

Gene copy number gain of *EGFR* is a poor prognostic biomarker in gastric cancer: evaluation of 855 patients with bright-field dual in situ hybridization (DISH) method

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Abstract

Background EGFR overexpression is a prognostic biomarker and is expected to be a predictive biomarker for anti-EGFR therapies in gastric cancer. However, few studies have reported the clinical impact of EGFR gene copy number (GCN) and its correlation with EGFR overexpression.

Methods We used dual in situ hybridization (DISH) to detect *EGFR* GCN and chromosome 7 centromere (CEN7) in a set of tissue microarrays representing 855 patients with gastric cancer. These data were compared with those of immunohistochemical (IHC) analysis of EGFR expression to evaluate prognostic value.

Results *EGFR* GCN gain (≥ 2.5 EGFR signals per cell) was detected in 194 patients (22.7 %) and indicated poor prognosis. Among 194 patients, *EGFR* amplification

($EGFR/CEN7 \geq 2.0$) was observed in 29 patients (14.9 %), which was almost identical to the IHC 3+ subgroup and worst prognostic subgroup. Patients with *EGFR* GCN gain but not amplification, including those exhibiting polysomy, also exhibited poorer prognosis than GCN non-gain patients and were distributed between IHC 0/1+ and 2+ subgroups. GCN gain was frequently observed in patients with more advanced disease, but served as an independent prognostic factor regardless of the pathological stage.

Conclusions *EGFR* GCN gain is a more accurate prognostic biomarker than EGFR overexpression in patients with gastric cancer.

Keywords EGFR · Gene copy number · Gastric cancer · Dual in situ hybridization · Amplification

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Introduction

Gastric cancer is the second leading cause of cancer-related death worldwide. Annually, 989,600 new patients are diagnosed, and 738,000 people die of this disease [1]. Despite recent improvements in surgical approaches combined with chemotherapy and radiotherapy, the benefits of chemotherapy for advanced gastric cancer are still limited. Thus, there is an urgent need for the development of more effective drugs, particularly those targeting tumor progression mechanisms.

In gastric cancer, mutations in genes encoding components of the receptor tyrosine kinase (RTK)/RAS-signaling pathway are infrequent. In contrast, evidence indicates that amplification, overexpression [2–6], or epigenetic deregulation [7] may have an important role in tumor progression. The results of a recent ToGA trial revealed that the anti-HER2 antibody trastuzumab improves the survival of patients with HER2-positive advanced gastric cancer [2]. The results of other studies suggest the potential efficacy of targeted therapy against gene alterations in gastric cancers [8–10], and several other therapeutic drugs against these RTKs, including EGFR, are currently under investigation [11].

Deng et al. [5] detected amplification of genes encoding RTKs, including *KRAS*, *HER2*, *EGFR*, *MET*, and *FGFR2*, in approximately 37 % of gastric cancers, and suggested that amplification of these genes may act as an oncogenic driver often related to poor prognosis.

EGFR resides on chromosome 7p12 and encodes a 170-kDa RTK that contributes to cancer progression by mediating cellular proliferation, migration, invasion, and metastasis [12]. *EGFR* GCN alterations occur in a wide range of cancers and serve as a marker of poor prognosis. Inhibitors of EGFR activity include specific monoclonal antibodies and small molecule tyrosine kinase inhibitors that are used for standard therapy of lung and colorectal as well as head and neck cancers [13–16]. Therefore, EGFR is a strong candidate for molecular-targeted therapy in gastric cancer. EGFR overexpression and gene amplification serve as effective biomarkers for predicting the clinical benefit of anti-EGFR monoclonal antibodies for treating colorectal [17] and lung cancer [18]. The recent REAL3 (panitumumab) [19] and EXPAND (cetuximab) [20] randomized controlled phase III trials failed to demonstrate the efficacy of the anti-EGFR monoclonal antibodies for treating gastric cancer, presumably because precise patient inclusion criteria were not set for the trials. Several studies suggest that EGFR overexpression or gene amplification may serve as a potential biomarker for the efficacies of anti-EGFR therapies in gastric cancer [9, 21–23].

We reported that EGFR overexpression detected using immunohistochemistry (IHC) is associated with poor outcome of gastric cancer after curative resection [4]. However,

the incidence of EGFR overexpression reported in previous studies varies from 2 % to 81 %, probably the result of differences in antibodies, scoring systems, and observers [4, 21, 24–32]. In contrast, the frequency of *EGFR* gene amplification in gastric cancers is consistently less variable (2–7 %), although most of these studies lacked a sufficiently large number of patients or a detailed *EGFR* GCN examination [3, 5, 22, 24, 26, 29, 30]. The correlation between overexpression and *EGFR* GCN has not been firmly established.

To address this issue, in the present study, we evaluated *EGFR* GCN in a large set of samples acquired from patients with gastric cancer and show that it correlates with protein expression and prognosis.

