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Extracellular Domain Mutation of *ErbB2* Status in Japanese Lung Cancer Patients

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Abstract

Purpose: The erbB pathway involves a family of tyrosine kinases and has contributed to resistance or sensitivity for chemotherapy in many tumor types. Somatic mutations of the erbB family receptor gene at kinase domain were found in lung cancer patients. However, the incidence of extracellular domain mutation of *erbB2* in Japanese patients has been rarely described. We report on the incidence of these mutations and clinical factors associated with these mutations.

Material and Methods: We have investigated extracellular domain mutations of *erbB2* status in non-small cell lung cancer (NSCLC) patients by reverse transcript polymerase chain reaction (RT-PCR) and direct sequencing. The study included 210 surgically removed *EGFR* or *ALK* gene wild type lung cancer cases from Nagoya City University Hospital.

Results: One *G309E erbB2* mutation case was found from squamous cell carcinoma. Within adenocarcinoma, no *erbB2* mutation was found in extracellular domain. We have previously detected six *erbB2* mutations at kinase domain all in adenocarcinomas. Among the 7 *erbB2* gene mutation cases, one case with kinase domain mutation was Hercep test positive.

Conclusion: The extracellular domain mutation of *erbB2* was rare in Japanese population. Although this mutation is rare, its identification could result in more precise treatment of patients.

Introduction

ErbB family signaling pathway plays a crucial role in many carcinogenic processes such as proliferation, angiogenesis, invasion, and metastasis, and resistance to apoptosis [1,2]. Because deregulation of erbB family pathway genes has been observed frequently in various types of tumors, including non-small cell lung cancer (NSCLC), the development of targeted agents for lung cancer therapy has focused mainly on erbBs and its downstream networks [3], such as RAS/RAF/MAP kinase and PI3K/AKT being the two major pathways [3,4].

The erbB family comprises 4 structurally related receptors: erbB1 (EGFR), 2, 3 and 4. On ligand stimulation, the receptor forms either homodimers or heterodimers, which activate their cytoplasmic domain. Several reports have shown that somatic mutations of the *EGFR* gene were found in about 25-40% of Japanese NSCLC patients [5,6], but only in about 10% of NSCLC patients in USA [7,8]. *EGFR* mutations were predominantly found in female, non-smoker with adenocarcinomas [5-9]. Actually, *EGFR* mutations in NSCLC have been correlated with clinical response to gefitinib therapy [10-12]. In addition, it has been reported that *erbB2* mutations at kinase domain were found in 2-4% of European-derived NSCLC patients [13,14]. The somatic *erbB2* mutations were more frequent in never smoker and adenocarcinoma history [14]. The *erbB2* mutation was also investigated in Japanese NSCLC [6,15,16].

The advent of next-generation sequencing technologies has enabled to compilation of large somatic mutation datasets from lung cancer sequencing studies [17]. To identify new lung cancer oncogenes, Greulich et al. [18] additionally assessed somatic alleles of significantly mutated receptor tyrosine kinase genes [17] for activity in cellular transformation assays. Although most receptor tyrosine kinase mutations tested failed to score, the extracellular domain mutations of *erbB2* were oncogenic. Additional reports of extracellular domain mutations of *erbB2* included G309E in 1/193 breast cancer samples and S310Y in 1/63 squamous lung cancer samples [19], S310F in 2/112 breast cancers [20], S310F in 1/65 breast cancers [21], and S310F in ovarian cancers [22]. These results indicate a unique therapeutic opportunity for patients with lung, breast and ovarian cancer who harbor extracellular domain mutations of *erbB2*.

Whereas the insertion mutation of the kinase domain of *erbB2* is already characterized [23,24], extracellular domain mutations of *erbB2* mutation frequency in Japanese NSCLC is not well known. We have previously described *erbB2* mutation cases at kinase domain [6,16], however, in this study, we have additionally investigated *erbB2* mutation status at extracellular domain in 210 surgically treated NSCLC cases.

