Significance of Tumor Heterogeneity of p-Smad2 and c-Met in HER2-positive Gastric Carcinoma with Lymph Node Metastasis

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Abstract

Background: Tumor heterogeneity has frequently been observed in gastric cancer (GC), but the correlation between patients' clinico-pathologic features and the tumoral heterogeneity of GC-associated molecules has not been clarified. We investigated the correlation between lymph node metastasis and the tumoral heterogeneity of driver molecules in GC.

Materials and Methods: We retrospectively analyzed the case of 504 patients who underwent a gastrectomy at the department of Gastroenterological Surgery Osaka City University and 389 cases of The Cancer Genome Atlas (TGCA) data. The clustering analysis was performed based on eight cancer-associated molecules including HER2, c-Met, and p-Smad2, using the protein expression revealed by our immunohistochemical study of the patients and TGCA cases. We determined the correlations between HER2 expression and the other molecules based on the degree of lymph node metastasis.

Results: Immunohistochemical staining data showed that a 43 of the 504 patients with GC (8.5%) were HER2-positive. In the HER2-positive cases, the expressions of c-Met and p-Smad2 were increased in accord with the lymph-node metastatic level. The overall survival of the HER2-positive GC patients with both p-Smad2 and c-Met expression was significantly (p=0.030) poorer than that of the patients with p-Smad2-negative and/or c-Met-negative expression. The results of the TCGA data analysis revealed that 58 of the 389 GC cases (14.9%) were ERBB2-positive. MET expression was more frequent in the N1 metastasis group than the N0 group. In the high lymph-node metastasis (N2 and N3) group, SMAD2 expression was more frequent in addition to ERBB2 and MET expression.

Conclusion: p-Smad2 and c-Met signaling might play important roles in lymph node metastasis in HER2-positive GC.

Background

Tumor heterogeneity of cancer cells is frequently present in various types of cancer and has been reported to be associated with therapeutic issues including chemo-resistance and metastasis[1-5]. Tumor heterogeneity has been observed both between tumors as inter-tumoral heterogeneity and within tumors as intra-tumor heterogeneity. The intra-tumoral heterogeneity of cancer-associated molecules might play an important role in metastasis [6-9]. However, the correlation between intra-tumoral heterogeneity patterns and the process of metastasis has not been determined. A clarification of intra-tumoral heterogeneity patterns may be useful, as this may help explain the manner of metastasis and contribute to the selection of therapeutic targets.

We previously demonstrated the clinicopathological significance of several cancer-associated molecules including fibroblast growth factor receptor 2 (FGFR2), transforming growth factor-beta 1 (TGFβ1), C-X-C chemokine receptor 2 (CXCR2), CXCR4, and phospho-Smad2 expressed at primary gastric tumors [10-14]. HER2 and c-Met are also known as driver protein of gastric cancer. The clarification of the correlation between tumoral heterogeneity of these molecules and the lymph node metastasis may contribute to

develop of new treatment strategies. Then we conducted the present study to (1) examine the correlation between the molecular intra-tumoral heterogeneity and lymph node metastasis in gastric tumors in order to clarify one of the mechanisms responsible for lymph node metastasis, and (2) investigating potential therapeutic targets against lymph node metastasis.

Materials And Methods

Patients

A total of 504 gastric cancer patients who underwent gastrectomy at the department of Gastroenterological Surgery Osaka City University between 2001 and 2006 was enrolled. Also, a total 389 cases of The Cancer Genome Atlas (TCGA) data were added in this study. The pathologic diagnoses and classifications were made according to the UICC TNM classification of malignant tumors.

