ERBB4*. We also analyzed *ERBB4* mRNA expression by real-time polymerase chain reaction and examined the relationship between the expression and clinicopathologic characteristics including prognosis.

PATIENTS AND METHODS

Cell Lines

Eight lung cancer cell lines were available. These comprised six adenocarcinomas (NCI-H23, NCI-H358, NCI-H3255, HCC78, A549, and ACC-LC-319), one adenosquamous cell carcinoma (NCI-H596), and one large cell carcinoma (ACC-LC-91). ACC-LC-319 and ACC-LC-91 were established in our institution. A549 and NCI-H596 were purchased from the American Type Culture Collection (Manassas, VA). The others were gifts from Dr. Adi F. Gazdar.

Patients

We studied 72 Japanese patients with lung cancer who underwent pulmonary resection at our institution. Tumor samples were rapidly frozen, and total RNA were extracted and genomic DNA was extracted from the blood sample using the Gene Tapping by Liquid Extraction kit (TAKARA BIO Inc., Otsu, Japan) after obtaining appropriate approval from the review board and written informed consent from the patients. The clinicopathologic characteristics of the patients were as follows: 44 were men and 28 were women, and the median age was 67 years at diagnosis (range, 38–85 years). Thirty-nine patients had pathologic stage I disease, 7 had stage II, 23 had stage III, and 3 had stage IV (TNM Classification of Malignant tumor, 6th Edition). There were 55 adenocarcinomas, 10 squamous cell

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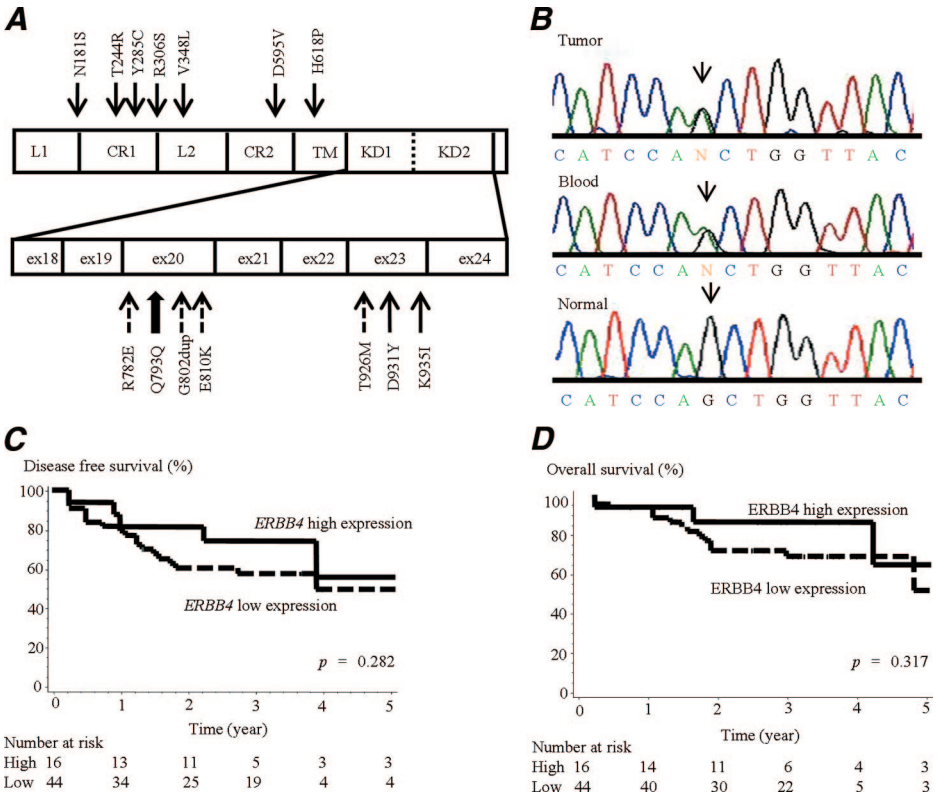

FIGURE 1. *A*, *ERBB4* mutations from the previous reports of Ding et al.[4] (arrow) and Soung et al.[2] (dotted arrow) and the Q793Q polymorphism identified in this study (thick arrow) are illustrated. *ERBB4* consists of four extracellular domains, a transmembrane domain (TM), and a kinase domain. The kinase domain is divided into two regions. L1, 2: large domain 1, 2 CR1: 2; CR domain 1, 2. *B*, Sequencing chromatograms for the synonymous mutation identified in exon 20 of *ERBB4* in the tumor and blood from the same patient. The nucleotide change was c.2379G>A, which did not lead to substitution of glutamine at position 793. *C* and *D*, Kaplan–Meier estimates of disease-free survival and overall survival in patients with high and low *ERBB4* expression. The median disease-free survival time and overall survival time were not significantly different among the two groups.

carcinomas, 1 adenosquamous cell carcinomas, 5 large cell carcinomas, and 1 small cell carcinoma. Twenty-four patients had never smoked, and 48 were current or former smokers.

Analysis of *ERBB4* Mutations

The *ERBB4* extracellular region and kinase domain, which was divided into *C*-terminal side (KD1) and *N*-terminal side (KD2; Figure 1*A*), were analyzed for mutations. By using total RNA or genomic DNA, *ERBB4* was analyzed by direct. Primer sequences were as follows: extracellular region, 5′-TCCTTTGTTATGCAGACACCAT-3′ and 5′-TTGTAAGGGTCCCCATGAATAC-3′; KD1, 5′-GGTGGAACCATTAACTCCCAGT-3′ and 5′-CAATGCTGATGGAGGAAAGATG-3′; and KD2, 5′-CAATGCTGATGGAGGAAAGATG-3′ and 5′-TGATCGTATGAAGCTTCCCAG-3′.