Patients and Methods

Patients

This is the retrospective study and the study group included 210 lung cancer patients who had undergone surgery at the Department of Surgery, Nagoya City University Hospital between 1997 and 2013. We have also investigated *EGFR*, *Kras*, *Braf*, *EML4-ALK*, and *KIF5B/RET* mutation status for the most of the same patients group. *EGFR*, *Braf*, *EML4-ALK*, or *KIF5B/RET* mutation positive samples were excluded from this study. All tumor samples were immediately frozen and stored at -80°C until assayed. The clinical and pathological characteristics of the 210 lung cancer patients are as follows; 123 cases at stage I, 38 at stage II, and 59 at stage III-IV. The mean age was 67.9 years (range, 44-86). Among the 210 lung cancer patients, 130 (61.9%)

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were diagnosed as adenocarcinoma, and 78 (37.1%) were squamous cell carcinoma. We have previously tested 269 patients to see if the *erbB2* kinase mutation was exclusive without containing overlapping mutations (*EGFR*, *Braf*, *EML4-ALK*, or *KIF5B/RET*) [16]. Of 269, 102 samples were overlapped with this study.

PCR assays for *erbB2*

Total RNA was extracted from lung cancer tissues and adjacent non-malignant lung tissues using Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturers' instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/ml. About 20 cases were excluded because tumor cells were too few to sufficiently extract tumor RNA. RNA (1μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 μg oligo $(dT)_{12\text{-}16}$ (Amersham Pharmacia Biotech Inc. Piscataway, NJ) . The reaction mixture was incubated at 42°C for 50 minutes and then at 72°C for 15 minutes. We then used 1 μl of each DNA for PCR analyses. The PCR reactions were performed using LA-Taq kit (Takara Bio Inc, Shiga, Japan) in a 50- μl reaction volume. The primer sequences for *erbB2* gene for extracellular domain (779-1070, 292bp) were as follows: the forward primer, 5-ACAGTGGCATCTGTGAGCTG -3 and the reverse primer, 5-GTAACTGCCCTCACCTCTCG -3. The cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 40 cycles at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 30 seconds. The primer sequences for *erbB2* gene for kinase domain (exon 19-22) were as follows: the forward primer, 5-CGCTTTTGGCACAGTCTACA -3 and the reverse primer, 5-GGGATCCCATCGTAAGGTTT -3 (594bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 40 seconds, 60°C for 40 seconds, 72°C for 45 seconds. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA). Amplified cDNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide and photographed under ultraviolet transillumination. These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review form forward and reverse, both side. EGFR, Kras and Braf sequencing methods were already submitted elsewhere [5,6,9,16,25].

ErbB2 Immunohistochemistry

7 cases were immunostained by methods for HercepTest II (Dako Japan Co., Tokyo, Japan) using the rabbit polyclonal antibody against HER2/neu. Unstained 4-μm sections of FFPE tumor tissue were submitted to the analysis. The Dako HercepTest™ system included pretreatment with Target Retrieval solution, pH 6 (Dako Co.) 97°C for 40 minutes, followed by incubation with rabbit polyclonal anti-HER2/neu at room temperature for 30 minutes. Antibody incubation was followed by standard signal amplification including HRP conjugated polymer solution at room temperature for 30 minutes, DAB reaction for 10 minutes and counter staining with hematoxylin for 3 minutes. An IHC score was assigned to each case according to the following criteria showing the designated staining pattern; 3+, intense staining; 2+, moderately staining; 1+, faint staining at membrane; and no staining. Tumors with intensity involving a minimum of 10% of the positive stained tumor cells were considered positive for erbB2 expression.

Statistical analysis

Statistical analyses were done using the Mann- Whitney U-test for unpaired samples. Correlation coefficients were determined by rank correlation using Spearman's test and $\chi 2$test. All analysis was done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA, USA), and was considered significant when the p -value was less than 0.05.

Results

ErbB2 gene mutation status in Japanese lung cancer patients

We have previously sequenced kinase domain of *erbB2* for 269 NSCLC patients [16]. Among 269 patients, 5 patients (2.0%) had *erbB2* mutations. All mutations were at exon 20. Three were female. All were non-smoker. Four had 12 amino acids insertion mutation (2324-2325 ins ATACGTGATGGC), located in the exon 20 at kinase domain (775-776 ins YVMA). One had amino acids insertion mutation (2326 G to TTGT), located in the exon 20 at kinase domain (776 Glycine to Leucine plus Cystein). We have additionally sequenced 17 samples, and one had ins YVMA. This case was male, smoker with adenocarcinoma. Totally, never smoker had significantly higher *erbB2* mutation rate than in smoker (p=0.0328). There was no significant difference of *erbB2* mutation rate in gender (p=0.4202), age (p=0.6849) and pathological stages (p=0.6803) (Table 1). All 6 are survived, however, 3 of them had relapsed and underwent conventional chemothepary.

