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ORIGINAL ARTICLE – CANCER RESEARCH

The prognostic impact of *EGFR*, *ErbB2* and *MET* gene amplification in human gastric carcinomas as measured by quantitative Real-Time PCR

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Abstract
Purpose Identification of critical genes which play pivotal roles in controlling tumor growth and survival will establish the basis for developing therapeutic targets. In this study, we focused on frequencies of *EGFR*, *ErbB2* and *MET* gene amplification in gastric cancer patients to develop personalized medicine to improve the treatment.
Method *EGFR*, *ErbB2* and *MET* gene amplification, and mRNA expression were analyzed by the quantitative Real-Time PCR in paraffin-embedded samples from 115 patients with gastric cancer.
Results *EGFR*, *ErbB2* and *MET* genes were amplified in 11.3 % (13/115), 6.1 % (7/115) and 19.1 % (22/115) of cancerous specimens, respectively. The correlation coefficient test clearly indicated that gene amplification in these three genes was positively correlated with mRNA transcription (*EGFR*: $R = 0.631$, $p = 0.009$; *ErbB2*: $R = 0.652$, $p = 0.023$; *MET*: $R = 0.715$, $p < 0.001$). *EGFR* and *MET* gene amplification was significantly associated with Ki-67 MI ($p = 0.022$ and $p = 0.015$). *MET* amplification was also significantly associated with age of ≥60 years ($p = 0.021$) and tumor size of ≥5 cm ($p = 0.032$). *MET* amplification, but not *EGFR* and *ErbB2*, was a significant prognostic factor in poor survival among patients with gastric cancer.
Conclusions *EGFR*, *ErbB2* and *MET* genes are frequently amplified in gastric carcinoma. *EGFR*, *ErbB2* and *MET* gene amplification is positively correlated with mRNA transcription. *MET* gene amplification correlates with a poor prognosis and poor survival in gastric carcinomas.

Keywords EGFR · ErbB2 · MET · Gene amplification · Gastric cancer

Introduction

Gastric cancer (GC) is the third common cause of cancer-related deaths worldwide (Jemal et al. 2011). Despite improvement in the therapeutic management, the prognosis for patients with advanced or recurrent GC still remains poor and with a median survival time of approximately 1 year (Van Cutsem et al. 2006; Al-Batran et al. 2008; Cunningham et al. 2010). Today, major efforts to discover biomarkers and new therapeutic options are carried out. These biomarkers can help to recognize subgroups of patients in order to develop a personalized medicine model for special treatments of GC patients.

Copy number alterations (CNAs) in genes are usually arise from genomic instability and are common in cancers (Stuart and Sellers 2009; Huang and Chuang 2013). The increasing gene copy number in the genome of malignant tumor cells can result from mainly two genetic mechanisms, polysomy and gene amplification. Polysomy leads to a copy number gain, due to extra copies of the entire chromosome. The gene amplification refers to the amplification of specific gene or a group of genes in a given chromosome (Albertson 2006). CNAs of specific genes may

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up-regulate signaling pathways involved in enhancing the cell proliferation and inhibit the apoptosis in tumor cells. Cumulative evidences show an association between CNA and prognosis in cancer patients (Beroukhim et al. 2010).

Epidermal growth factor receptors (EGFR) are members of the ErbB family, a group of transmembrane receptor tyrosine kinase with key roles in embryonic development and cell growth. This family consists of four homolog receptors, including EGFR (HER1, ErbB1), HER2 (Neu, ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (Yarden and Sliwkowski 2001; Casalini et al. 2004). Aberrant EGFR signaling pathway plays a central role in the development and progression of different human cancers. EGF receptors phosphorylate several cellular proteins and regulate multiple signaling cascades, which lead to cell proliferation, angiogenesis, migration, invasion, metastasis and apoptosis inhibition (Atmaca et al. 2012). Today, EGFR inhibitors for advanced GC are under investigation; however, their prognostic role in GC types remains controversial. While some first reports have demonstrated an unfavorable outcome for EGFR overexpression in the cases with resectable GC, some studies could not report a significant correlation between EGFR overexpression, survival, and favorable outcome in patients with resected GC (García et al. 2003; Gamboa-Dominguez et al. 2004; Song et al. 2004; Galizia et al. 2007; Matsubara et al. 2008; Lieto et al. 2008; Kim et al. 2009). Some earlier studies have shown that FGFR amplification can increase sensitivity to anti-FGFRs in GC both in vitro and in vivo (Takeda et al. 2007; Kim et al. 2008; Matsumoto et al. 2012; Kato et al. 2013). Their data strongly suggest that FGFR amplification might be a promising target for treatment of GC.

