HER2 Gene Mutations in Non-Small Cell Lung Carcinomas: Concurrence with *HER2* Gene Amplification and HER2 Protein Expression and Phosphorylation

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ABSTRACT

Background: Dysregulation of human epidermal growth factor receptor 2 (HER2) signaling pathways results in tumor progression in several types of carcinomas. The aim of the current study was to identify clinicopathological characteristics of *HER2*-mutated non-small cell lung carcinomas (NSCLCs) in conjunction with HER2 protein expression, gene amplification, and phosphorylation.

Materials and Methods: We investigated 1275 patients including 1055 adenocarcinomas (ADCs), 146 squamous cell carcinomas, 2 large cell carcinomas, 8 sarcomatoid carcinomas, and 64 adenosquamous carcinomas. High-resolution melting analysis of *HER2* mutations, chromogenic *in situ* hybridization for *HER2* amplification, and immunostaining of wild-type and phosphorylated HER2 were performed.

Results: *HER2* mutations were detected in 46 (3.6%) of the NSCLCs, with mutations only present in the ADC. When analyzing ADC cases alone, the incidence of *HER2* mutation was increased to 4.3%. All *HER2*-mutated tumors were negative for other driver gene alterations. *HER2* mutation status correlated with younger patient age, never-smoker status, and patients with smaller tumor size. *HER2* amplifications were also identified in approximately half of the tumors with *HER2* mutations. The overall survival rate was not significantly different between patients without and with *HER2* mutations. Amongst the 46 patients harboring *HER2* mutations, univariate and multivariate analysis revealed that *HER2* amplification was an unfavorable prognostic factor, while HER2 phosphorylation was a favorable prognostic factor.

Conclusion: In conclusion, *HER2* mutations were observed in 3.6% of NSCLCs, particularly in younger patients, those with no history of smoking, and those with small tumors. Although all *HER2*-mutated cases were ADC, distinct histological features were not detected. Amongst the patients with HER2 mutations, *HER2* amplification and phosphorylation were independent prognostic factors.

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2/ERBB2) is a receptor tyrosine kinase that is a member of the epidermal growth factor receptor (EGFR) subfamily. Unlike EGFR, HER3, and HER4, no ligand for HER2 has been found. Instead, HER2 is activated ligand-independently by homo- and hetero-dimerization with other members of the ERBB family, resulting in phosphorylation of intracellular tyrosine residues and activation of diverse signaling pathways. Dysregulation of HER2 by overexpression and/or gene amplification plays an important role in the development and progression of many cancers, notably breast and gastric cancer.

In lung tumors, HER2 protein overexpression and gene amplifications are present in 6–36% and 10–20% of NSCLCs, respectively. [1-6] *HER2*-activating mutations in NSCLCs were first described in 2004. [2] These mutations occur in the first 4 exons of the tyrosine kinase domain (exons 18–21), including the most frequently observed alteration, a 12-bp duplication/insertion of the amino acid sequence YVMA in exon 20 at codon 776 (HER^{YVMA}).[3, 7-17] Both *in vitro* and *in vivo* studies confirm the oncogenic potential of these mutations.[18-20] *HER2* mutations are present in 1–4.8% of lung NSCLC,[2, 7, 10-14, 16, 21-25], particularly in Asians, never-smokers,[7-12, 16] women,[7, 10] and adenocarcinomas (ADCs).[2, 7, 10-14, 16, 21-25]

The frequency and genotype-specific phenotypic consequences of *HER2* mutations in NSCLC have not been fully investigated in conjunction with HER2 protein expression or gene amplification. We also evaluated which of the abovementioned HER2 receptor dysregulations contributed to the activation of HER2 signaling pathways, as assessed by immunohistochemical detection of HER2 phosphorylated at tyrosine (pHER2-tyr) 1221/1222, one of the tyrosine residues associated with ERBB2 downstream interactions.

MATERIALS AND METHODS

Patient selection

This study was approved by the institutional review board of the National Cancer Center Hospital (Tokyo, Japan). Specimens were obtained from 1275 patients who underwent surgical resection for primary lung cancer at the National Cancer Center Hospital between 1993 and 2009. Clinicopathological information was collected for each patient, including age, sex, smoking history, outcome, maximum tumor size (in cm), and pathologic stage (in p-stage).

Histological diagnoses were based on the most recent World Health Organization classification[26] and the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) for ADC. [27]

Analysis of *HER2, BRAF, EGFR,* and *KRAS* mutations and *ALK* rearrangement status

Molecular-based mutation status was analyzed in fresh-frozen and formalin or methanol-fixed paraffin-embedded tissue samples of surgically resected lung cancer specimens. Fresh-frozen samples were provided by the National Cancer Center Biobank (Tokyo, Japan). Methods for mutation detection by high-resolution melting analysis (HRMA) and verification by Sanger sequencing are summarized in the Supplementary Note. HRMA was also used to detect common *BRAF* (V600E), *EGFR* (DEL and L858R), and *KRAS* mutations. This HRMA analysis is routinely performed at our institution. Anaplastic lymphoma kinase (*ALK*) gene fusions were analyzed by immunohistochemistry. In cases with ALK expression, further confirmation of *ALK* rearrangement was accomplished by reverse transcriptase (RT)-PCR and/or fluorescence *in situ* hybridization assays.

Immunohistochemistry

For analysis of HER2 protein expression, we used the BenchMark® XT automated slide processing system (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocol (I-VIEW pathway HER2/neu kit; Ventana Medical Systems).

The 249 cases with HER2 protein expression, gene amplification, or gene mutations were further analyzed for HER2 receptor activation using pHER2-tyr1221/1222 immunostaining. Heat-induced epitope retrieval with Target Retrieval Solution 9 (Dako Corporation, Carpinteria, CA, USA) was performed. The slides were subsequently incubated with primary antibody against pHER2-tyr1221/1222 (6B12; 1:200 dilution; Cell Signaling Technology, Danvers, MA, USA). Immunoreactions were detected using EnVision-FLEX and LINKER (Dako). Evaluation of immunostaining was performed by 2 independent observers (MS and KT). An immunopositive case was defined as that in which weak to strong complete, basolateral, or lateral membrane staining was detected in 10% or more of the tumor cells. Samples with cytoplasmic-only staining were excluded, irrespective of staining intensity.

HER2 gene copy number evaluation by bright-field *in situ* hybridization

The BenchMark® XT automated slide processing system (Ventana Medical Systems) was used for the bright-field *in situ* hybridization assay of the *HER2* gene (INFORM Dual IHC HER2 kit; Ventana Medical Systems). *HER2* amplification was defined as an average *HER2* gene copy number/centromere ratio of ≥2.0.

Statistical analyses

Statistical analysis was performed using the SPSS Statistics 21 program (IBM Corporation, Somers, NY, USA). Student's *t*-test was used to analyze continuous variables, and chi-square tests were used to analyze categorical variables. Additional statistical analysis of HER2 expression, amplification, and mutation amongst the different histological subtypes of ADCs was performed using the Fisher exact test. After applying the Bonferroni correction, the level of significance was set at <0.00833.

Cumulative survival rates were calculated by the Kaplan–Meier method. Statistical differences

in survival status were calculated using the log-rank test. The Cox proportional hazards model was used in the multivariate analysis and expressed by the hazard ratio (HR) with a 95% confidence interval (CI).

