Evaluation of Fibroblast Growth Factor Receptor 2 Expression, Heterogeneity and Clinical Significance in Gastric Cancer

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Key Words

Fibroblast growth factor receptor 2 · Fluorescence in situ hybridization · Immunohistochemistry · mRNA in situ hybridization · Stomach cancer

Abstract

Background: We aimed to evaluate the protein and mRNA expression of fibroblast growth factor receptor 2 (FGFR2) by immunohistochemistry (IHC) and mRNA in situ hybridization (ISH), respectively, and to assess the heterogeneity of FGFR2 expression in gastric cancer (GC). **Methods:** A tissue microarray containing 362 surgically resected GC tissues and 135 matched metastatic lymph nodes was evaluated using FGFR2b IHC and *FGFR2* ISH. *FGFR2* fluorescence ISH was also performed in 188 cases. **Results:** All *FGFR2*-amplified cases (5 of 188) showed FGFR2b protein and *FGFR2* mRNA overexpression ($p < 0.001$), and *FGFR2* amplification was not identified in FGFR2b IHC- and *FGFR2* mRNA ISH-negative cases. Kaplan-Meier survival analysis revealed that FGFR2b protein and *FGFR2* mRNA overexpression was significantly associated with a poor overall survival ($p < 0.001$ and $p = 0.012$, respectively), and multivariate analyses showed that *FGFR2* mRNA overexpression was an independent biomarker of a poor overall survival. Intratumoral heterogeneity of FGFR2b protein and *FGFR2* mRNA overexpression was observed in 5

of 9 (55.5%) and 18 of 21 (85.7%) cases, respectively. Discordant FGFR2b and *FGFR2* expression results between primary and matched metastatic lymph nodes were observed in 5 of 9 (55.5%) and 4 of 14 (28.6%) cases, respectively. **Conclusions:** Intratumoral heterogeneity and discordant FGFR2b expression in primary tumors and metastatic lymph nodes are common in GC.

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Introduction

Gastric cancer (GC) is the fourth most commonly diagnosed cancer and the third most frequent cause of cancer death in men worldwide [1]. Its early detection and complete excision results in a 5-year survival rate of over 90% for patients with early-stage disease in both Western countries and Japan [2]. However, the outcome of advanced GC is still bleak [3], and targeted agents have, therefore, been investigated for these patients, including trastuzumab, which has been used to successfully treat metastatic GC [4]. Fibroblast growth factor receptor 2 (FGFR2) is one of four *FGFR* family members that encode transmembrane receptor tyrosine kinases [5]. Alternative splicing of the *FGFR2* transcript gives rise to the IIIb isoform (*FGFR2b*) that is preferentially expressed in epithe-

lial cells and determines ligand specificity [6, 7]. *FGFR2* gene amplification in GC was first identified in the human GC cell line KATO-III [8], and can activate the *FGFR2* signaling pathway and thus promote the proliferation and survival of GC cells [9]. *FGFR2* amplification has been reported to occur in 2–9% of GC and is associated with shorter survival [10–16]. Recent studies of *FGFR* inhibitors in GC cell lines have given promising results [9, 17–20], suggesting that *FGFR2* is a potential molecular target for the treatment of a subset of GC.

In previous studies, fluorescence in situ hybridization (FISH) and quantitative real-time PCR were used to evaluate *FGFR2* status [10, 11]. FISH is regarded as the gold standard for the detection of *FGFR2* gene amplification [12], but it is expensive and time-consuming to perform. Quantitative real-time PCR does not allow in situ evaluation, and the presence of adjacent stromal cells and/or inflammatory cells make it difficult to obtain pure tumor samples. Immunohistochemistry (IHC) and mRNA in situ hybridization (ISH) are in situ methods using light microscopy to assess protein and mRNA expression, respectively. Both are faster and more economical than FISH and they make it easier to evaluate large areas of the tumor, which makes them appealing techniques for screening for *FGFR2* gene amplification or *FGFR2b* protein overexpression. However, mRNA ISH and IHC are currently not standard, validated methods for assessing the *FGFR2* and the *FGFR2b* status, respectively.

