

HER2 somatic mutations are associated with poor survival in HER2-negative breast cancers

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It is well documented that human epidermal growth factor receptor 2 (*HER2*) overexpression/amplification is associated with poor survival in breast cancer patients. However, it is largely unknown whether *HER2* somatic mutations are associated with survival in *HER2*-negative breast cancer patients. Here, we identified *HER2* somatic mutations in tumors from 1348 unselected breast cancer patients by sequencing the entire *HER2* coding region. All of these mutations were tested for in corresponding blood samples to determine whether they were somatic or germline mutations. We further investigated the associations between *HER2* somatic mutations and recurrence-free survival and distant recurrence-free survival in this cohort of patients. We found that 27 of 1348 (2.0%) of these patients carried a *HER2* somatic mutation. *In vitro* experiments indicated that some of the novel mutations and those with unknown functions increased *HER2* activity. *HER2* status was available for 1306 patients, and the *HER2* somatic mutation rates in *HER2*-positive ($n = 353$) and *HER2*-negative breast cancers ($n = 953$) were 1.4% and 2.3%, respectively. Among the *HER2*-negative patients, those with a *HER2* somatic mutation had a significantly worse recurrence-free survival (unadjusted hazard ratio = 2.67; 95% confidence interval, 1.25–5.72, $P = 0.002$) and distant recurrence-free survival (unadjusted hazard ratio = 2.50; 95% confidence interval, 1.10–5.68, $P = 0.004$) than those with wild-type *HER2*. Taken together, our findings suggested that *HER2* somatic mutations occur at a higher frequency in *HER2*-negative breast cancer, and *HER2*-negative breast cancer patients with these mutations have poor survival. Therefore, *HER2*-negative patients with a *HER2* somatic mutation are potentially good candidates for *HER2*-targeted therapy.

Human epidermal growth factor 2 is a major proliferative stimulator that activates downstream signaling through the phosphoinositide 3-kinase/AKT and MAPK pathways.^(1–5) Amplification/overexpression of *HER2* occurs in 20%–25% of breast cancers, and is associated with poor survival.^(6,7) The use of *HER2*-targeted drugs, which currently include trastuzumab, pertuzumab, and lapatinib, have dramatically improved the outcomes in *HER2*-positive breast cancers.^(8–13)

Human epidermal growth factor receptor 2 somatic mutations were initially identified in the tyrosine kinase domain of the *HER2* gene in breast cancer patients in 2006.⁽¹⁴⁾ Recently, such mutations have been identified in *HER2*-negative breast cancer patients by sequencing assays, including whole-cancer genome sequencing.^(14–21) Although the mutation rate of this gene is very low (<2%), *in vitro* and *in vivo* experiments have shown that some somatic mutations can activate the *HER2* signaling pathway in *HER2*-negative cells and that these cells are sensitive to some *HER2*-targeted drugs.^(22–25) These findings suggest that *HER2* somatic mutations represent an alternative mechanism for the activation of *HER2* in *HER2*-negative breast cancers, raising an interesting question of whether somatic mutations in *HER2*-negative breast cancer patients

influence the clinical outcome. Therefore, the purposes of this study were as follows: (i) to identify *HER2* somatic mutations in tumor tissues from a large cohort of 1348 patients; (ii) to investigate whether the somatic mutations with novel or unknown functions identified in this study affect *HER2* function by undertaking *in vitro* experiments; and (iii) to investigate whether *HER2* somatic mutations influence patient survival in the entire study population and, specifically, in *HER2*-negative breast cancer patients.

Materials and Methods

Patients. From November 2003 to July 2012, fresh-frozen tumor tissues were obtained by core-needle biopsy prior to therapy or were procured during surgery from 1496 primary breast cancer patients (stages I–III) at the Breast Center, Peking University Cancer Hospital (Beijing, China). Human epidermal growth factor receptor 2 somatic mutation status was successfully determined in 1348 of these patients. Tumor stage was classified according to the TNM classification of the Union for International Cancer Control. Tumor size was defined as the maximum tumor diameter as measured by

ultrasound at the time of diagnosis. Tumors were graded histologically according to the modified Bloom–Richardson grading system. This study was carried out in accordance with the ethics principles of the Declaration of Helsinki and approved by the Research and Ethics Committee of Peking University Cancer Hospital. All patients provided written informed consent.