RESULTS

Patient characteristics

A total of 1275 patients with surgically resected NSCLC were included in the present analysis. All patients were Asian. The NSCLC cases comprised 1055 ADCs, 146 squamous cell carcinomas, 2 large cell carcinomas, 8 sarcomatoid carcinomas, and 64 adenosquamous carcinomas. The median follow-up time was 75 months after surgical resection. Amongst the 1055 ADCs, the most common histological subtype was papillary predominant (35%), followed by lepidic predominant (17.6%), solid predominant (17.4%), acinar predominant (11.4%), minimally invasive ADC/ADC *in situ* (MIA/AIS; 8.0%), micropapillary predominant (6.4%), invasive mucinous ADC (IMA; 4.1%), and colloid carcinoma (0.09%).

EGFR, *KRAS*, and *BRAF* mutations were analyzed in 99.8%, 98.3%, and 97.5% of the total cases, respectively. *EGFR*, *KRAS*, and *BRAF* mutations were present in 395 (31.1%), 105 (8.4%), and 8 (0.6%) cases, respectively. *ALK* rearrangement was analyzed in 1261 (98.9%) cases, and translocation was present in 40 (3.2%) cases.

HER2 mutations

The clinical features of patients carrying *HER2* mutations were analyzed (Table 1). *HER2* mutations were detected in 46 out of 1275 NSCLCs (3.6%). All 46 tumors were of the ADC histology ($P = 0.041$). Analysis of ADC cases alone revealed 46 *HER2* mutations out of 1055 ADC cases (4.3%). Four mutation genotypes were identified: 776_779insYVMA was the most prevalent genotype (40 cases; 87%), followed by 4 cases (8.8%) of G776VCins, and only 1 case each (2.2%)

of 775_778insAYVM and 781_783insGSP.

Clinicopathological features associated with *HER2* mutations in NSCLC

HER2 mutations were found most frequently in patients of younger age (63.1 vs. 59.9 years old, $P = 0.048$), patients who had never smoked (41.4% vs 60.9%, $P = 0.010$), and in patients with smaller tumor size (3.1 vs. 2.4 cm, $P = 0.013$). *HER2* mutation status was not associated with sex, lymph node status, or tumor stage. *HER2* mutations were found to be mutually exclusive of other driver genetic alterations (*EGFR*, *KRAS*, and *BRAF* mutational status and *ALK* rearrangements). When the analysis was further restricted to patients with never-smoking history, ADC histology, and no other driver gene alterations (*EGFR*, *KRAS*, *BRAF*, or *ALK*), *HER2* mutations were present in 27 out of 196 patients (13.8%).

Detailed histological analysis for ADC subtypes were performed all but 1 colloid carcinoma and revealed that *HER2* mutations were most prevalent in papillary predominant (34.8%; Figure 1A) followed by AIS/MIA (21.7%), lepidic predominant (13.0%), solid predominant (13.0%), acinar predominant (8.7%), micropapillary predominant (6.5%), and IMA (2.2%). Although the Fisher exact test indicated a statistically significant correlation ($P = 0.038$) between histological subtype and *HER2* mutations, after the Bonferroni correction, there was no statistical correlation.

HER2 protein expression by immunohistochemistry

The clinical features of patients with HER2 protein expression were analyzed (Figure 1B and Table 1). HER2 expression was observed in 31 (2.4%) out of 1266 NSCLCs (Figure 1 A,B). The HER2-positive cases presented with a high proportion of lymph node metastasis (30.5% vs. 53.3%, $P = 0.015$) and higher tumor stage (19.0% vs. 36.7 %, $P = 0.031$). Although there were no statistically significant correlations between HER2 expression and histological types ($P = 0.863$), the majority of cases with HER2 expression were ADCs (90.3%). HER2 expression was not associated with sex, occurrence age, smoking status, tumor size, or the presence of other driver genetic alterations (*EGFR*,

KRAS, and *BRAF* mutational status and *ALK* rearrangement).

Cases with *HER2* amplification

The clinical features of patients carrying *HER2* gene amplifications were analyzed (Figure 1C and Table 1). Out of the NSCLC cases, 222 (19.0%) of 1170 contained *HER2* amplifications (Figure 2). *HER2*-amplification occurred most frequently in patients who were female (40.0% vs. 48.2%, $P = 0.028$), of younger age (63.4 years vs. 60.8 years, $P < 0.001$), and who had ADC histology (78.8% vs. 97.3%, $P < 0.001$). Amongst the histological subtypes of ADC, significant differences in rates of *HER2* amplification were detected between papillary and IMA ($P = 0.006$) and acinar and IMA ($P = 0.001$).

HER2 amplification was not associated with smoking status, tumor size, lymph node status, tumor stage, or other driver genetic alterations (*EGFR*, *KRAS*, and *BRAF* mutational status and *ALK* rearrangement).

Association between HER2 overexpression, amplification, and mutation

Statistically significant associations between HER2 overexpression and amplification ($P < 0.001$) and between HER2 overexpression and mutation ($P = 0.001$) were observed. However, the sensitivity and specificity for detecting *HER2* amplification were 10.3% and 74.2%, respectively, and 15% and 19.4%, respectively, for detecting *HER2* mutation.

HER2 phosphorylation status amongst HER2 overexpression, amplification, and mutation

Amongst the 249 analyzed cases, pHER2-tyr1221/1222 expression was observed in 83 (33.3%) cases (Figure 1D and Table 3). The pHER2-tyr1221/1222 expression was significantly associated with HER2 overexpression (19/30, 63.3%, $P < 0.001$), amplification (66/220, 30.0%, $P = 0.002$), and mutation (29/46, 63.0%, $P < 0.001$). Multivariate analysis revealed that HER2 overexpression and mutation were independently associated with pHER2-tyr1221/1222 expression (Table 3B).

Survival analysis of *HER2* dysregulations in NSCLC

Amongst the 1275 patients, the median follow-up time was 74.8 months after surgical resection. The number of patients who were still alive at the time of the most recent follow-up out of the original 31 patients with HER2 overexpression, 222 patients with *HER2* amplification, and 46 patients with *HER2* mutations were 13 (41.9%), 83 (37.4%), and 20 (43.5%), respectively. Postoperative survival curves, estimated using the Kaplan–Meier method, are shown in Figure 2. Overall survival was not significantly different between patients with or without HER2 overexpression (Figure 2A; P = .390), between patients with or without *HER2* amplification (Figure 2B; P = .627), and between patients with wild-type vs. mutated *HER2* (Figure 2C; P = .369).

Although HER2 phosphorylation status was not correlated with overall survival in the 249 analyzed cases (P = .103), cases with HER2 expression (P = .107), or cases with *HER2* amplification (P = .159), a statistically significant association between favorable outcome and HER2 phosphorylation status was observed in *HER2*-mutated cases (P = 0.003; Table. 4).

Amongst the 46 cases harboring *HER2* mutations, univariate analysis revealed that male sex, blood vessel invasion, lymph vessel invasion, advanced p-stage (III or IV), and *HER2* amplification (Figure 2D) were significantly associated with a higher risk of death (Table 4). Multivariate analysis revealed that advanced p-stage (III or IV) (HR 11.774, 95% CI 1.983–69.919; P = 0.007), *HER2* amplification (HR 11.213, 95% CI 2.177–57.760; P = 0.004), and HER2 phosphorylation were independent prognostic factors (HR 0.137, 95% CI 0.040–0.468; P = 0.001) (Table 4).