Immunohistochemical staining

The immunohistochemical determination of the tumor-associated molecules expression in gastric tumors was performed as follows. Bond Oracle™ HER2 IHC System (Leica Biosystems, Newcastle Upon Tyne, UK) was used for HER2 staining, according to the manufacturer's instructions. HER2 expression were considered positive when intensity scores were ≥ 2 and proportion score were 10%. Immunohistochemical staining for CXCR2 (1:50; R&D Systems, Minneapolis, MN, USA), CXCR4 (1:100; Abcam, Cambridge, MA, USA), and FGFR2□b (1:333; Cell Signaling, Danvers, MA, USA) was performed as previously reported [10, 12, 13]. Immunohistochemical staining for c-Met (1:200; Santa Cruz Biotechnology, Dallas, TX, USA), IGF1R (Insulin-like growth factor 1 receptor) (1:500; Abcam, Cambridge, MA, USA), p-smad2 (1:2000; Chemicon International, Temecula, CA, USA), and TGFβ1 (1:100; Lab vision, Fremont, CA, USA) was performed as follows. Shortly, we performed deparaffinization and slides were heated. After blocking endogenous peroxidase activity, the sample were incubated with each antibody for 1 hour at room temperature. The sample were incubated with biotinylated second antibody. The samples were treated with streptavidin-peroxidase reagent, and counterstaining with Mayer’s hematoxylin. The expression level was analyzed by both intensity of staining and percentage of stained cancer cells at the invading tumor front. Evaluation was made by two double-blinded independent observers who were unaware of clinical data and outcome. When a different evaluation between two independent observers was found, the evaluation was rechecked and discussed. The p-smad2 and c-Met expression were considered positive when intensity cores were ≥ 2 and proportion score were ≥ 40%.

Clustering analysis of protein and gene expressions

The clustering analysis based on protein expression and gene expression of 8 molecules, including HER2 (*ERBB2*), c-Met (*MET*), CXCR2 (*CXCR2*), CXCR4 (*CXCR4*), FGFR2□b (*FGFR2*), IGF1R (*IGF1R*), TGFβ1 (*TGFB1*), p-smad2 (*SMAD2*) were conducted. The correlations among these molecules were analyzed according to the degree of lymph node metastasis. The mRNA expression Z-score of genes (RNA-Seq V2

RSEM normalized, RNA-Seq data) were obtained from 389 cases of TCGA stomach adenocarcinoma dataset (PanCancer Atlas) through cBioPortal [15]. Z-scores were used for clustering analysis.

Protein expression for clustering analysis

Protein expression was evaluated with the intensity of staining and percentage of stained tumor cells. Intensity was scored 0-3 (0: negative, 1: weak, 2: moderate, and 3: intense immunoreactivity), and proportion was scored 0-100%. When the intensity was scored 2-3, the proportion score (%) were used for final score. When the intensity was scored 0-1, final score was scored 0. The final scores were used for clustering analysis. Evaluation was made by double blinded independent observers who were unaware of clinical data and histologic diagnoses.

Statistical analysis

Comparative analyses of the clinicopathologic features of gastric cancer and HER2 expression between with and without p-Smad2 and c-Met were performed using the chi-squared test or Fisher's exact test. The survival durations were calculated using the Kaplan-Meier method and analyzed by the log-rank test to compare the cumulative survival durations in the patient group. JMP 13 software (SAS Institute Japan, Tokyo, Japan) was used for the analyses.

Results

HER2, p-smad2, and c-Met expression on gastric cancer

Figure 1 shows representative intra-tumoral heterogeneity of HER2 expression in a primary gastric tumor. Well-differentiated gastric cancer cells showed HER2 overexpression, whereas poorly-differentiated cancer cells were HER2-negative. Different HER2 expression levels were also observed among the well-differentiated cancer cells. **Figure 2** shows a HER2-positive gastric tumor with lymph node metastasis. HER2 heterogeneity was detected in the primary tumor, and p-Smad2 and c-Met were expressed in both HER2-negative and HER2-positive areas. In metastatic lymph nodes, the presence of both p-Smad2 expression and c-Met expression revealed heterogeneity of HER2 expression, the same as in the primary tumor **(Fig. 2B,C)**.