Analysis of ERBB4 Expression

Total RNA from 60 patients and 2 normal lung tissue samples were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). We analyzed *ERBB4* expressions according to the protocol of the TaqMan Gene Expression assay, using 18S rRNA as the internal reference gene. The primer IDs for *ERBB4* and 18S rRNA were Hs00171783_m1 and Hs99999901_s1 (Applied Biosystems), respectively. The 60 patients were divided into 2 groups on the basis of the average *ERBB4* expression value of 2 normal lung tissue samples.

Statistical Analysis

For comparison of proportions, a χ^2 test or Fisher's exact test was applied. For quantitative variables, Student *t* test was used. Disease-free survival (DFS) was measured from the date of first operation until the date of radiologic recurrence or death. Overall survival (OS) was measured from the date of first operation until the date of death. The Kaplan–Meier method was used to estimate the probability of survival as a function of time, and survival differences were analyzed by the log-rank test. We defined the significance level at $p < 0.05$.

RESULTS

In the *ERBB4* mutation analysis, one patient with adenocarcinoma had a c.2379G>A synonymous genetic change resulting in Q793Q in the *ERBB4* kinase domain (exon 20). The synonymous change was confirmed as a polymorphism by DNA sequencing a matched blood sample (Figure 1*B*). However, somatic mutation of *ERBB4* was not detected in this study.

The average *ERBB4* expression level was not significantly different between tumor samples and normal lung tissue samples ($p = 0.384$). The high- and low-expression groups were 27% (16 of 60 patients) and 73% (44 of 60 patients), respectively. The two groups were compared for clinicopathologic characteristics (Table 1), but we did not identify any significant difference. In the survival analysis, the median DFS and OS time was not significantly different between the patients with high and low *ERBB4* expression (Figures 1*C*, *D*).

DISCUSSION

We have previously analyzed *EGFR*, *KRAS*, *MET*, and *ERBB2* mutations and *MET* amplification, which are mutually

TABLE 1. Clinicopathologic Data for 60 Patients with Lung Cancer

Characteristic	*ERBB4* Expression (%) High (n = 16)	Low (n = 44)	p
Age (yr)			
>67	7 (43.8)	23 (52.3)	0.559
≤67	9 (56.2)	21 (47.7)	
Sex			
Male	8 (50.0)	31 (70.5)	0.142
Female	8 (50.0)	13 (29.5)	
Pathologic stage			
I, II	13 (81.3)	25 (56.8)	0.130
III, IV	3 (18.7)	19 (43.2)	
Smoking			
Never	6 (37.5)	12 (27.3)	0.445
Current or former	10 (62.5)	32 (72.7)	
Tumor size (cm)			
>3	6 (43.8)	28 (63.6)	0.071
≤3	10 (56.2)	16 (36.4)	
Histology			
Adenocarcinoma	11 (68.9)	32 (72.7)	0.763
Nonadenocarcinoma	5 (31.1)	12 (27.3)	

exclusive, in lung cancers.[6,7] The aims of this study were to identify mutations of the *ERBB4* extracellular and kinase domains in lung cancers and to confirm their mutual exclusivity with mutations in the above genes. Recently, activating point mutations at the *EGFR* extracellular domains were found in 12.9% of glioblastoma.[8] Mutations of the *ERBB4* extracellular domain also have been reported in NSCLC.[4] We considered that there was need for analysis of mutations of the *ERBB4* extracellular domain in East Asian patients with lung cancer. In this mutation analysis, only a Q793Q polymorphism was detected in the *ERBB4* kinase domain. The *ERBB4* polymorphism was analogous to the Q787Q polymorphism of *EGFR*. In East Asia, two mutation analyses of *ERBB4* have been reported.[2,3] Considering those together with this study, 5 of 394 patients with lung cancer (1.3%) harbored *ERBB4* mutations, all of which were present in the *ERBB4* kinase domain.

Gene amplification and expression of the *ERBB* family have been reported in lung cancer. *EGFR* gene amplifications are frequently observed in squamous cell carcinoma with poor prognosis.[9,10] Synchronous protein overexpression of *EGFR* and *ERBB2* significantly predicted increased recurrence risk and decreased survival.[11] In *ERBB4* expression assays of lung cancers, the expression levels are remarkably lower than those of other members of the *ERBB* family, and there is no relationship between *ERBB4* expression and metastasis.[12] Conversely, Starr et al.[13] reported that the proliferation of the *ERBB4*-transfected human adenocarcinoma cell line H1299 was 2-fold higher than that of the parental cells, and in mice injected with the *ERBB4*-transfected cells, the tumors were larger,[13] suggesting that *ERBB4* is associated with metastasis and inferior survival.[13]

As indicated earlier, opinions vary concerning the relationship of *ERBB4* expression with metastasis and prognosis in lung cancers. In this study, the patients with regional lymph node or distant metastasis at diagnosis made up 40% (6 of 16 patients) of the *ERBB4* high-expression group and 44% (20 of 44 patients) of the *ERBB4* low-expression group (p = 0.582). The DFS and OS were not significantly different between the high- and low-expression groups. There was no significant relationship between *ERBB4* expression and metastasis or prognosis, and we did not detect a significant relationship between *ERBB4* expression and any clinicopathologic factors.

In conclusion, mutation of the *ERBB4* kinase domain and CR1 domain were not detected. High *ERBB4* expression was infrequent in Japanese patients with lung cancer, and the clinical significance of *ERBB4* was negligible.

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