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Original Article

Differential expression of PI3K isoforms and correlation with clinicopathological factors in patients with gastric cancer

Hyoun Wook Lee[1], Eun Hee Lee[1], Moon-il Park[1], Mee-Seon Kim[1], Jin Sook Jeong[2], Mee Sook Roh[2], Dae Cheol Kim[2], Su Jin Kim[2], Min Gyoung Pak[2], Seo Hee Rha[2]

[1]Department of Pathology, Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, Changwon, South Korea; [2]Department of Pathology, Dong-A University College of Medicine, Busan, South Korea

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Abstract: Activation of phosphoinositide 3-kinase (PI3K) is pivotal for the oncogenic PI3K/AKT signaling pathway. This study assessed the differential expression of PI3K isoforms in gastric cancer and correlation with clinicopathological parameters. Tissue microarray blocks were generated from 153 gastric cancers and immunohistochemically stained for 4 PI3K isoforms: p110α, p110β, p110γ and p110δ. Isoform p110α was more highly expressed in tumors with a diameter less than 4 cm (P=0.009); lower pathological T (pT) stage (P<0.001), N stage (P=0.001), or TNM stage (P<0.001); and no lymphovascular invasion (P=0.001); p110β was significantly associated with advanced pT stage (P=0.028) and lymphovascular invasion (P=0.014). High p110α expression significantly correlated with longer overall survival (OS) (P=0.007) and recurrence-free survival (RFS) (P=0.048), whereas high p110β expression correlated with shorter OS (P=0.008) and RFS (P=0.058). In addition, p110β was an independent factor for poor prognosis in multivariate analysis for OS. Neither p110γ nor p110δ expression showed clinicopathological significance. These results suggested that p110β might be more important for the development and progression of gastric cancer than other PI3K isoforms and had potential as a prognostic biomarker in patients with gastric cancer.

Keywords: Gastric cancer, p110α, p110β, immunohistochemistry, prognosis

Introduction

The incidence and mortality of gastric cancer have steadily decreased over the past several decades [1, 2]. However, gastric cancer remains the fourth most common cancer worldwide and the second most common cause of cancer-related death [3]. A considerable proportion of patients with gastric cancer present with unresectable advanced disease at diagnosis and have a poor prognosis. Although surgical resection of primary gastric cancers can be curative for patients with early stage disease, over 60% eventually have a relapse [4]. For patients with advanced or recurrent disease, fluoropyrimidin-based chemotherapy is widely used. This treatment has a prolonged median overall survival of less than 12 months [5-8]. To overcome the limits of current chemotherapy, the development of novel targeted agents has been intensively investigated to improve outcomes of gastric cancer patients [9-11].

The phosphoinositide 3-kinase (PI3K)/AKT pathway is involved in the regulation of cell survival, growth, proliferation and migration [12] and is constitutionally activated by aberrations in the molecular components of the pathway. These aberrations include mutation or amplification of *PIK3CA*, a gene encoding p110α, a catalytic subunit of PI3K, and genetic or epigenetic inactivation of phosphatase and tensin homolog (PTEN) protein, a negative regulator of the PI3K/AKT pathway [13]. The constitutional activation of the PI3K/AKT pathway contributes to oncogenic cellular transformation in different cancers, including gastric cancer [13-17]. Thus, the PI3K/AKT pathway is a promising therapeutic target [13, 18].

To date, most studies on PI3K/AKT pathway aberrations in gastric cancer have focused on amplification or mutation of *PIK3CA*, not PI3K protein expression [17, 19-21]. The PI3K family is divided into three classes according to struc-

tural features and substrate specificity. Of these, only class I PI3Ks are involved in oncogenesis [16]. Class I PI3Ks are heterodimeric proteins that consist of a catalytic subunit and a regulatory subunit. The four isoforms of the catalytic subunit are p110α, p110β, p110γ and p110δ [16]. Some studies have reported an association between overexpression of these isoforms and clinicopathological factors in human cancers [22-26]. However, no data are available on gastric cancer.

This study examined the differential expression of PI3K isoforms in gastric cancer and the correlation between their expression and clinicopathological factors, including patient survival. This study aimed to assess the potential of PI3K expression as a predictive biomarker for PI3K inhibitor therapy or a prognostic biomarker of gastric cancer.

Materials and methods

Patients and tissue samples

Included were 153 patients with gastric cancer who underwent radical gastrectomy at Samsung Changwon Hospital from January 2002 through December 2005. No patients had neoadjuvant chemotherapy. All pathological slides were reviewed and histological type, Lauren classification, location, depth of invasion, regional lymph node metastasis, lymphatic invasion and venous invasion were reevaluated. Other clinicopathological data such as age, sex, distant metastasis and survival data were obtained from medical records. Clinical stage was redetermined according to the 7th edition of the American Joint Committee on Cancer TNM staging system [27]. Follow-up data were included through July 2013 or until death or loss to follow-up.