DISCUSSION

In the present study, we investigated the incidence of *HER2* mutations, amplifications, and protein expression and their associations with clinicopathological features and prognosis in surgically resected NSCLC. We found that the incidence of *HER2* gene mutations, amplification, and protein expression in NSCLC was 3.6 %, 17.4%, and 2.4%, respectively. *HER2* mutations were observed in

younger patients and patients with no smoking history. In addition, amongst the patients harboring *HER2* mutations, *HER2* amplification was observed in 56.8% of cases and was an independent unfavorable factor.

Consistent with our results, most lung cancer patients harboring *HER2* mutations are reported to have ADC histology. [2, 7-14, 16, 21-25, 28, 29] In agreement with previous studies, [7-12, 16] we also found a significant association of *HER2* mutations with never-smoking status and younger age. Female sex, as previously reported,[7, 10] was marginally correlated with *HER2* mutation-positive status. We and others reported that *HER2* mutations are present exclusive of other major driver gene mutations. [2, 3, 8-14, 30, 31] Thus, when our analysis was further restricted to patients with never-smoking history, ADC histology, and absence of other driver gene alterations (*EGFR*, *KRAS*, *BRAF*, or *ALK*), the frequency of *HER2* mutations increased from 3.6% to 13.8%.

In the present study, 4 genotypes of *HER2* gene mutation were found in NSCLC cases. In accordance with other studies,[7, 9-12, 14, 15, 21-25, 28, 29, 31] all *HER2* mutations were insertion mutations in a small stretch of exon 20. Amongst these, the most prevalent genotype was 776-779insYVMA. Compared with *EGFR* mutations, *HER2* insertions are less heterogeneous, with over 80% of cases containing the A775_G776insYVMA insertion/duplication. Only 5 missense mutations in the *HER2* gene were previously found in exons 19 through 20.[2, 3, 8, 13, 16, 17, 32]

We found that approximately half of the *HER2*-mutated tumors showed *HER2* gene amplification. The correlation between *HER2* mutation and amplification has been reported to be between 0%[12] and 87.5%.[9] The variability of these reports may be due to differences in criteria defining amplification levels or methods for detecting amplifications. Similar to the *EGFR* gene, where a portion of *EGFR*-mutated tumors also harbor *EGFR* gene amplifications,[12, 33] our current data indicate that mutated allele amplification also occurs for the *HER2* gene.

Our data indicate that HER2 overexpression, amplification, and mutation do not affect postoperative overall survival in NSCLC patients. However, on analysis of only *HER2*-mutated cases,

HER2-amplified cases demonstrated unfavorable outcomes. Most of the reports analyzing patients with *HER2* mutations did not evaluate the patients' survival because of the paucity of the mutation-positive cases. Two reports did not indicate statistically significant survival differences between patients with and without *HER2* mutation.[8, 10] Although there are conflicting reports on the correlation between *HER2* amplification and patients' survival, a recent meta-analysis of *HER2* amplification determined by FISH concluded that *HER2* amplification is not a prognostic factor.[34] Thus, *HER2* gene amplification should be analyzed in clinical trials for HER2-targeted therapy for patients with *HER2* mutations.

HER2 expression, amplification, and mutation were associated with HER2 phosphorylation at Tyr1221/1222. Because there were some overlaps amongst HER2 expression, amplification, and mutation, a multivariate analysis revealed that only HER2 expression and mutation were associated with HER2 phosphorylation at Tyr1221/1222. Unexpectedly, in contrast to breast carcinoma,[35] a statistically significant association between favorable outcome and HER2 phosphorylation status was observed in *HER2*-mutated cases. Assessment of the expression of phosphorylated proteins by immunohistochemistry is subject to limitations, as multiple HER2 tyrosine residues exist and the anti-phospho-HER2 antibodies are site-specific; thus, not all activated forms of the receptor can be detected.

Despite HER2 overexpression and mutation were prevalent in papillary-predominant ADCs, there were no statistical differences, whereas *HER2* gene amplification was less prevalent in invasive mucinous ADCs. There are few reports of the histological subtypes of *HER2*-mutated ADCs. Some reports noted lepidic features (formerly the bronchioloalveolar feature)[3] or invasive mucinous ADC,[15] while another report noted the significant heterogeneity and predominance of high-grade morphologic features.[8] *EGFR* mutations are most prevalent in ADCs formerly classified as nonmucinous bronchioloalveolar carcinomas, likely corresponding to AIS/MIA, and lepidic-predominant ADC, with no mutations found in invasive mucinous ADC.[36] We and others

reported that *HER2*-mutated ADCs include heterogeneous histological subtypes.[13] In addition, we and others detected *HER2* mutations in invasive mucinous ADC.[37] Detailed histological typing of ADC does not contribute to the enrichment of *HER2*-mutation bearing patients.

In conclusion, *HER2* mutations were observed in 3.6% of NSCLC cases. *HER2* mutations were observed in younger patients and patients with no smoking history. In addition, *HER2* mutations did not coexist with other driver gene alterations. Although all *HER2*-mutated cases were ADC, distinct histological features were not detected. HER2 phosphorylation at Tyr1221/1222 was associated with *HER2* mutation and favorable outcome in patients harboring *HER2* mutation.

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FIGURE LEGENDS

Figure 1. Histological features of the *HER2*-mutated adenocarcinomas

(A) Papillary growth was the most prevalent histological subtype in *HER2*-mutated adenocarcinomas (original magnification, ×100).

(B) Moderate to strong intensity of basolateral or lateral membrane staining for HER2 (original magnification, ×400).

(C) Amplified *HER2* gene signals appear as clusters of black dots, and 2-4 CEN 17 signals as red dots in the nuclei (original magnification, ×400).

(D) Complete, basolateral, or lateral membrane staining for phosphorylated-HER2 (original magnification, ×200).

Figure 2. Overall survival curves in 1275 patients with non-small cell lung carcinoma

(A) Overall survival curves for patients with HER2 expression-negative (blue line) and HER2 expression-positive (green line) tumors ($P = 0.390$).

(B) Overall survival curves for patients with *HER2* amplification-negative (blue line) and *HER2* amplification-positive (green line) tumors ($P = 0.627$).

(C) Overall survival curves for patients with *HER2* mutation-negative (blue line) and *HER2* mutation-positive (green line) tumors ($P = 0.369$).

(D) Overall survival curves for patients with *HER2* amplification-negative (blue line) and *HER2* amplification -positive (green line) tumors containing *HER2* mutations ($P = 0.004$).

Supplementary Figure 1. Electropherograms demonstrating mutational patterns.

(A) 12-bp insertion (A776_G779insYVMA) in primary tumor.

(B) 9-bp insertion (P780_Y781insGSP) in primary tumor.

(C) 3-bp insertion (G776V,Cins) in primary tumor.

(D) 12-bp insertion (775_G778insAYUM) in primary tumor.

Supplementary Figure 2. Overall survival curves based on HER2 phosphorylation status

(A) Overall survival curves for patients with HER2 phosphorylation-negative (blue line) and HER2 phosphorylation-positive (green line) tumors in the 249 analyzed cases ($P = 0.103$).

(B) Overall survival curves for patients with HER2 phosphorylation-negative (blue line) and HER2 phosphorylation-positive (green line) tumors containing HER2 overexpression ($P = 0.107$).

(C) Overall survival curves for patients with HER2 phosphorylation-negative (blue line) and HER2 phosphorylation-positive (green line) tumors containing *HER2* amplifications ($P = 0.159$).

(D) Overall survival curves for patients with HER2 phosphorylation-negative (blue line) and HER2 phosphorylation-positive (green line) tumors containing *HER2* mutations ($P = 0.003$).