Intratumoral heterogeneity is frequently observed in GC, and clonal and phenotypic divergence of histologically mixed carcinoma has been reported [21]. Human epidermal growth factor receptor 2 (HER2) was shown to be expressed heterogeneously and at a higher level in GC compared to breast cancer [22]. Heterogeneity of the gene amplification status within a tumor or between primary and paired metastatic lesions is considered to be an important potential cause of targeted therapy failure [22–24]. Heterogeneous *FGFR2* amplification has recently been described in GC [12, 15, 16], but it has not yet been comprehensively studied. Additional studies with survival analysis are needed to evaluate the prevalence of heterogeneous *FGFR2* expression in GC.

To validate *FGFR2* mRNA ISH and *FGFR2b* IHC methods and to elucidate the intratumoral heterogeneity and the discordance between primary and metastatic GC, we performed *FGFR2b* IHC, *FGFR2* mRNA ISH and *FGFR2* FISH in 362 primary GC cases and 135 associated synchronous metastatic lymph nodes. In addition, we evaluated the frequency and clinical implication of *FGFR2b* overexpression in GC.

Material and Methods

Patients

A total of 362 primary GC patients who underwent surgical resection at the Department of Pathology, Seoul National University Hospital, in 2005 were examined. Age, gender, WHO and Lauren's classification, pathological TNM stage (according to the 7th UICC/AJCC manual) [25], and lymph node and distant metastases were evaluated by reviewing medical charts, pathological records and glass slides. The median follow-up period was 58.7 months (range, 0–80 months). The cases lost to follow-up were regarded as censored data for the analysis of survival rates. No patient received preoperative chemotherapy or radiotherapy before surgery. Patients with stage II, III or IV disease received postoperative chemotherapy using a 5-fluorouracil-based regimen (either alone, or combined with mitomycin C or cisplatin). The retrospective study protocol was reviewed and approved by the Institutional Review Board of Seoul National University Hospital under the condition of anonymization (IRB No. H-1309-087-522).

Tissue Microarrays

Array blocks obtained from patients with GC were prepared as described previously (Superbiochips Laboratories, Seoul, Korea) [26]. Briefly, representative tissues cores (2 mm in diameter) were taken from individual paraffin-embedded GC samples and arranged in new tissue array blocks using a trephine apparatus. Non-neoplastic gastric mucosa specimens were included in each of the array blocks, which contained up to 60 cores. To evaluate intratumoral heterogeneity, tissue arrays were constructed by sampling 3 cores from each primary tumor in multiple paraffin blocks. Two cores were obtained from submucosal or deeper invasive sites and 1 core from the mucosal site. In cases of lymph node metastases, tissue arrays were constructed separately. One core for each case was obtained from the paraffin block containing the largest metastatic tumor if the tumor occupied more than 10% of the core area.

FISH

Dual color Zytolight kits (Zytovision, Bremerhaven, Germany) were used for FISH. Briefly, 2- to 3- μ m-thick sections were deparaffinized and dehydrated, and then incubated in 20% sodium bisulfate/ $\times 2$ standard saline citrate at 43°C for 20 min. This was followed by treatment with proteinase K at 37°C for 25 min. Denaturation, hybridization and washing after hybridization were carried out according to the manufacturer's instructions. Slides were counterstained with 4',6-diamidino-2'-phenylindole dihydrochloride and examined under a fluorescence microscope (Olympus, Tokyo, Japan). After counting at least 50 tumor cell nuclei per core, *FGFR2* amplification was defined as a ratio of *FGFR2* (green) to chromosome 10 centromere signals (red) of ≥ 2.0 .