The *mesenchymal–epithelial transition* (MET) gene is a proto-oncogene that encodes the receptor tyrosine kinase c-MET. The binding of hepatocyte growth factor protein to MET leads to tyrosine phosphorylation of intracellular domain of the receptor and activation of downstream proteins of several signaling pathways (Kawakami et al. 2013). Oncogenic activation of MET promotes cell proliferation, survival, migration, angiogenesis and apoptosis inhibition (Birchmeier et al. 2003). Recently, several studies have shown the *MET* gene amplification is one of the causes of *MET* overexpression in GC; however, the frequency of *MET* amplification is various among previous studies (Lee et al. 2011, 2012; Huang and Chuang 2013; Kawakami et al. 2013).

According to these controversial findings which are based on different assay methods and various patient populations, there is a need for evaluation of *EGFR*, *ErbB2* and *MET* gene amplification in GC patients in different populations. Therefore, in the current study, we investigated the CANs and gene overexpression status of these three genes in GC patients. We also analyzed the association of *EGFR*, *ErbB2* and *MET* gene amplification with some clinical and pathological features.

Materials and methods

Subjects

Patient samples included 115 formalin-fixed paraffin-embedded (FFPE) tissues of stomach cancer collected from the sporadic GC patients who had surgery from October 2009 to March 2013. The clinical data of patients were obtained from medical records. The overall survival was recorded from the time of diagnosis to the time of last follow-up. This study was approved by the Clinical Research Ethics Committee in Mazandaran University of Medical Science.

DNA and RNA extraction

The tumor samples were cross-checked by a pathologist, and the percentage of tumor cells was at least 90 %. Genomic DNA was extracted from microdissected tumor tissue samples using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's method. Total RNA was isolated from 50 mg microdissected FFPE using the ReliaPrep FFPE Total RNA Miniprep System Kit (Promega, Madison, USA). The first-strand cDNA was synthesized using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Hitchin, UK) according to the manufacturer's instruction. The concentration and quality of DNA and RNA were determined using Nanophotometer (Implen, Munich, Germany).

Primer design

The qPCR primers were designed for specific recognizing genomic DNA of *EGFR*, *ErbB2*, *MET* and *Ribonuclease P* (RNase P) genes, as well as the qRT-PCR primers for specific recognizing complementary DNA (cDNA) of ErbB1, ErbB2, MET and Hypoxanthine–guanine phosphoribosyltransferase (HPRT) mRNAs. Primer design was performed by Allele ID software (Premier Biosoft, Palo Alto, USA) and is analyzed in NCBI and BLAST websites (Table 1).

Gene copy number assay

The quantitative polymerase chain reaction (qPCR) was performed to quantify gene copy number in tumor DNA samples using primers set and Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Sankt Leon-Rot, Germany) in 7500 Real-Time PCR system (Applied Biosystem,

Table 1 Used primers in this study

Amplified factors	Primers 5′–3′		AT (°C)	Genbank accession number
Gene copy number				
EGFR	S	CAAGGCACGAGTAACAAGC	56	NG_007726.3
	AS	GAGGCTGAGAAAATGATCTTC		
ErbB2	S	GAACTGGTGTATGCAGATTGC	56	NG_007503.1
	AS	AGCAAGAGTCCCCATCCTA		
MET	S	ATCAACATGGCTCTAGTTGTC	56	NM_000314
	AS	GGGAGAATATGCAGTGAACC		
RNase P	S	TTCTGACTTTGCTGCAGATAC	56	NC_018930
	AS	AGGAATGCAACTTACGTGTC		
mRNA expression				
EGFR	S	GCTTGCATTGATAGAAATGG	55	NM_005228.3
	AS	GTCGTCTATGCTGTCCTC		
ErbB2	S	GCTTTGTGGTCATCCAGAA	55	NM_004448.3
	AS	CTCCAGCCCTAGTGTCAG		
MET	S	AGCACTGCTTTAATAGGACAC	55	NM_000245
	AS	GATCGAGAAACCACAACCTG		
HPRT	S	TGGACTAATTATGGACAGGAC	55	NM_000194
	AS	CCTGTTGACTGGTCATTACAA		

