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ORIGINAL ARTICLE

Association of *HER2* gene amplification and tumor progression in early gastric cancer

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Abstract

Overexpression of human epidermal growth factor receptor 2 (HER2) protein in association with *HER2* gene amplification is found in 7–34% of gastric cancers. In breast cancer, HER2 overexpression is a prognostic factor in advanced cases and is associated with tumor progression in ductal carcinoma in situ. However, the biological and clinical significance of HER2 status in early gastric cancer is unknown. Here, we aimed to examine the correlation between *HER2* gene amplification and tumor progression in early gastric cancer. The HER2 status was evaluated in 149 lesions from 141 consecutive patients with early gastric cancer who underwent endoscopic resection by immunohistochemistry and dual color in situ hybridization. *HER2* gene amplification was detected in 35 (23.5%) of 149 lesions, and of those, 26 cases (74.3%) showed intratumoral heterogeneity. *HER2* gene amplification was found in noninvasive carcinoma, and there was a significant correlation between HER2 status and T factor ($P = 0.0290$). Our study demonstrated that *HER2* gene amplification occurred during the early stages of gastric cancer and showed heterogeneity in several cases. *HER2* gene amplification may be involved in tumor progression in early gastric cancer.

Keywords Early gastric cancer · *HER2* gene amplification · HER2 homogeneity · HER2 heterogeneity · Tumor progression

Introduction

In breast cancer, human epidermal growth factor receptor 2 (HER2) overexpression is a powerful prognostic factor. In addition, HER2 overexpression in ductal carcinoma in situ has been reported to be associated with tumor progression [1, 16]. On the other hand, overexpression of HER2 protein in association with *HER2* gene amplification has been found in 7–34% of patients with gastric cancer [7, 8, 13]. The therapeutic effects of trastuzumab in the treatment of gastric cancer were confirmed in the trastuzumab for gastric cancer study (ToGA study). Subsequently, this anticancer agent has been applied in the management of patients with advanced gastric cancer. A recent study showed improvement in the overall survival of patients with HER2-positive advanced gastroesophageal and gastric adenocarcinoma who were treated with chemotherapy plus trastuzumab compared with overall survival in patients who received conventional chemotherapy alone [19]. Therefore, HER2 overexpression in advanced gastric cancer may be important for the treatment and prognosis of this disease. However, the biological and clinical significance of HER2 status in early gastric cancer is unknown.

Overexpression of HER2 protein and amplification of the *HER2* gene occur mainly in the intestinal subtype of gastric cancer, as defined by Lauren's classification [11, 24]. Intestinal-type gastric cancers result from phenotypic dedifferentiation and accumulation of genetic dysregulations. These conditions are also triggered by gastritis and metaplastic changes [18]. In particular, intestinal metaplasia may develop into intraepithelial neoplasia and invasive adenocarcinoma [3, 6, 17]. Overexpression of p53, MYC, Cdx2, and hTERT has been suggested to be involved

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in the risk of malignant transformation in previous studies [2, 22, 25]. However, the molecular events underlying this malignant transformation are not fully understood. Our previous study has shown that *HER2* gene amplification can occur in early noninvasive gastric cancer, and we reported that such cases showed HER2 heterogeneity [10]. Thus, *HER2* gene amplification could be a nonlocalized private mutation that may occur in the early stage of gastric cancer.

In the present study, we examined the frequency and patterns of *HER2* gene amplification in early gastric cancer and evaluated whether HER2 status was associated with tumor progression.

Materials and methods

Case selection and tissue preparation

This study was approved by the Ethics committee of Mie University Hospital (Tsu, Japan, approval no. 2746). A total of 149 lesions from 141 consecutive patients with primary early gastric cancer who underwent endoscopic submucosal dissection (ESD) or endoscopic mucosal resection (EMR) at Mie University Hospital between 2009 and 2011 were enrolled in this study. All clinicopathological parameters, including patient age, sex, tumor size, macroscopic classification, existence of ulcer scars, histological classification, Vienna classification (Fig. 1) [20], T factor in TNM classification, and nuclear grade, were reviewed. The nuclear grade was classified as follows: grade 1, uniform nucleus size and shape; grade 2, nuclear atypia showing an intermediate state between grades 1 and 3; and grade 3, pleomorphic nuclei and enlarged nuclei of various sizes as well as prominent large nucleoli [9, 15]. Histological classification was determined according to Lauren's classification. All tissues were fixed with neutral buffered formalin for 24–48 h, routinely processed, and embedded in paraffin. The tumor tissue was cut from the most representative block of the lesion, avoiding areas with massive ulceration and necrosis. Serial tissue sections measuring 3 μm thick were used with hematoxylin and eosin staining, whereas sections measuring 4 μm thick were used for immunohistochemistry (IHC), and sections measuring 5 μm thick were used for dual color in situ hybridization (DISH).

