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Overexpression of *FGFR1* Promotes Peritoneal Dissemination *Via* Epithelial-to-Mesenchymal Transition in Gastric Cancer

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Abstract. *Background: Peritoneal dissemination (PD) is one of the most common causes of cancer-related mortality in gastric cancer (GC). We aimed to identify PD-associated genes and investigate their role in GC. Materials and Methods: We identified FGFR1 as a putative PD-associated gene using a bioinformatics approach. The biological significance of FGFR1 in epithelial-to-mesenchymal transition (EMT) was evaluated according to the correlation with genes that participated in EMT and FGFR1 knockdown experiments. The associations between FGFR1 expression and the clinicopathological features were examined. Results: FGFR1 expression positively correlated with SNAI1, VIM and ZEB1 expression, and negatively correlated with CDH1 expression. Knockdown of FGFR1 suppressed the malignant phenotype of GC cells. High FGFR1 expression significantly correlated with the peritoneal lavage cytology and synchronous PD positivity as well as poor prognosis. Conclusion: High FGFR1 expression was associated with PD via promotion of EMT and led to a poor prognosis of GC patients.*

Gastric cancer (GC) is the third leading cause of cancer-related mortality in both sexes worldwide, and the prognosis of patients with GC is dismal (1, 2). In most patients with advanced GC, GC-related mortality is caused by distant metastasis, and PD is the most frequent metastasis from GC (3). Once GC metastasizes to the peritoneal cavity, there is no therapeutic strategy to cure GC radically. Understanding the mechanisms of PD and the consequent development of sensitive biomarkers and therapeutic targets is necessary to conquer PD and improve the prognoses of patients.

Fibroblast growth factor receptor 1 (*FGFR1*) is a member of the FGFR family, and it is a receptor tyrosine kinase that activates mitogen activated protein kinase signaling and phosphoinositide-3-kinase/AKT signaling (4, 5). Aberrant expression and somatic mutation of *FGFR1* have been reported in several cancers, and *FGFR* inhibitors have been focused on as anticancer agents (6-8). In GC tissue, *FGFR1* has been reported to be overexpressed and to be associated with poor prognosis (9, 10). However, the molecular mechanisms of *FGFR1* contributing to metastasis and poor prognosis in GC are not fully known. Establishment of a metastatic focus requires a multistep process, and epithelial to mesenchymal transition (EMT) plays a pivotal role in several steps, including vessel invasion, detachment from primary lesion and anoikis resistance (11). To the best of our knowledge, there are currently no studies on the association between *FGFR1* and EMT in GC. Therefore, the aim of the present study was to assess the clinical significance of *FGFR1* expression in GC focusing on EMT and PD.

Materials and Methods

Identification of putative PD-associated genes. To identify genes that mediate PD, we performed extraction-of-expression modules (EEM) analysis as previously described (12, 13). EEM indicated that the gene set that was up-regulated in the 58As9 cell line, that was established from the HSC-58 cell line and is considered to be a highly peritoneal-metastatic cell line compared to the HSC-58 cell line, was most significantly associated with poor overall survival (OS), and *FGFR1* was included in the upregulated gene set.

Cell lines and knockdown (KD) of FGFR1. The human scirrhous GC cell lines HSC-58 and 58As9Luc and the culture conditions have been previously described (13-16). The pcDNA6.2-GW/EmGFP-miR

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Key Words: Gastric cancer, *FGFR1*, peritoneal dissemination, epithelial to mesenchymal transition.