S sense, *AS* antisense, *AT* annealing temperature

Foster City, USA). The reactions for each sample were performed in triplicate. Ribonuclease P (RNase P) was applied as the endogenous reference which has two copies in the cell. After amplification, the copy number of the target gene was calculated from threshold cycle (CT) values by $\Delta\Delta C_t$ method [$\Delta\Delta C_t = (C_t$ of target gene, tumor sample $- C_t$ of RNase P, tumor sample) $-$ (average C_t of target gene, control samples $-$ average C_t of RNase P, control samples)]. Because there are two copies of the gene in each cell, the copy number is equal to $2 \times 2^{-\Delta\Delta C_t}$. The cutoff value for normal gene copy number was established as mean $\pm$ 2 standard deviation (SD) derived from normal DNA of the ten control samples. A value >2 was considered as the increasing copy number.

QRT-PCR assay

The quantitative reverse transcriptase PCR (QRT-PCR) was performed to determine mRNA levels of ErbB1, ErbB2 and MET using primers set and Maxima SYBR Green/ROX qPCR Master Mix. Each sample was tested in triplicate and was normalized to HPRT cDNA in 7500 Real-Time PCR system. For each gene, the mRNA level was calculated using the $2^{\Delta C_t}$ ($\Delta C_t = C_t$ of target gene $- C_t$ of HPRT gene) method.

Statistical analysis

The associations between gene copy number and clinicopathological parameters were analyzed by χ^2 test and Fisher's exact test. The correlation of between CNA and mRNA level was analyzed by the Spearman correlation test. The survival curves were drawn by Kaplan–Meier method, and the differences between curves were compared by the log-rank test. Multivariate analysis was performed by the Cox proportional hazards model. Data analyses were performed by SPSS software version 19 (SPSS Inc., Chicago, USA). A p value less than 0.05 was considered statistically significant.

Results

Gene amplification results

In order to determine the gene amplification of *EGFR*, *ErbB2* and *MET* in tissue samples, we used a Real-Time PCR-based copy number assay. Gene amplification was defined as values equal or more than three copies. As Table 2 shows, *EGFR*, *ErbB2* and *MET* genes were amplified in 11.3 % (13/115; range 1.1–27.4 copies), 6.1 % (7/115; range 1.6–18.5 copies) and 19.1 % (22/115; range 2.6–47.8 copies) of cancerous specimens, respectively.

The correlation between gene expression and gene amplification in GC samples

In order to compare the mRNA level in groups with $\geq$3 copies and <3 copies, we used a QRT-PCR assay on EGFR, ErbB2 and MET cDNAs acquired of GC tissue samples.

Table 2 Relationship between gene copy number and clinicopathological features in patients

