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A SINGLE NUCLEOTIDE POLYMORPHISM IN THE TRANSMEMBRANE DOMAIN CODING REGION OF *HER-2* IS ASSOCIATED WITH DEVELOPMENT AND MALIGNANT PHENOTYPE OF GASTRIC CANCER

Kazuya KURAOKA[1], Shunji MATSUMURA[1], Yoichi HAMAI[1], Kei NAKACHI[2], Kazue IMAI[2], Keisuke MATSUSAKI[3], Naohide OUE[1], Reiko ITO[1], Hirofumi NAKAYAMA[1] and Wataru YASUI[1]*

[1]*Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan*
[2]*Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, Hiroshima, Japan*
[3]*Department of Surgery, Hofu Institute of Gastroenterology, Yamaguchi, Japan*

Alterations of the *HER-2 (erbB-2/neu)* proto-oncogene have been associated with carcinogenesis and poor prognosis of certain cancers. A single nucleotide polymorphism (*Ile/Val,* A/G) in the transmembrane domain was reported to be associated with a risk of breast cancer. In our study, we examined the association between the *HER-2* polymorphism and gastric carcinoma. The *Ile/Ile, Ile/Val* and *Val/Val* genotypes were found in 146 (68.9%), 56 (26.4%) and 10 (4.7%) of 212 gastric cancer patients and in 234 (81.5%), 48 (16.7%) and 5 (1.8%) of 287 control subjects, respectively. The *Ile/Val* or *Val/Val* genotype was significantly more frequent in patients than in controls ($p = 0.005$ and 0.033, respectively). The OR of *Val/Val* genotype then revealed a significantly enhanced risk of 3.25 (95% CI 1.09–9.70) compared to *Ile/Ile* genotype; heterozygous *Ile/Val* genotype showed an intermediate risk of 1.97 (1.27–3.06). In patients, carcinomas of advanced stage were significantly more frequent in patients with *Ile/Val* or *Val/Val* genotype than those with *Ile/Ile* genotype ($p < 0.001$). The logistic regression analysis for tumor invasion, lymph node metastasis and distant metastasis revealed that lymph node metastasis was most closely associated with the *HER-2* genotype. These results suggest that this nucleotide polymorphism in the transmembrane domain-coding region of *HER-2* could be associated with development of gastric carcinoma and may serve as a predictor of risk for a malignant phenotype of gastric cancer. The association of *HER-2* genotype with clinicopathologic characteristics of gastric cancer was also suggested, which has to be confirmed with a larger sample size.

Key words: *single nucleotide polymorphism;* HER-2; *transmembrane domain coding region; gastric carcinoma; case-control study*

HER-2 (also known as *erbB-2* or *neu*) proto-oncogene, a member of the epidermal growth factor (EGF) receptor family, located at chromosome 17q21, encodes a transmembrane glycoprotein (p185) with tyrosine kinase activity.[1–5] Dimerization of the HER-2 leads to tyrosine kinase activation and subsequent downstream signaling events.[6–9] Amplification of *HER-2* gene has been found in some human cancers including carcinomas of the breast, ovary and stomach.[10,11] In breast cancer, *HER-2* amplification and/or overexpression has been associated with steroid hormone receptor-negative tumors, increased tumor aneuploidy, high growth rate, reduced response to chemotherapy and hormonal therapy and poor prognosis.[12] In gastric cancer, overexpression of HER-2 caused by amplification has been closely related to liver metastasis and poor prognosis.[13,14] HER-2 is now being paid more attention because recently, trastuzumab, a humanized murine monoclonal antibody directed against the extracellular domain of HER-2, was introduced for the treatment of patients with HER-2-overexpressing advanced breast cancer.[15–17] Point mutations in the *HER-2* gene have not been identified,[18–20] and a major mechanism of HER-2 activation is thought to be gene amplification.