Clustering analysis of primary tumor *in silico* TCGA data or immunohistochemical staining data

We performed a clustering analysis of the primary tumors by using *in silico* data from The Cancer Genome Atlas (TCGA) and immunohistochemical staining data based on the degree of lymph node metastasis. We divided the TCGA data regarding gastric cancer into clusters **(Fig. 3)**. The gastric cancer group with N0 or N1 metastasis formed clusters with high expressions of *ERBB2* mRNA and *MET* mRNA. In the cluster of *MET* and *ERBB2* co-expressed, the average Z-scores of *MET* m-RNA expression was 1.24 in the N0 group and 1.92 in the N1 group. Moreover, in the distant lymph-node metastatic group of N2 and N3 metastasis, *SMAD2* expression was frequently observed in addition to high *ERBB2* and high

MET expression (Z-scores of *SMAD2* m-RNA expression was 1.98 in the cluster of *MET, ERBB2* and *SMAD2* co-expressed).

We also divided the immunohistochemical staining data of the primary tumors into clusters based on the protein expression (**Fig. 4**). In the N0 group, c-Met expression and p-Smad2 expression were relatively low in HER2-over expressed cluster (the average of protein expression score of c-Met and p-Smad2 were 23.1 and 43.1). In contrast, in the N2/N3 group, high c-Met expression, and high p-Smad2 expression were frequently identified in HER2-over expressed cluster (the average of protein expression score of c-Met and p-Smad2 were 54.3 and 51.1) which is consistent with results of the *in silico* analysis of TCGA data.

Relationship between the clinicopathologic features of gastric cancer and HER2 expression with or without p-Smad2 and c-Met

The details of the relationships among HER2 expression, p-Smad2 expression, and c-Met expression are provided in **Table 1**. A total of 43 of the 504 (9.53%) cases of primary GC were HER2-positive. In the HER2-positive gastric cancer cases, the co-expression of p-Smad2/c-Met was significantly correlated with the pathological stage (p=0.042) and tended to be correlated with the T stage (p=0.104) and N stage (p=0.098). In the cases of HER2-negative gastric cancer, there was a significant correlation between the co-expression of p-Smad2 and c-Met and the microscopic type (p=0.006).

Survival

In the HER2-positive gastric cancers, p-Smad2 and c-Met expression was associated with a poorer outcome. The overall survival (OS) of the HER2-positive gastric cancer patients with p-Smad2 and c-Met expression (n=13) was significantly poorer than that of the patients with p-Smad2-negative and/or c-Met-negative expression (n=30) (p=0.030) (**Fig. 5A**). On the other hand, there was no significant difference on the OS of the HER2-negative gastric cancer patients with both p-Smad2 and c-Met expression (n=79) and the patients with p-Smad2-negative and/or c-Met-negative expression (n=382) (p=0.478) (**Fig.** 5B).

Discussion

HER2 overexpression is found in about 15% of gastric cancers, and HER2 is the only predictive biomarker of response to targeted therapy in gastric cancer. The HER2 expression pattern in the primary tumor was dependent on the differentiation of the cancer cells. Most of the well-differentiated cancer cells showed HER2-positive expression, and most of the poorly-differentiated cancer cells showed HER2-negative expression, which is similar to previous findings [16-18]. In the present cases of HER2-positive GC, the prognosis of the patients with both p-Smad2-positive and cMet-positive gastric cancer was significantly poorer than another cases. The results of the cluster analysis also indicated that in HER2-positive gastric cancer, the co-expression of p-Smad2 and c-Met was related to lymph node metastasis. These findings suggest that HER2-positive gastric tumors co-expressing p-Smad2 and c-Met might have higher malignant potential than those with either negative expression.

Not only the inter-patient heterogeneity of HER2-positive cases but also the inter-tumoral heterogeneity of HER2 was also frequently observed in the HER2-positive tumors. In the HER2-positive gastric tumors, the expression status of both p-Smad2 and c-Met was positive in both the primary tumor and metastatic lymph nodes, and the p-Smad2 and c-Met expression levels in the metastatic lymph nodes tended to be higher than those in the primary tumors. These results suggest that the expression of c-Met and that of p-Smad2 might be correlated with lymph node metastasis in HER2-positive gastric cancer. The heterogeneity may provide the opportunity for clonal evolution, and the intra-tumoral heterogeneous clonal evolution of signaling such as p-Smad2 and c-Met might be associated with the acquisition of lymph-node metastatic ability in gastric cancer cells. These findings may contribute to the development of new molecular target therapy against lymph-node metastasis in patients with HER2-positive gastric cancer.