IHC and DISH

IHC and DISH were performed as previously described [10]. Briefly, HER2 IHC was carried out using an automated slide stainer (Bench-Mark XT; Ventana Medical Systems, Tucson, AZ, USA), anti-HER2 antibodies (clone 4B5; Ventana Medical Systems), and an iView DAB detection kit (Ventana Medical Systems). In HER2 IHC scoring criteria [8, 10], an IHC score of 0 was given if there was no reactivity or membranous reactivity in more than 10% of tumor cells. An IHC score of 1+ was given if there was faint or barely detectable membranous reactivity in more than 10% of tumor cells, and stained tumor cells

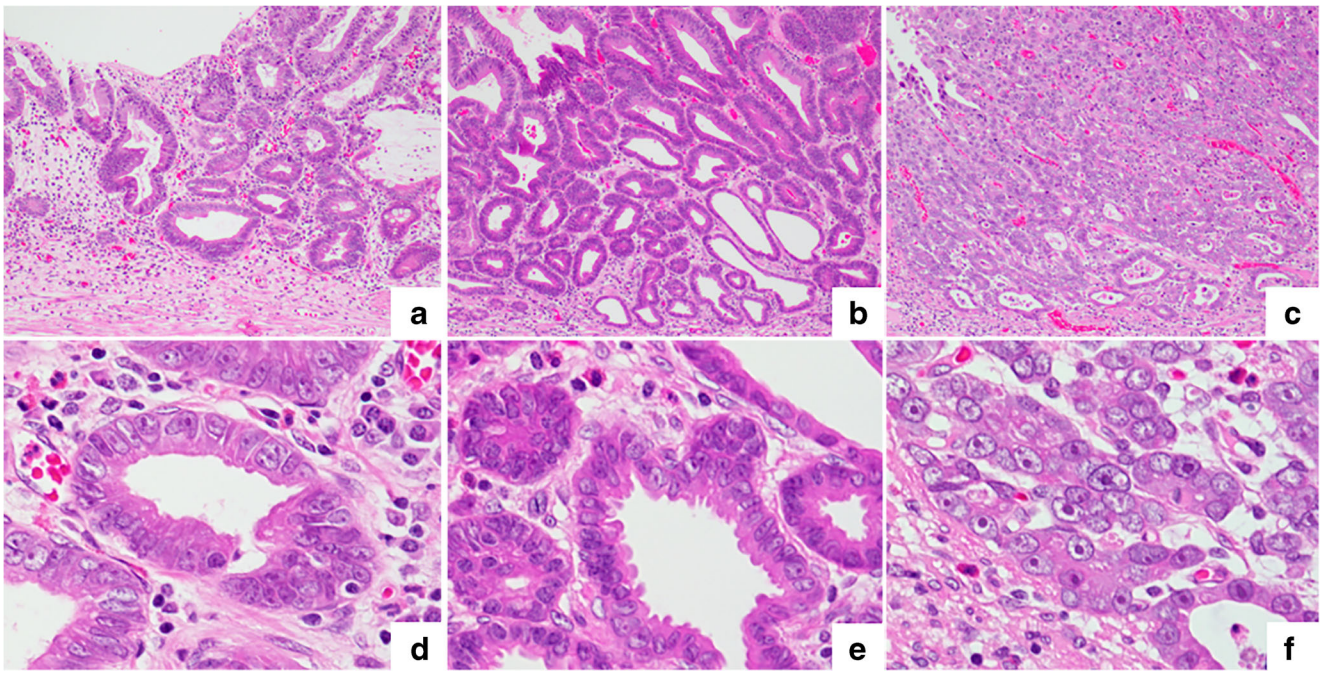

Fig. 1 Representative cases of the Vienna classification categories 4.2, 4.3, and 5.1. **a**–**c** HE (× 10). **d**, **e** HE (× 40). Category 4.2 is shown in panels **a** and **d** (case no. 122). The tumor cells were cuboidal with a high nucleus to cytoplasm ratio. The nuclei were round with prominent nucleoli, and nuclear polarity was lost, but no distinct structural abnormalities, such as glandular crowding and excessive branching, were observed. Category 4.3 had distinct structural abnormalities, such as glandular crowding and excessive branching, but no obvious invasion into the lamina propria (**b** and **e**, case no. 40). Category 5.1 showed obvious invasion into the lamina propria (**c** and **f**, case no. 46)