Analysis of HER2 mutations in tumor tissue. Tumor samples were obtained by core-needle biopsy or at the time of surgery and immediately stored at -80°C . Total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) and reverse-transcribed to cDNA using the standard procedure. The complete HER2 coding sequence was amplified with nine sets of primers. All fragments were sequenced using a BigDye Terminator Cycle Sequencing Kit and an ABI3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequence variants were confirmed in duplicate.

Analysis of HER2 germline mutations. Genomic DNA was extracted from peripheral blood leukocytes using a Whole Blood Genome DNA Isolation Kit (Biotek, Beijing, China). Patients with a HER2 mutation in their breast tumor were further investigated to determine whether the mutation was present in their blood DNA.

Estrogen receptor, PR, and HER2 status. Estrogen receptor, PR, and HER2 status were determined using breast cancer tissue obtained from the core-needle biopsy or tumor tissues after surgery. Estrogen receptor or PR immunostaining was considered positive when $\geq 1\%$ of tumor cells showed positive

nuclear staining. The HER2 staining was scored according to the standard method. Scores of 0 and 1+ were considered negative, and a score of 3+ was considered positive. If a score was 2+, we further evaluated the HER2 status using FISH (Vysis, Downers Grove, IL, USA) of core biopsies according to the manufacturer's instructions.

Functional characterization of somatic HER2 mutations in vitro. MCF-7 (human breast cancer cells) and HEK293T (human embryonic kidney cells) cells were recultured in DMEM (HyClone, Logan, UT, USA) with 10% FBS (HyClone). The HER2^{WT}-pXJ40-myc plasmid was kindly provided by Dr. Qinong Ye (Academy of Military Medical Sciences, Beijing, China). Fifteen missense mutations (L12R, E139G, E139D, A466V, C515R, T526A, L755S, G776R, S783P, T862R, L869R, P885S, R897G, F1030C, and P1074S) were introduced by site-directed mutagenesis and confirmed by sequencing. V777L, a well-characterized activating mutation, served as a positive control. Transient transfection of the plasmids into the MCF-7 and HEK293T cells was carried out using VigoFect (Vigorous Biotechnology, Beijing, China), according to the manufacturer's protocol. After 24 h, the cells were washed twice with $1 \times$ PBS and starved in serum-free medium for another 12 h.

The following antibodies were used: AKT (C67E7), phospho-AKT (Ser473) (D9E), ERK1/2 (137F5), and phospho-ERK1/2 (Thr202/Tyr204) (D13.14.4E) (Cell Signaling Technology, Boston, MA, USA). Phospho-HER2 (pY1248, 06-229) was from Millipore (CA, USA); HER2 (C-18) and GAPDH

Table 1. Clinical information of breast cancer patients with HER2 somatic mutations ($n = 27$)