Materials and methods

Patients and pathological specimens

Formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissues from consecutive patients who underwent surgical resection at the National Cancer Centre Hospital East in Japan from January 2003 to July 2007 were used. Construction of tissue microarrays (TMA) using these tumors is described elsewhere [33]. In brief, two separate tumor areas were selected based on depth and tumor histology, and a tissue core of 2 mm in diameter was obtained from each of the areas from each patient. Serial 4- μ m sections of TMA blocks were analyzed using hematoxylin and eosin (H&E) staining, IHC, and dual in situ hybridization (DISH). Clinicopathological parameters including age, sex, tumor location, macroscopic type, histological type, lymphatic invasion, venous invasion, tumor depth (T), lymph node metastasis (N), HER2 status, resection margin, and adjuvant chemotherapy were acquired from medical records. Disease stage was classified using the TNM criteria of International Union Against Cancer (UICC), seventh edition. Overall survival was determined from the dates of surgical resection to death. The Institutional Review Board of the National Cancer Centre approved the study protocol.

EGFR immunohistochemistry

All reagents and instruments for IHC were manufactured by Ventana Medical Systems (Tucson, AZ, USA). FFPE sections were stained with Ventana Benchmark XT using a primary antibody against EGFR (clone 5B7) and Ventana iVIEW DAB Universal Kit, according to the manufacturer's protocol. Stained slides were examined under light microscopy and were interpreted by E.H. and T.K. Membranous staining was scored (0, 1+, 2+, or 3+) as follows: 0 = undetectable membrane staining or <20 cancer cells with any intensity; 1+ = at least 20 cancer cells with faint

membranous staining; 2+ = at least 20 cancer cells with weak to moderate membrane staining; and 3+ = at least 20 cancer cells with strong membrane staining. If the two cores from the same tumors had different IHC scores, the higher score was employed as the final IHC result.

Dual in situ hybridization (DISH) detection of *EGFR* and CEN7

All reagents and instruments for DISH were manufactured by Ventana. DISH analyses for *EGFR* were carried out with Ventana Benchmark XT, using DNA cocktail probes [*EGFR* and chromosome 7 centromere (CEN7)] according to the manufacturer's protocol. The number of *EGFR* signals (silver ISH detection, black dots) and CEN7 signals (red ISH detection, red dots) of 20 representative cells were counted in each core, and the higher score of two cores from each sample was employed as the final result. The *EGFR*/CEN7 ratio was calculated by dividing the total number of *EGFR* GCN signals by that of the CEN7 signals. Amplification of *EGFR* was defined as $EGFR/CEN7 \geq 2.0$. Chromosome 7 polysomy was defined as the average of CEN7 signals ≥ 3.0 per cell. Cancer tissue cores that had no more than 1 signal for either *EGFR* or CEN7 per cell were excluded from further analyses.

Statistical analyses

For the multiple subgroup analyses of overall survival, the hazard ratio (HR) and 95 % confidence interval (CI) within each subgroup were summarized and displayed in a forest plot using the Cox regression model. An analysis of overall survival between subgroups was performed using the Kaplan–Meier method, and differences were compared using the log-rank test. The clinical characteristics of the two groups were compared using the chi-square test, Fisher's exact test, and Cramér's V. Univariate and multivariate analyses were conducted using the Cox regression model. Variables for multivariate analysis were selected by a backward stepwise approach using a significance level of $P < 0.05$ for variables remaining in the model. All reported P values are two sided, and the level of significance was $P < 0.05$. All statistical analyses were performed using IBM SPSS statistics version 18 for Windows (IBM, Armonk, NY, USA).

Results

Characteristics of patients and TMA specimens

After examining 2,012 cores from 1,006 patients using IHC and DISH, we excluded 151 patients from further analysis because no gastric cancer cells or deficient DISH signals were observed, leaving 855 patients for further analyses.

The median (range) follow-up period of the surviving patients was 61.2 (1–121) months. The profile of clinicopathological treatment-related characteristics of these patients is shown in Online Resource 1.

EGFR expression and prognosis

IHC scores for the 855 patients were as follows: 463 (54.1 %) were IHC 0, 251 (29.3 %) were 1+, 114 (13.3 %) were 2+, and 27 (3.2 %) were 3+. The hazard ratios of IHC 1+, 2+, and 3+ subgroups compared with those of IHC 0 are described in Online Resource 2a. IHC 2+ and IHC 3+ results were significant indicators of poor prognosis, and IHC 3+ was identified as the strongest prognostic value in overall survival curve estimates (Online Resource 2b).