were reactive only in a part of the membrane. An IHC score of 2+ was given if weak to moderate complete or basolateral membranous reactivity was observed in more than 10% of tumor cells. An IHC score of 3+ was given if there was moderate to strong complete or basolateral membranous reactivity in more than 10% of tumor cells. HER2 positivity was defined as either an IHC score of 3+ or an IHC score 2+ with positive results for DISH. The intratumoral heterogeneity of HER2 was defined as samples with 10–60% of tumor cells showing HER2 positivity. In the current study, HER2 heterogeneity was defined as tumors mixed with HER2-negative tumor cells regardless of the percentage of HER2-positive tumor cells. A HER2 homogeneous pattern was defined as samples with all HER2-positive tumor cells.

DISH was carried out using an automated slide stainer. HER2 signals were detected using an INFORM Dual ISH HER2 kit (Ventana Medical Systems). The HER2/CEP17 ratio was determined by counting the HER2 signals and CEP17 signals in more than 40 nuclei for each tissue section. Amplification of the *HER2* gene was defined as a HER2/CEP17 ratio of higher than 2.2. Negativity for *HER2* gene amplification was defined as a HER2/CEP17 ratio of less than 1.8 [10]. When the HER2/CEP17 ratio was between 1.8 and 2.2, adding signals in another 20 nuclei was counted, and HER2/CEP17 ratios of 2.0 or higher were defined as positivity for *HER2* gene amplification. Because IHC scores 0 and 1+ did not represent *HER2* gene amplification in our previous study [10], DISH was performed in cases in which the IHC score was 2+ or 3+ in this study.

Statistical analysis

All statistical analyses were performed using Statcel 3. χ^2 tests, *t* tests, and Mann-Whitney tests were performed to assess the correlations between clinicopathological parameters and *HER2* gene amplification. All *P* values were two-sided. Results with *P* values of less than 0.05 were considered significant.

Results

Comparison of HER2 IHC and DISH in early gastric cancer

The results of HER2 IHC and DISH in the 149 lesions are shown in Table 1. *HER2* gene amplification was observed in 35 lesions (23.5%; 26 with an IHC score of 3+, and 9 with an IHC score of 2+) by DISH. Among the 35 lesions, HER2 heterogeneity and homogeneity were found in 26 lesions (74.3%) and 9 lesions (25.7%), respectively (Fig. 2).

Table 1 Comparison of IHC and DISH results for HER2 status in early gastric cancers

DISH	HER2 IHC score			Total
	0 or 1+ (%)	2+ (%)	3+ (%)	
Unamplified (< 1.8)	Not determined	19 (67.9)	1 (3.7)	114
Amplified (< 2.2)	Not determined	9 (32.1)	26 (96.3)	35
Total	94	28	27	149

Comparison of HER2 status and clinicopathological factors in patients with early gastric cancer

Comparisons of HER2 status, Vienna classifications, and T factors are shown in Table 2. *HER2* gene amplification was observed in 1 case of noninvasive carcinoma (Vienna classification category 4.2), 4 cases of suspicious of invasive carcinoma (Vienna classification category 4.3), 21 cases of intramucosal invasive carcinoma (Vienna classification category 5.1), and 9 cases of submucosal carcinoma or beyond (Vienna classification category 5.2). *HER2* gene amplification tended to correlate with the Vienna classification ($P = 0.0536$) and showed a significant correlation with the T factor ($P = 0.0290$). However, there was no significant association between *HER2* gene amplification and musclaris mucosae or submucosal invasion (Supplementary Table 1 and Supplementary Fig. 1). In addition, *HER2* gene amplification tended to correlate with tumor size ($P = 0.0533$) and Lauren's classification ($P = 0.0821$) but was not associated with age, sex, macroscopic classification, papillary structure, ulcer scars, or nuclear grade ($P > 0.05$; Table 3).