Single nucleotide polymorphism (SNP) in the transmembrane coding region of the *HER-2* gene at codon 655, encoding either isoleucine (Ile: ATC) or valine (Val: GTC), has been identified.[21] Xie *et al.*[22] first reported that this *Ile/Val* SNP is associated with significantly increased risk of breast cancer development. However, several studies have shown that this association is controversial. Positive correlation between the *Ile/Val* SNP and breast cancer risk was reported to be associated with stage of disease,[23] whereas no association has been found in breast cancer among the British,[24] German[25] and Japanese[26] populations. In colorectal cancer, *Ile/Val* SNP was not associated with cancer risk in Caucasians.[27] The mechanistic role of this SNP in possible involvement of tumorigenesis has not been fully understood. Fleishmann *et al.*[28] recently reported that the *Val* allele enhanced active dimeric conformations of HER-2, resulting in increased autophosphorylation, tyrosine kinase activation and cell transformation, even under conditions of HER-2 overexpression. Although a role of HER-2 in gastric cancer has been acknowledged, there have been no studies done on the correlation between *HER-2* SNP and gastric cancer. In our present study, we investigated whether the *Ile/Val* SNP of *HER-2* is associated with the development and malignant phenotype of gastric cancer.

MATERIAL AND METHODS

Study subjects

The 287 controls we analyzed were randomly selected from those visiting hospitals in Hiroshima for regular health checks or symptoms such as appetite loss or epigastralgia. They were proven to be free from malignancy by medical examination with gastric endoscope and biopsy. Representative biopsy samples of mucosa confirmed histologically to be benign were used for genotype analysis. We analyzed 212 patients with primary gastric cancer, who underwent surgical operation at Hiroshima University Hospital in 1990–2001, at Hiroshima Memorial Hospital in 1998–2000 or at Hofu Institute of Gastroenterology in 2000–2001. We confirmed microscopically that all gastric cancer patients have gastric adenocarcinomas, and the corresponding nonneoplastic mucosae did not exhibit any tumor-cell invasion or show signifi-

Abbreviations: A, adenine; CI, confidence interval; G, guanine; Ile, isoleucine; OR, odds ratio; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; Val, valine.

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*Correspondence to: Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan. Fax: +81-82-257-5149.
E-mail: wyasui@hiroshima-u.ac.jp

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cant inflammatory involvement. The clinicopathologic staging and histologic classification were made according to the criteria of the TNM classification (UICC), the 5th edition, 1997, stomach (ICD-O C16). The demographic characteristics of 212 gastric cancer patients and 287 controls are summarized in Table I. There were no significant differences in gender and age at recruitment between the patients and controls. Because written informed consent was not obtained, for strict privacy protection, all samples were unidentified before correlation with genotype. This procedure is in accordance with Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and approved by the Ethical Review Committee of the Hiroshima University School of Medicine.

DNA extraction

DNA was extracted from freshly frozen nonneoplastic gastric mucosae of 84 patients and also from paraffin-embedded mucosae of 128 patients using a genomic DNA purification kit (Promega, Madison, WI) and proteinase K as described previously, respectively.[29] DNA was extracted from paraffin-embedded gastric mucosae of all controls using proteinase K.

PCR/restriction fragment length polymorphism (PCR-RFLP)-based assay

Genotypes of the *HER-2* gene were analyzed by PCR-RFLP as previously described using the DNA extracted from nonneoplastic gastric mucosae.[22] PCR fragments were generated from 10–20 ng of genomic DNA in a 25 μl reaction mixture containing 0.75 units Ampli Taq Gold (Perkin Elmer, Norwalk, CT), 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl and 200 μM of each deoxynucleotide triphosphate (dNTP). PCR was performed at 94°C for 30 sec followed by 35 cycles at 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR primers used, based on the published sequence of human complementary DNA of the *HER-2* gene,[22] were 5′-AGAGCGCCAGCCCTCTGACGTCCAT-3′ (HER-2/U) and 5′-TCCGTTTCCTGCAGCAGTCTCCGCA-3′ (HER-2/L). The 148 bp of PCR products (7 ′l) were digested with *BsmAI* (New England BioLabs, Beverly, MA) at 55°C for 2 hr in a total reaction volume of 10 μl followed by heat inactivation at 80°C for 20 min. *BsmAI* gives 116 bp and 32 bp fragments for the *Val* (GTC) allele and a single 148 bp fragment for the *Ile* (ATC) allele.[22] Fragments digested with *BsmAI* (6 μl) were subjected to electrophoresis with 8% nondenaturing polyacrylamide gels, stained with ethidium bromide and visualized under UV light. The genotyping was made by 2 investigators (K.K. and S.M.) without knowledge of case-control status. About 10% of the samples were randomly selected for repeated assays.