It has been reported that c-Met is frequently overexpressed in HER2-positive breast cancer cells and that c-Met contributes to trastuzumab resistance in HER2-positive breast cancer [19]. The overexpression of c-Met was observed in HER2-positive gastric cancer patients [20, 21]. *MET* amplification has been proposed as a resistance mechanism against HER2 therapy in gastric cancer [22, 23]. This resistance mechanism may be caused by the activation of MET RTK and which restores downstream signaling pathways such as MAPK and AKT [22]. These findings suggest that a combination of a HER2-inhibitor and a c-Met-inhibitor might exert a greater effect than a HER2-inhibitor alone in HER2-positive gastric cancer patients with lymph node metastasis.

Smad-2 is phosphorylated via TGFβ1/TGFβ1 receptor signaling, which acts in tumor progression[14, 24, 25]. It has been reported that HER2 and the TGFβ1-SMAD pathway are correlated with each other and that there are synergistic effects of TGFβ and HER2 in the progression of breast cancer [26, 27]. It was also reported that HER2 activated the tumor progressive effect by TGFβ1 [26, 28]. Several mechanisms that HER2 regulates TGFβ signaling have been reported. ERK, p38 MAPK and PI3K/AKT have been suggested to mediate the TGFβ–induced migration and invasion in HER2-positive breast cancer[29]. These findings suggest that the co-expression of HER2 and p-Smad2 signaling might promote tumor progression and lead to distant lymph node metastasis at the N2 and N3 levels.

In our cluster analysis of the TCGA data of gastric cancer patients with N2 and N3 metastasis, the cases with high *ERBB2* expression formed clusters with high *SMAD2* expression, but not with the cases with a high expression of *TGFB1*, which is an upstream cytokine of *SMAD2* signaling. We evaluated the expression of TGFβ1 in cancer cells alone in the immunohistochemical staining, and the TCGA data included the information from both cancer cells and tumor stromal cells. One of the reasons for the discrepancy between the TCGA data and the immunohistochemical data regarding *SMAD2* expression and TGFβ1 expression might be due to the difference in the evaluated cells between the TCGA and immunohistochemical data.

In conclusion, the intra-tumoral heterogeneity of HER2 was frequently present in HER2-positive gastric cancer. p-Smad2 and c-Met signaling might play important roles in lymph node metastasis in HER2-

positive gastric cancer. The use of a c-Met inhibitor or a p-Smad2 inhibitor in combination a HER2 inhibitor might be promising against lymph node metastasis in patients with HER2-positive gastric cancer. In HER2-positive gastric cancer, the expression of p-Smad2 and c-Met might be predicting marker for prognosis and targeting therapy.

Declarations

Ethics approval and concept to participate

The present study was approved by the Medical Ethics Committee of Osaka City University (approval no. 924).

Patients provided written informed consent, and ethical approval was obtained from the institutional review boards of Osaka City University (reference number 912). This retrospective study was conducted in accordance with the principles of the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The data supporting these findings during the current study are not publicly available since data contain potentially sensitive information. However, this data could be available from the corresponding author or the Ethics Committee (ethics@med.osaka-cu.ac.jp) on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

G.T. and M.Y. designed and performed the experiments and co-wrote the manuscript; G.T. and M.Y. contributed equally; K.M., Y.Y., T.S., A.S., H.K., Y.M., M.Y., T.T., T.T., H.T., K.M., collected tumor specimens and contributed to the in vitro experiments; M.O. suggested and co-designed the study. All authors read and approved the final manuscript.

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Tables

Table 1. Association between clinicopathologic factors of 504 gastric cancers and p-Smad2 and/or c-Met expression in accordance to HER2 expression.