Comparisons of *HER2* amplification patterns, Vienna classifications, and T factors showed that HER2 heterogeneity was already identified in noninvasive carcinomas (Vienna classification category: 4.2), whereas HER2 homogeneity was observed in intramucosal invasive carcinomas (Vienna classification category: 5.1; Table 4). The frequencies of HER2 homogeneity in categories 5.1 and 5.2 were 33.3% (7/21) and 22.2% (2/9), respectively. In both categories, the frequency of HER2 heterogeneity was higher than that of HER2 homogeneity. There were no significant associations between *HER2* amplification patterns and tumor size, musclaris mucosae, or submucosal invasion (Supplementary Table 2 and Supplementary Figs. 2, 3).

Discussion

Several studies have investigated *HER2* gene amplification in patients with advanced gastric cancers [7, 8, 13]; however, few have examined HER2 dysregulation in gastric intraepithelial neoplasia (IEN) and early cancer. Lee et al. [13] reported that *HER2* gene amplification was observed in 4.3% of high-grade

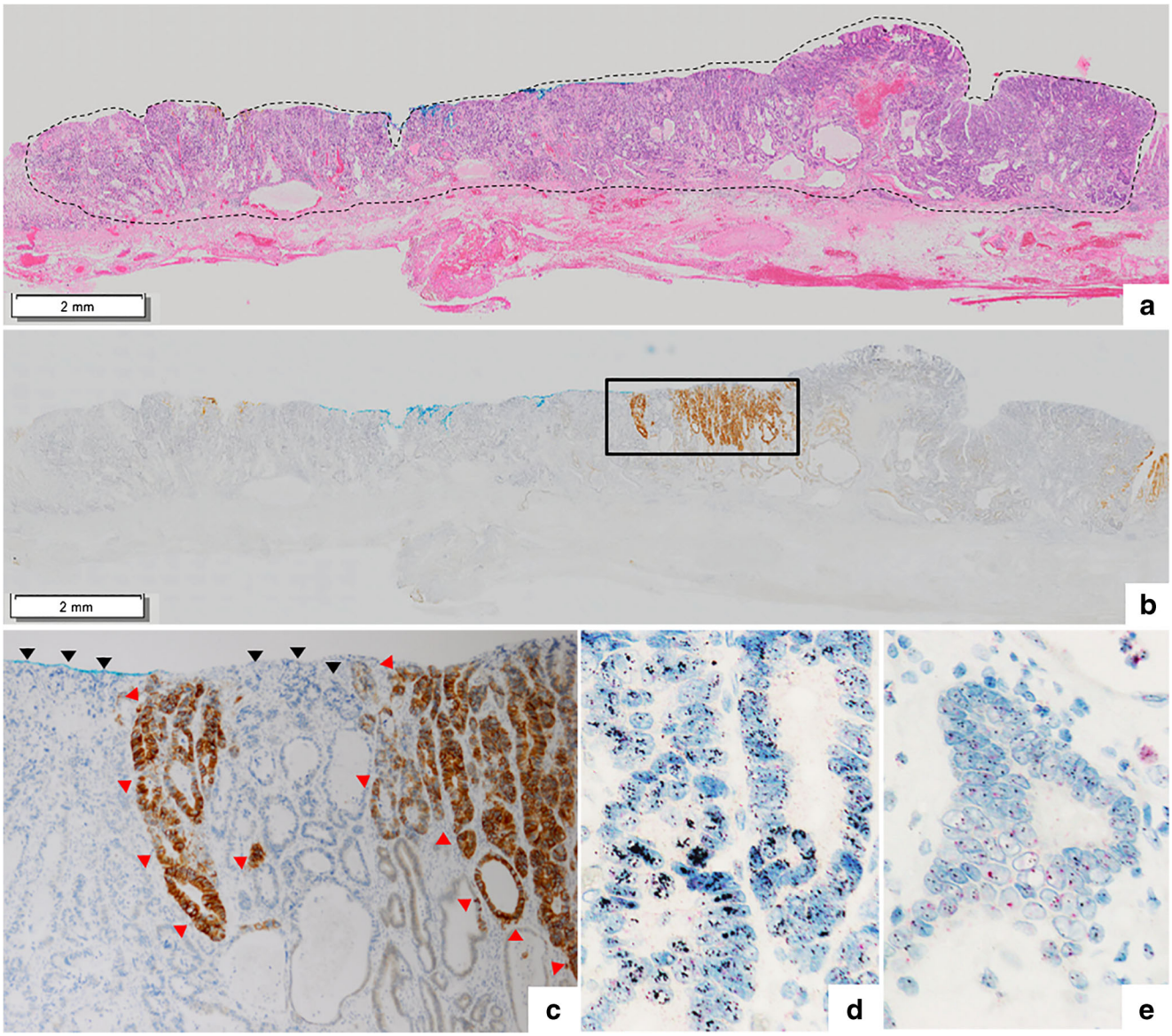

Fig. 2 A representative case of early gastric cancer showing heterogeneous *HER2* amplification (Vienna classification category 5.1; case no. 123). **a** HE (whole section). **b** HER2 IHC (whole section). **c** HER2 IHC (× 10). **d**, **e** HER2 DISH (× 40). The circled region with a dotted line in panel **a** represents a tumor site. *HER2* gene amplification is shown in regions with black rectangles in panel **b**, and red arrow heads in panel **c**. The red arrow heads correlate with amplified areas in panel **d**. The black arrow heads also correlate with nonamplified areas in panel **e**. Black dots and red dots in the nuclei are the *HER2* gene and chromosome 17 centromere in panels **d** and **e**, respectively

IEN. Additionally, Fassan et al. [5] reported that *HER2* gene amplification was found in 4.0% of low-grade IEN and 16.0% of high-grade IEN. In our study, *HER2* gene amplification was detected in 23.5% of early gastric cancer, and there was good concordance between IHC and DISH (Table 1). *HER2* gene amplification was found in noninvasive carcinoma (Table 2), whereas 25 cases of gastric adenoma HER2 amplification were not observed (Vienna classification category: 4.1; data not shown). These findings suggested that *HER2* gene amplification occurred during the early stages of gastric cancer and could be involved in malignant transformation.