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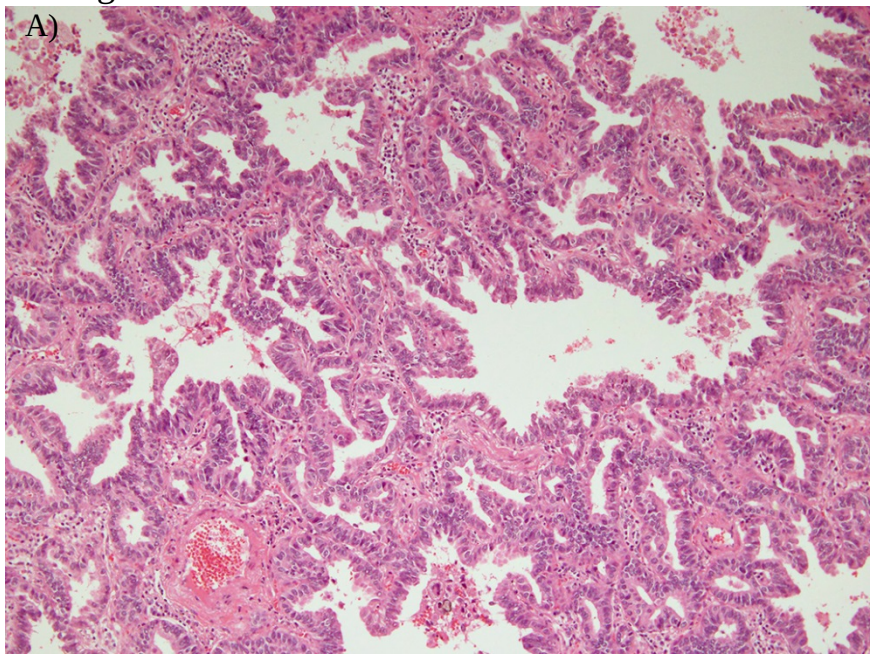
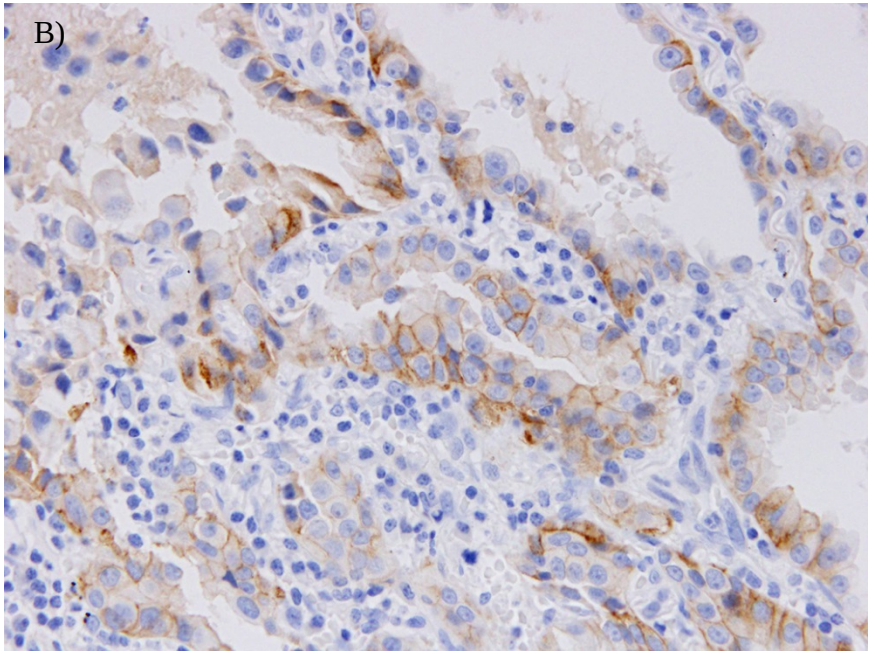
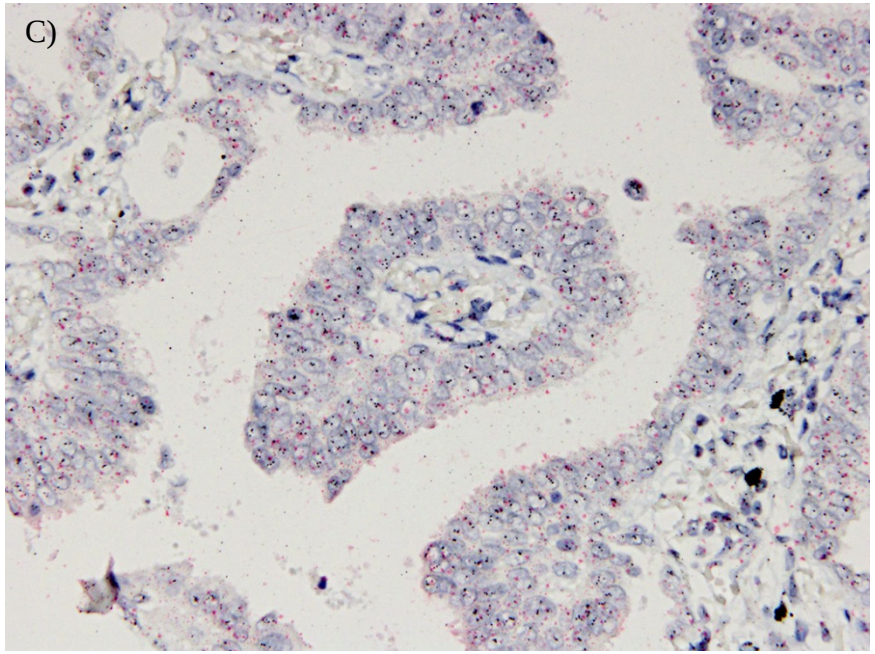
Figure 1