Patient ID	Protein change	Impact	Tumor type	Grade	ER	PR	HER2	Lymph nodes status
469	p.S310F	Activating ⁽²⁶⁾	IDC	1	–	–	+	–
3044	p.S310F	Activating ⁽²⁶⁾	IDC	2	+	–	–	–
3456	p.S310F	Activating ⁽²⁶⁾	IDC	2	+	+	–	–
5547	p.S310F	Activating ⁽²⁶⁾	ILC	–	+	+	–	–
9603	p.D769H	Activating ⁽²²⁾	IDC	2	–	–	+	–
4393	p.A775-G776insYVMA	Activating ⁽²⁷⁾	IDC	2	+	+	–	–
2619	p.A775-G776insYVMA	Activating ⁽²⁷⁾	IDC	2	+	+	–	+
2373	p.V777L	Activating ⁽²²⁾	ILC	–	–	–	–	+
3624	p.V777L	Activating ⁽²²⁾	IDC	2	+	–	–	–
4223	p.V777L	Activating ⁽²²⁾	IDC	1	+	+	–	–
5669	p.V777L	Activating ⁽²²⁾	IDC	2	–	–	+	+
6795	p.V777L	Activating ⁽²²⁾	IDC	2	+	–	+	–
3943	p.V777L	Activating ⁽²²⁾	IDC	2	–	–	–	–
3943	p.T862A	Unknown†	IDC	2	–	–	–	–
1510	p.L12R	Novel	IDC	2	–	–	–	+
327	p.E139D	Novel	IDC	3	+	–	–	–
930	p.E139G	Novel	IDC	2	+	+	–	–
3860	p.A466V	Novel	IDC	3	+	–	–	+
146	p.C515R	Novel	IDC	2	–	–	–	–
407	p.T526A	Novel	ACC	–	–	–	–	–
10001	p.G776R	Novel	IDC	2	+	+	–	+
1028	p.L869R	Unknown†	IDC	2	+	+	+	–
3733	p.L869R	Unknown†	IDC	2	–	–	–	+
3733	p.R897G	Novel	IDC	2	–	–	–	+
4137	p.P885S	Novel	IDC	2	–	–	–	–
4892	p.F1030C	Novel	IDC	3	+	+	–	–
4663	p.P1074S	Novel	IDC	2	+	+	–	–
2476	p.L755S	Unknown ⁽²²⁾	IDC	2	+	–	–	+
3896	p.L755S	Unknown ⁽²²⁾	IDC	3	+	+	–	+

†Reported in COSMIC (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>). ACC, adenoid cystic carcinoma; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; PR, progesterone receptor.

Table 2. Association of patient/tumor characteristics with *HER2* somatic mutation in the full cohort of breast cancer patients (n = 1348)

Characteristic	No. of patients	Non-carriers (n = 1321)		Carriers (n = 27)		†P-value
		No.	%	No.	%	
Age, years						
≤50	596	589	44.6	7	25.9	<0.001
>50	752	732	55.4	20	74.1	
Tumor size, cm						
≤2	515	504	38.2	11	40.7	0.789
>2	831	815	61.8	16	59.3	
Unknown	2	2		0		
Tumor grade						
1	146	144	12.0	2	8.0	0.790
2	907	888	74.1	19	76.0	
3	170	166	13.9	4	16.0	
Unknown	125	123		2		
Tumor stage						
I	316	308	24.1	8	29.6	0.793
II	764	749	58.7	15	55.6	
III	224	220	17.2	4	14.8	
Unknown	44	44		0		
Lymph node status						
Negative	742	724	56.7	18	66.7	0.298
Positive	563	554	43.3	9	33.3	
Unknown	43	43		0		
ER status						
Negative	388	378	28.7	10	37.0	0.347
Positive	954	937	71.3	17	63.0	
Unknown	6	6		0		
PR status						
Negative	530	514	39.4	16	59.3	0.037
Positive	802	791	60.6	11	40.7	
Unknown	16	16		0		
HER2 status						
Negative	953	931	72.8	22	81.5	0.314
Positive	353	348	27.2	5	18.5	
Unknown	42	42		0		
Triple-negative status						
No	1144	1124	85.5	20	74.1	0.098
Yes	198	191	14.5	7	25.9	
Unknown	6	6		0		
Surgery type						
BCS	478	472	36.7	6	22.2	0.122
Mastectomy	835	814	63.3	21	77.8	
Unknown	35	35		0		
Trastuzumab use						
No	1286	1260	95.4	26	96.3	1.000
Yes	62	61	4.6	1	3.7	
Adjuvant therapy						
C	374	362	28.6	12	44.4	0.060
E	246	239	18.9	7	25.9	
C plus E	672	664	52.5	8	29.6	
None	56	56		0		

†Patients with *HER2* somatic mutations versus patients with wild-type. BCS, breast-conserving surgery; C, chemotherapy; E, endocrine therapy; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

(FL-335) were from Santa Cruz Biotechnology (CA, USA). GAPDH was used as a protein loading control. The relative intensities of individual protein bands were quantified by analysis of

digitized images using ImageJ software (<https://imagej.nih.gov/ij/download.html>). For the phospho-specific detection of proteins, the acquired density was compared with the corresponding total antibody signal. All data shown are representative of at least three experiments.