Table I. *Sequences of shRNAs and primers.*

Oligo	Number/Gene	Type	Sequence
shRNA	#1	Top	5'-TGCTGAGTTGATGCTCTGCACATCGTGTTTTG GCCACTGACTGACACGATGTGGAGCATCAACT-3'
		Bottom	5'-CCTGAGTTGATGCTCCACATCGTGTCAGTCAG TGGCCAAAACACGATGTGCAGAGCATCAACTC-3'
	#2	Top	5'-TGCTGAAGTGAAGCACCTCCATCTCTGTTTTG GCCACTGACTGACAGAGATGGGTGCTTCACTT-3'
		Bottom	5'-CCTGAAGTGAAGCACCCATCTCTGTCAGTCAG TGGCCAAAACAGAGATGGAGGTGCTTCACTTC-3'
	#3	Top	5'-TGCTGAAAGTCTGCTATCTTCATCACGTTTTG GCCACTGACTGACGTGATGAATAGCAGACTTT-3'
		Bottom	5'-CCTGAAAGTCTGCTATTCATCACGTCAGTCAG TGGCCAAAACGTGATGAAGATAGCAGACTTTC-3'
Primer	*FGFR1*	Forward	5'-ACAACCTGCCTTATGTCCAGA-3'
		Reverse	5'-TCCATCTCTTTGTCGGTGGT-3'
	CDH1	Forward	5'-TGGAGGAATTCTTGCTTTGC-3'
		Reverse	5'-CGCTCTCCTCCGAAGAAAC-3'
	SNAI1	Forward	5'-GCTGCAGGACTCTAATCCAGA-3'
		Reverse	5'-ATCTCCGGAGGTGGGATG-3'
	VIM	Forward	5'-GACAATGCGTCTCTGGCACGTCTT-3'
		Reverse	5'-TCTTCTGCCTCCTGCAGGTTCTT-3'
	ZEB1	Forward	5'-TTTTTCCTGAGGCACCTGAA-3'
		Reverse	5'-AAAATGCATCTGGTGTTCCAT-3'

plasmid from the Block-iT Pol II miR RNAi Expression Vector Kit (Invitrogen, Carlsbad, CA, USA) was obtained for KD of *FGFR1*. Three double-stranded shRNAs specific for *FGFR1* (sh#1, #2 and #3) were designed using an online software (http://www.invitrogen.com/rnai) (Table I). shRNAs specific for *FGFR1* and negative control shRNA (shNC) were transfected into 58As9Luc cells using Lipofectamine 2000 (Invitrogen). Stable transfectants were selected by 10 μg/mL blasticidin and fluorescence activated cell sorting for GFP.

Clinical GC samples. Primary GC samples of the Singapore dataset were obtained from 198 patients who underwent gastric resection at the Singapore Health Services and deposited in National University Hospital System tissue repositories. Primary GC samples of the Beppu dataset were obtained from 197 patients who underwent gastric resection at the Oita Prefectural Hospital and the Kyushu University Beppu Hospital. All patients provided written informed consent, and the study protocol was approved by the appropriate ethics committee. Experiments with these samples were performed in accordance with the approved guidelines.

RNA extraction, gene expression array and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). The gene expression array of the Singapore dataset has been previously described (13). Using the Beppu dataset and GC cell lines, qRT-PCR was performed as previously described, and glyceraldehyde-3-phosphate dehydrogenase mRNA expression was quantified for standardization (13). The specific primers are listed in Table I.

Protein extraction and western blotting. Cells were lysed in lysis buffer, and protein expression was evaluated by WB as previously described (17). A primary rabbit monoclonal antibody against *FGFR1* (#9740; Cell Signaling Technology, Beverly, MA, USA) was used at a dilution of 1:1,000 at 4˚C overnight. A primary mouse monoclonal antibody against β-actin (sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA) was used at a dilution of 1:1,000 at room temperature for 1 h.

Cell proliferation, migration and invasion assay. The cell proliferation capacity was evaluated by the MTT assay using the Cell Proliferation Kit 1 (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol in biological sextuplicates. The cell migration capacity and invasion capacity were assessed using Corning BioCoat Control Inserts (#354578; BD Biosciences, Bedford, MA, USA) and Matrigel Invasion Chambers (#354480; BD Biosciences) according to manufacturers' protocol in biological quadruplicates. After an appropriate incubation time (migration, 24 h; invasion, 96 h), we used a light microscope to count the cells present on the surface of the membrane.

Gene Set Enrichment Analysis (GSEA). The associations between *FGFR1* expression and previously defined gene sets were analyzed by GSEA using gene expression profiles from The Cancer Genome Atlas (TCGA) dataset as previously described (18).