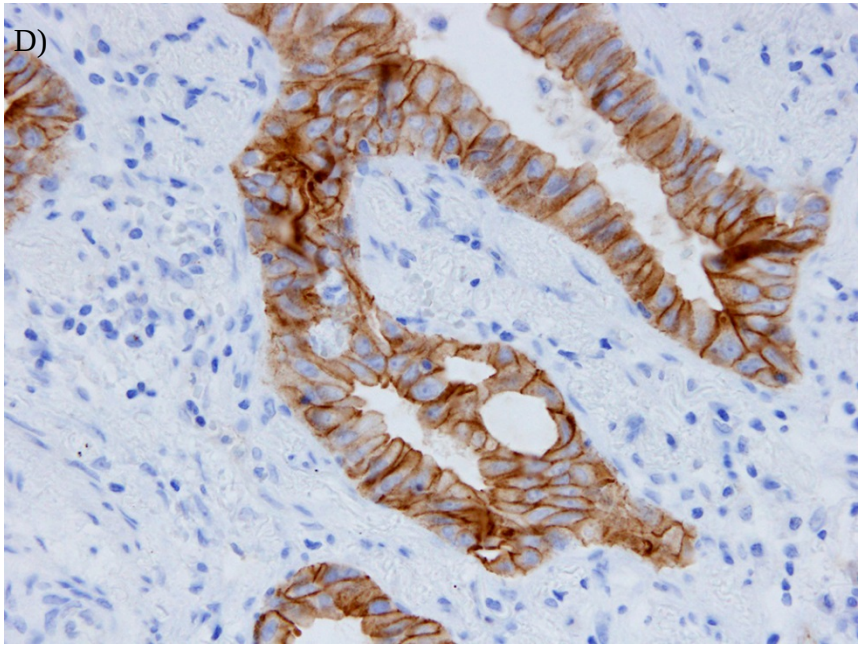
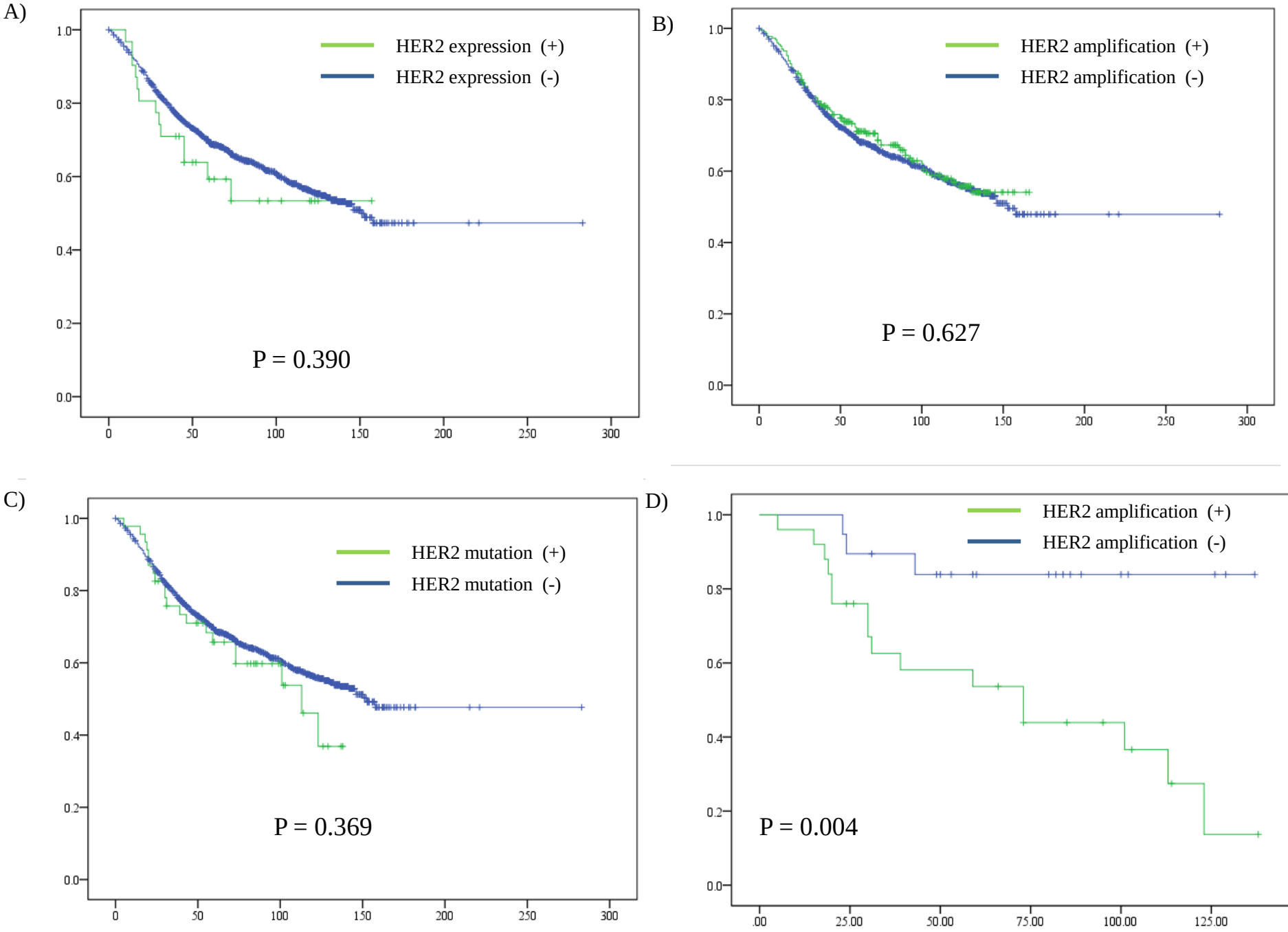
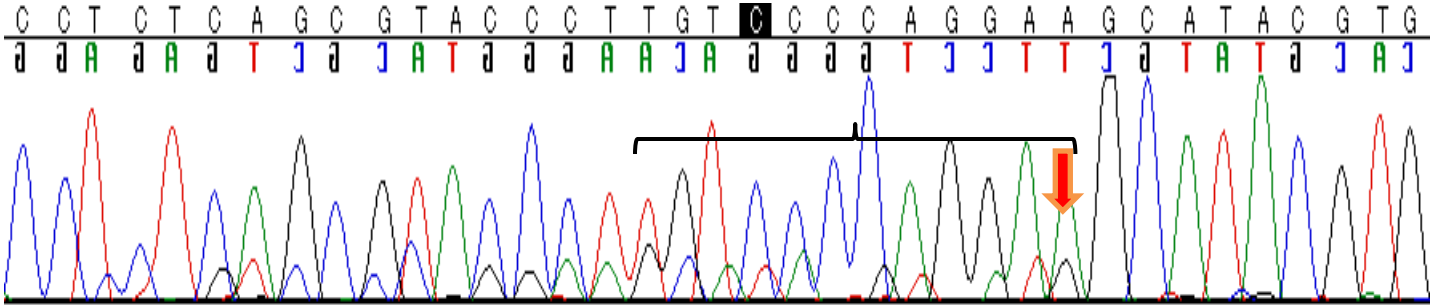


Figure 2

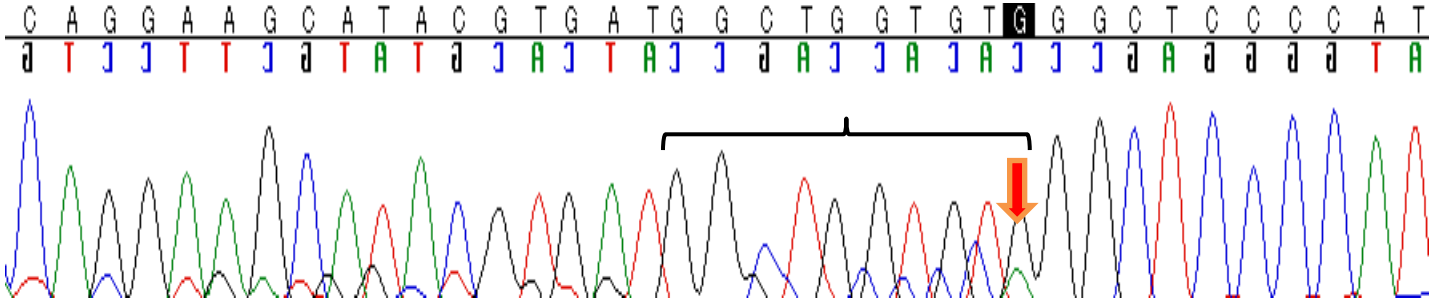

A)
HER2 expression (+)
HER2 expression (-)
P = 0.390
B)
HER2 amplification (+)
HER2 amplification (-)
P = 0.627
C)
HER2 mutation (+)
HER2 mutation (-)
P = 0.369
D)
HER2 amplification (+)
HER2 amplification (-)
P = 0.004