Statistical analysis

Fisher's exact test was used to test whether the distribution of *HER-2* genotypes was significantly different between gastric cancer patients and controls. In gastric cancer patients, correlation between the genotypes and clinicopathologic characteristics was also examined by Fisher's exact test (InStat Ver. 2.01, GraphPad Software, San Diego, CA). The logistic regression model calculated odds ratios for the genotypes, adjusting for age and gender; the logistic regression analysis was performed for the association between the genotypes and clinicopathologic characteristics (SPSS software, Ver .11.0).

TABLE I – CHARACTERISTICS OF STUDY SUBJECTS

	Patients (n = 212)	Controls (n = 287)
Gender[1]		
Male	152 (72.2%)	193 (67.2%)
Female	60 (27.8%)	94 (32.8%)
Age (years, ± SD)	66.0 ± 12.0	64.2 ± 11.8

[1]p = 0.3 for differences between patients and controls.

RESULTS

Risk of gastric cancer by HER-2 *genotyping*

Representative PCR-RFLP patterns of *HER-2* genotypes are shown in Figure 1. Digestion of PCR product (148 bp) with *BsmAI* resulted in a single fragment of 148 bp for the *Ile* allele or 2 fragments of 116 bp and 32 bp for the *Val* allele, as reported previously.[22] We confirmed that each PCR product had no nonspecific bands corresponding to these fragments before digestion with *BsmAI* by polyacrylamide gel electrophoresis. Genotypes *Ile/Ile, Ile/Val* and *Val/Val* were found in 146 (68.9%), 56 (26.4%) and 10 (4.7%) of 212 gastric cancer patients and in 234 (81.5%), 48 (16.7%) and 5 (1.8%) of 287 controls, respectively (Table II). The genotype distribution among controls was in good agreement with Hardy-Weinberg equilibrium ($p < 0.05$). Genotypes *Ile/Val* and *Val/Val* were more frequent in gastric cancer patients than those in controls ($p = 0.005$ and 0.033, respectively); *Val* allele frequencies were 0.179 and 0.101 in patients and controls, respectively ($p < 0.001$). The OR of the *Val/Val* genotype then revealed a significantly enhanced risk of 3.25 (95% CI 1.09–9.70) compared to the *Ile/Ile* genotype; the heterozygous *Ile/Val* genotype showed an intermediate risk of 1.97 (1.27–3.06). Adjustment for age and gender did not make a substantial change.