IHC

IHC staining was performed using an automated immunostainer (Bond-Max; Leica Microsystems, Wetzlar, Germany) according to the manufacturer's instructions. Anti-*FGFR2b* monoclonal antibody (FPR2-D; Five Prime Therapeutics, Inc., San Francisco, Calif., USA), which is specific for the *FGFR2*-IIIb splice variant of human *FGFR2*, was used as a primary antibody. Another commercial anti-*FGFR2* monoclonal antibody (ab59201; Abcam, Cambridge, UK) was tested for comparisons of *FGFR2* immunoreactivity. Cancer cells showing membranous staining,

Fig. 1. Representative photomicrographs of FGFR2b protein and *FGFR2* mRNA expression in primary GC. **a** Histological features of *FGFR2* amplified GC. HE. ×200. **b** *FGFR2* gene amplification evaluated by FISH using dual-color break-apart probes. ×1,000. **c** *FGFR2* mRNA transcript expression evaluated by mRNA ISH. ×200. **d** FGFR2b protein expression evaluated by IHC. ×200.

regardless of the presence of cytoplasmic staining, were considered to be positive. Immunostaining of FGFR2b was evaluated according to the HercepTest™ scoring guideline [27]: score 3, strong, complete membrane staining in more than 10% of the malignant cells; score 2, weak to moderate, complete membrane staining in more than 10% of the malignant cells, and score 0/1, less intense staining or less than 10% of cells. A score of 2+ or 3+ was considered positive and scores of 0 or 1+ were considered negative (2-grade system). Intratumoral heterogeneity was defined as different results between tissue array cores from the same tumor [24].

mRNA ISH

ISH detection of *FGFR2* mRNA was performed using a manual method with the RNAscope 2.0 FFPE assay kit (Advanced Cell Diagnostics, Hayward, Calif., USA) according to the manufacturer's instructions, as previously described [28]. Positive staining was indicated by brown punctate dots present in the tumor cells. *FGFR2* mRNA expression was scored according to the RNAscope 2.0 FFPE assay kit instructions [29, 30]: no staining or less than 1 dot/cell under a ×40 objective lens (score 0); 1–3 dots/cell under a ×20–40 objective lens (score 1); 4–10 dots/cell and no or very few clusters of dots under a ×20–40 objective lens (score 2); >10 dots/cell and <10% of positive cells with dot clusters under a ×20 objective lens (score 3), and >10 dots/cell and >10% of positive cells with dot clusters under a ×20 objective (score 4). Tissue microarrays with a score of 3 or 4 were considered to show *FGFR2* mRNA overexpression. Each tissue array block was stained for ubiquitin C (*UBC*) mRNA as a positive control, and only those with a score of 4 were used in the analysis.

Statistical Analysis

Survival curves were plotted using the Kaplan-Meier product limit method, and significant differences between survival curves

were determined using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazard model. The χ^2 or Fisher's exact test (2-sided) was used to determine associations between parameters. Results were considered significant when *p* values were <0.05. All statistical analyses were conducted using the SPSS 21.0 statistical software package (IBM Corp., Armonk, N.Y., USA).

Results

Validation of FGFR2b IHC and FGFR2 mRNA ISH by Comparison with Dual Color FISH

Of 362 GC cases with ISH and IHC results, FISH was performed in 188 cases (fig. 1). *FGFR2* gene amplification was found in 5 of 188 (2.7%) primary GC using FISH. All *FGFR2* gene-amplified cases showed FGFR2b protein overexpression and a *FGFR2* mRNA ISH score of 4 (*p* < 0.001). *FGFR2* gene amplification was not identified in FGFR2b IHC- or *FGFR2* mRNA ISH-negative carcinomas (table 1).