	N	Gene copy number					
		EGFR		*ErbB2*		*MET*	
		≥3 copies (%)	*p* value	≥3 copies (%)	*p* value	≥3 copies (%)	*p* value
Total cases	115	13 (11.3)		7 (6.1)		22 (19.1)	
Sex			0.446		0.838		0.395
Female	38	3 (23.1)		2 (28.6)		9 (40.9)	
Male	77	10 (76.9)		5 (71.4)		13 (59.1)	
Age			0.281		0.437		**0.021**
<60	33	2 (15.4)		1 (14.3)		2 (9.1)	
≥60	82	11 (84.6)		6 (85.7)		20 (90.9)	
Tumor size (cm)			0.717		0.985		**0.032**
<5	50	5 (38.5)		3 (42.8)		5 (22.7)	
≥5	65	8 (61.5)		4 (57.2)		17 (77.3)	
Depth of invasion			0.555		0.677		0.525
Tis–T1	16	1 (7.7)		1 (14.3)		4 (18.2)	
T2–T4	99	12 (92.3)		6 (85.7)		18 (81.8)	
Lymph node metastasis			0.581		0.629		0.506
Negative	44	4 (30.8)		2 (28.6)		7 (31.8)	
Positive	71	9 (69.2)		5 (71.4)		15 (68.2)	
TNM staging			0.918		0.531		0.729
I	9	1 (7.7)		0 (0)		1 (4.6)	
II	33	3 (23.1)		1 (14.3)		5 (22.7)	
III	66	9 (61.5)		5 (71.4)		14 (63.6)	
IV	7	1 (7.7)		1 (14.3)		2 (9.1)	
Tumor differentiation			0.083		0.594		0.107
High	39	2 (15.4)		2 (28.6)		4 (18.2)	
Moderate	56	6 (46.1)		5 (71.4)		15 (68.2)	
Low	20	5 (38.5)		0 (0)		3 (13.6)	
Ki-67 MI (%)			**0.022**		0.255		**0.015**
<15	41	1 (7.7)		1 (14.3)		3 (13.6)	
≥15	74	12 (92.3)		6 (85.7)		19 (86.4)	

Significant *p* values are in bold

N, number; Tis, carcinoma in situ; T1, lamina propria and submucosa; T2, muscularis propria and subserosa; T3, exposure to serosa; T4, invasion into serosa; TNM, tumor, node, metastases staging system

Our results have shown, and the mRNA level averages of *EGFR*, *ErbB2* and *MET* (3.21 ± 1.01, 4.31 ± 1.21 and 3.91 ± 0.71) in ≥3 copies group were significantly higher compared with <3 copies group (2.18 ± 0.52, 2.93 ± 0.43 and 2.33 ± 0.61) ($p < 0.001$) (Fig. 1). The correlation coefficient test clearly indicated that gene amplification in these three genes was positively correlated with mRNA transcription (*EGFR*: $R = 0.631$, $p = 0.009$; *ErbB2*: $R = 0.652$, $p = 0.023$; *MET*: $R = 0.715$, $p < 0.001$) (Table 3).

The correlation between gene amplification and clinicopathological features in GC patients

All the patients evaluated in this study had undergone surgery with histologically confirmed stage. Some of the patient features are shown in Table 2. The median follow-up period was 23 months (range 0–55 months).

In this study, the correlations between *EGFR*, *ErbB2* and *MET* gene amplification and clinicopathological features were evaluated. These clinicopathological features included sex, age, tumor size, depth of invasion, lymph node metastasis, TNM staging, tumor differentiation and Ki-67 mitotic activity index (MI). As Table 2 shows, *EGFR* and *MET* gene amplification was significantly associated with Ki-67 MI ($p = 0.022$ and $p = 0.015$). *MET* amplification was also significantly associated with age of ≥60 years ($p = 0.021$) and tumor size of ≥5 cm ($p = 0.032$). No correlation was seen between *ErbB2* gene amplification and clinicopathological parameters ($p > 0.05$). The results of the multivariate analysis indicated that *MET* gene amplification, tumor size and

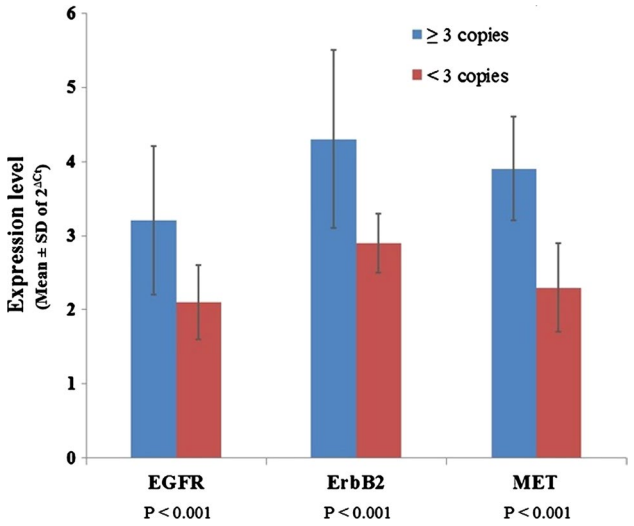

Fig. 1 QRT-PCR analysis of EGFR, ErbB2 and MET mRNA in patients with GC. The cDNA levels of genes were normalized to *HPRT* cDNA. The expression levels of *EGFR*, *ErbB2* and *MET* in ≥3 copies groups were significantly higher than <3 copies groups in patients with GC