Statistical analysis. Differences in clinicopathological characteristics between patients with a *HER2* somatic mutation and those with wild-type *HER2* were determined using Pearson's chi-square-test or Fisher's exact test. Survival curves were generated and compared using the Kaplan–Meier method and Breslow tests. Recurrence-free survival was calculated from the time of diagnosis to the first recurrence (local or distant) or death from breast cancer (for patients without a recorded relapse) or to the date of the last follow-up. Distant recurrence-free survival was calculated from the time of diagnosis to the first distant metastasis or death from breast cancer (for patients without a recorded relapse) or to the date of the last follow-up. The Cox proportional hazards model was used to determine the association of *HER2* somatic mutation status with the risk of local or distant recurrence after adjustments for patient and tumor characteristics. Two-sided *P*-values less than 0.05 were considered statistically significant. All analyses were carried out using spss 20.0 software (Chicago, IL, USA).

Results

Somatic mutations in the *HER2* gene. A total of 33 patients carried *HER2* mutations in their tumor tissues in this cohort of

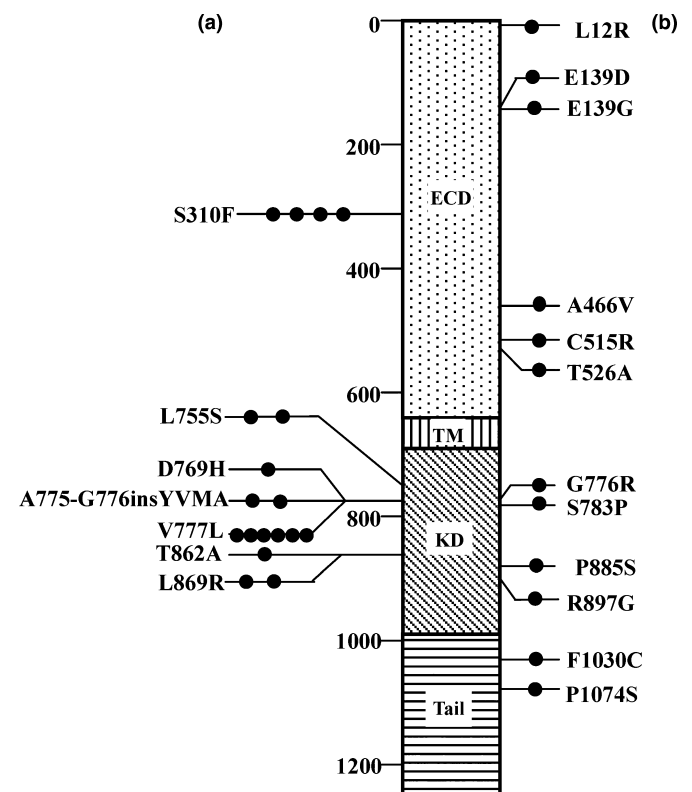


Fig. 1. *HER2* somatic mutations observed in 27 patients with primary breast cancer. (a) Mutations described previously. (b) Novel mutations. The black circles represent each case of the indicated mutation. Three patients had two *HER2* somatic mutations each, resulting in a total of 30 mutations in 27 patients. ECD, extracellular domain; KD, kinase domain; TM, transmembrane region.

1348 breast cancer patients (Table S1). Four patients had two *HER2* mutations, resulting in a total of 37 mutations in 33 patients. The mutations in seven patients (one of the seven patients also had a somatic *HER2* mutation) were also present in the corresponding blood DNA samples, indicating that they were germline (Table S1). The remaining 30 mutations (27 patients) were absent from matched blood samples, indicating that they were somatic (Table S1). Thus, we finally confirmed that 27 of 1348 patients (2.0%) carried a *HER2* somatic mutation in this cohort (Table 1).