Sup. Figure 1-1

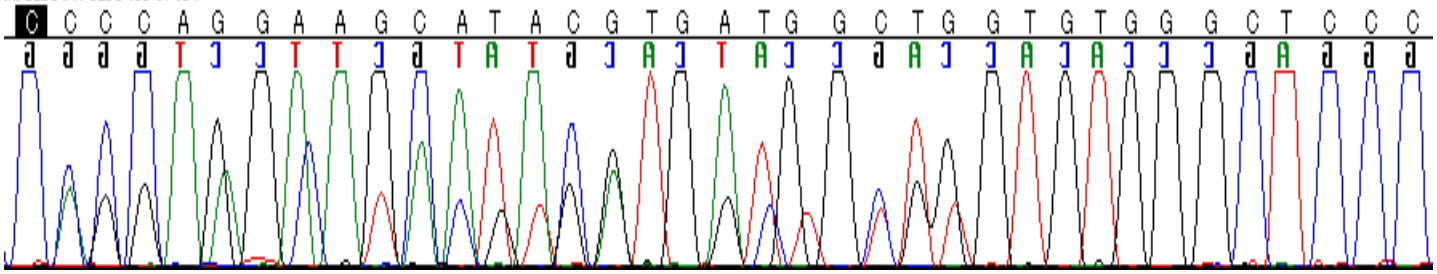
(A) A776_G779insYVMA



(B) P780_Y781insGSP

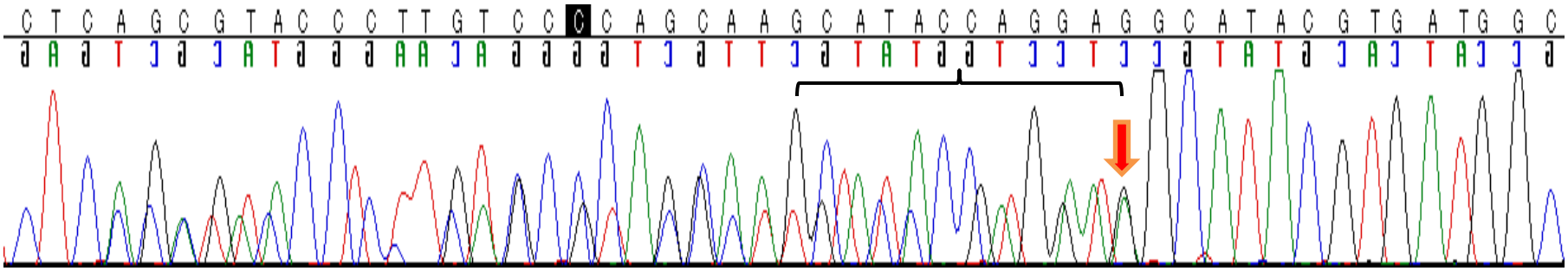


(C) G776V,Cins

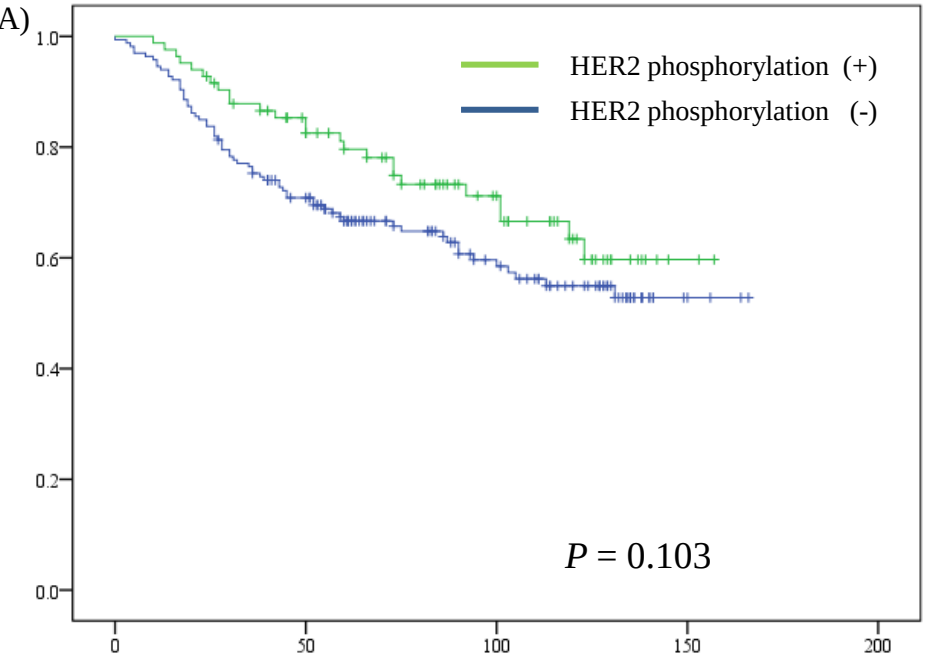


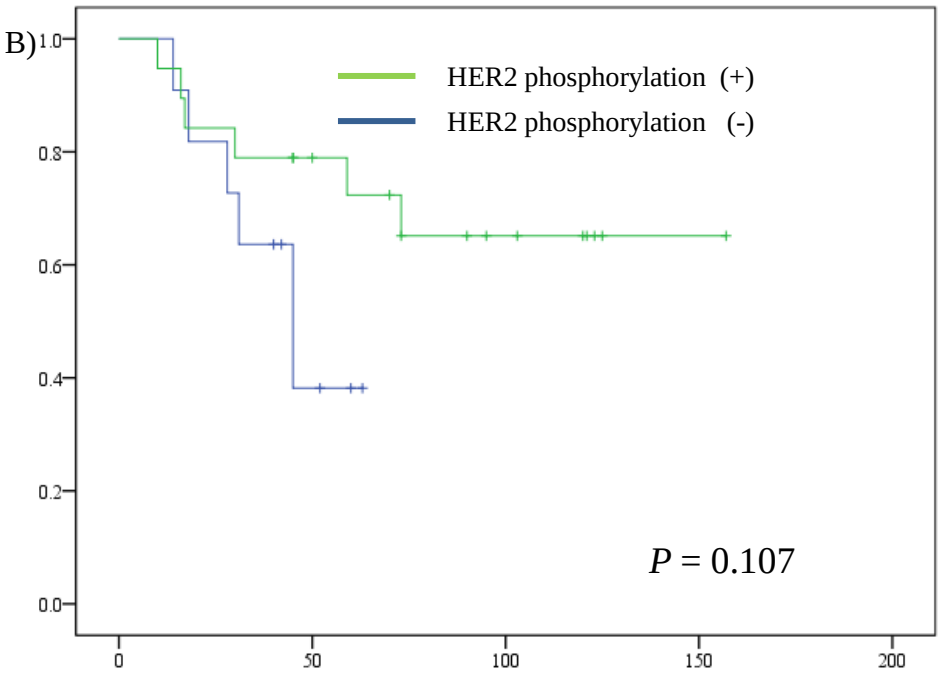
Sup. Figure 1-2

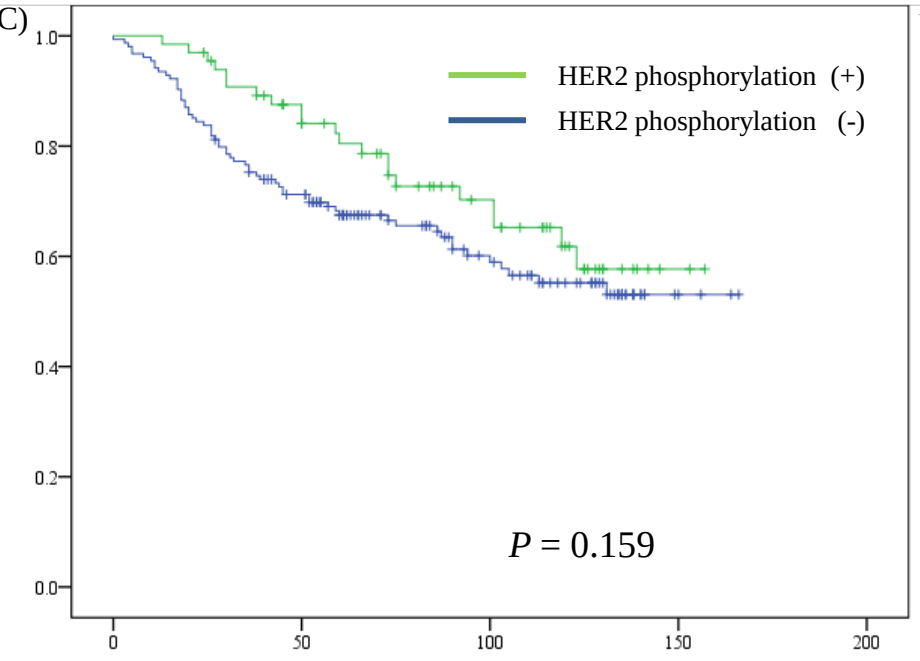
(D) 775_G778insAYUM



Supplementary Figure 2







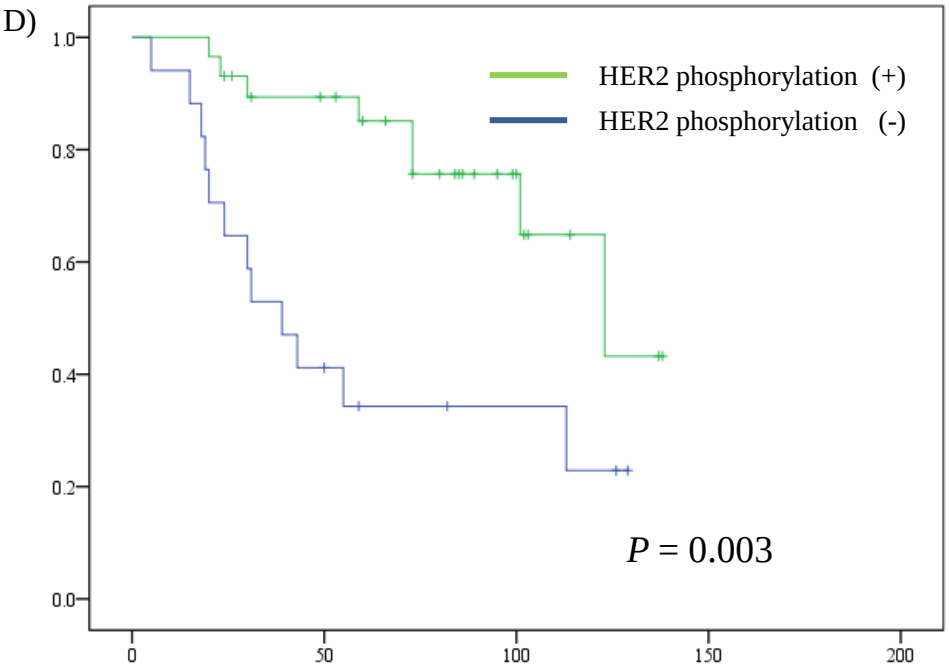


Table 1. Patient characteristics according to HER2 expression, amplification, and mutation status

Variables	HER2 expression			HER2 amplification		
	Negative (%)	Positive (%)	*P*	Negative (%)	Positive (%)	*P*
Gender			0.357			0.028
Male	716 (58.0)	21 (67.6)		569 (60.0)	115 (51.8)	
Female	519 (42.0)	10 (32.3)		379 (40.0)	107 (48.2)	
Age (Year)			0.867			0.001
Median	63.0	62.7		63.4	60.8	
Range	23−89	39−81		23−89	31−83	
Smoking			0.144			0.083
Never	526 (42.6)	9 (29.0)		387 (40.8)	105 (47.3)	
Former/Present	709 (57.4)	22 (71.0)		561 (59.2)	117 (52.7)	
Tumor size (cm)			0.221			0.079
Median	3.1	3.7		3.2	2.9	
Range	0.4−17.5	2.0−11.0		0.4−17.5	0.5−13.0	
N status			0.015			0.329
Negative	826 (69.5)	14 (46.7)		634 (69.6)	144 (66.1)	
Positive	363 (30.5)	16 (53.3)		277 (30.4)	74 (33.9)	
Stage			0.031			0.130
I+II	992 (81.0)	19 (63.3)		766 (81.5)	170 (76.9)	