Ki-67 MI were significant independent predictors ($p < 0.05$) in poor overall survival in GC patients (Table 4). The Kaplan–Meier curves for overall survival, according to the gene copy number, indicated that *MET* amplification, but not *EGFR* and *ErbB2*, was a significant prognostic factor for poor survival among GC patients (Fig. 2). Furthermore, comparison of overall survival among patients with *EGFR*, *ErbB2* and *MET* high copy number indicated that *MET* amplification has significantly poorer outcome (Fig. 2d).

Discussion

In recent years, many studies performed to understand the association of the biological characteristics of GC with clinical outcomes of the patients. Current studies have focused more on understanding the molecular mechanisms of gastric carcinomas to help a more accurate diagnosis and selecting a more effective treatment. With regard to the discovery of novel specific targeted therapeutics, these molecular mechanisms may be helpful to achieve more efficient therapeutic outcomes (Yk et al. 2011). Recently, changes in the expression of growth factor receptors and their legends have been reported in various tumors that may be a biological marker in cancer malignancy. To date, many genetic changes have been reported in gastric carcinoma; among these, abnormalities of tyrosine kinas receptors are more noteworthy. Alterations in *EGFR*, *ErbB2* and *MET* genes are considered to be the most frequent genetic alterations in various cancers (Tahara 1995; Nakajima et al. 1999). Presently, no available knowledge is about *EGFR*, *ErbB2* and *MET* gene amplification or overexpression status in Iranian population. Hence, in the present study, we determined amplification and the overexpression status of these three genes in GC patients by Real-Time PCR-based assay. The method used in this study is according to YK et al.'s (2011) study method, but in contrast to some other studies such as Bang et al.'s (2010) study method, in which overexpression of HER2 protein determined by immunohistochemistry or HER2 gene amplification determined by fluorescence in situ hybridization represents the eligibility criteria for trastuzumab therapy of gastric cancer patients; Nakajima et al.'s (1999) study method in which amplification and overexpression of *ErbB2* and *MET* were investigated by immunohistochemistry and Southern blot hybridization; and YK et al.'s (2011) study that used FISH method. EGF receptors mediate multiple signal transduction pathways in

Table 4 Multivariate analysis of predictive prognostic factors

	p value		
	EGFR	*ErbB2*	*MET*
EGFR gene amplification	0.215	–	–
ErbB2 gene amplification	–	0.129	–
MET gene amplification	–	–	**0.002**
Sex	0.651	0.364	0.235
Age	0.164	0.712	0.631
Tumor size	**0.007**	**0.009**	**0.013**
Depth of invasion	0.211	0.294	0.622
Lymph node metastasis	0.365	0.327	0.347
TNM staging	0.468	0.631	0.164
Tumor differentiation	0.862	0.169	0.239
Ki-67 MI	**0.003**	**0.006**	**0.002**

Significant *p* values are in bold

Table 3 Correlation between mRNA expression and gene copy numbers in cancerous tissue samples

	Gene copy number					
	EGFR		*ErbB2*		*MET*	
	R	*p* value	*R*	*p* value	*R*	*p* value
mRNA level ($2^{\Delta C_t}$)	0.631	0.009	0.652	0.023	0.715	<0.001