Information regarding *HER2* status was available for 1306 of the 1348 patients in this study. Of these, 353 (27.0%) were *HER2*-positive, and 953 (73.0%) were *HER2*-negative (Table 2). Among the 27 patients with a *HER2* somatic mutation, the frequencies of these mutations in those with *HER2*-negative and *HER2*-positive breast cancers were 2.3% (22/953) and 1.4% (5/353), respectively.

HER2 somatic mutations were not significantly associated with tumor size, tumor grade, lymph node status, ER, PR, or

HER2 status. However, patients with a *HER2* somatic mutation were older than those with wild-type *HER2* (Table 2). Trastuzumab use, adjuvant chemotherapy, and breast-conserving therapy did not significantly differ between the patients with a *HER2* somatic mutation and those with wild-type *HER2* (Table 2).

The majority of patients with a *HER2* mutation had missense mutations (92.6%, 25/27), and only two showed the same insertion mutation (A775_G776insYVMA). Examination of the locations of the somatic mutations in the *HER2* domains revealed that they were clustered into two major areas: 37.0% of the patients (10/27) had an ECD mutation and 55.6% (15/27) had a KD mutation (Fig. 1). Five recurrent mutations, V777L, S310F, L755S, A775_G776insYVMA, and L869R, were found in this cohort (Table 1). Sixteen of the 27 patients (59.3%) carried a recurrent mutation (Table 1). All five recurrent mutations have been reported previously, and all were located in the KD, with the exception of S310F (Fig. 1).