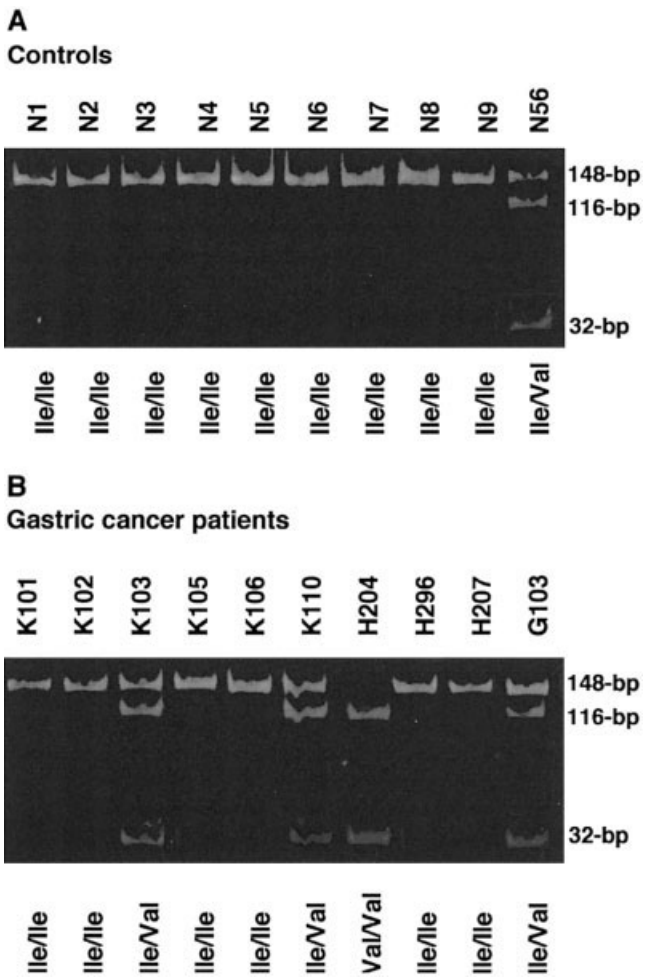

FIGURE 1 – Genotype of the *HER-2* transmembrane domain coding region at codon 655 analyzed by PCR-RFLP in healthy control subjects *(a)* and in gastric carcinoma cases *(b)*. Numbers above the panel are case numbers. Genotypes are shown below each panel.

TABLE II – *HER-2* GENOTYPE DISTRIBUTION OF STUDY SUBJECTS

	Patients (n = 212)	Controls[1] (n = 287)	P[2]	OR (95% CI)	
				Crude	Adjusted[3]
Ile/Ile	146 (68.9%)	234 (81.5%)		1 (ref.)	1 (ref.)
Ile/Val	56 (26.4%)	48 (16.7%)	0.005	1.97 (1.27–3.06)	2.00 (1.28–3.10)
Val/Val	10 (4.7%)	5 (1.8%)	0.033	3.25 (1.09–9.70)	3.25 (1.08–9.76)
Allele frequencies					
Ile	0.821	0.899	<0.001		
Val	0.179	0.101			

[1]The observed genotype distribution of controls was in agreement with Hardy-Weinberg equilibrium.–[2]*P*-values were for the difference in genotype frequencies between patients and controls.–[3]ORs were adjusted for age and gender.

TABLE III – ASSOCIATION BETWEEN GENOTYPING OF HER-2 AND CLINICOPATHOLOGIC CHARACTERISTICS

	Genotypes		p
	Ile/Ile (n = 146)	*Ile/Val* or *Val/Val* (n = 66)	
TNM classification[1]			
T			
Tis or T1	54 (37.0%)	14 (21.2%)	0.026
T2, 3 or 4	92 (63.0%)	52 (78.8%)	
N			
N0	87 (59.6%)	23 (34.8%)	0.001
N1, 2 or 3	59 (40.4%)	43 (65.2%)	
M			
M0	142 (97.3%)	60 (90.9%)	0.074
M1	4 (2.7%)	6 (9.1%)	
Stage			
0 or I	80 (54.8%)	17 (25.8%)	<0.001
II, III or IV	66 (45.2%)	49 (74.2%)	
Histopathologic grading			
Well	46 (31.5%)	16 (24.2%)	0.068
Moderately	45 (30.8%)	15 (22.7%)	
Poorly	55 (37.7%)	35 (53.1%)	

[1]TNM classification: T, primary tumor; N, regional lymph node metastasis; M, distant metastasis.

Association between HER-2 *genotyping and clinicopathologic characteristics*

We analyzed the association between the *HER-2* genotypes and clinicopathologic characteristics in gastric cancer patients. Patients with *Ile/Val* or *Val/Val* genotype showed deeper invasion over T2 ($p = 0.026$) and more lymph node metastasis ($p = 0.001$) than those with *Ile/Ile* genotype (Table III). Carcinomas of advanced stage were significantly more frequent in patients with the *Ile/Val* or *Val/Val* genotype than those with the *Ile/Ile* genotype ($p < 0.001$). Moreover, poorly differentiated adenocarcinoma tended to be more frequently found in patients with the *Ile/Val* or *Val/Val* genotype than those with the *Ile/Ile* genotype ($p = 0.068$). The logistic regression analysis then revealed that clinicopathologic staging was significantly associated with the *HER-2* genotype ($p = 0.004$), but histopathologic grading was not ($p = 0.6$); a subsequent analysis for T, N and M showed that N was most closely associated with the genotype ($p = 0.054$).