FGFR2 Expression Status by IHC and mRNA ISH and Its Clinical Implication

FGFR2b protein overexpression was identified in 9 of 362 primary GC (2.5%) by IHC, and *FGFR2* mRNA overexpression was identified in 21 of 362 primary GC (5.8%)

Table 1. FGFR2b IHC and *FGFR2* mRNA ISH results according to *FGFR2* dual-color FISH findings

	FISH		p value
	nonamplified	amplified	
IHC (n = 188)			<0.001
Negative	183	0	
Positive	0	5	
mRNA ISH (n = 188)			<0.001
Negative	180	0	
Positive (score 3 or 4)	3	5	
Fisher's exact test was used in each analysis.			

Table 2. Relationship between FGFR2b IHC and *FGFR2* mRNA ISH results

	<i>FGFR2</i> mRNA ISH			p value
	negative	positive	total	
FGFR2b IHC				<0.001
Negative	340 (93.9%)	13 (3.6%)	353 (97.5%)	
Positive	1 (0.3%)	8 (2.2%)	9 (2.5%)	
Total	341 (94.2%)	21 (5.8%)	362 (100%)	

by mRNA ISH. There was a strong association between FGFR2b protein expression and *FGFR2* mRNA expression ($p < 0.001$; table 2).

The clinicopathological features of FGFR2b- and *FGFR2*-positive and -negative tumors are summarized in table 3. FGFR2b protein overexpression was associated with perineural invasion ($p = 0.015$; table 3). However, FGFR2b protein and mRNA expression status showed no significant differences with respect to patient age, gender, histological subtype, lymphatic invasion, venous invasion, and lymph node and distant metastases ($p > 0.05$). In univariate survival analysis, FGFR2b protein and *FGFR2* mRNA overexpression was significantly associated with a poor outcome ($p < 0.001$ and $p = 0.012$, respectively; fig. 2). When the analysis was restricted to the advanced disease cohort (stage III–IV GC), FGFR2b protein and *FGFR2* mRNA overexpression was also significantly associated with a poor survival ($p = 0.019$ and 0.014 , respectively). Multivariable Cox hazard models revealed that *FGFR2* mRNA overexpression was an independent biomarker of poor survival ($p = 0.016$; table 4) after adjusting for tumor invasion and lymph node and distant metastases, which were all significant prognostic factors in the univariate analyses.

Fig. 2. Kaplan–Meier survival plots for the 362 GC patients according to FGFR2b protein overexpression (a) and *FGFR2* mRNA overexpression (b).

Intratumoral Heterogeneity of FGFR2 in Primary GC

FGFR2 mRNA and FGFR2b protein expression was examined in 3 different areas of each primary cancer. Intratumoral heterogeneity was defined as different results between tissue array cores from the same tumor [24] and was observed in 5 of 9 FGFR2b IHC-positive cases (55.5%) and 18 of 21 *FGFR2* mRNA ISH-positive

Table 3. Clinicopathologic characteristics related to FGFR2b protein and *FGFR2* mRNA expression