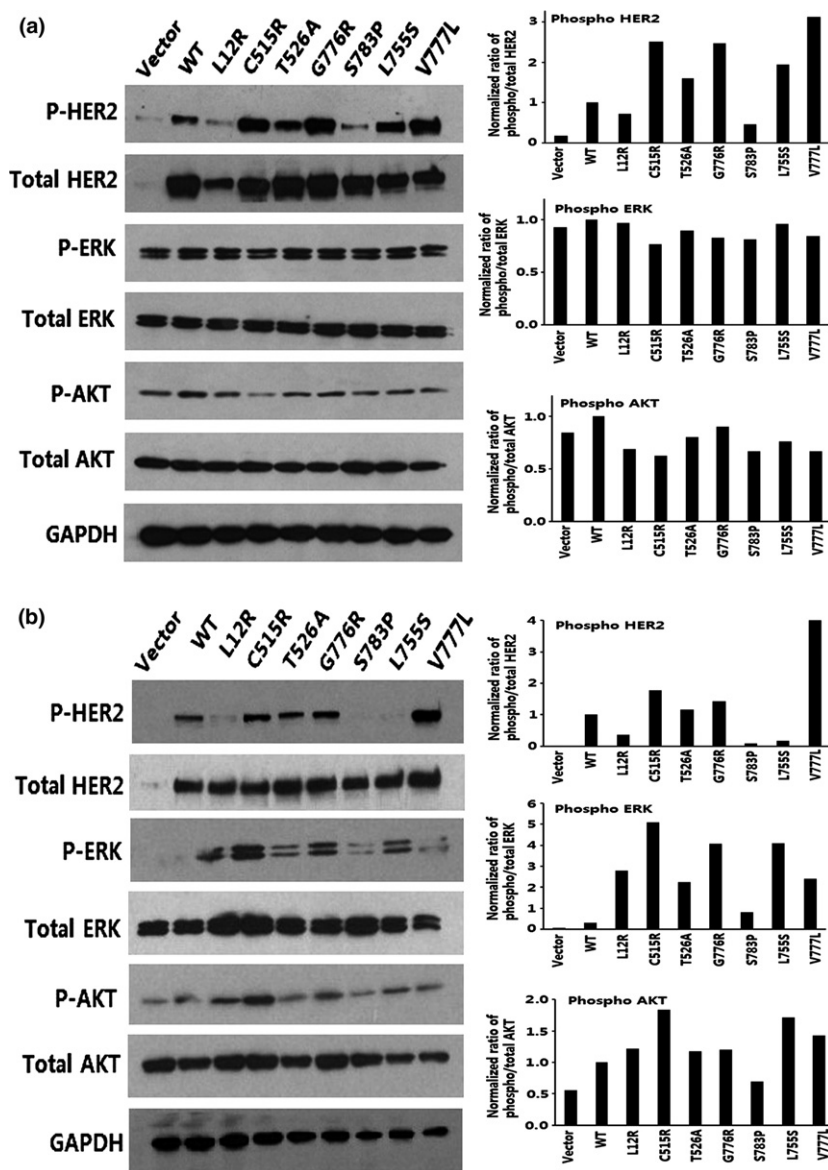


Fig. 2. MCF-7 (a) and HEK293T (b) cells were transfected with wild-type *HER2* or L12R, C515R, T526A, G776R, S783P, L755S, and V777L mutants, and lysates were probed with the indicated antibodies. The bar graphs show the quantifications of Western blot bands. AKT, protein kinase B; HER2, human epidermal growth factor receptor 2; P-, phosphorylated.

Functional effects of *HER2* somatic mutations. In this cohort, 27 patients carried 30 somatic mutations (Table 1, Fig. 1). Of these, 18 have been reported previously^(22,26,27) and 12 are novel (Fig. 1). Based on published reports,^(22,26,27) we estimated that at least 13 of the 27 patients carried an activating *HER2* mutation that was likely to be a driver event in breast cancer (Table 1). The functional effects of the remaining 15 mutations were not determined or were not fully elucidated. We therefore further characterized these mutations by undertaking *in vitro* experiments.

Functional analyses of novel and unknown mutations *in vitro*. The level of P-HER2 was markedly higher in MCF-7 cells with C515R, T526A, G776R, L755S, A466V, T862R, and P1074S mutations than in cells with wild-type *HER2* (Fig. 2a, Fig. S1a). No significant differences were observed in the level of P-HER2 between cells with the remaining mutations and wild-type *HER2* (Fig. 2a, Fig. S1a). In HEK293T cells, the C515R, A466V, T862R, L869R, and R897G mutations strongly increased the level of P-HER2 (Fig. 2b, Fig. S1b). The C515R mutation also increased the levels of P-ERK and P-AKT (Fig. 2b). In addition, the L12R, T526A, and G776R mutations increased the level of P-ERK but had little effect on P-HER2 and P-AKT levels. The L755S mutation increased the phosphorylation of ERK and AKT but decreased the phosphorylation of *HER2* (Fig. 2b). These results showed that the C515R, A466V, and T862R mutations increased *HER2* activity in both cell lines, suggesting that they are activating mutations. The T526A, G776R, L755S, L869R, R897G, and P1074S mutations increased *HER2* activity in either MCF-7 or HEK293T cells, suggesting that they are also activating mutations. The other mutations (L12R, E139G, E139D, S783P, P885S, and F1030C) seemed to be neutral. Thus, nine of the 15 mutations are activating mutations.

***HER2* somatic mutations and survival in the entire study cohort.** To investigate the association between survival and *HER2* somatic mutations, a total of 1348 breast cancer patients were evaluated. The median length of follow-up was 60 months (range, 1–116 months). Two hundred and sixteen patients experienced recurrence (local or distant) or died of breast cancer in this cohort of patients during the follow-up period.

Patients with *HER2* mutations had a significantly worse RFS (unadjusted HR = 1.91; 95% CI, 0.90–4.05, $P = 0.025$; Fig. S2a) and DRFS (unadjusted HR = 1.91; 95% CI, 0.85–4.32, $P = 0.033$; Fig. S2b) than those with wild-type *HER2* in the entire study cohort.