Tissue microarrays and immunohistochemistry

Representative areas of tumors were marked on hematoxylin-and-eosin-stained slides and used for tissue microarrays (TMAs). Two tissue cores per tumor with a diameter of 1 mm were taken from donor paraffin blocks and put in blank recipient paraffin blocks. TMA blocks were sectioned at 4 μm for immunohistochemical staining using a Ventana Benchmark XT (Roche-Ventana, Tucson, AZ, USA). All sections were deparaffinized and subjected to pretreatment with CC1 (Roche-Ventana) for 30 minutes at 100°C. Sections were washed with reaction buffer followed by incubation with primary antibodies for 32 or 60 minutes at 37°C. Primary antibodies were against p110α (clone C73F8, 1:100, Cell Signaling Technology, Danvers, MA, USA), p110β (clone EPR5515, 1:300, Epitomics, Burlingame, CA, USA), p110γ (clone D-12, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p110δ (clone A-8, 1:100, Santa Cruz Biotechnology). An UltraView Universal DAB kit (Roche-Ventana) was used according to the manufacturer's recommendations to detect primary antibody followed by counterstaining with hematoxylin (Roche-Ventana). Breast carcinoma was used as the positive control. The negative control was buffer instead of primary antibody.

Assessment of immunohistochemical results

Immunostained slides were evaluated without clinicopathological information. Assessment of immunohistochemical results used the modified H-score method [28, 29]. Specifically, staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). Staining extent was expressed as percent positive cells (0-100%) in 5% increments. H-score was calculated as (% of cells stained at intensity 1 × 1) + (% of cells stained at intensity 2 × 2) + (% of cells stained at intensity 3 × 3). H-scores ranged from 0 to 300. For isoform expression, median H-score was used as the cutoff for dividing into low-expression and high-expression groups.

Statistical analysis

Statistical analyses were performed with SPSS Ver. 18 (SPSS Inc., Chicago, IL, USA). Correlations among PI3K isoform H-scores were estimated with the Spearman correlation test. To evaluate relationships between expression of PI3K isoforms and clinicopathological parameters, we used Fisher's exact test for categorical variables or the Mann-Whitney test for ordinal variables. Impacts of parameters on overall survival (OS) and recurrence-free survival (RFS) were analyzed by the Kaplan-Meier method and differences compared by the log-rank test. Multivariate analyses for OS and RFS used the Cox proportional hazards model. A P-value <0.05 was considered statistically significant.

Table 1. Proportion of patients with high PI3K isoform expression according to clinicopathological characteristics

Variables	No. of cases	p110α		p110β		p110γ		p110δ	
		No. (%)	P	No. (%)	P	No. (%)	P	No. (%)	P
Total	153	78 (51)		88 (58)		77 (50)		96 (63)	
Age (years)									
<60	56	31 (55)	0.502	33 (59)	0.866	25 (45)	0.317	35 (63)	1.000
≥60	97	47 (49)		55 (57)		52 (54)		61 (63)	
Sex									
Male	103	52 (51)	0.865	63 (61)	0.224	49 (48)	0.390	62 (60)	0.378
Female	50	26 (52)		25 (50)		28 (56)		34 (68)	
Location									
Antrum	104	49 (47)	0.114	57 (55)	0.574	51 (49)	0.239	64 (62)	0.952
Body	28	20 (71)		16 (57)		18 (64)		19 (68)	
Cardia	5	2 (40)		4 (80)		1 (20)		3 (60)	
Two or more areas	16	7 (44)		11 (69)		7 (44)		10 (63)	
Histological type									
Tubular	131	67 (51)	0.358	78 (60)	0.499	69 (53)	0.535	85 (65)	0.169
Signet ring cell	17	10 (59)		7 (41)		6 (35)		10 (59)	
Mucinous	3	0 (0)		2 (67)		1 (33)		1 (33)	
Others	2	1 (50)		1 (50)		1 (50)		0 (0)	
Lauren classification									
Intestinal	84	42 (50)	0.871	50 (60)	0.624	43 (51)	0.872	51 (61)	0.616
Diffuse	69	36 (52)		38 (55)		34 (49)		45 (65)	
Size (cm)									
<4	64	41 (64)	0.009	36 (56)	0.869	34 (53)	0.624	36 (56)	0.178
≥4	89	37 (42)		52 (58)		43 (48)		60 (67)	
Pathological T stage									
pT1	46	36 (78)	<0.001	21 (46)	0.028	26 (57)	0.202	24 (52)	0.205
pT2	24	12 (50)		15 (63)		13 (54)		14 (58)	
pT3	33	13 (39)		17 (52)		16 (49)		27 (82)	
pT4	50	17 (34)		35 (70)		22 (44)		31 (62)	
Pathological N stage									
pN0	57	39 (68)	0.001	28 (49)	0.079	31 (54)	0.243	29 (51)	0.060
pN1	41	18 (44)		24 (59)		21 (51)		28 (68)	
pN2	19	9 (47)		12 (63)		11 (58)		16 (84)	
pN3	36	12 (33)		24 (67)		14 (39)		23 (64)	
Distant metastasis									
No metastasis	144	74 (51)	0.742	84 (58)	0.496	74 (51)		89 (62)	0.485
Metastasis	9	4 (44)		4 (44)		3 (33)		7 (78)	
TNM stage									
I	54	40 (74)	<0.001	28 (52)	0.229	30 (56)	0.191	28 (52)	0.067
II	36	15 (42)		19 (53)		19 (53)		25 (69)	
III	54	19 (35)		37 (69)		25 (46)		36 (67)	
IV	9	4 (44)		4 (44)		3 (33)		7 (78)	
Lymphatic invasion									
Negative	49	35 (71)	0.001	21 (43)	0.014	27 (55)	0.489	30 (61)	0.858
Positive	104	43 (41)		67 (64)		50 (48)		66 (64)	