Statistical analysis. For continuous variables, statistical analyses were performed using Student's *t*-tests. Categorical variables were compared using Pearson's correlation coefficients and χ^2 tests or Fisher's exact tests. Survival time was evaluated using the Kaplan-Meier method,

Table II. *Association between FGFR1 expression and the clinicopathological parameters in the Beppu dataset.*

Clinicopathological parameters	*FGFR1* high n=99	*FGFR1* low n=98	*p*-Value
Age			
<65	44	35	0.246
≥65	54	63	
Gender			
Male	37	35	0.883
Female	62	63	
T			
1/2	53	69	0.019
3/4	46	29	
N			
Negative	29	41	0.074
Positive	69	56	
M			
Negative	69	81	0.044
Positive	30	17	
H			
Negative	92	92	1.000
Positive	7	6	
CY/P			
Negative	78	89	0.028
Positive	21	9	
Differentiation			
Differentiated	34	57	0.002
Undifferentiated	64	41	
Lymphatic invasion			
Negative	32	39	0.301
Positive	67	59	
Venous invasion			
Negative	65	73	0.214
Positive	34	25	

T, Tumor depth; N, lymph node metastasis; M, distant metastasis; H, hepatic metastasis; CY/P, peritoneal lavage cytology and synchronous peritoneal dissemination. NA values were omitted.

Table III. *Association between FGFR1 expression and the clinicopathological parameters in the Singapore dataset.*

Clinicopathological parameters	*FGFR1* high n=99	*FGFR1* low n=99	*p*-Value
Age			
<65	41	34	1.000
≥65	53	46	
Gender			
Male	38	32	0.457
Female	61	67	
T			
1/2	24	30	0.102
3/4	70	50	
N			
Negative	23	15	0.364
Positive	71	66	
M			
Negative	78	69	0.837
Positive	16	12	
CY/P			
Negative	71	70	0.053
Positive	23	10	
Differentiation			
Differentiated	29	44	0.039
Undifferentiated	70	55	
Lymphovascular invasion			
Negative	24	22	0.419
Positive	34	21	
H. pylori			
Negative	18	14	0.497
Positive	37	20	
Lauren's classification			
Intestinal	42	59	0.015
Diffuse	48	28	
Mixed/other	9	12	
UICC pathological stage			
I/II	27	36	0.222
III/IV	72	63	

T, Tumor depth; N, lymph node metastasis; M, distant metastasis; CY/P, peritoneal lavage cytology and synchronous peritoneal dissemination. NA values were omitted.

and survival curves were compared using log-rank tests. Multivariate analysis for survival time was estimated by the Cox proportional hazard model. All statistical analyses were performed using R version 3.4.1 (Vienna, Austria. URL: http://www.R-project.org/).

Results

Identification of FGFR1 as a putative PD-associated gene. EEM analysis detected module genes for PD in a gene set obtained from comparison between HSC-58 and 58As9 cells. In this study, we focused on *FGFR1*, which was overexpressed in 58As9 cells by 4.03 $\log_2$-fold compared to HSC-58 cells, and its high expression was significantly associated with poor OS in the Singapore dataset.

High FGFR1 expression is positively correlated with EMT markers. GSEA revealed that high *FGFR1* expression was positively correlated with the gene set associated with EMT (Figure 1A). We established stable *FGFR1* KD cell lines, and inhibition of *FGFR1* expression was confirmed by qRT-PCR and WB (Figure 1B). sh#2 and sh#3 worked well, and sh#1 did not have a KD effect. *FGFR1* in the GC cell lines negatively correlated with *cadherin 1* (*CDH1*) and positively correlated with *snail family transcriptional repressor 1* (*SNAI1*), *vimentin* (*VIM*) and *zinc finger E-box binding homeobox 1* (*ZEB1*) (Figure 1C-F). A significant negative correlation with *CDH1* and significant positive correlations with *SNAI1, VIM* and *ZEB1* were observed in both the Singapore dataset (Figure 1G-J) and TCGA dataset (Figure 1K-N) with statistical significance. In subsequent experiments, sh#1-transfected cells were excluded due to an insufficient KD effect.