Clinico-pathologic features	HER2-positive cases (n=43)			HER2-negative cases (n=461)		
	Both p-smad2 and c-Met positive (n=13)	Either p-smad2 or c-Met negative (n=30)	p-value	Both p-smad2 and c-Met positive (n=79)	Either p-smad2 or c-Met negative (n=382)	p-value
Age						
<60	4 (33.3%)	8 (66.7%)		22 (16.1%)	115 (83.9%)	
≧60	9 (29.0%)	22 (71.0%)	1.000	57 (17.6%)	267 (82.4%)	0.690
Macroscopic type						
Bormann type 4	1 (50%)	1 (50%)		7 (13.2%)	46 (86.8%)	
Other types	12 (29.3%)	29 (70.7%)	0.518	72 (17.7%)	336 (82.3%)	0.420
Microscopic type						
Differentiated	11 (32.4%)	23 (67.6%)		48 (22.3%)	167 (77.7%)	
Undifferentiated	2 (22.2%)	7 (77.8%)	0.699	31 (12.6%)	215 (87.4%)	0.006
T stage						
T1/T2	5 (20%)	20 (80%)		40 (15.7%)	215 (84.3%)	
T3/T4	8 (44.4%)	10 (55.6%)	0.104	39 (18.9%)	167 (81.1%)	0.358
Lymph node metastasis						
Negative	3 (15.8%)	16 (84.2%)		37 (14.5%)	218 (85.5%)	
Positive	10 (41.7%)	14 (58.3%)	0.098	32 (20.4%)	164 (79.6%)	0.096
Metastasis						
Negative	12 (28.6%)	30 (71.4%)		78 (17.4%)	371 (82.6%)	
Positive	1 (100%)	0 (0%)	0.302	1 (8.3%)	11 (91.7%)	0.700

Pathological stage						
I/II	5 (18.5%)	22 (81.5%)		50 (17.0%)	245 (83.0%)	
III/IV	8 (50%)	8 (50%)	0.042	29 (17.5%)	137 (82.5%)	0.887

Figures

Figure 1

A representative gastric cancer case with intra-tumoral heterogeneity. A: Hematoxylin and eosin (H&E) staining. Histologic intra-tumoral heterogeneity is shown. *Arrows:* poorly-differentiated cancer cells, *arrowheads:* well-differentiated cancer cells. **B:** Intra-tumoral heterogeneity of HER2. *Arrowheads:* HER2-positive, *arrows:* HER2-negative. In this case, the HER2 expression was dependent on the differentiation level of the gastric cancer. The well-differentiated gastric cancer cells showed HER2-positive expression, while the poorly-differentiated cancer cells were HER2-negative (A-1, A-2, B-1, B-2). The well-differentiated cancer cells showed different HER2-positive expression levels (A-3, B-3).

Figure 2

HER2-positive gastric cancer with lymph node metastasis. A: In the primary tumor, the HER2 expression presented intra-tumoral heterogeneity. The p-Smad2 expression and c-Met expression were both high regardless of the HER2 expression **B,C:** In this metastatic lymph node, HER2 expression presented intra-tumoral heterogeneity that was similar to that of the primary tumor. The expressions of both p-Smad2 and c-Met were high regardless of the HER2 expression, as in the primary tumor.

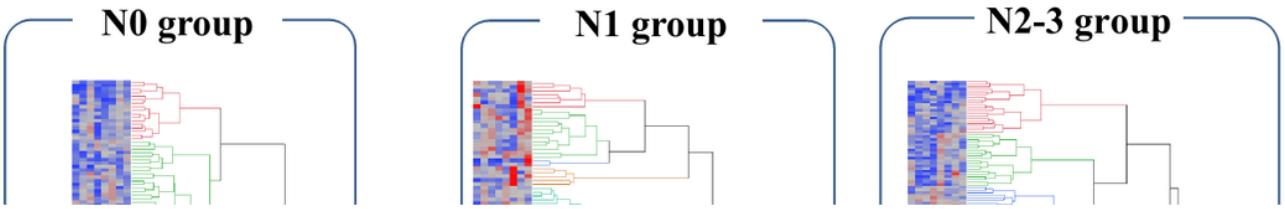

Figure 3

Clustering analysis of primary tumors by TCGA data with each degree of lymph node metastasis. A: In the N0 group, the *MET* amplification cases formed a cluster within the cluster with *ERBB2* amplification. **B:** In the N1 group, the *MET* amplification cases formed a cluster within the cluster showing *ERBB2* amplification. The expression level in the cluster showing *ERBB2* amplification was higher than that in the N0 cases. **C:** The *SMAD2* amplification cases formed a cluster within the cluster showing *ERBB2* amplification, as did the *MET* amplification cases.