***HER2* somatic mutations and survival in *HER2*-negative breast cancer patients.** *HER2* somatic mutations were detected at a higher frequency in *HER2*-negative breast cancer patients. *HER2*-negative patients with a *HER2* somatic mutation ($n = 22$) had a significantly worse RFS (unadjusted HR = 2.67; 95% CI, 1.25–5.72, $P = 0.002$; Fig. 3a) and DRFS (unadjusted HR = 2.50; 95% CI, 1.10–5.68, $P = 0.004$; Fig. 3b) than those with wild-type *HER2* ($n = 953$). Multivariate analysis revealed that *HER2* somatic mutation was a borderline unfavorable factor for RFS (HR = 2.47; 95% CI, 0.99–6.16, $P = 0.051$) (Table S2), and it was also a non-significantly unfavorable factor for DRFS (HR = 2.29; 95% CI, 0.92–5.70, $P = 0.075$) (Table S2) after adjusting for age, lymph node, tumor size, tumor grade, ER, PR, and *HER2* status.

Only five patients with a *HER2* somatic mutation were *HER2*-positive, and none of them received trastuzumab treatment. Given the small sample size, we did not undertake survival analysis in this subgroup.

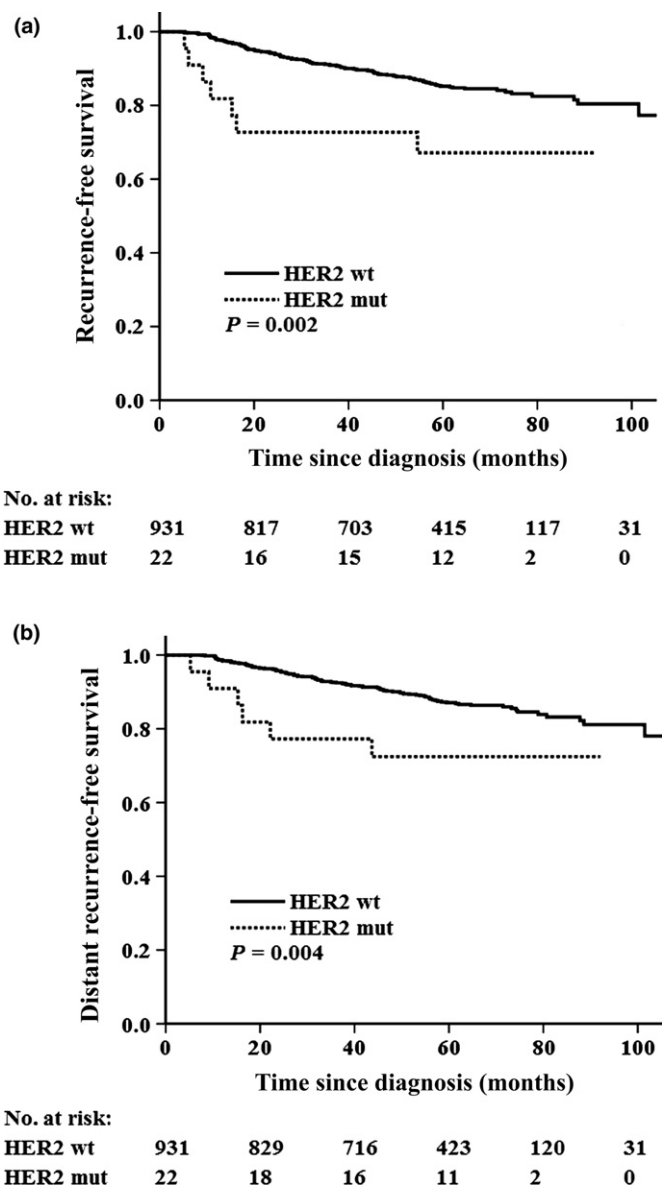


Fig. 3. Kaplan–Meier curves for survival based on *HER2* somatic mutations in *HER2*-negative patients with primary breast cancer ($n = 953$). (a) Recurrence-free survival. (b) Distant recurrence-free survival. *HER2*, human epidermal growth factor receptor 2; mut, mutant; wt, wild-type.

Discussion

In this study, by sequencing tumor cDNA, we found that 27 patients (2.0%) carried a *HER2* somatic mutation in a cohort of 1348 operable primary breast cancer patients. These mutations were more common in *HER2*-negative breast cancer patients (2.3%) than in those who were *HER2*-positive (1.4%). *HER2*-negative patients with a *HER2* somatic mutation had a significantly worse survival than those with wild-type *HER2*.

