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HER2 Gene Amplification in ER-positive HER2 Immunohistochemistry 0 or 1+ Breast Cancer With Early Recurrence

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Abstract. *Background/Aim: In estrogen receptor (ER)-positive, human epidermal growth factor receptor 2 (HER2)-negative breast cancer, standard chemotherapies as well as adjuvant endocrine therapy might not be enough for prevention of early relapse. Materials and Methods: We focused on ER-positive, HER2 immunohistochemistry (IHC) 0 or 1+ breast cancer, and retrospectively examined HER2 gene amplification and TP53 mutation in breast cancer tissues in patients with or without early recurrence. Post-relapse survival in patients with early recurrence was also analyzed by mutation status of HER2 and TP53. Results: Surprisingly, amplification of the HER2 gene was found in 15% of patients with early recurrence. None of the patients without relapse had HER2-amplified tumors. Post-relapse survival in patients with HER2 gene amplification and/or TP53 mutation in primary tumors was shorter than that in patients without these mutations, especially among postmenopausal women. Conclusion: HER2 gene amplification exists in ER-positive, HER2 IHC 0 or 1+ breast cancer in patients who developed early distant metastasis.*

Endocrine therapy is the most important treatment option for women with estrogen receptor (ER)-positive breast cancer (1, 2). We and others have previously reported that tumor grade and/or Ki67 expression are predictors of early recurrence in ER-positive, human epidermal growth factor receptor 2 (HER2)-negative breast cancer (3-5). Adjuvant chemotherapy has proven to be effective in reducing the risk of early recurrence, which is defined as relapse within the first 5 years after diagnosis (6). Nevertheless, our previous study revealed that approximately two thirds of patients with ER-positive, HER2-negative breast cancer who relapsed within 5 years had received anthracyclines and/or taxanes as adjuvant or neoadjuvant chemotherapy (3). These standard chemotherapies as well as adjuvant endocrine therapy might not be enough for prevention of early relapse. Furthermore, our previous study also indicated short post-relapse survival in patients with early recurrence (7).

The *HER2* gene is amplified and/or overexpressed in approximately 15% of primary breast cancers (8), and more than half of HER2-positive breast cancers are ER-positive (9). Since 2007, the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) has issued clinical practice guidelines on HER2 testing (10-12). *HER2* gene amplification assessed by *in situ* hybridization (ISH) or protein overexpression assessed by immunohistochemistry (IHC) remains the primary predictor of responsiveness to HER2-targeted therapies in breast cancer. A previous study reported that a large percent of tumors with *HER2* gene amplification by ISH showed a HER2 IHC score of 0 or 1+, although the concordance rate of HER2 testing by IHC and ISH was more than 95% (13). In clinical practice, tumors with HER2 IHC 0 or 1+ have not been tested for amplification by ISH. Therefore, the clinical significance for *HER2* gene amplification in HER2-non-overexpressing (score 0 or 1+) breast cancer has not been identified. On the other hand, some patients with ER-positive, HER2 IHC 0 or 1+ primary tumors develop early relapse with HER2-positive metastasis. In these cases, *HER2* gene amplification might have existed in the primary tumors.

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The *TP53* gene is the most frequently mutated gene in cancer, including breast cancer (14). A *TP53* gene mutation has been found to be present in 12% of luminal A and 32% of luminal B breast cancers, although the frequency of *TP53* gene mutations in luminal tumors is lower compared to basal-like (84%) or HER2-positive (75%) breast cancers (14). Functional p53 plays an important role in maintaining genomic stability, regulating the cell cycle and inducing apoptosis (15). Since mutated p53 accumulates in the nucleus of tumor cells, IHC staining for p53 is frequently used as a surrogate marker for *TP53* mutational status. The association between overexpression of p53 and poor prognosis has been reported in premenopausal breast cancer patients treated with tamoxifen after chemotherapy (16). A recent study has also demonstrated that *TP53* wild-type status conferred superior 5-year overall survival in patients treated with adjuvant endocrine therapy (17). We previously reported that 20% of ER-positive breast cancer patients showed p53 accumulation by IHC (18), and that p53 accumulation predicted resistance to endocrine therapy and decreased post-relapse survival in metastatic breast cancer (19). We also demonstrated that p53 accumulation was a strong predictor, especially of early recurrence in postmenopausal ER-positive breast cancer (20, 21).