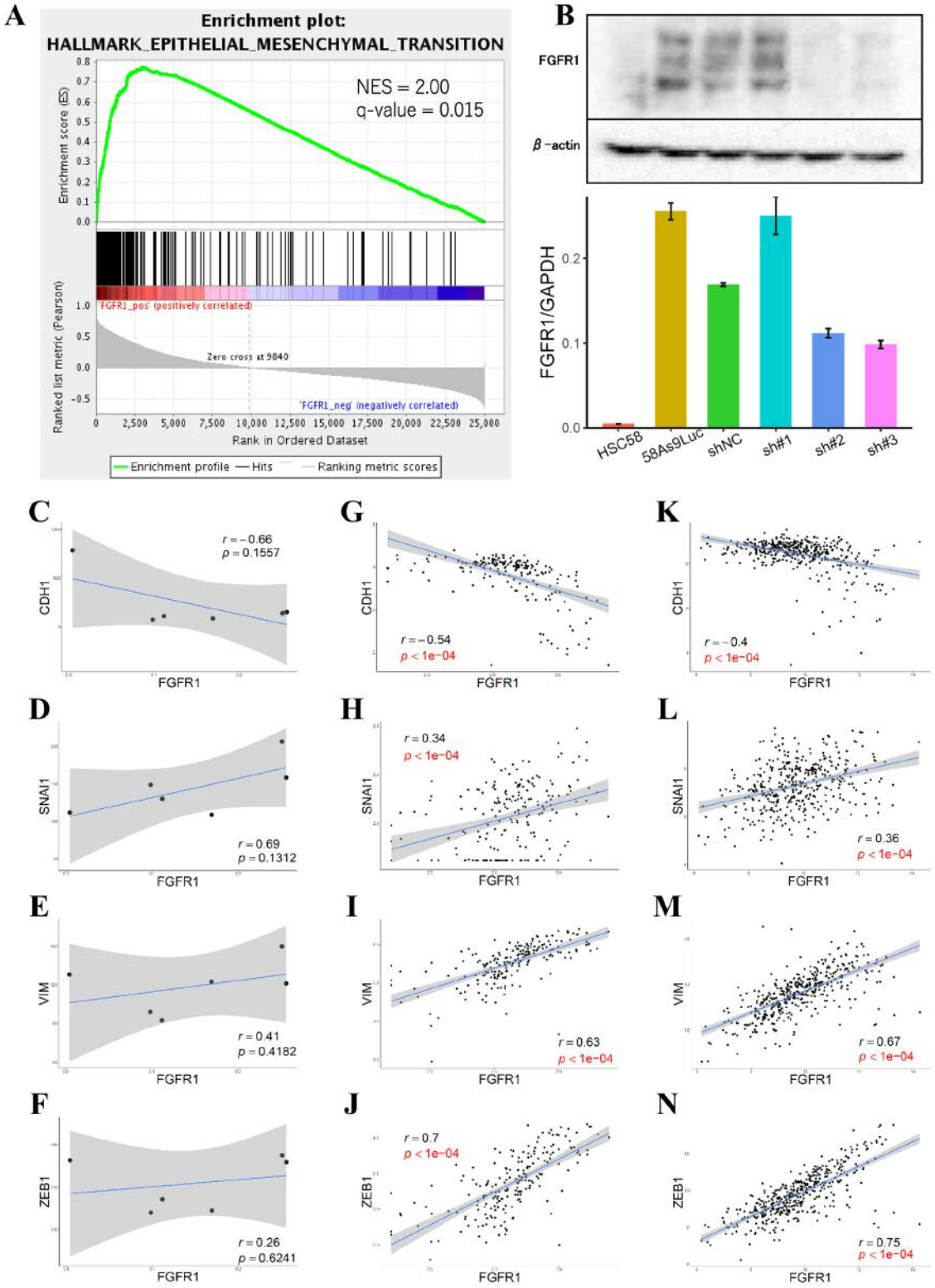

Figure 1. *FGFR1 expression is associated with EMT-associated genes. A: GSEA revealed that high FGFR1 expression positively correlated with a gene set associated with EMT. B: KD of FGFR1 was confirmed by qRT-PCR and western blotting. C-F: Correlation analysis between FGFR1 and EMT-associated genes in the GC cell line. G-J: Correlation analysis between FGFR1 and EMT-associated genes in the Singapore dataset. K-N: Correlation analysis between FGFR1 and EMT-associated genes in the TCGA dataset.*