R correlation coefficient

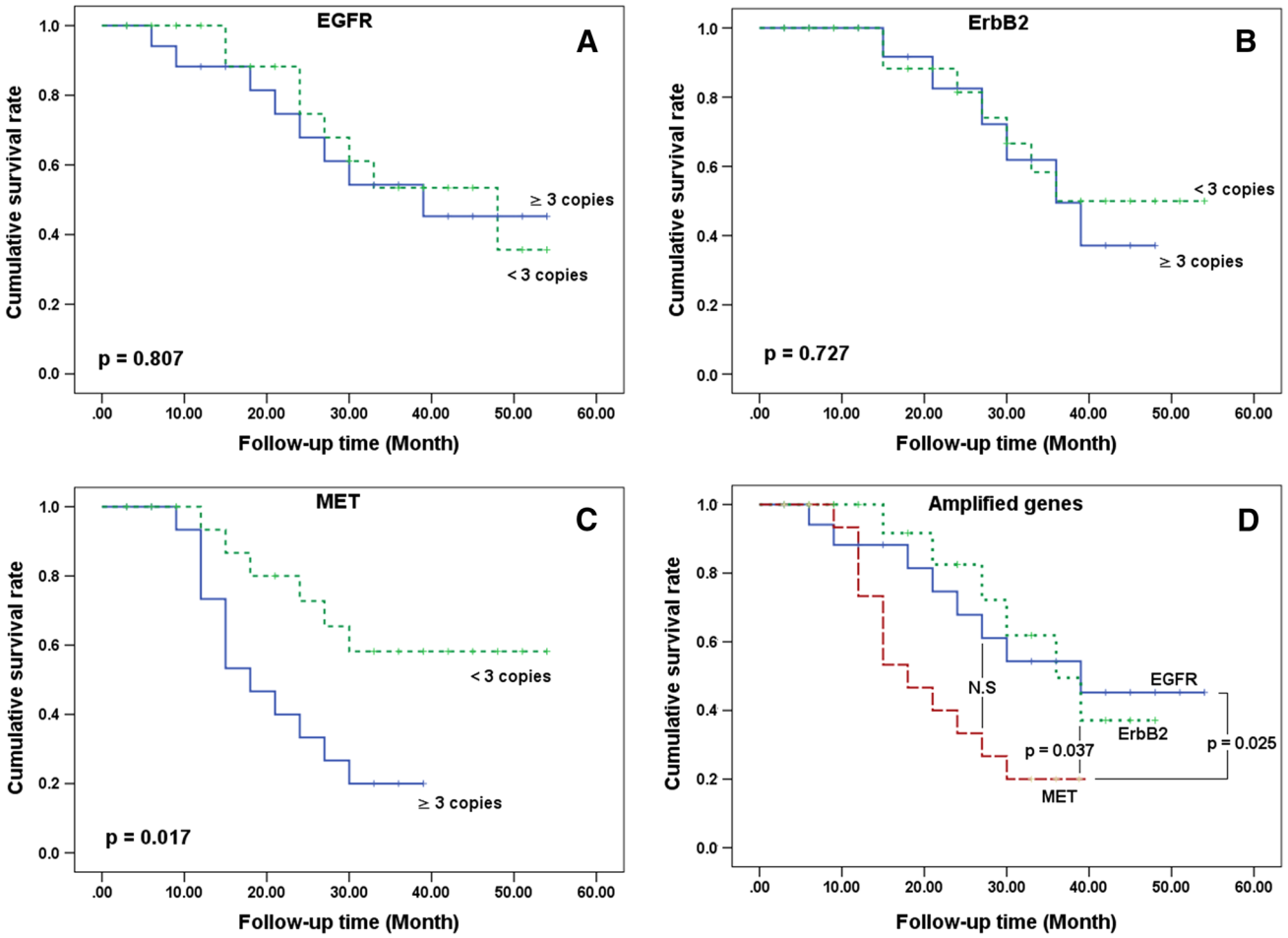

Fig. 2 Kaplan–Meier curves for overall survival in patients with GC. **a, b** Cumulative survival shows no significant poor outcome in patients with *EGFR* and *ErbB2* copy number ≥3 copies and <3 copies. **c** A significant poorer outcome is seen in patients with *MET* copy number ≥3 copies than those with <3 copies. **d** *MET* amplification has significantly poorer outcome among patients with *EGFR*, *ErbB2* and *MET* high copy number. *p* values were estimated using the log-rank test. N.S: not significant