Within these NSCLC, five genes (*EGFR*, *erbB2*, *Kras*, *Braf* and *KIF5B/RET*) mutations were exclusively existed. Only one patient had *EGFR* and *EML4/ALK* mutations (data not shown). In the same period, 232 had *EGFR* mutations within kinase domain, including 107 exon19 deletions and 102 L858R mutations. 7 had *ALK* translocations and 3 had *KIF5B/RET* translocations. 7 *Braf* mutations were within kinase domain (data not shown).

Extracellular domain of erbB2 gene mutation status in Japanese lung cancer patients

Of 210 patients, only one patient had G309E (926 guanine to adenine; Glycine to Glutamic acid). This case was male, smoker with stage I well differentiated squamous cell carcinoma. Adjacent normal lung tissue was available and the sequence result showed the wild type, suggesting that the mutation was somatic (Figure 1). The kinase domain of the *erbB2* gene sequencing result showed the case was wild type. The patient had no recurrence for 48 months. No mutation at 310 amino acid position was found. In 6 *erbB2* mutation cases at kinase domain, the sequences of extracellular domain were wild type.

Immunohistochemistry

An immunohistochemical approach, Hercep Test was used to study erbB2 protein expression in 7 NSCLC cases, including 6 mutants at kinase domain and one mutant at extracellular domain. One erbB2 ins YVMA mutant adenocarcinoma showed more than 10% membrane signal with 1+ (Figure 2). In addition, another ins YVMA case had the 1+ staining intensity, less than 10% of tumors. In other cases, including extracellular domain mutant case, there were no-stained.

Discussion

In this study, we found that an extracellular domain mutation of *erbB2* case out of 210 NSCLC. The *erbB2* mutation was exclusively

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Table 1: Clinico-pathological data of 286 lung adenocarcinoma patients and *erbB2* kinase domain mutations.

			erbB2 gene kinase domain status		
			Mutant	Wild type	p-value
Factors			patients (n=6)	patients (n=280)	
Mean age (years)		65.6±8.9	68.2±4.5	65.5±9.0	0.4435
Stage					
	I II-IV		3(1.7%) 3(2.7%)	172(98.3%) 108(97.3%)	0.6803
Lymph node metastasis					
	N0		3(1.5%)	196(98.5%)	0.3728
N+			3(3.4%)	84(96.6%)	
Smoking	status				
Never smoker			5(4.5%)	105(95.5%)	0.0328
	Smoker		1(0.6%)	175(99.4%)	
Kras mutation					
	Mutation		0(0%)	14(100%)	0.9999
Wild type			6(3.6%)	220(96.7%)	
Pathology					
	Adeno Non-adeno		6(2.7%) 0(0%)	241(97.3%) 39(100%)	0.9999
Age					
	≤65		2(1.4%)	137(98.6%)	0.6849
>65			4(2.7%)	143(97.3%)	
Gender					
Male			3(1.6%)	184(98.4%)	0.4202
	Female		3(3.0%)	96(97.0%)	

*N0: lymph node metastasis negative, N+; lymph node metastasis positive, Adeno; adenocarcinoma.

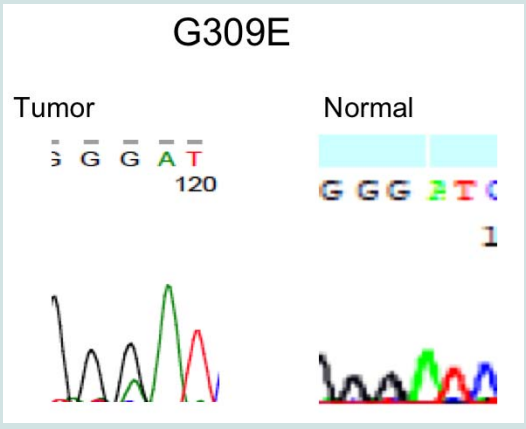

Figure 1: G309E (926 guanine to adenine; Glycine to Glutamic acid) mutation at extracellular domain of erbB2. Adjacent normal lung tissue was available and the sequence result showed the wild type, suggesting that the mutation was somatic.

found without *erbB2* mutation at kinase domain. Extracellular domain mutation of *erbB2* was found in squamous cell carcinomas but not in adenocarcinoma. On the other hands, in our analysis, *erbB2* genes mutations at kinase domain were predominantly found in non-smoker with adenocarcinomas.