In breast cancer, HER2 overexpression in ductal carcinoma in situ has been reported to be associated with the risk of cancer progression to invasive ductal adenocarcinoma [1, 16]. Fassan et al. [5] reported that *HER2* gene amplification increased significantly from low-grade IEN to high-grade IEN and advanced gastric cancer. In our study, *HER2* gene amplification showed an increasing trend from category 4.2 to 5.2 in Vienna classification and a significant correlation with T factor (Table 2). These findings suggested that *HER2* gene amplification may be related to tumor progression in early gastric cancer. Furthermore, we found a higher proportion of HER2 homogeneity in advanced

Table 2 Comparison of HER2 status, Vienna classification, and T factor

	HER2 status		*P* value
	Unamplified (%) (*n* = 114)	Amplified (%) (*n* = 35)	
Vienna classification			0.0536
Category 4.2	15 (93.7)	1 (6.3)	
Category 4.3	20 (83.3)	4 (16.7)	
Category 5.1	67 (76.1)	21 (23.9)	
Category 5.2	12 (57.1)	9 (42.9)	
T factor			0.0290
pTis	35 (87.5)	5 (12.5)	
pT1a	67 (76.1)	21 (23.9)	
pT1b	12 (57.1)	9 (42.9)	

P values were calculated using χ^2 tests

Table 3 Comparison of HER2 status and clinicopathological factors in early gastric cancers

	HER2 status		*P* value
	Unamplified (%) (*n* = 114)	Amplified (%) (*n* = 35)	
Age (years)[a]	70.9	78.9	0.5382[b]
Sex			0.2504
Male	79 (74.5)	27 (25.5)	
Female	35 (81.4)	8 (18.6)	
Macroscopic classification			0.2815
I	4 (100)	0 (0)	
IIa	22 (75.9)	7 (24.1)	
IIb	12 (92.3)	1 (7.7)	
IIc	72 (72.7)	27 (27.3)	
IIa + IIc	4 (100)	0 (0)	
Tumor size (mm^2) [a]	309.2	482.2	0.0533[c]
Lauren's classification			0.0821
Intestinal type	100 (74.6)	34 (25.4)	
Diffuse/mixed type	14 (93.3)	1 (6.7)	
Papillary structure			0.3999
Absent	107 (75.9)	34 (24.1)	
Present	7 (87.5)	1 (12.5)	
UL			0.3165
Absent	94 (77.7)	27 (22.3)	
Present	20 (71.4)	8 (28.6)	
Nuclear grade			0.2116
1	26 (86.7)	4 (13.3)	
2	69 (74.2)	24 (25.8)	
3	19 (73.1)	7 (26.9)	