We hypothesized *HER2* gene amplification and/or *TP53* mutation might exist in some ER-positive, HER2 IHC 0 or 1+ breast cancer patients who developed early distant recurrence. In this study, we retrospectively examined *HER2* gene amplification and *TP53* mutation in ER-positive, HER2 IHC 0 or 1+ primary breast cancer in patients who relapsed within five years after initial treatment.

Materials and Methods

Patients and breast cancer tissues. A total of 27 consecutive women with ER-positive, HER2 IHC 0 or 1+ breast cancer who relapsed with distant metastasis within five years after initial treatment were recruited to this study (Table I). All patients had undergone breast surgery for Stage I to III breast cancer between 2002 and 2011 at Hokkaido University Hospital. For each recurrence patient, approximately one age-matched control patient without relapse for more than six years who was initially treated in 2011, was randomly selected using RAND in combination with Excel software. The study protocol was approved by the institutional review board and conformed to the guidelines of the 1996 Declaration of Helsinki. Written informed consent for the use of surgically resected tumor tissues was provided by all patients prior to treatments. All patients had undergone mastectomy or lumpectomy. Patients who were positive for axillary lymph nodes received neoadjuvant or adjuvant chemotherapy. Pretreatment specimens obtained by core needle biopsies were used for IHC and mutation analyses in patients treated with neoadjuvant chemotherapy. Of the remaining patients, tumor samples were obtained during surgery. As standard adjuvant systemic treatment, all patients received endocrine therapy. Patients with positive axillary lymph nodes and/or high-grade tumors received pre- or post-operative chemotherapies including anthracyclines and taxanes or TC (docetaxel and cyclophosphamide), as well as adjuvant endocrine therapy.

Immunohistochemical analysis. IHC status of ER, progesterone receptor (PgR), and HER2 was determined using the PATHWAY rabbit monoclonal antibodies (clone SP1, 1E2, and 4B5, respectively) and iView DAB Detection Kit (Ventana Medical Systems, Inc., Tucson, AZ, USA) (22). Tumors with ≥1% of cells showing positive nuclear staining for the expression of ER and PgR were evaluated as ER/PgR-positive (23). To determine the level of HER2 expression, the membrane staining pattern was estimated and scored on a scale of 0 to 3+ (12). Tumors with a score of 0 or 1+ were recruited in this study. For Ki67 and p53 staining, antigens were retrieved in Dako EnVision FLEX Target Retrieval Solution, high pH (pH9.0) using Dako PT Link (Dako, Glostrup, Denmark) (20, 21). IHC for Ki67 was performed using a mouse monoclonal anti-human Ki67 antibody (MIB-1, Dako) and the Dako Envision FLEX system was used for visualization (20, 21). The labeling index (LI) was assessed as the percentage of tumor cells showing definite nuclear staining among >1000 invasive tumor cells (24). IHC for p53 was performed using a mouse monoclonal anti-human p53 antibody (DO-7, Dako) with the Dako Envision FLEX system for visualization. Expression of p53 protein was measured as the percentage of cells showing definite nuclear staining (20, 21). Three researchers including two pathologists (N.I., H.K-O. and K.C.H) who were blinded to the clinical information of patients independently examined and scored each case. Differences in interpretation were resolved by the consensus agreements of these three researchers.