Figure 4

Clustering analysis of primary tumors based on the protein expression with each degree of lymph node metastasis. A: In the clusters with high HER2 expression, the expression levels of c-Met and p-Smad2 were not very high. **B:** In the clusters with high HER2 expression, the expressions of c-Met and p-Smad2 were high. **C:** In the clusters with high HER2 expression, the c-Met and p-Smad2 expressions were high.

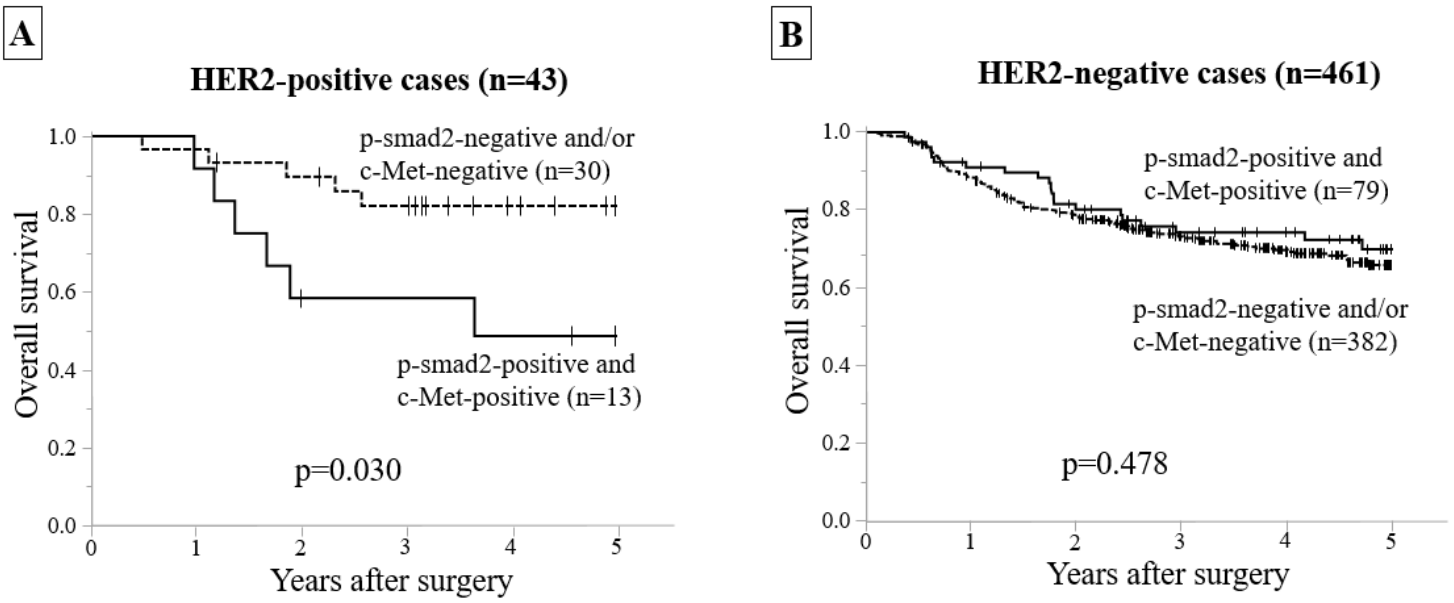

Figure 5

Survival curves. A: The overall survival (OS) of the HER2-positive gastric cancer patients with a p-Smad2-positive and c-Met positive tumor was significantly poorer (p=0.030) than that of the patients with a p-Smad2-negative and/or c-Met-negative tumor. **B:** There was no significant difference (p=0.478) in the OS of the HER2-negative gastric cancer patients with a p-Smad2-positive and c-Met positive tumor and that of the patients with a p-Smad2-negative and/or c-Met-negative tumor.

Supplementary Files

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