All of the recurrent mutations identified in this study have been previously reported, and of these, V777L, S310F, and A775_G776insYVMA were determined to be activating mutations.^(22,26,27) Four of the five recurrent mutations were located in the KD, and S310F was localized to the ECD. The five recurrent mutations and other mutations in the KD accounted

for 74.1% (20/27) of the mutations in this study. Therefore, screening for recurrent mutations and mutations in the KD is a fast and effective assay to quickly detect *HER2* somatic mutations in breast cancer patients.

We also identified 12 novel mutations, which were located throughout the entire gene. Functional analysis of the novel mutations and those with unknown function (a total of 15 mutations) showed that 9 of the 15 mutations activated the *HER2* signaling pathway. Our findings, and those of previous reports,^(22,26,27) suggest that the majority of *HER2* somatic mutations are disease-associated.

In this study, we also found six *HER2* germline mutations (R157W, A466V, K681N, R849W, R1146W, and E1195G) in seven breast cancer patients. Among them, R157W was previously reported as a somatic mutation,⁽²⁸⁾ which was found in micropapillary urothelial carcinoma located in the ECD and was predicted to be pathogenic by FATHMM prediction (Cosmic database). The A466V mutation was also determined as somatic in this cohort and increased *HER2* activity in MCF-7 and HEK293T cell lines. The remaining germline mutations (K681N, R849W, R1146W, and E1195G) were novel and need further functional studies.

Clinically, *HER2*-negative breast cancers are not typically treated with *HER2*-targeted therapy. Recent studies have suggested that the *HER2*-targeted drugs trastuzumab and lapatinib inhibit the growth of cells with *HER2* somatic mutations *in vitro* and *in vivo*.^(22–24) In addition, neratinib, a novel *HER2*-targeted drug, has shown a strong inhibitory effect in patients with a *HER2* somatic mutation.^(22,29,30) Therefore, given the poor survival of *HER2*-negative breast cancer patients who carry a *HER2* somatic mutation, they are good candidates for receiving *HER2*-targeted therapy or for recruitment into ongoing clinical trials. In addition, five *HER2*-positive patients carried an activating *HER2* somatic mutation. Two of them had contralateral breast cancer and one died from suicide. Given the small sample size, we did not undertake survival analysis in the *HER2*-positive subgroup.

The 2.3% somatic mutation rate in *HER2*-negative breast cancer patients was remarkable, considering that 70%–80% of all patients had *HER2*-negative breast cancer. Therefore, it will

not be difficult to gather enough patients for clinical trials. *HER2*-negative patients had a generally favorable survival compared with the *HER2*-positive patients, and when the *HER2*-negative patients were grouped according to the presence/absence of a *HER2* somatic mutation, those without a somatic mutation had better survival.

In summary, we found that *HER2* somatic mutations occurred more frequently in the *HER2*-negative breast cancer patients compared with those with *HER2*-positive breast cancer, and that approximately 2.3% of these *HER2*-negative patients harbored a *HER2* somatic mutation. *HER2*-negative patients carrying a *HER2* somatic mutation were determined to have an unfavorable outcome. Therefore, *HER2*-negative patients with this type of mutation are potentially good candidates for *HER2*-targeted therapy.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

AKT	protein kinase B
CI	confidence interval
DRFS	distant recurrence-free survival
ECD	extracellular domain
ER	estrogen receptor
HER2	human epidermal growth factor receptor 2
HR	hazard ratio
KD	kinase domain
PR	progesterone receptor
RFS	recurrence-free survival

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. MCF-7 (a) and HEK293T (b) cells were transfected with wild-type *HER2* or mutants.

Fig. S2. Kaplan–Meier curves for survival.

Table S1. *HER2* somatic and germline mutations in the entire cohort of primary breast cancer

Table S2. Multivariate analyses of recurrence-free survival (RFS) and distant recurrence-free survival (DRFS) in *HER2*-negative subgroup.