III+IV	232 (19.0)	11 (36.7)		174 (18.5)	51 (23.1)	
Histology			0.863			<0.001
Adenocarcinoma	1023 (82.8)	28 (90.3)		747 (78.8)	216 (97.3)	
Squamous cell carcinoma	139 (11.3)	2 (6.5)		129 (13.6)	4 (1.8)	
Large cell carcinoma	2 (0.2)	0		2 (0.2)	0	
Sarcomatoid carcinoma	8 (0.6)	0		6 (0.6)	2 (0.9)	
Adenosquamous carcinoma	63 (5.1)	1 (3.2)		64 (6.8)	0	
HER2 expression						<0.001
Negative				934 (99.3)	197 (89.5)	
Positive				7 (0.7)	23 (10.5)	
HER2 amplification			<0.001			NA
Negative	934 (82.6)	7 (23.3)				
Positive	197 (17.4)	23 (76.7)				
HER2 mutation			0.001			<0.001
Wild type	1195 (96.8)	25 (80.6)		929 (98.0)	197 (88.7)	
Mutation	40 (3.2)	6 (19.4)		19 (2.0)	25 (11.3)	
EGFR			0.695			1.00
Wild type	848 (68.8)	23 (74.2)		650 (68.6)	152 (68.8)	
Mutation	384 (31.2)	8 (25.8)		297 (31.4)	69 (31.2)	
KRAS			0.734			1.00
Wild type	1115 (91.7)	27 (90.0)		860 (91.8)	198 (92.1)	
Mutation	101 (8.3)	3 (10.0)		77 (8.2)	17 (7.9)	
BRAF (V600E)			1.00			0.174
Wild type	1195 (99.3)	31 (100)		923 (99.5)	210 (98.6)	
Mutation	8 (0.7)	0		5 (0.5)	3 (1.4)	
ALK			0.621			0.124
Wild type	1181 (96.7)	31 (100)		911 (97.4)	211 (95.5)	
Rearranged	40 (3.3)	0		24 (2.6)	10 (4.5)	

Abbreviations: EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma. kinase; TKI, tyrosine kinase inhibitor