EGFR GCN and prognosis

The median copy numbers of *EGFR* and CEN7 per cell were 1.9 and 2.3, respectively, and the median *EGFR*/CEN7 ratio was 0.90. Chromosome 7 polysomy was observed in 154 patients (Online Resource 1). We divided the patients into four grades according to *EGFR* GCN per cell: grade 1 ($GCN < 1.5$), grade 2 ($1.5 \leq GCN < 2.0$), grade 3 ($2.0 \leq GCN < 2.5$), and grade 4 ($2.5 \leq GCN$). The hazard ratio of each *EGFR* GCN subgroup for grade 1 is described in Fig. 1a. There were stepwise increases in the hazard ratio with increases in the GCN grade, and GCN grade 4 (HR = 2.51, $P < 0.001$) was the most informative prognostic factor.

Receiver operating characteristic (ROC) analysis was performed to define the optimal cutoff value that discriminates patients with poor prognosis (Fig. 1b). The ROC curve identified 2.35 signals of *EGFR* per cell as the optimal cutoff value discriminating between favorable and poor prognosis for 5-year survival. For further examination, we defined 2.5 *EGFR* signals per cell as the cutoff value for the definition of GCN gain, and 194 patients were assigned to this category.

Among the 194 patients with GCN gain, the *EGFR*/CEN7 ratio was < 2.0 for 165 patients, whereas *EGFR* was amplified in only 29. Among the 29 patients with *EGFR* amplification, 20 patients possessed > 20 copies of *EGFR* per cell and formed a subgroup distinguished from the others (Fig. 2). Further, a clustered pattern of *EGFR* signals was observed in the nucleus for 27 of 29 patients with *EGFR* amplification (Fig. 3a). In the remaining 165 GCN patients without detectable gene amplification, chromosome 7 polysomy (Fig. 3b) was observed in 103 (53.1 %). The number of *EGFR* ISH signals without amplification or polysomy of the remaining 62 patients was slightly increased (Fig. 3c). The prognoses of patients with *EGFR* GCN gain were significantly worse compared with those without this gain. *EGFR* amplification was the

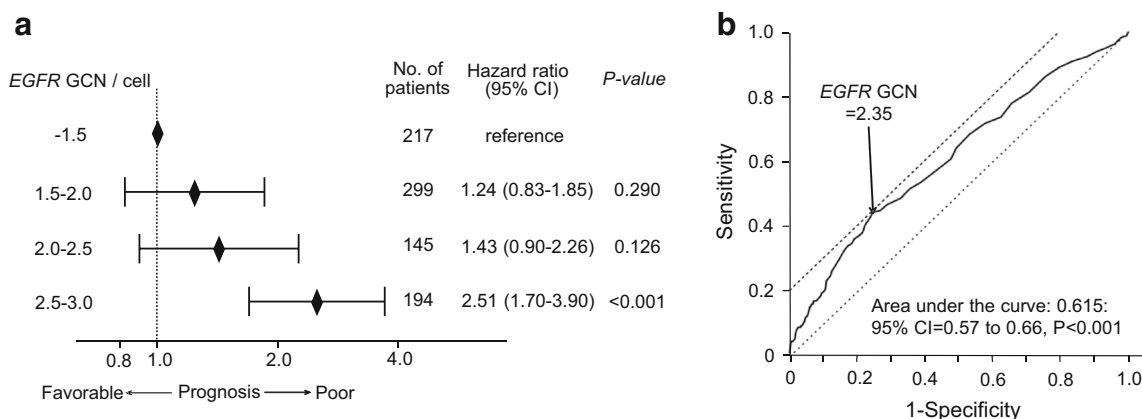


Fig. 1 Definition of the cutoff value of *EGFR* gene copy number (GCN) gain in patients with gastric cancer. **a** Hazard ratios and 95 % confidence intervals (CIs) for overall survival according to *EGFR*

strongest prognostic marker in the *EGFR* GCN subgroups (Fig. 4a). The prognostic value of *EGFR* GCN gain without amplification was still significant to predict poor clinical outcome from the absence of *EGFR* GCN gain.

Correlations between *EGFR* expression and *EGFR* GCN gain

EGFR was amplified in all 27 patients with IHC 3+, and GCN gain was detected in 59.6 % (68/114) of IHC 2+ patients (Table 1). GCN gain was a significant indicator of poor prognosis in the IHC 0/1+ ($P = 0.014$; Fig. 4b) and IHC 2+ subgroups ($P = 0.042$; Fig. 4c).

Correlation between clinicopathological characteristics and *EGFR* GCN gain

The correlations between clinicopathological characteristics and *EGFR* GCN gain are summarized in Table 2.