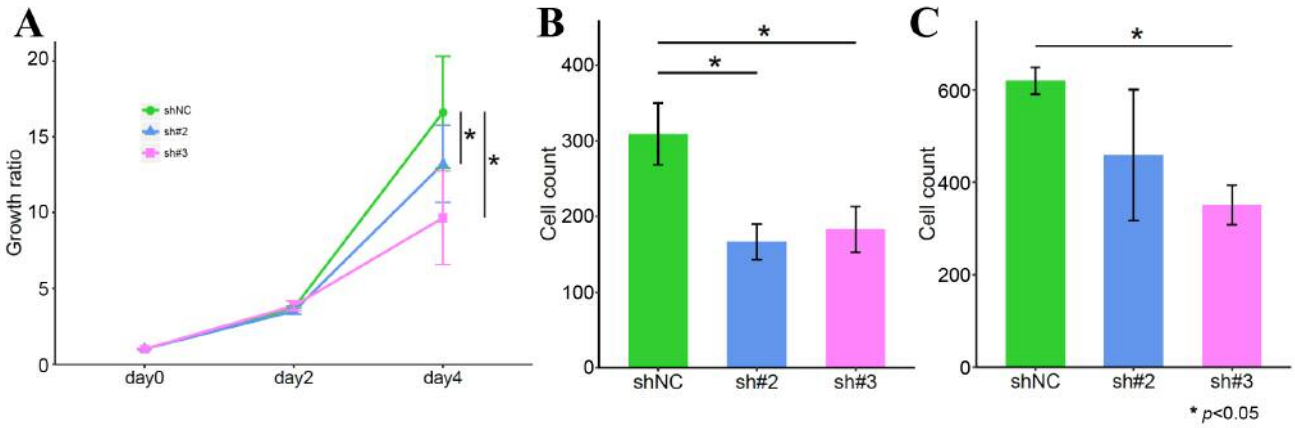

Figure 2. *Effect of KD of FGFR1 on the cell phenotype. A; MTT assay. B; Migration assay. C; Invasion assay.*

KD of FGFR1 suppresses the malignant phenotype of GC cells. To determine the influence of *FGFR1* on the cell phenotype, we evaluated the proliferation, migration and invasion capacities using *FGFR1* KD cells. KD of *FGFR1* significantly decreased the cell proliferation capacity (Figure 2A). The number of GC cells migrating across the micropore membrane decreased in *FGFR1* KD cells (Figure 2B). Moreover, there was significant change in the invasion capacity in sh#3-transfected cells (Figure 2C).

Clinical significance of FGFR1 expression. Patients were divided into two groups according to the median value of *FGFR1* expression in the Beppu and Singapore datasets. High *FGFR1* expression significantly correlated with tumor depth, distant metastasis, undifferentiated type and the peritoneal lavage cytology and synchronous PD (CY/P) positivity in the Beppu dataset (Table II), and high *FGFR1* expression significantly correlated with undifferentiated type and Lauren's classification in the Singapore dataset (Table III). Patients with high *FGFR1* expression had the following results for each dataset: significantly short disease-specific survival time in the Beppu dataset, significantly short OS and recurrence free survival (RFS) in the Singapore dataset, and short OS in the TCGA dataset (Figure 3). High *FGFR1* expression was an independent prognostic factor for OS and RFS in the Singapore dataset (Table IV).