Table. 1 continued

Variables	*HER2 mutation*		
	Negative (%)	Positive (%)	*P*
Gender			0.093
Male	722 (58.7)	21 (45.7)	
Female	507 (41.3)	25 (54.3)	
Age (Year)			0.048
Median	63.1	59.9	
Range	23−89	30−78	
Smoking			0.010
Never	509 (41.4)	28 (60.9)	
Former/Present	720 (58.6)	18 (39.1)	
Tumor size (cm)			0.013
Median	3.1	2.4	
Range	0.4−17.5	0.8−6.0	
N status			0.313
Negative	821 (69.2)	26 (61.9)	
Positive	365 (30.8)	16 (38.1)	
Stage			0.324
I+II	987 (80.9)	32 (74.4)	
III+IV	233 (19.1)	11 (25.6)	
Histology			0.041
Adenocarcinoma	1009 (82.1)	46 (100)	
Squamous cell carcinoma	146 (11.9)	0	
Large cell carcinoma	2 (0.2)	0	
Sarcomatoid carcinoma	8 (0.7)	0	
Adenosquamous carcinoma	64 (5.2)	0	
HER2 expression			0.001
Negative	1195 (98.0)	40 (87.0)	
Positive	25 (2.0)	6 (13.0)	
HER2 amplification			<0.001
Negative	929 (82.5)	19 (43.2)	
Positive	197 (17.5)	25 (56.8)	
HER2 mutation			NA
Wild type			
Mutation			
EGFR			<0.001
Wild type	831 (67.8)	46 (100)	
Mutation	395 (32.2)	0	
KRAS			0.028
Wild type	1105 (91.3)	45 (100)	
Mutation	105 (8.7)	0	
BRAF (V600E)			1.000
Wild type	1190 (99.3)	45 (100)	
Mutation	8 (0.7)	0	
ALK			0.397
Wild	1175 (96.7)	46 (100)	
Rearranged	40 (3.3)	0	

Table. 2 Distribution of HER2 expression, amplifications, and mutations in the predominant adenocarcinoma histology

	Total	HER2 expression (%) $P = 0.496$	HER2 amplification (%) $P = 0001$	HER2 mutation (%) $P = 0.038$
AIS or MIA	84 (8.0)	3 (10.7)	11 (5.1)	10 (21.7)
Lepidic predominant	186 (17.6)	1 (3.6)	27 (12.5)	6 (13.0)
Papillary predominant	370 (35.1)	12 (42.9)	84 (38.9)	16 (34.8)
Acinar predominant	121 (11.5)	2 (7.1)	35 (16.2)	4 (8.7)
Micropapillary predominant	67 (6.4)	2 (7.1)	14 (6.5)	3 (6.5)
Solid predominant	183 (17.4)	6 (21.4)	42 (19.4)	6 (13.0)
Invasive mucinous	43 (4.1)	2 (7.1)	3 (1.4)	1 (2.2)
Total	1054	28	216	46

HER2: human epidermal growth factor receptor 2, AIS: adenocarcinoma in situ, MIA: minimally invasive adenocarcinoma

Table. 3 A Association of pHER2 expression with HER2 expression, amplification, and mutation

		HER2 expression ($P < 0.001$)		*HER2* amplification($P = 0.002$)		*HER2* mutation ($P < 0.001$)	
		Negative	Positive	Negative	Positive	Negative	Positive
pHER2 expression	Negative	154 (70.6)	11(36.7)	10 (38.5)	154 (70.0)	149 (73.4)	17 (37.0)
	Positive	64 (29.4)	19 (63.3)	16 (61.5)	66 (30.0)	54 (26.6)	29 (63.0)

Table. 3B Multivariate analysis of pHER2 expression amongst HER2 expression, amplification, and mutation

	Reference	OR	95% CI	*P* value
HER2 expression	Negative/Positive	0.346	0.172−0.530	< 0.001
HER2 amplification	Negative/Positive	-0.44	-0.254−0.165	0.677
HER2 mutation	Negative/Positive	0.342	0.176−0.508	< 0.001

HER2: human epidermal growth factor 2, OR: odds ratio, CI: confidence interval,

Table. 4: Univariate and multivariate analysis of overall survival of patients with HER2 mutations

	Reference	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Gender	Female	3.363	1.329−8.509	0.006	0.662	0.108−4.052	0.655
Blood vessel invasion	Negative	3.700	1.462−9.362	0.003	0.812	0.242−2.721	0.736
Lymph vessel invasion	Negative	5.827	2.102−16.149	<0.001	3.620	0.527−24.857	0.191
Lymph node metastasis	Negative	5.425	2.138−13.776	<0.001	0.773	0.128−4.678	0.779
Pathological stage	I&II/III&IV	9.503	3.507−25.747	<0.001	11.774	1.983−69.919	0.007
HER2 gene amplification	Negative	5.068	1.472−17.446	0.004	11.213	2.177−57.760	0.004
HER2 phosphorylation	Negative	0.275	0.111−0.680	0.003	0.137	0.040−0.468	0.001

HER2: human epidermal growth factor 2, HR: hazard ratio, CI: confidence interval,

Supplementary Note

HER2 Gene Mutations and Amplification, and Protein Expression in Non-Small Cell Lung Carcinomas

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Supplementary Method

Analysis of HER2, EGFR, KRAS, and BRAF mutation status and ALK rearrangement

Fresh-frozen and formalin-fixed, paraffin-embedded samples were provided by the National Cancer Center Biobank (Tokyo, Japan). DNA was extracted from the specimens with a QIAamp DNA Mini kit (QIAGEN, Venlo, Netherlands). The HRMA of primer set A was 5 -ctctcagcgtacccttgtccc-3 (forward) 5 -cagaaggcgggagacatatgg-3 (reverse). Primer set A was designed to amplify a region containing codon 776 (HERYVMA). PCR was performed using LightScanner Master Mix (Idaho Technology, Salt Lake City, UT, USA) with the LightCycler System (Roche Diagnostics). The samples were denatured at 95°C for 10 min. For primer set A, samples were subjected to 45 cycles of denaturing for 10 s at 95°C, annealing for 10 s at 65°C, and extension for 5 s at 72°C. These samples were heated at a transition rate of 0.3°C/s. The acquired data were analyzed using the provided software (Idaho Technology: Salt Lake City, Utah, USA) [1]. The graph normalized by the software demonstrated the degree of the reduction in fluorescence over a temperature range of 70–98°C. A difference plot was generated using serial dilutions of DNA from a mutated cell line compared to wild-type DNA to assess HRMA sensitivity. The melting profiles of each sample were compared with those of the reference samples. HRMA-positive cases where DNA was extracted from frozen tissue were subjected to independent PCR amplification and Sanger sequencing. The Sanger sequencing of primer set B was 5 -GCCATGGCTGTGGTTTGTGATGG-3 (forward) 5 -ATCCTAGCCCCTTGTGGACATAGG-3 (reverse) [2]. detect common EGFR (DEL and L858R), KRAS, and BRAF mutations. This HRMA analysis is routinely performed at our institution [3, 4].

Reference

1. Takano T, Ohe Y, Tsuta K et al. Epidermal growth factor receptor mutation detection using high-resolution melting analysis predicts outcomes in patients with advanced non small cell lung cancer treated with gefitinib. Clin Cancer Res 2007; 13: 5385-5390.
2. Fukui T, Ohe Y, Tsuta K et al. Prospective study of the accuracy of EGFR mutational analysis by high-resolution melting analysis in small samples obtained from patients with non-small cell lung cancer. Clin Cancer Res 2008; 14: 4751-4757.
3. Kinno T, Tsuta K, Shiraishi K, Mizukami T, Suzuki M, Yoshida A, Suzuki K, Asamura H, Furuta K, Kohno T, Kushima R. Clinicopathological features of nonsmall cell lung carcinomas with BRAF mutations. Ann Oncol. 2014; 25(1):138-42.
4. Shigematsu H, Takahashi T, Nomura M, Majmudar K, Suzuki M, Lee H, Wistuba II, Fong KM, Toyooka S, Shimizu N, Fujisawa T, Minna JD, Gazdar AF. Cancer Res. 2005; 65(5):1642-6.