Table 1 Correlation between immunohistochemistry (IHC) score and *EGFR* gene copy number (GCN) status

	IHC 0 ($n = 463$) No. (%)	IHC 1+ ($n = 251$) No. (%)	IHC 2+ ($n = 114$) No. (%)	IHC 3+ ($n = 27$) No. (%)
GCN non-gain ^a ($n = 661$)	406 (87.7)	209 (83.3)	46 (40.4)	0 (0.0)
GCN gain				
Non-amplification ^b ($n = 165$)	56 (12.1)	42 (16.7)	67 (58.8)	0 (0.0)
Amplification ^c ($n = 29$)	1 (0.2)	0 (0.0)	1 (0.9)	27 (100)

IHC immunohistochemistry, GCN gene copy number

^a Gene copy number <2.5 per cell

^b Gene copy number ≥ 2.5 per cell without amplification

^c Gene copy number ≥ 2.5 per cell with amplification

GCN. **b** Optimal cutoff values determined using a receiver operating characteristics curve

EGFR GCN gain was more prevalent for patients with characteristics as follow: male, age >65 years, macroscopic type 2 or 3, differentiated type, presence of lymphatic or venous invasion, advanced tumor depth, lymph node metastasis, advanced stage, HER2-positive, R2 of resection margin, and adjuvant chemotherapy.

Multivariate analysis of prognostic factors for survival

Clinicopathological factors of 855 patients were analyzed using Cox regression to determine their prognostic significance. Thirteen factors were significant for overall survival as follows: age >65 years, macroscopic type 4, undifferentiated type, presence of lymphatic invasion, presence of venous invasion, T4 tumor depth, lymph node

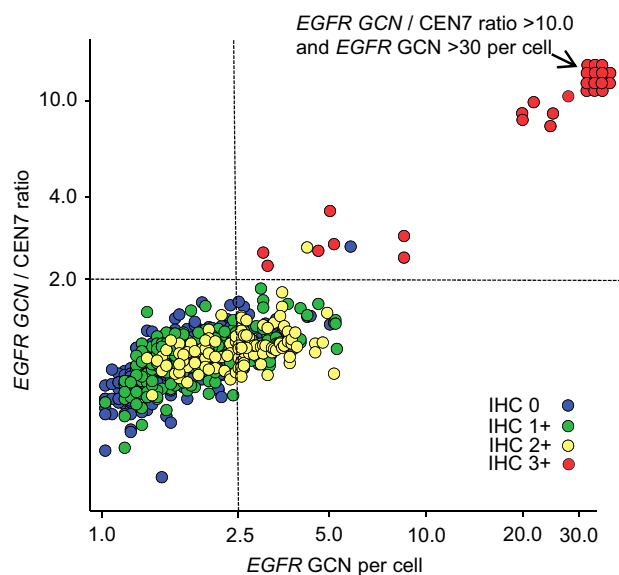


Fig. 2 Distribution of *EGFR* GCN per cell and *EGFR*/chromosome 7 centromere (CEN7) ratios according to immunohistochemical (IHC) scores of 855 patients with gastric cancer

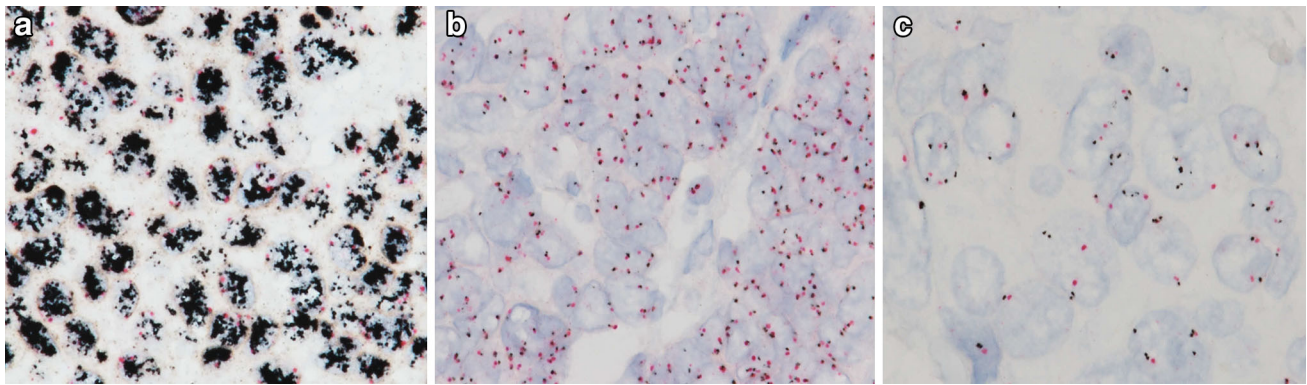


Fig. 3 Representative images of GCN gain with amplification (a), GCN gain without amplification derived from polysomy of CEN7 (b), and GCN gain without amplification showing slightly amplified *EGFR* signals and an *EGFR*/*CEN7* ratio <2.0 (c)