HER2 gene testing by dual color in situ hybridization. *HER2* gene testing by dual color *in situ* hybridization (DISH) was performed with the invasive cancer nests stained most strongly by IHC (25). Hybridization was performed using an Inform HER2 Dual ISH DNA Probe Cocktail (Ventana Medical Systems). Hapten labeling of the *HER2* gene was visualized with an UltraView SISH DNP Detection Kit (Ventana Medical Systems), and hapten labeling of CEP17 was visualized with an UltraView Red ISH DIG Detection Kit (Ventana Medical Systems). Signal counting in DISH was performed in accordance with the Interpretation Guide for the Ventana Inform HER2 Dual ISH DNA Probe Cocktail Assay. *HER2* gene amplification status was classified according to the ASCO/CAP guidelines for HER2 testing (11, 12). *HER2* gene and CEP17 signals were counted for 20 cancer cells, and *HER2*/CEP17 ratio ≥2.0 with an average *HER2* copy number ≥4.0 signals per cell were taken to indicate *HER2* gene amplification. *HER2*/CEP17 ratio <2.0 with an average *HER2* copy number <4.0 signals per cell was considered to indicate *HER2* gene non-amplification. *HER2*/CEP17 ratio <2.0 with an average HER2 copy number ≥6.0 signals per cell indicated *HER2* gene amplification.

TP53 mutation analysis. Genomic DNA was extracted from formalin-fixed paraffin-embedded tumor blocks using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany). Four to eight pieces of a deparaffinized 10 μm section were used for the extraction process. The amount of genomic DNA was spectrophotometrically determined (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA, USA) before use.

The *TP53* gene was amplified by means of polymerase chain reaction using primers for exons 5-9 as described in the detection

Table I. *Comparison of clinicopathological characteristics and treatments between patients with early and no recurrence.*

	Early recurrence (n=27)	No recurrence (n=29)	*p*-Value
Age (at the time of the initial therapy), mean±SD (range)	53.6±10.2 (35-74)	57.3±10.3 (35-78)	0.61
Menopausal status (at the time of the initial therapy)			
Premenopausal	9 (33%)	10 (34%)	0.93
Postmenopausal	18 (67%)	19 (66%)	
Tumor category			
T1	13 (48%)	20 (69%)	0.04*
T2	9 (33%)	9 (31%)	
T3	5 (19%)	0 (0%)	
Lymph node status			
pN0	17 (63%)	15 (52%)	0.40
pN1-pN3	10 (37%)	14 (48%)	
Tumor grade			
1	7 (26%)	14 (48%)	0.05
2	14 (52%)	14 (48%)	
3	6 (22%)	1 (4%)	
ER (%), mean±SD (range)	54.8±36.8 (1-100)	98.8±3.2 (90-100)	<0.001*
PgR (%), mean±SD (range)	28.6±39.5 (0-100)	55.3±40.5 (0-100)	0.08
HER2 IHC score			
0	16 (59%)	20 (69%)	0.45
1+	11 (41%)	9 (31%)	
HER2 gene amplification			
Present	4 (15%)	0 (0%)	0.048*
Absent	23 (85%)	29 (100%)	
Ki67 LI (%), mean±SD (range)	19.8±12.7 (4-44.4)	17.2±11.4 (1.9-45.5)	0.51
p53 protein (%), mean±SD (range)	19.5±32.4 (0-100)	15.3±24.6 (0-95)	0.84
p53 <10%	18 (67%)	19 (66%)	0.93
p53 ≥10%	9 (33%)	10 (34%)	
TP53 mutation			
Present	6 (23%)	7 (26%)	0.81
Absent	20 (77%)	20 (74%)	
Invalid	1	2	
Neoadjuvant/adjuvant chemotherapy			
Yes	12 (44%)	8 (28%)	0.19
No	15 (56%)	21 (72%)	
Adjuvant endocrine therapy			
Yes	24 (89%)	29 (100%)	0.07
No	3 (11%)	0 (0%)	
Follow-up time, mean±SD (range) (months)	72.7±41.6 (14-146)	63.0±5.8 (40-70)	

*$p<0.05$ is considered significant.

of *TP53* mutations by direct sequencing [IARC protocol (26)]. Amplification was performed using MightyAmp DNA Polymerase Ver. 2 (Takara Bio Inc., Kusatsu, Japan). PCR samples were purified with the ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific, Tokyo, Japan). Direct sequencing was performed using each of the primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). After purification of the samples with the BigDye XTerminator Purification Kit (Thermo Fisher Scientific), DNA sequencing was carried out using the 3730xl DNA Analyzer (Thermo Fisher Scientific).