DISCUSSION

In our study, we examined whether the risk of gastric cancer is associated with the *Ile/Val* SNP of *HER-2* transmembrane domain coding region at codon 655. We found significant differences in genotype distribution between gastric cancer patients and controls, suggesting that the individuals with *Val/Val* or *Ile/Val* genotype, which may account for about 3% or 21%, respectively, in a Japanese general population, have an enhanced risk of gastric cancer development, with an OR of 1.97 or 3.25, respectively. Furthermore, this genotyping was associated with invasion, lymph node metastasis and poor differentiation in gastric cancer patients. These observations imply that this *HER-2* SNP may participate in not only development but also progression of gastric cancer.

However, the molecular mechanism of the association between the *Ile/Val* SNP at codon 655 and cancer has not been fully clarified. Several studies showed that a missense point mutation (Val664Glu) in the transmembrane domain of the *neu* proto-oncogene (*HER-2* human homologue) greatly enhanced its kinase activity and cell transformation properties.[7,30,31] It has been proposed that tyrosine kinase activity of HER-2 protein was stimulated by reorientation of the cytoplasmic domain within receptor dimers, resulting in increased transautophosphorylation and enzymatic activity, and the conformations of transmembrane domain affected this dimerization.[30,32] Recently, Fleishmann *et al.*[28] found 2 stable conformations, active or inactive, of the HER-2 transmembrane domain, using a computational exploration of conformation space of the transmembrane segments of a HER-2 homodimer; the *Val* allele was associated with active dimeric conformations of the HER-2 transmembrane domain, resulting in increased autophosphorylation, tyrosine kinase activation and cell transformation. We recently examined the association between this *HER-2* genotyping and autophosphorylation levels of HER-2 protein and found that the *Val* allele did not show higher kinase activity than the *Ile* allele in human gastric cell lines (data not shown). The *Ile/Val* genotyping may influence the ability of HER-2 protein to promote cell proliferation and transformation through other mechanisms than autophosphorylation such as dimerization capacity with other EGFR families and interactions with tumor-specific human leukocyte antigen (HLA)-A2-restricted CTLs.[33] Activation of the HER-2 signal transduction pathway is known to result in subsequent activation of the mitogen-activated protein kinase (MAPK) signaling pathway.[34] Stress-activated protein kinase-2 (SAPK2/p38), one of MAPKs, was reported to play an important role in cancer metastasis.[35] In our present study, the logistic regression analysis for tumor invasion, lymph node metastasis and distant metastasis revealed that lymph node metastasis was most closely associated with the *HER-2* genotype, suggesting that this SNP may affect the interaction between HER-2 and SAPK2/p38.

This SNP has been reported to be associated with significantly increased risk of breast cancer development.[22,23] Our present study also showed the association of this SNP with development and progression of gastric carcinoma. However, no association of this SNP with cancer development was found among other populations. These conflicting reports might be attributed partly to the small number of subjects with the homozygous *Val* genotype, leading to a decreased statistical power to detect the association between the SNP and cancer risk. Another possibility is difference of environmental factor for cancer etiology in different populations. In addition, the distribution of this *HER-2* polymorphism has been reported to vary considerably between ethnic groups. The *Val* allele has a frequency of 13% in Japanese (this study), 20% in Caucasians and 24% in African-Americans but was not detected in an African population.[36] Another HER-2 polymorphism (A to G, A23275G, where the positions are numbered from the translation initiation site; Genebank accession no. AC087491) at the intron 3′ to the transmembrane domain-coding region may also affect cancer risk.[37]

In conclusion, our study suggests that this nucleotide polymorphism in the transmembrane domain-coding region of *HER-2*

could be associated with development of gastric carcinoma and may serve as a predictor of risk of malignant phenotype of gastric cancer. Although the association of the *HER-2* genotype with clinicopathologic characteristics, especially with malignant phenotype, was also suggested, this has to be confirmed with a larger sample size.

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