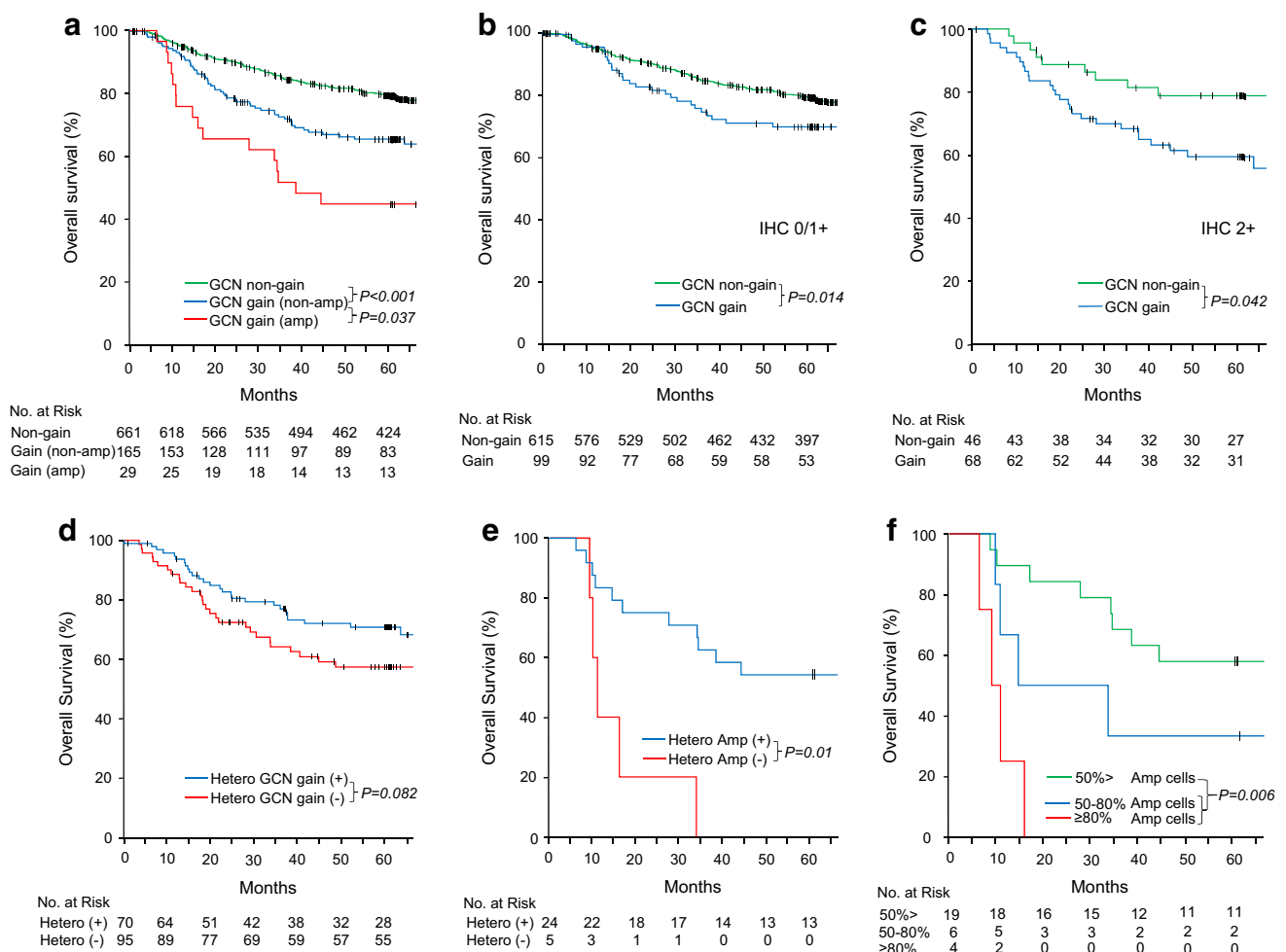


Fig. 4 Kaplan-Meier analysis of overall survival. **a** Comparison between patients without GCN gain (Non-gain, $n = 661$), with GCN gain but without amplification [Gain (non-amp), $n = 165$] and with both GCN gain and amplification [Gain (amp), $n = 29$] among 855 total patients. **b** Comparison between patients without GCN gain (Non-gain, $n = 615$) and with GCN gain (Gain, $n = 99$) among 714 patients with IHC scores of 0/1+. **c** Comparison between patients without GCN gain (Non-gain, $n = 46$) and with GCN gain (Gain, $n = 68$) among 114 patients with IHC scores of 2+.

d Comparison between patients without heterogeneous GCN gain [Hetero GCN (-), $n = 95$] and with heterogeneous GCN gain [Hetero GCN (+), $n = 70$] among 165 patients with GCN gain but without amplification. **e** Comparison between patients without heterogeneous amplification [Hetero Amp (-), $n = 5$] and with heterogeneous amplification [Hetero Amp (+), $n = 24$] among 29 patients with amplification. **f** Comparison between patients with *EGFR* amplification observed in <50% ($n = 19$), 50–80% ($n = 6$), and ≥80% ($n = 4$) of all tumor cells among 29 patients with amplification

metastasis, TNM stage III/IV, R2 resection margin, IHC 2+, IHC 3+, *EGFR* GCN gain without amplification, and *EGFR* GCN gain with amplification (Table 3). The eight factors, which were identified using multivariate analysis of overall survival, correlated independently with worse prognosis as follows: age >65 years, macroscopic type 4, undifferentiated type, venous invasion, TNM stage III/IV, R2 of resection margin, *EGFR* GCN gain without amplification (HR = 1.53; 95 % CI = 1.11–2.10, $P = 0.009$), and *EGFR* GCN gain with amplification (HR = 2.46; 95 % CI = 1.44–4.21, $P < 0.001$) (Table 3). Note that IHC 2+ was not demonstrated as an independent indicator of poor prognosis. Further, IHC 3+ was excluded by multivariable analyses because of confounding GCN gain results with amplification. Even when survival analyses were adjusted for stage and resection margin, *EGFR* GCN gain was a significantly worse prognostic indicator in patients compared with the absence of GCN gain (Online Resource 3a/b).