	FGFR2b IHC (n = 362)			<i>FGFR2</i> mRNA ISH (n = 362)		
	positive	negative	p value	positive	negative	p value
Age (mean $\pm$ SD), years	55 $\pm$ 17	58 $\pm$ 12	0.585	58 $\pm$ 15	58 $\pm$ 12	0.945
Gender			0.501			0.459
Males	7 (77.8%)	227 (64.3%)		12 (57.1%)	222 (65.1%)	
Females	2 (22.2%)	126 (35.7%)		9 (42.9%)	119 (34.9%)	
WHO classification			0.529			0.339
WD	0	14 (4.0%)		0	14 (4.1%)	
MD	1 (11.1%)	115 (32.6%)		4 (19.0%)	112 (32.8%)	
PD	7 (77.8%)	162 (45.9%)		14 (66.7%)	155 (45.5%)	
SRC	1 (11.1%)	38 (10.8%)		3 (14.3%)	36 (10.6%)	
Others	0	24 (6.6%)		0	24 (6.6%)	
Laurén classification			0.756			0.230
Intestinal	3 (33.3%)	172 (48.7%)		7 (33.3%)	168 (49.3%)	
Diffuse	5 (55.6%)	136 (38.5%)		10 (47.6%)	131 (38.4%)	
Mixed	1 (11.1%)	41 (11.6%)		3 (14.3%)	39 (11.4%)	
Indeterminate	0	4 (1.1%)		1 (4.8%)	3 (0.9%)	
Tumor invasion			1.000			1.000
Early GC	0	22 (6.2%)		1 (4.8%)	21 (6.2%)	
Advanced GC	9 (100.0%)	331 (93.8%)		20 (95.2%)	320 (93.8%)	
Lymphatic invasion			0.055			0.712
Not identified	0	122 (34.6%)		6 (30.0%)	116 (34.0%)	
Present	8 (100.0%)	231 (65.4%)		14 (70.0%)	225 (66.0%)	
Venous invasion			0.149			0.113
Not identified	6 (66.7%)	300 (85.0%)		15 (71.4%)	291 (85.3%)	
Present	3 (33.3%)	53 (15.0%)		6 (28.6%)	50 (14.7%)	
Perineural invasion			0.015			0.174
Not identified	0	139 (39.4%)		5 (23.8%)	134 (39.3%)	
Present	9 (100.0%)	214 (60.6%)		16 (76.2%)	207 (60.7%)	
Pathological TNM stage			0.059			0.690
I	0	67 (19.0%)		3 (14.3%)	64 (18.8%)	
II	1 (11.1%)	124 (35.1%)		7 (33.3%)	118 (34.6%)	
III	6 (66.7%)	136 (38.5%)		8 (38.1%)	134 (39.3%)	
IV	2 (22.2%)	26 (7.4%)		3 (14.3%)	25 (7.3%)	
Lymph node metastasis			0.062			0.435
Absent	0	114 (32.3%)		5 (23.8%)	109 (32.0%)	
Present	9 (100.0%)	239 (67.7%)		16 (76.2%)	232 (68.0%)	
Distant metastasis			0.148			0.215
Absent	7 (77.8%)	327 (92.6%)		18 (85.7%)	316 (92.7%)	
Present	2 (22.2%)	26 (7.4%)		3 (14.3%)	25 (7.3%)	
Total	9 (2.5%)	353 (97.5%)		21 (5.8%)	341 (94.2%)	

χ^2 or Fisher's exact test was used in the analysis of each factor except for age (Student's t test). WD = Well differentiated; MD = moderately differentiated; PD = poorly differentiated; SRC = signet ring cell.

cases (85.7%; table 5). All FGFR2b IHC-positive cases also showed *FGFR2* mRNA ISH positivity. One representative paraffin tumor block ('whole section') was tested for each FGFR2b-positive tumor. Whole section staining could be performed in all 9 cases by IHC and 19 of 21 cases by mRNA ISH. In the former, the average

proportion of positively stained area was 45.9% (81.2% in concordant cases and 17.6% in discordant cases; fig. 3a). There was no residual tumor tissue to obtain whole sections in 2 of the 21 *FGFR2* ISH-positive cases, but the average proportion of positively stained area in the other 19 tumors was 21.4% (63.3% in concordant

Table 4. Multivariable Cox proportional hazard models for predictors of overall survival

Variables	Hazard ratio	p value
FGFR2b IHC		
IHC expression (positive vs. negative)	1.869 (0.905–3.860)	0.091
Tumor invasion (early vs. advanced GC)	3.133 (1.151–8.534)	0.025
Lymph node metastasis (no vs. yes)	4.245 (2.462–7.319)	<0.001
Distant metastasis (no vs. yes)	4.592 (2.918–7.227)	<0.001
FGFR2 mRNA ISH		
mRNA ISH expression (positive vs. negative)	2.028 (1.141–3.603)	0.016
Tumor invasion (early vs. advanced GC)	3.219 (1.183–8.763)	0.022
Lymph node metastasis (no vs. yes)	4.303 (2.499–7.409)	<0.001
Distant metastasis (no vs. yes)	4.832 (3.074–7.595)	<0.001

95% confidence intervals are indicated in parentheses.