Statistical analysis. The chi-squared test, Student's *t*-test and the Mann-Whitney *U*-test were used to compare clinicopathological characteristics and treatments among patients with early and no recurrence. Estimation of post-relapse survival was performed using the Kaplan–Meier method, and differences between survival curves were assessed using the log-rank test. Cox's proportional hazards model was used for univariate and multivariate analyses of prognostic values. Statistical analysis was performed using Excel software (Microsoft corp., Albuquerque, MX, USA).

Results

Comparison of clinicopathological characteristics and treatments between patients with early and no distant recurrence. We collected data from a total of 27 women with early distant recurrence and 29 women without relapse for more than six years, all of whom had ER-positive, HER2 IHC 0 or 1+ breast cancer (Table I). Patients with early recurrence had larger clinical tumor size than those without recurrence ($p=0.04$). Expression levels of ER were

Table II. *Tumor characteristics of four patients with HER2 gene amplification without HER2 overexpression.*

Patient no.	*HER2*/CEP17 ratio	Average *HER2* copy number per cell	HER2 IHC score	*TP53* mutation	p53 protein	Ki67 LI	ER	Recurrence status
18	2.0	4.3	1+	G262S Missense	1%	10%	3%	Early recurrence
3	2.3	4.5	0	Wild-type	5%	4%	90%	Early recurrence
16	2.3	4.9	1+	S313N Missense	5%	20%	70%	Early recurrence
8	3.2	6.8	1+	Wild-type	3%	18%	1%	Early recurrence

Table III. *Tumor characteristics of thirteen patients with TP53 mutations.*

Patient no.	Exon	Nucleotide change	Amino acid change	Mutation type	p53 protein	Ki67 LI	*HER2* amplification	ER	Recurrence status
Single mutation									
33	5	c.424G>A	P142S	Missense	2%	8%	Non-amplified	100%	No recurrence
37	5	c.430G>A	Q144*	Nonsense	25%	15%	Non-amplified	100%	No recurrence
51	5	c.489G>A	Y163	Silent	1%	11%	Non-amplified	100%	No recurrence
21	8	c.839G>C	R280T	Missense	100%	37%	Non-amplified	100%	Early recurrence
35	9	c.920G>C	A307G	Missense	8%	18%	Non-amplified	100%	No recurrence
16	9	c.938C>T	S313N	Missense	5%	20%	Amplified	70%	Early recurrence
27	9	c.971G>A	Q331*	Nonsense	100%	44%	Non-amplified	10%	Early recurrence
Double mutations									
52	6	c.594delC	E198	Frameshift	3%	14%	Non-amplified	90%	No recurrence
	6	c.600delA	N200	Frameshift					
18	8	c.784G>A	G262S	Missense	1%	10%	Amplified	3%	Early recurrence
	8	c.829T>C	C277	Silent					
47	8	c.814C>T	V272M	Missense	95%	23%	Non-amplified	100%	No recurrence
	8	c.859C>T	E287K	Missense					
Triple mutations									
17	5	c.425G>T	P142H	Missense	30%	21%	Non-amplified	100%	Early recurrence
	5	c.432C>G	Q144H	Missense					
	8	c.813C>T	E271	Silent					
49	5	c.550G>A	D184N	Missense	25%	25%	Non-amplified	100%	No recurrence
	7	c.696C>T	I232	Silent					
	7	c.729G>C	M243I	Missense					
2	7	c.694A>G	I232V	Missense	5%	4%	Non-amplified	40%	Early recurrence
	7	c.718G>C	S240T	Missense					
	7	c.746G>T	R249M	Missense					

significantly lower in patients with early recurrence than in those without relapse ($p<0.001$).