Intratumoral *EGFR* GCN alteration heterogeneity

In the present study, a tumor was defined as heterogeneous if different *EGFR* GCN gain or amplification results were obtained from two separate TMA cores from the same tumor. Heterogeneity status of the *EGFR* GCN alteration was detected for 70 (42.4 %) of 165 patients with *EGFR* GCN gain and for 24 (82.8 %) of 29 patients with amplification. As shown in Fig. 4d, e, a heterogeneous *EGFR* GCN status tended to have better prognostic impact upon *EGFR* gene amplification as well as GCN gain tumors ($P = 0.01$ and $P = 0.082$, respectively). Among the 29 patients with amplification, the proportion of tumor cells exhibiting *EGFR* amplification in two TMA cores was <50 % in 19 patients (65.5 %), 50–80 % in 6 patients (20.7 %), and ≥ 80 % in 4 patients (13.8 %). The patients in whom ≥ 50 % of tumor cells exhibited *EGFR* amplification had a significantly poor prognosis ($P = 0.006$; Fig. 4f), and an increased proportion of cells exhibiting amplification was suggested to be associated with a poor prognosis.

Discussion

EGFR gene amplification results from an aberrant DNA replication and leads to a large number of gene copies integrated into either intrachromosomal tandem repeats or extrachromosomal double minutes and is a key mechanism of *EGFR* signaling activation [34]. In contrast, *EGFR* GCN gain is defined as GCN ≥ 2.5 per cell and results from various causes such as segmental chromosomal duplications or polysomy. *EGFR* GCN gain may accelerate

EGFR-associated signaling, although the biological significance of *EGFR* GCN gain may differ from *EGFR* gene amplification in *EGFR* signaling activation.

We showed that *EGFR* amplification was a rare event (3.4 %) and indicated the worst prognostic impact. Although the criteria for *EGFR* amplification are not formally established and a wide range of *EGFR*/CEN7 ratios (1.7–6.0) were used in previous studies [3, 5, 22, 24, 26, 29, 30], the frequency of *EGFR* amplification did not vary among these studies, which may be attributed to the fact that the *EGFR*/CEN7 ratio is >10.0 in most patients with *EGFR* amplification, and a defined cutoff value would not influence the results (Fig. 2). For example, in the present study, 27 of 29 patients with *EGFR* amplification exhibited large clusters of signals in the nucleus.

Few studies have revealed a correlation between clinical outcome and *EGFR* GCN gain without amplification in gastric cancer. In the present study, we found that prognosis worsened as *EGFR* GCN increased. We defined the cutoff value of GCN gain as 2.5 *EGFR* signals per cell in the present study, and this value effectively delineated between poor and favorable prognoses. Thus, *EGFR* GCN gain without amplification was also a poor prognostic factor. The pathological characteristics of patients with *EGFR* GCN gain, including lymphatic invasion, venous invasion, tumor depth, and lymph node metastasis, were more severe and frequent in advanced stages, suggesting that *EGFR* GCN gain increases *EGFR* RTK signaling and contributes to tumor progression. Therefore, *EGFR* GCN gain may serve as a potential biomarker for guiding anti-*EGFR* therapies.

Evidence indicates that polysomy of chromosome 17 in patients with breast cancer may represent a clinical response to trastuzumab, even in patients without amplification of *HER2* [35]. Polysomy of chromosome 17 indicates some degree of clinical benefit for the use of trastuzumab in gastric cancer as well [36]. Our data raise the possibility that chromosome 7 polysomy may represent a potential biomarker for *EGFR* therapy, although further studies are needed for corroboration.