Table 5. Summary of FGFR2b IHC and *FGFR2* mRNA ISH findings in 3 different areas of primary GC

	IHC (n = 362)	mRNA ISH (n = 362)
Negative	353 (97.5)	341 (94.2)
Positive	9 (2.5)	21 (5.8)
Homogeneous	4 (44.4)	3 (14.3)
Heterogeneous	5 (55.5)	18 (85.7)

Table 6. Comparison of FGFR2b protein and *FGFR2* mRNA expression in primary GC and synchronous metastatic lymph nodes (LN)

	IHC (n = 135)	mRNA ISH (n = 135)
Negative	126 (93.3%)	121 (89.6%)
Positive in primary GC or metastatic LN	9 (6.7%)	14 (10.4%)
Concordant cases	5 (55.5%)	4 (28.6%)
Discordant cases	4 (44.4%)	10 (71.4%)

cases and 14.8% in discordant cases; fig. 3b). A representative image of intratumoral heterogeneity is shown in figure 4.

Discordance of FGFR2 Expression between Primary GC and Synchronous Metastatic Lymph Nodes

We evaluated 135 paired primary tumors and synchronous metastatic lesions in regional lymph node tissues (table 6). Using IHC, 6.7% (9/135) of tumors over-

expressed FGFR2b, and discordant FGFR2b expression between primary tumors and metastatic lymph nodes was noted in 4 of these 9 cases (44.4%). Of these 4 cases, 3 showed FGFR2b protein overexpression in primary tumors but not in their corresponding synchronous lymph nodes (negative conversion), whereas 1 case was negative for primary tumor FGFR2b expression but showed FGFR2b protein overexpression in the metastatic lymph node (positive conversion). In whole section staining for the 3 negative conversion cases, FGFR2b expression was observed in 10, 5 and 3% of the primary tumor area, respectively. In contrast, FGFR2b expression was not observed in whole section staining of the single positive conversion case, although dual color FISH revealed *FGFR2* gene amplification in the metastatic lymph nodes.

FGFR2 mRNA was expressed in 10.4% (14/135) of the metastatic lymph nodes, and discordant *FGFR2* mRNA expression between the primary tumor and metastatic lymph nodes was noted in 10 of these 14 cases (71.4%). Of the 10 discordant cases, 6 cases showed *FGFR2* mRNA overexpression in the primary tumor only, but not in synchronous lymph nodes (negative conversion), while in the other 4 cases the primary tumor was negative for *FGFR2* and the metastatic lymph nodes were positive (positive conversion). We performed mRNA ISH in the whole sections of 6 negative conversion cases, and *FGFR2* mRNA overexpression was observed in 85, 5, 5, 3, 3 and 0% of the whole primary tumor area. In contrast, only 1 out of 4 positive conversion cases showed focal (3%) positivity in the whole section, and the other 3 cases were negative. *FGFR2* dual color FISH on metastatic lymph nodes was performed in 3 out of 4 positive conversion cases, 2 of which showed *FGFR2* amplification.

Fig. 3. Areas of FGFR2 overexpression in a whole section of a primary tumor evaluated semiquantitatively using IHC (**a**) and mRNA ISH (**b**). Con = Homogeneous (concordant) overexpression of FGFR2b (i.e. FGFR2b overexpression in 3 different tissue array cores of each case; shaded); Dis = heterogeneous (discor-

dant) overexpression of FGFR2b (i.e. different FGFR2 expression intensities were observed between the tissue array cores in each case; not shaded); upper line = average extent of homogeneous overexpression in the tissue array study; lower line = average extent of heterogeneous overexpression in the tissue array study.