HER2 gene amplification in breast cancer tissues without HER2 overexpression. We examined *HER2* gene amplification in 56 HER2 non-overexpressing (IHC 0 or 1+) tumors from patients with or without early recurrence. Surprisingly, DISH confirmed amplification of the *HER2* gene in four tumors, and all four patients with *HER2*-amplified tumors relapsed within five years after initial treatment (Tables I and II). Thus, four (15%) of the 27 patients with early distant recurrence had *HER2*-amplified tumors in ER-positive, HER2 IHC 0 or 1+ breast cancer. Of the four tumors with *HER2* gene amplification, *HER2*/CEP17 ratio was 2.0 to 3.2, and average *HER2* copy number per cell was 4.3 to 6.8 (Table II). *TP53* missense mutations were also detected in two tumors (G262S and S313N, respectively). In contrast, p53 expression was less than 10% in all four *HER2*-amplified tumors. The Ki67 LI was 4% to 20%, and expression levels of ER were 3% to 90% in these four *HER2*-amplified tumors.

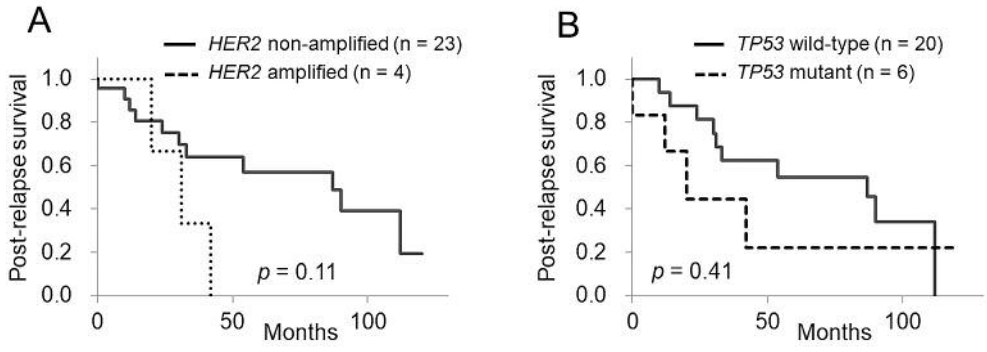

Figure 1. *Kaplan–Meier curves of the effect of HER2 gene amplification (A) and TP53 mutation (B) on post-relapse survival in postmenopausal women with early recurrence.*

TP53 mutations in breast cancer tissues. We next evaluated the genomic DNA of primary breast cancer specimens for *TP53* mutations. Specimens from one tumor with early recurrence and two tumors without relapse were invalid. Of the 53 tumors that we were able to evaluate, seven tumors had a single mutation, three tumors had mutations at two sites, and three tumors had mutations at three sites (Table III). All of the mutations were present at different sites. Of the 22 mutations, 14 were missense, four were silent, two were nonsense, and two were frameshift mutations. Ki67 LI values were from 4% to 44% (median 18%) and expression levels of ER ranged from 3% to 100% (median 100%) in *TP53*-mutant tumors. Among the thirteen patients with *TP53*-mutant tumors, six patients relapsed within 5 years and seven patients remained disease-free. *TP53* mutation status did not differ between patients with early distant recurrence and those without recurrence (Table I).

Of the 53 tumors with and without *TP53* mutations, p53 expression was significantly higher in *TP53*-mutant tumors (n=13) than in *TP53*-wild-type tumors (n=40) (mean±SD 30.8%±40.0% *versus* 11.8%±20.0%, mutant *versus* wild-type, p=0.001). However, expression levels of p53 varied from 1% to 100% in *TP53*-mutant tumors (Table III). Moreover, p53 expression did not differ between patients with early distant recurrence and those without recurrence (Table I).