P values were calculated using χ^2 tests, *t* tests, and Mann-Whitney tests for categorical variables. Percentages show the ratios of HER2 unamplified or amplified patients for each item

HER2 human epidermal growth factor receptor 2

[a] Age and tumor size were reported as means

[b] *t* tests

[c] Mann-Whitney tests

Table 4 Comparison of *HER* amplification patterns, Vienna classification, and T factor in *HER2*-positive early gastric cancers

	HER2 amplification pattern		*P* value
	Homogeneous (%) (*n* = 9)	Heterogeneous (%) (*n* = 26)	
Vienna classification			0.4887
Category 4.2	0 (0)	1 (100)	
Category 4.3	0 (0)	4 (100)	
Category 5.1	7 (33.3)	14 (66.7)	
Category 5.2	2 (22.2)	7 (77.8)	
T factor			0.2972
pTis	0 (0)	5 (100)	
pT1a	7 (33.3)	14 (66.7)	
pT1b	2 (22.2)	7 (77.8)	

P values were calculated using χ^2 tests

gastric cancers than early gastric cancers in comparison with our previous study (Supplementary Table 3). HER2 homogeneity in early gastric cancer could be associated with the risk of tumor progression to advanced gastric cancer. In contrast, HER2 heterogeneity decreased in advanced gastric cancer. This finding suggests that HER2 heterogeneity may be changed to HER2 homogeneity by dominant proliferation of *HER2* gene amplification tumor cells. However, because of the concordances of HER2 heterogeneity or homogeneity in both invasive and intramucosal lesions in each case, as observed in our previous study [10], the possibility that HER2 heterogeneity may change into homogeneity is considered to be low. In cases of HER2 heterogeneity, some HER2-positive cell subpopulations may not be directly involved in tumor progression. However, there was no significant difference in *HER2* amplification patterns between early gastric cancers and advanced gastric cancers (Supplementary Table 3). In order to clarify the risk of cancer progression to advanced cancer with regard to HER2 homogeneity and heterogeneity in early gastric cancer, further large-scale studies are necessary.

The mechanism of HER2 heterogeneity is poorly understood. Seol et al. [21] reported that HER2 heterogeneity is caused by chromosomal instability. In a recent study of intratumoral heterogeneity, both public and private mutations arise at an early stage and become pervasive during tumor growth [14, 23]. Additionally, minor cell subpopulations in intratumoral heterogeneity can drive tumor growth and contribute to treatment resistance [4]. Kurozumi et al. [12] reported that patients with triple negative breast cancer (TNBC) showing HER2 intratumoral heterogeneity had significantly worse survival than patients with TNBC without HER2 heterogeneity. In our study, HER2 heterogeneity was observed in noninvasive carcinoma (Table 4). In addition, the frequencies of HER2 heterogeneity in categories 5.1 and 5.2 were higher

than HER2 homogeneity (Table 4). These findings suggested that HER2 heterogeneity was formed by a subpopulation of cells with *HER2* gene amplification appearing at an early stage in gastric cancer and may be developed while maintaining HER2 heterogeneity. Moreover, HER2-positive cells in the tumor were present at a low ratio, but cases with large tumor sizes and submucosal invasion were included (Supplementary Fig. 2B and 3B). In such cases, HER2-positive minor cell subpopulations may be related to tumor growth and tumor progression. The molecular mechanisms of tumor progression may differ between HER2 homogeneity and heterogeneity, and further studies are needed to elucidate these mechanisms.

In summary, *HER2* gene amplification was observed in 35 of 149 (23.5%) lesions in patients with early gastric cancer and showed intratumoral heterogeneity in a number of cases. Our study demonstrated that *HER2* gene amplification occurred in the early stages of gastric cancer. Thus, *HER2* gene amplification may be involved in tumor progression in early gastric cancer.

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Compliance with ethical standards

This study was approved by the ethics committee of Mie University Hospital (Tsu, Japan, approval no. 2746).

Conflict of interest The authors declare that they have no conflicts of interest.

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