The recurrent mutated erbB2 residue 309-310 lies in domain II of the extracellular region has considered to mediate receptor dimerization [26]. Furthermore, residue S310 is involved in interactions with a therapeutic antibody that prevents erbB2 dimerization [27]. Hence the recurrent mutation of this site probably alters the function of erbB2. *ErbB2* mutations have reported in breast

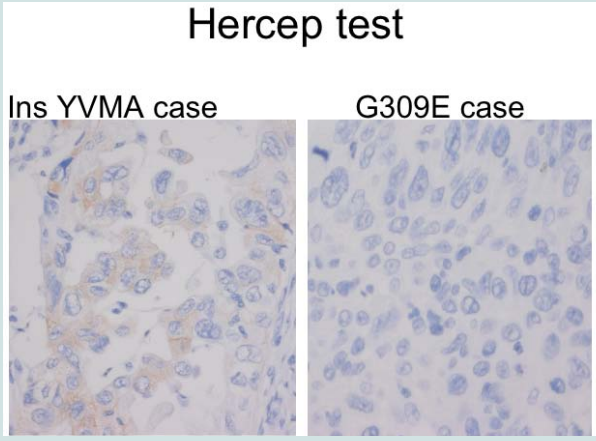

Figure 2: Hercep test results for erbB2 mutant case at kinase domain (775-776 ins YVMA) (left) and at extracellular domain (right).

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cancers, with two being identical S310F mutations [20]. These two samples were distinct on the basis of their germline and somatic genotypes [20], suggested the mutation was somatic. Neither sample with the S310F activating mutation had *erbB2* amplification. This is consistent with our results showing Hercep-test negative for our G309E sample. However, the two mutant breast cancer samples belong to the erbB2-enriched and luminal B subtypes, which typically have *erbB2* amplification, indicated the observed mutations have a driving role in these tumors. The G309E, S310F and S310Y mutants over expressed NIH 3T3 cells supported robust colony formation in soft agar [18]. AALE human lung epithelial cells were similarly transformed to anchorage independence by the extracellular mutants of *erbB2* [18]. These mutants have two distinct mechanisms: elevation of C-terminal phosphorylation and formation of disulfide-linked dimmers [18]. Whereas only a slight increase in phosphopeptide ratio was seen in the *erbB2* G309E-expressing cells over wild type, the cells expressing erbB2 S310F exhibited a more substantial increase in peptide phosphorylation [18]. Ba/F3 cells transformed with the *erbB2* extracellular domain mutants had effective results using the irreversible erbB2 inhibitor neratinib and afatinib [18], however the sensitivity was lower than ins YVMA cells. The reversible inhibitor afatinib had sensitive effect for the lung cancer cell line NCI-H1781, haboring an erbB2 kinase domain mutation [28]. A combination of erbB2 inhibition and Mek inhibition was necessary for abrogation of 5637 bladder cancer cell line, haboring an erbB2 S310F mutation [18,29].

Mutations in *erbB2* gene were found in approximately 2% of primary non-small cell lung cancers, dominantly in never smoker like *EGFR* mutations [4-9]. It has shown that the common *erbB2* mutation, A775insYVMA, lead to oncogenic transformation in a cellular assay [23]. Because one co-existed case with *erbB2* and *Kras* mutation was reported [30], we have also examined the *Kras* mutant cases. However, in our cohort, *Kras* and *erbB2* mutations were exclusive. The mutant cells [28] and patients [31] exhibited exquisite sensitivity to the irreversible dual-specificity EGFR/ERBB2 kinase inhibitor BIBW2992, afatinib. Trastuzumab-besed therapies effective cases were previously reported [30]. However in our cohort, only one case had Hercep Test 1+. Usually in breast cancer, 2+ or 3+ cases were administered with trastuzumab, and it may not useful for our patients.

In our analysis, extracellular domain mutation of *erbB2* was only found from squamous cell carcinoma but not from adenocarcinoma. In contrast, *erbB2* mutations at kinase domain were found only in 6/279 adenocarcinomas dominantly in never smokers. Our data showed that mutations of *erbB2* gene as a mechanism of tumorigenesis is unlikely to be associated with many of Japanese NSCLC. Despite the promise of irreversible erbB inhibitors, our findings indicated that a small percentage of NSCLC actually harbor *erbB2* mutations and, in turn that a few patients with these tumors likely benefit from these anticancer agents. However, completely exclusive *EGFR*, *erbB2, Kras* and *Braf* mutation status would help us to choose order-made molecular target therapy for NSCLC. Further studies are needed to confirm the mechanisms of *erbB2* mutations for the sensitivity or resistance of targeted therapy for the lung cancer. Now that in 2013, afatinib has been approved for lung cancer treatment in USA [32].

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