Post-relapse survival according to HER2 and TP53 status in patients with early recurrence. We then analyzed whether *HER2* gene amplification and *TP53* mutation affected post-relapse survival in patients with early distant recurrence. Median post-relapse survival in patients with HER2-non-amplified and HER2-amplified tumors were 33 months and 25.5 months, whereas median post-relapse survival in patients with *TP53*-wild-type and *TP53*-mutant tumors were 36.5 months and 17 months, respectively (Table IV). Kaplan–Meier analysis showed that patients with *HER2*-amplified tumors had shorter post-relapse survival compared to those with *HER2*-non-amplified tumors (Figure 1A). Moreover, patients with *TP53*-mutant tumors had shorter post-relapse survival compared to those with *TP53*-wild-type tumors (Figure 1B). In 18 postmenopausal patients with early relapse, one tumor was invalid for *TP53* mutation analysis. Univariate analysis demonstrated a significant association between decreased post-relapse survival and low ER expression (p=0.045), presence of *TP53* mutation and/or *HER2* amplification (p=0.02) and short total duration of endocrine therapies after relapse (p=0.04) (Table V). However, presence of *TP53* mutation or *HER2* amplification was not significantly correlated with post-relapse survival. In multivariate analysis, the presence of *TP53* mutation and/or *HER2* amplification was the only prognostic factor for decreased post-relapse survival in postmenopausal patients with early distant recurrence (p=0.02, Table V).

Table IV. *Post-relapse survival according to HER2 gene and TP53 status in patients with early recurrence.*

	No. of patients	Post-relapse survival, median (range) (months)
All patients	27	31 (0-120)
HER2 gene amplification		
Non-amplified	23 (85%)	33 (0-120)
Amplified	4 (15%)	25.5 (9-42)
TP53 mutation*		
Wild-type	20 (77%)	36.5 (0-112)
Mutant	6 (23%)	17 (0-120)

*One tumor was invalid.

Discussion

In this study, we focused on ER-positive, HER2 IHC 0 or 1+ breast cancer patients who relapsed within five years after initial treatment. Assessment of HER2 protein expression by

Table V. *Univariate and multivariate analyses of factors predicting post-relapse survival in postmenopausal patients with early recurrence.*

	Univariate			Multivariate		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Age	0.91	0.77-1.07	0.25			
Tumor category	1.23	0.53-2.89	0.63			
Lymph node status	1.06	0.99-1.14	0.10			
Tumor grade	1.07	0.37-3.08	0.90			
ER (%)	0.97	0.95-0.99	0.045*	0.96	0.93-1.00	0.05
Ki67 LI (%)	1.03	0.96-1.09	0.47			
p53 protein (%)	1.00	0.98-1.03	0.72			
Presence of *TP53* mutation	1.65	0.50-5.45	0.41			
Presence of *HER2* amplification	2.90	0.74-11.32	0.12			
Presence of *TP53* mutation and/or *HER2* amplification	8.41	1.35-52.3	0.02*	20.4	1.55-268	0.02*
Neoadjuvant/adjuvant chemotherapy	1.36	0.34-5.47	0.66			
Disease-free interval	1.06	1.00-1.13	0.07			
Duration of first-line endocrine therapy	1.00	0.95-1.04	0.85			
Total duration of endocrine therapies after relapse (months)	0.97	0.95-0.99	0.04*	0.98	0.96-1.01	0.24

*$p<0.05$ is considered significant.