Discussion

PD from GC greatly affects the quality of life of patients and is the main cause of cancer-related mortality. Understanding the mechanism and management of PD should contribute to the improvement of patient prognosis. We aimed to identify the PD-associated genes and investigate how those genes are associated with GC progression. Some studies have reported that high expression of *FGFR1* is associated with poor prognosis in GC patients, and the utility of *FGFR1* inhibitors has been reported in GC *in vitro* and *in vivo* (9, 10, 19-22). However, the underlying mechanism of the exacerbation of GC patient prognosis *via* high *FGFR1* expression is not fully understood. We performed GSEA to explore the *FGFR1* contribution to poor prognosis in GC and revealed that high FGFR1 expression was positively correlated with a gene set associated with EMT. The association between *FGFR1* and EMT has been reported in prostate cancer, bladder cancer, head and neck squamous cell carcinomas and chordoma, but not in digestive cancers, including GC (23-27). We demonstrated that *FGFR1* negatively correlated with *CDH1* and positively correlated with *SNAI1, VIM* and *ZEB1* in GC cells and clinical GC samples. KD of *FGFR1* significantly suppressed the malignant phenotype of GC cells. These results supported the relevance of *FGFR1* to EMT. In the Beppu dataset, high *FGFR1* expression significantly correlated with CY/P positivity, although there was no correlation with hepatic metastasis. In the Singapore dataset, high *FGFR1* expression correlated with CY/P positivity. Therefore, these data suggest that *FGFR1* specifically contributes to the establishment of PD *via* promoting EMT. Moreover, high FGFR1 expression correlated with the undifferentiated type in both datasets. The comprehensive analyses of large cohorts indicated that FGFR1 specifically associates with PD because the undifferentiated type has been reported to be a risk factor for PD in GC (28). Furthermore, patients with high FGFR1 expression had poor prognosis, and high FGFR1 expression was an independent prognostic factor for OS and RFS in the Singapore dataset. Thus, FGFR1 might be a putative prognostic biomarker for GC patients.