which extracellular signals are converted into intracellular changes in expression of the given genes where control cellular growth and differentiation. Among the HER family receptors, EGFR and ErbB2 have been more considered in human cancers (Yk et al. 2011). Quantitative information about HER family expression levels in tumorous cells is necessary, not only for knowing the biological roles of EGF receptors, but also for clinical applications (Junttila et al. 2003). The expression level of EGF receptors may correlate with prognosis, responses to chemotherapy and hormonal therapy (Arteaga 2002). In addition, treatment with EGFR inhibitors may provide a good results if applied in patients with overexpression of these receptors (Junttila et al. 2003). The best example for this is herceptin therapy that provides clinical benefit almost in patients with HER2 overexpression (Vogel et al. 2002). Gene amplification and expression status of *EGFR* and *ErbB2* in GC have been analyzed in many previous studies (Sakai et al. 1986; Nakajima et al. 1999; Yk et al. 2011; Atmaca et al. 2012; Kato et al. 2013). The reported frequencies of *EGFR* and *ErbB2* gene expression in this cancer type were ranged from 5 to 30 %. The correlation between *EGFR* and *ErbB2* gene amplification with prognosis in GC patients remains controversial. In this study, *EGFR* and *ErbB2* gene amplification was, respectively, present in 11.3 and 6.1 % of gastric tumor samples (Table 2). These frequencies are similar to the frequency of *ErbB2* gene amplification in Nakajima et al.'s (1999) study on the Japanese population, significantly higher than Kato et al.'s (2013) study on the Japanese population, and significantly lower than Yk et al.'s (2011) study on Chinese population. Our results also demonstrated that *EGFR* and *ErbB2* gene amplification was positively correlated with *EGFR* and *ErbB2* gene expression status (Table 3). Furthermore, *EGFR* gene amplification was significantly associated with Ki-67 MI in GC patients. In contrast to previous studies (Nakajima et al. 1999; Yk et al. 2011; Kato et al. 2013),

no associations were found between *ErbB2* gene amplification and clinicopathological features in GC patients. *EGFR* and *ErbB2* gene amplification, and overexpression status in gastric epithelial cells may be useful markers for malignancy and poor prognosis in gastric cancer. Activation of MET signaling modulates tumor cell growth, invasion, survival and tumor angiogenesis (Liu et al. 2010). In gastric cancer, mutations of *MET* gene are extremely rare (Park et al. 2000; Lee et al. 2000; Chen et al. 2001) On the other hand, MET activation may have been mostly attributed to gene amplification and overexpression status. Previous studies have reported an increase in *MET* gene copy number 10–20 % in GC patients by a Real-Time PCR-based assay (Lee et al. 2011; Graziano et al. 2011) or by Southern blot analysis (Seruca et al. 1995; Tsujimoto et al. 1997). In the current study, *MET* gene amplification was detected in 19.1 % of gastric tumors (Table 2), which is close to Nakajima et al.'s (1999) study and significantly higher than Kato et al.'s (2013) study. Our results also shown that *MET* gene amplification was positively correlated with *MET* gene expression status (Table 3). Furthermore, *EGFR* gene amplification was significantly associated with age, tumor size and Ki-67 MI in GC patients, in contrast to Kuniyasu et al.'s (1993) and Nakajima et al.'s (1999) studies that gene amplification and overexpression of *MET* were significantly associated with lymph node metastasis and depth of tumor invasion in gastric carcinoma. We found that *MET* gene amplification was correlated with poor prognosis and poor survival in patients with gastric carcinoma (Fig. 2) (Table 4). These data suggest that the overexpression of these receptors may play an important role in the early stages of tumorigenesis. Comparing our results with other studies indicates the frequencies of *EGFR*, *ErbB2* and *MET* gene amplifications are various among the Asian populations.

EGFR, *ErbB2* and *MET* genes are frequently amplified in gastric carcinoma. *EGFR*, *ErbB2* and *MET* gene amplification is positively correlated with mRNA transcription. *MET* gene amplification correlates with a poor prognosis and poor survival in gastric carcinomas.

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Conflict of interest We declare that we have no conflict of interest rests.

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