No published data have been available to show a correlation between *EGFR* GCN and *EGFR* overexpression in patients with gastric cancer. Although there are no acknowledged consensus criteria for the histological determination of *EGFR* status in gastric cancer, some studies show that strong IHC staining of the tumor cell membrane (corresponding to IHC 3+ or IHC 2+/3+ in the present study) indicates poor prognosis [4, 26]. In the present study, we used a monoclonal anti-*EGFR* antibody (clone 5B7) raised against the functionally active intracellular domain of *EGFR*. When used for studies on colon cancer, this antibody exhibited higher sensitivity and specificity for *EGFR* overexpression than other anti-*EGFR*

Table 2 Comparison of clinicopathological characteristics according to *EGFR* GCN status in 855 patients with gastric cancer

	GCN non-gain (<i>n</i> = 661)		GCN gain				<i>P</i> value ^a (GCN non-gain vs. GCN gain)
			Non-amplification (<i>n</i> = 165)		Amplification (<i>n</i> = 29)		
	No.	(%)	No.	(%)	No.	(%)	
Age (years)							<i>P</i> = 0.001
≤65	366	(55.4)	71	(43.0)	11	(37.9)	
>65	295	(44.6)	94	(57.0)	18	(62.1)	
Gender							<i>P</i> < 0.001
Male	428	(64.8)	126	(76.4)	26	(89.7)	
Female	233	(35.2)	39	(23.6)	3	(10.3)	
Location							<i>P</i> = 0.450
Junction	20	(3.0)	6	(3.6)	2	(6.9)	
Stomach	641	(97.0)	159	(96.4)	27	(93.1)	
Macroscopic tumor type							<i>P</i> < 0.001
Type 0	351	(53.1)	75	(45.5)	3	(10.3)	
Type 1	17	(2.6)	4	(2.4)	0	(0.0)	
Type 2	69	(10.4)	30	(18.2)	8	(27.6)	
Type 3	157	(23.8)	40	(24.2)	17	(58.6)	
Type 4	58	(8.8)	10	(6.1)	1	(3.4)	
Type 5	9	(1.4)	6	(3.6)	0	(0.0)	
Histological type							<i>P</i> < 0.001
Differentiated	295	(44.6)	114	(69.1)	22	(75.9)	
Undifferentiated	366	(55.4)	51	(30.9)	7	(24.1)	
Lymphatic invasion							<i>P</i> < 0.001
Absent	397	(60.1)	70	(42.4)	5	(17.2)	
Present	264	(39.9)	95	(57.6)	24	(82.8)	
Venous invasion							<i>P</i> < 0.001
Absent	375	(56.7)	66	(40.0)	3	(10.3)	
Present	286	(43.3)	99	(60.0)	26	(89.7)	
Tumor depth ^b							<i>P</i> < 0.001
pT1	345	(52.2)	68	(41.2)	2	(6.9)	
pT2	88	(13.3)	13	(7.9)	5	(17.2)	
pT3	135	(20.4)	54	(32.7)	13	(44.8)	
pT4	93	(14.1)	30	(18.2)	9	(31.0)	
Lymph node metastasis							<i>P</i> < 0.001
Absent	422	(63.9)	76	(46.1)	8	(27.6)	
Present	239	(36.2)	89	(53.9)	21	(72.4)	
TNM stage ^c							<i>P</i> < 0.001
I	380	(57.5)	61	(37.0)	4	(13.8)	
II	127	(19.2)	46	(27.9)	9	(31.0)	
III	112	(16.9)	39	(23.6)	12	(41.4)	
IV	42	(6.4)	19	(11.5)	4	(13.8)	
HER2 status ^d							<i>P</i> = 0.001
Negative	610	(92.3)	138	(83.6)	25	(86.2)	
Positive	51	(7.7)	27	(16.4)	4	(13.8)	
Resection margin							<i>P</i> = 0.027
R0, R1	628	(95.0)	150	(90.9)	26	(89.7)	
R2	33	(5.0)	15	(9.1)	3	(10.3)	

Table 2 continued

	GCN non-gain (<i>n</i> = 661)		GCN gain				<i>P</i> value ^a (GCN non-gain vs. GCN gain)
			Non-amplification (<i>n</i> = 165)		Amplification (<i>n</i> = 29)		
	No.	(%)	No.	(%)	No.	(%)	
Adjuvant chemotherapy							<i>P</i> = 0.027
Absent	617	(93.3)	150	(90.9)	26	(89.7)	
Present	44	(6.7)	15	(9.1)	3	(10.3)	

^a Clinical characteristics of the two groups were compared using the chi-square test, Fisher's exact test, and Cramér's V

^b Pathological tumor depth

^c Disease stage was classified using the TNM criteria of the International Union Against Cancer (UICC), seventh edition

^d HER2-positive was defined as IHC score 3+ or IHC 2+ plus *HER2*:chromosome 17 ratio ≥ 2.0

Table 3 Hazards analysis of prognostic factors in 855 patients with gastric cancer