Fig. 4. Heterogeneous FGFR2 expression in different areas of the same primary tumor. FGFR2b protein expression was evaluated by IHC (**a**) and *FGFR2* mRNA expression was assessed by mRNA ISH (**b**).

Patient Survival Is Not Associated with Tumor Heterogeneity

The heterogeneity of FGFR2b protein and *FGFR2* mRNA tumor expression was not significantly associated with overall survival (data not shown). We evaluated the survival of the 135 GC patients with lymph node metastasis. The median survival for patients with and without FGFR2b protein overexpression was 17.3 and 41.9 months, respectively. The overall survival rate of patients

with FGFR2b protein overexpression was significantly lower than that of patients without FGFR2b protein overexpression, as determined by the log-rank test ($p = 0.039$; fig. 5a). However, on multivariate analysis, FGFR2b overexpression was not associated with reduced overall survival (data not shown). Neither *FGFR2* mRNA expression ($p = 0.241$; fig. 5b) nor lymph node discrepancy was significantly associated with overall survival (data not shown).

Fig. 5. Kaplan-Meier survival plots for the 135 GC patients with lymph node metastasis according to FGFR2b protein overexpression (a) and *FGFR2* mRNA overexpression (b).

Discussion

We performed simultaneous analyses by FISH, mRNA ISH and IHC for FGFR2 expression in GC, which is a particularly powerful approach as all of these assays are performed in situ. The proportion of positive tumors was 2.7, 5.8 and 2.5% by FISH, mRNA ISH and IHC, respectively, and, if positive in one of these assays, tumors were significantly more likely to be positive in the other two. The *FGFR2* gene amplification rate in this study is similar to those previously reported by studies using FISH [10].

Targeted drugs are currently being developed for patients with GC that exhibit *FGFR2* gene amplification [9, 17–20, 31]. A phase II FGFR2-targeted trial has been completed (although not yet published) [32] and another study is ongoing [33]. FISH is accepted as the gold standard for assessing gene amplification in tumors. However, as described previously, this technique has a number of limitations, especially in the evaluation of resected samples from large tumors. Pathologists frequently use IHC as a practical screening tool for genetic alterations and for selecting patients for clinical trials. HER2 protein overexpression in breast cancer [34] and GC [35], and anaplastic lymphoma kinase (ALK) protein expression in lung cancer [36] are examples of therapeutically signifi-

cant proteins that can be assessed by IHC. However, it was unknown whether previously reported FGFR2 IHC data reflected the *FGFR2* gene amplification rate. Six previous studies reported that FGFR2 protein overexpression assessed using IHC occurred in 21–57% of tumors [21, 37–41], but they did not assess the relationship between IHC and FISH results. Our study is the first to use an FGFR2IIIb-specific antibody. We also performed IHC with a commercial anti-FGFR2 antibody (Abcam) using the same primary GC tissue array, and the FGFR2-positive rate was 22.6%. This difference may be due to the low specificity of the Abcam antibody.

The incidence of *FGFR2* gene amplification detected using FISH is reportedly 2–9% in GC [10–16, 42]. Our IHC and mRNA ISH data concur with previously reported results using FISH, and all of the tumors with *FGFR2* gene amplification scored positive for IHC and mRNA ISH (table 1). These two methods can, therefore, potentially be used as primary evaluation tools for screening *FGFR2*-amplified GC. In scoring the *FGFR2* expression using mRNA ISH, the difference between score 3 and 4 is related only to the proportion of positive cells in the tumor. We, therefore, classified tumor samples with an mRNA ISH score of 3 as positive given the heterogeneity of FGFR2b protein expression. To establish a scoring guideline, such as that used for HER2 IHC

evaluation in breast cancer [34] and GC [35], further research on FGFR2b IHC and/or *FGFR2* mRNA ISH is warranted.