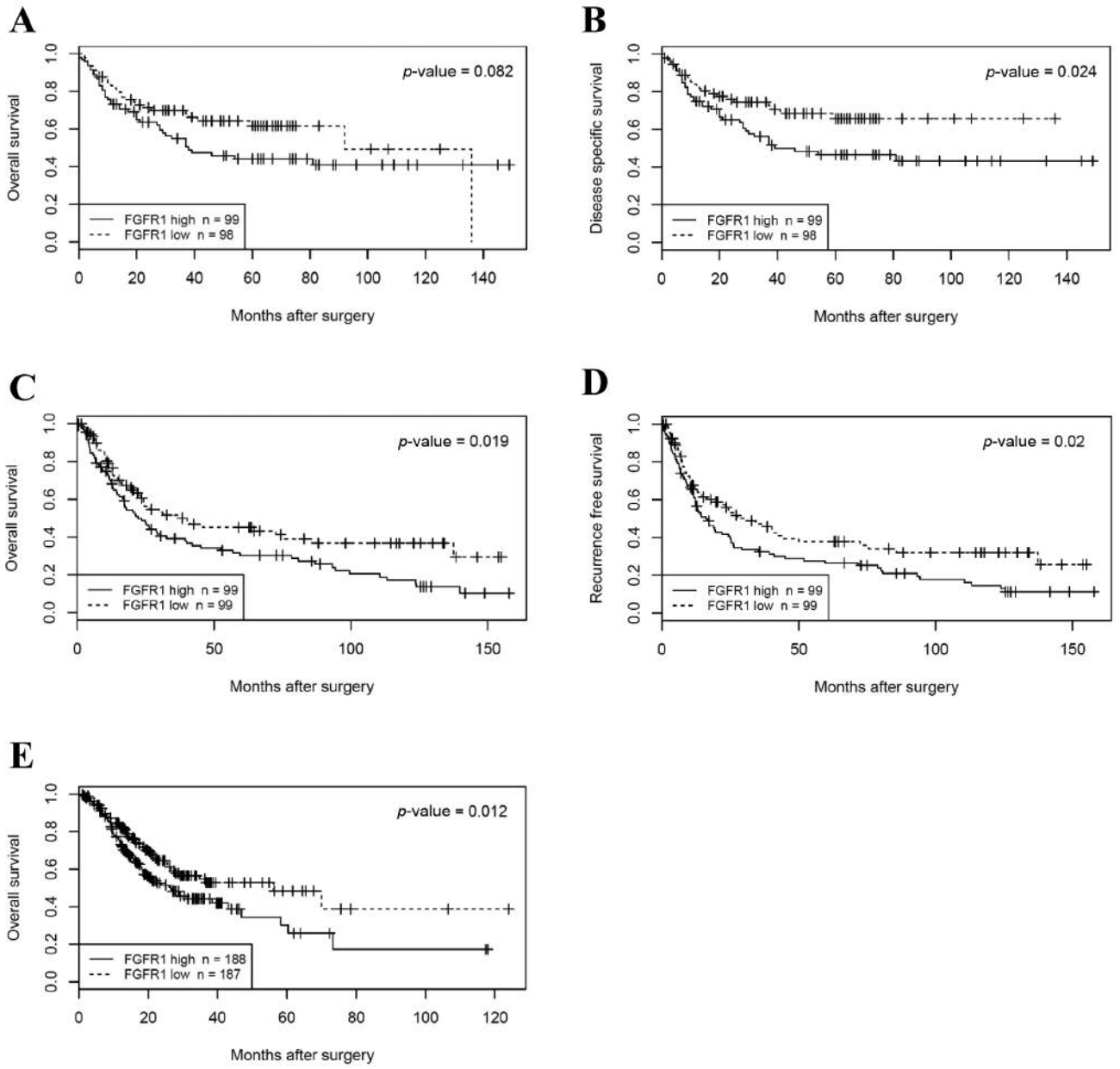

Figure 3. *Survival analysis according to FGFR1 expression. A, B: OS and DSS in the Beppu dataset. C-D: OS and RFS in the Singapore dataset. E: OS in the TCGA dataset.*

One limitation of our study was that the molecular mechanism through which *FGFR1* promoted EMT remains unknown. Receptor tyrosine kinase has been reported to activate the *PI3K/AKT* signaling pathway, which is essential for EMT (5, 29, 30). Additionally, it has been reported that *AKT* phosphorylates and suppresses *GSK3β*, which leads to the degradation of β-catenin *via* promoting ubiquitination (31-34). Another limitation was the lack of an *in vivo* study. Further investigation into the effect of *FGFR1* on downstream pathways and *in vivo* using an appropriate orthotopic xenograft model would lead to a better understanding of the association between *FGFR1* and PD. In conclusion, our study demonstrated that *FGFR1* associates with PD *via* promoting EMT in GC and that FGFR1 might be a putative prognostic biomarker.

Conflicts of Interest

The Authors declare no competing financial interests.

Table IV. *Multivariate analysis for survival time in the Singapore dataset*.

Variables	OS			RFS		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Older than 65 yo	1.75	0.82-3.73	0.149	1.78	0.87-3.64	0.114
Male gender	0.66	0.32-1.36	0.261	0.76	0.37-1.56	0.458
T3/4	4.94	1.71-14.28	0.003	4.56	1.59-13.07	0.005
N (+)	5.42	0.86-34.23	0.073	10.03	1.85-54.28	0.007
M (+)	5.63	1.55-20.42	0.009	2.64	0.77-9.03	0.121
CY/P (+)	2.33	0.89-6.12	0.085	3.84	1.44-10.18	0.007
Undifferentiated type	2.33	0.92-5.91	0.075	2.01	0.85-4.73	0.112
Lymphovascular invasion (+)	1.67	0.77-3.62	0.191	1.93	0.90-4.14	0.091
H. pylori (+)	0.37	0.16-0.83	0.016	0.42	0.20-0.92	0.030
Lauren's classification	0.63	0.32-1.22	0.171	0.80	0.44-1.45	0.457
UICC stage III/IV	0.67	0.13-3.49	0.632	0.35	0.08-1.53	0.165
FGFR1 high	3.00	1.40-6.42	0.005	3.02	1.45-6.27	0.003

Yo, Years old; T, tumor depth; N, lymph node metastasis; M, distant metastasis; H, hepatic metastasis; CY/P, peritoneal lavage cytology and synchronous peritoneal dissemination.

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