IHC might vary according to tissue handling including fixation, the operating procedure, and the evaluation method (10). Moreover, a large percent of tumors without HER2 protein overexpression (IHC score 0 or 1+) have *HER2* gene amplification (13, 27, 28). However, the clinical significance of *HER2* gene amplification in HER2-non-overexpressing breast cancer has not been identified. Because tumors with HER2 IHC 0 or 1+ are not tested for amplification by ISH in clinical practice, anti-HER2 treatments are not administered to patients with *HER2* gene amplification without HER2 overexpression. In the present study, *HER2*/CEP17 ratios and average *HER2* copy numbers per cell were not very high (2.0-3.2 and 4.3-6.8, respectively) in all four *HER2*-amplified tumors. The Ki67 LI was 4% to 20% in four tumors with *HER2* gene amplification, indicating that the risk of early recurrence was probably low (3). On the other hand, expression levels of ER were very low (1% and 3%, respectively) in two *HER2*-amplified tumors. Although the sample size of this study is small, ER-positive tumors with *HER2* gene amplification, HER2 IHC 0 or 1+ breast cancer are present especially in patients with early recurrence. Moreover, not just early relapse, but also shorter post-relapse survival was observed in patients with *HER2* gene amplification, suggesting that endocrine therapies and chemotherapies might not have been effective for early and metastatic breast cancers. Since these patients were treated as having HER2-negative breast cancer, anti-HER2 therapies were not given. It is not clear whether HER2 IHC 0 or 1+ breast cancer with *HER2* gene amplification responds to adjuvant anti-HER2 therapy. A re-biopsy against the metastatic sites to examine HER2 protein overexpression and/or multigene panel testing of the primary or metastatic sites might be useful when planning treatment options for ER-positive, HER2-negative metastatic breast cancer, especially in the context of early recurrence.

In this study, we also examined *TP53* mutation in primary breast cancer tissues with ER-positive and HER2 IHC 0 or 1+ breast cancer. We previously demonstrated that p53 accumulation was correlated with an aggressive phenotype, such as high tumor grade and high Ki67 expression, and that p53 accumulation was a strong predictor of both early and late recurrence in ER-positive breast cancer patients treated with aromatase inhibitors as adjuvant endocrine therapy (20, 21). The correlation between p53 accumulation measured by IHC and *TP53* mutation detected by sequencing has been estimated to be less than 75% in breast cancer (29). Not all mutations yield a stable protein and some mutations lead to a truncated protein not detected by IHC. Done and colleagues demonstrated strong p53 nuclear staining in all tumors known to have missense mutations but in none of the tumors with truncation mutations (30). On the other hand, wild-type p53 may accumulate in some tumors in response to DNA damage or by binding to other cellular proteins, giving a positive IHC result (15). Our present study showed that *TP53* mutations in breast cancer tissues were present both in patients with early relapse and in patients without recurrence, and expression levels of the p53 protein were significantly higher in *TP53* mutant tumors than in *TP53* wild-type tumors. It is not clear whether mutations of the *TP53* gene present in a primary tumor function as a driver for progression of breast cancer. Uji and colleagues analyzed *TP53* mutation both by next-generation sequencing and Sanger sequencing and the *TP53* mutation-associated gene expression signature by DNA microarray in 115 ER-positive

breast cancers (31). They reported that next-generation sequencing was more sensitive in the detection of *TP53* mutations than Sanger sequencing, and that only the *TP53* mutation-associated gene expression signature proved to be a powerful prognostic indicator. On the other hand, our present study showed that post-relapse survival was significantly worse in postmenopausal patients with *HER2* gene amplification and/or *TP53* mutation compared to that in patients without these mutations. In addition to *HER2* gene amplification, *TP*53 gene alteration might be a key biological characteristic of ER-positive breast cancer.

In conclusion, *HER2* gene amplification exists in ER-positive, HER2 IHC 0 or 1+ breast cancer in patients who developed early distant metastasis. Post-relapse survival in patients with *HER2* gene amplification and/or *TP53* mutation in primary tumors was found to be shorter than in patients without these mutations, especially among postmenopausal women. A re-biopsy of the metastatic sites to examine HER2 protein overexpression and/or multigene panel testing of the primary or metastatic sites might be useful when planning treatment options for ER-positive, HER2-negative metastatic breast cancer, especially in the context of early recurrence.

Conflicts of Interest

All Authors declare no conflicts of interest regarding this study.

Authors' Contributions

HY conceived of the study, analyzed the data, and participated in manuscript writing. NI carried out experiments and analyzed the data. YH and KCH carried out mutation experiments. KH, TO and TT provided tissue samples and clinical data. HK-O, AS, KCH and YM carried out immunostaining experiments. All Authors read and approved the final manuscript.

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