Heterogeneity of *FGFR2* gene amplification has been reported previously. Su et al. [12] reported intratumoral heterogeneity of *FGFR2* gene amplification in 7 of 29 (24%) GC cases, and Ooi et al. [16] reported that 5 of 6 *FGFR2* gene-amplified GC cases showed focal (30–70%) amplification, and only 1 case showed homogeneous staining. We have also demonstrated that FGFR2b protein overexpression is highly heterogeneous in GC. This discordance seems to be due to intratumoral heterogeneity because the majority of discordant cases showed negative conversion. Given that we only found 1 negative conversion case, it seems more likely that GCs are originally heterogeneous and metastasize randomly, rather than clonally obtaining FGFR2b IHC positivity and then metastasizing. A similar example is the heterogeneous expression of HER2 in GC [22, 24], and it has been suggested that heterogeneous HER2 expression could be a cause of Herceptin® (trastuzumab) resistance [43, 44]. Similarly, heterogeneity might make FGFR2b less attractive as a drug target.

The problem of heterogeneity might mean that evaluating FGFR2 expression in small biopsy samples from inoperable cases is inadequate, and Das et al. [15] found that a GC case showed heterogeneous *FGFR2* gene amplification in sample blocks despite none being found in the biopsy specimen. However, even cases that showed discordance in 3 tissue array cores were found to have an average positivity rate of 14.8% in mRNA ISH and 17.6% in IHC in a whole tissue section, with a positivity rate over 80% in some cases (fig. 3). Given that cases with only 10% HER2-positive GC are eligible for targeted treatment [4, 35], just a small portion of FGFR2b-positive cells in a biopsy might reflect a significant number of clones with *FGFR2* gene amplification in the whole tumor. Multiple biopsies or a deep biopsy would be good alternatives in these situations.

In our study, the FGFR2b protein and *FGFR2* mRNA overexpression rates increased from 2.5 to 6.7 and from 5.8 to 10.4%, respectively, when lymph node metastasis was present (table 6). This suggests that many potential candidates for targeted therapy will have tumors with *FGFR2* gene amplification, and concurs with the findings of our earlier study in which *FGFR2* gene amplification was shown to be associated with a high nodal stage [10]. Tumors carrying FGFR2b protein and *FGFR2* mRNA overexpression were not associated with other clinicopathological parameters, which is also consistent with the

findings of a published multicenter study [12] and our previous results [10].

This study has a number of limitations. We used tissue microarray to sample the tumor specimens, which may not fully represent and could underestimate the true number of FGFR2b-overexpressing cases. In addition, our study was conducted in an Asian population, and the rate of FGFR2b-overexpressing cases may differ with ethnicity. Considering the low overall rate of FGFR2 overexpression and its heterogeneity, it seems advisable to perform screening with IHC or mRNA ISH rather than FISH for surgically resected specimens. We suggest that both the primary tumor and lymph node metastases should be analyzed using FGFR2 IHC or mRNA ISH to correctly stratify patients and thus fully examine the efficacy of FGFR2-targeted therapy in GC.

Patients with FGFR2b protein- and *FGFR2* mRNA-overexpressing tumors have a significantly worse prognosis (fig. 2), as do those with lymph node metastases (fig. 5). This is consistent with former studies evaluating *FGFR2* gene amplification as a prognostic factor using FISH [10–12]. Conducting a FISH study for evaluating prognosis has poor cost-effectiveness, and IHC can be a reasonable and more economical alternative. Hence, evaluation of FGFR2b status by IHC or *FGFR2* status using mRNA ISH can potentially form the basis of both a diagnostic and a prognostic test.

In summary, a small subset of GC overexpresses FGFR2 and does so in a highly heterogeneous manner. Both FGFR2b IHC and *FGFR2* mRNA ISH predicted *FGFR2* gene amplification with high accuracy. These methods, therefore, offer a practical and economical alternative to FISH for detecting *FGFR2* gene amplification, and could be used for selecting patients in further studies of FGFR2-targeted therapy in GC.

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Disclosure Statement

The authors declare that they have no competing interests.

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