	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	<i>P</i> value	Hazard ratio (95 % CI)	<i>P</i> value
Age (years)				
≤65	Reference			
>65	2.09 (1.58–2.76)	<i>P</i> < 0.001	1.74 (1.31–2.31)	<i>P</i> < 0.001
Gender				
Male	1.38 (0.99–1.91)	<i>P</i> = 0.057		
Female	Reference			
Macroscopic tumor type				
Non-type4	Reference			
Type 4	6.21 (4.54–8.49)	<i>P</i> < 0.001	2.70 (1.89–3.84)	<i>P</i> < 0.001
Histological type				
Differentiated	Reference			
Undifferentiated	1.70 (1.29–2.25)	<i>P</i> < 0.001	1.52 (1.11–2.06)	<i>P</i> = 0.008
Lymphatic invasion				
Absent	Reference			
Present	4.24 (3.11–5.80)	<i>P</i> < 0.001		
Venous invasion				
Absent	Reference			
Present	5.03 (3.58–7.06)	<i>P</i> < 0.001	1.86 (1.26–2.76)	<i>P</i> = 0.002
Tumor depth				
pT1, 2, 3	Reference			
pT4	6.16 (4.67–8.12)	<i>P</i> < 0.001		
Lymph node metastasis				
Absent	Reference			
Present	5.38 (3.92–7.38)	<i>P</i> < 0.001		
TNM stage				
I, II	Reference			
III, IV	8.80 (6.56–11.81)	<i>P</i> < 0.001	4.14 (2.90–5.91)	<i>P</i> < 0.001
HER2 status				
Negative	Reference			
Positive	0.79 (0.48–1.30)	<i>P</i> = 0.354		
Resection margin				
R0, R1	Reference			

Table 3 continued

	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	<i>P</i> value	Hazard ratio (95 % CI)	<i>P</i> value
R2	11.30 (8.05–15.87)	<i>P</i> < 0.001	2.85 (1.95–4.15)	<i>P</i> < 0.001
EGFR IHC score				
IHC 0, 1+	Reference			
IHC 2+	1.70 (1.20–2.43)	<i>P</i> = 0.003		
IHC 3+	3.18 (1.87–5.40)	<i>P</i> < 0.001	No examination ^a	
EGFR gene copy number status				
GCN non-gain ^b	Reference			
GCN gain (non-amp) ^c	1.75 (1.29–2.38)	<i>P</i> < 0.001	1.53 (1.11–2.10)	<i>P</i> = 0.009
GCN gain (amp) ^d	2.87 (1.72–4.78)	<i>P</i> < 0.001	2.46 (1.44–4.21)	<i>P</i> < 0.001
CEN7 polysomy ^e				
Negative	Reference			
Polysomy	1.38 (1.00–1.91)	<i>P</i> = 0.052		

IHC immunohistochemistry, GCN gene copy number, CEN7 chromosome 7 centromere, CI confidence interval

^a IHC 3+ was also excluded in multivariable analyses because of confounding GCN gain results with amplification

^b Gene copy number <2.5 per cell

^c Gene copy number \geq 2.5 per cell without amplification

^d Gene copy number \geq 2.5 per cell with amplification

^e Chromosome 7 centromere signals \geq 3 per cell

antibodies that bound to the external domain of the EGFR [37]. As expected, in the present study, EGFR amplification was detected in all IHC 3+ samples. The sensitivity and specificity of EGFR amplification corresponding to EGFR IHC 3+ were 100 % and 99.8 %, respectively. Moreover, we detected GCN gain in more than half the samples with IHC 2+, and this gain was a significant prognostic factor for the IHC 0/1+ and IHC 2+ subgroups. These results suggest that the evaluation of GCN gain is a more effective biomarker than the IHC score for identifying poor prognosis.

In the present study, we used the DISH assay to evaluate the EGFR and CEN7 copy numbers. Notably, in some patients, the number of EGFR signals per cell was less than 1, possibly because the sizes of the dots representing EGFR were occasionally smaller than those representing CEN7 and were thus more difficult to identify. Accordingly, the frequency of GCN gain observed in the present study was lower than the frequencies in previous reports that incorporated the fluorescence in situ hybridization (FISH) method [22]. Therefore, we employed a cutoff value of 2.5 EGFR signals per cell. Nevertheless, we assumed that evaluation of EGFR GCN status using DISH must have an advantage over evaluation using FISH, primarily because DISH particles can be evaluated via conventional light microscopy, facilitating the identification of tumor cells. In the present study, we showed that cells exhibiting EGFR amplification were distributed heterogeneously throughout

tumors (82.8 %) and that an increased proportion of EGFR-amplified cells was associated with a poor prognosis. Therefore, it is important to examine the EGFR GCN status throughout the tumor area and to evaluate the proportion of tumor cells exhibiting EGFR amplification. DISH is recommended for HER2 testing by the ASCO/CAP 2013 guidelines for breast cancer [38] and proves advantageous because it enables examination of tumors exhibiting more heterogeneity, such as gastric cancer.

In conclusion, we showed here that EGFR amplification was relatively rare in gastric cancer, although EGFR GCN gain was observed in 22.7 % of the patients. EGFR GCN gain correlated significantly with the IHC results and may serve as a biomarker with increased sensitivity and specificity for predicting prognosis. EGFR GCN gain, especially if combined with amplification status and IHC score, may provide a superior method for distinguishing among patients who may benefit from therapies that target EGFR.

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