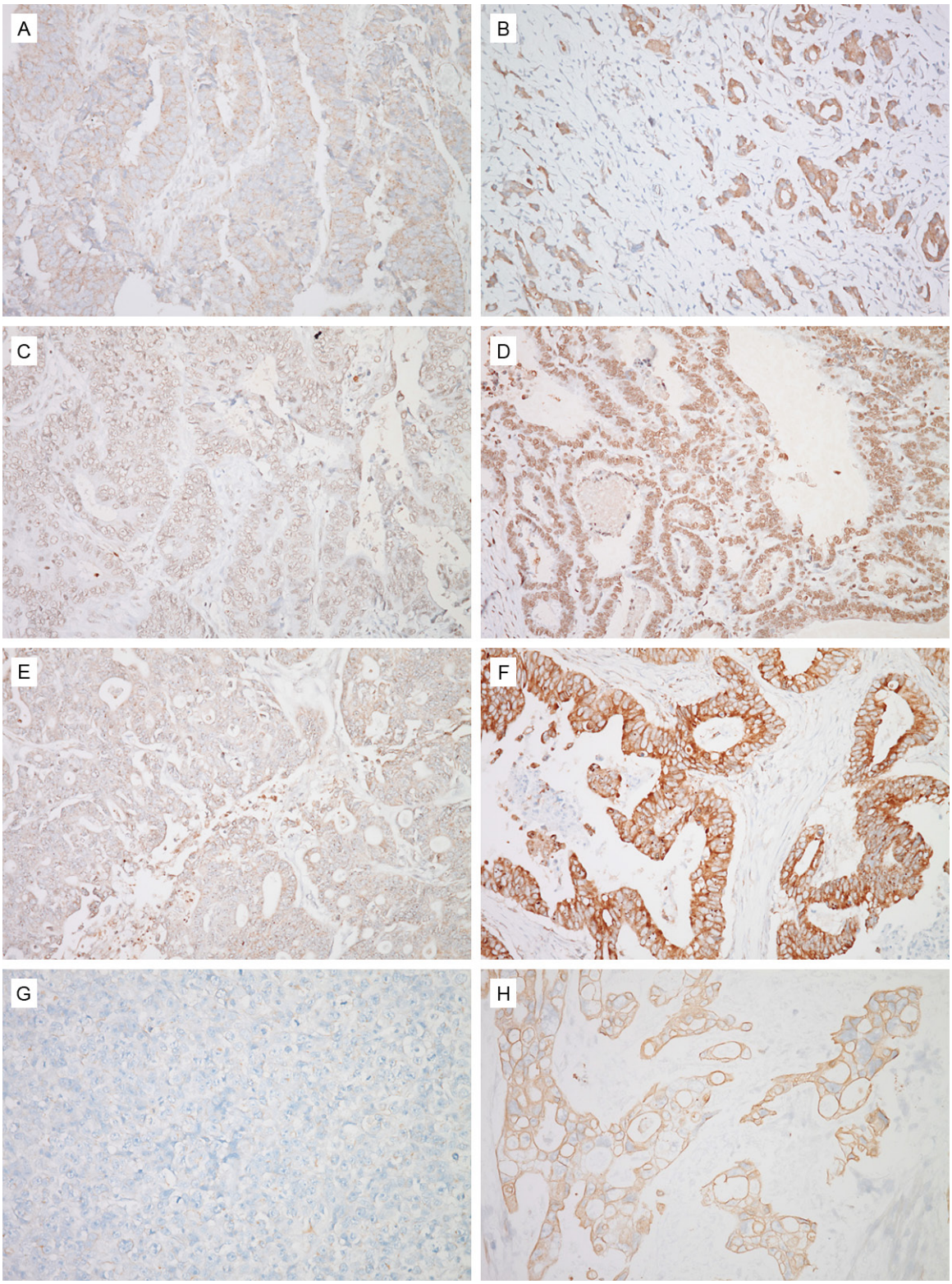

Figure 1. Immunohistochemical staining of PI3K isoforms in gastric cancers. Low (A) and high (B) expression of p110α, low (C) and high (D) expression of p110β, low (E) and high (F) expression of p110γ, and negative (G) and high (H) expression of p110δ.

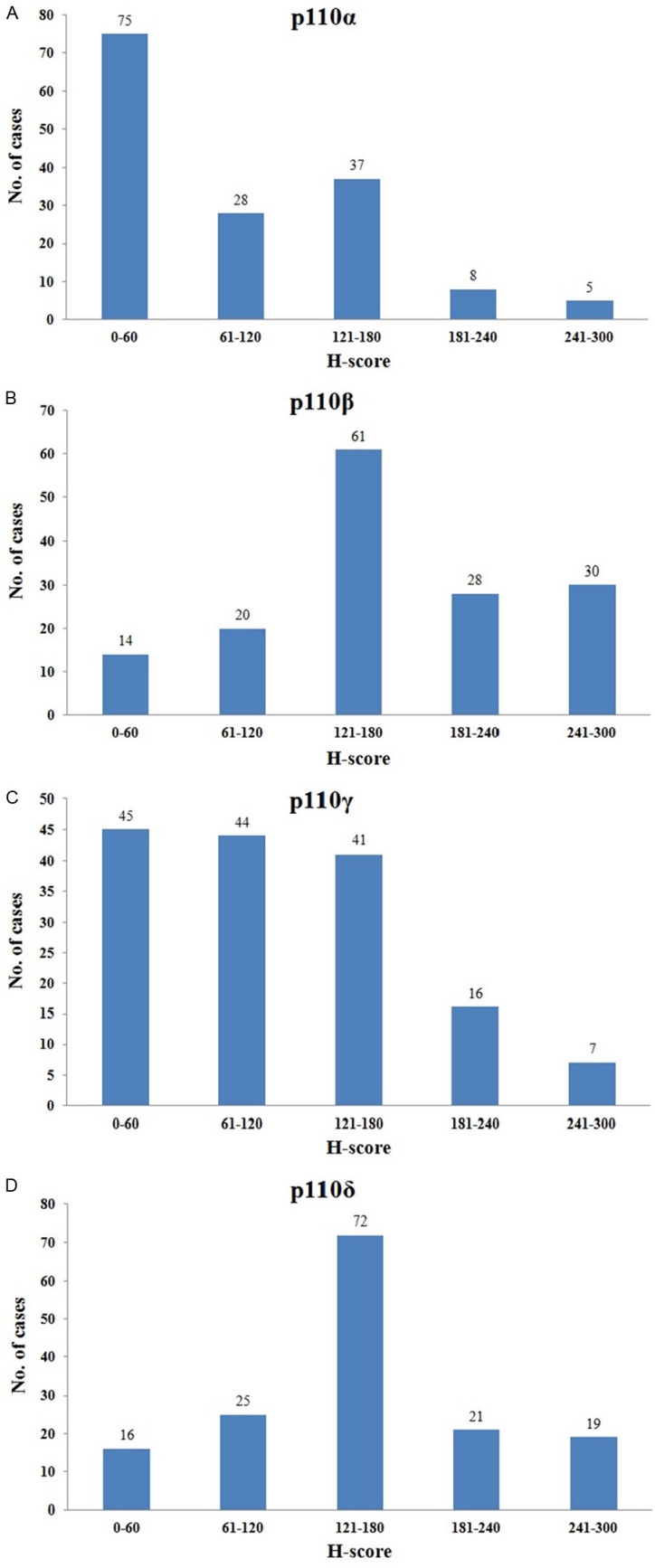

Figure 2. Distribution of H-scores for PI3K isoforms.

Results

Clinicopathological characteristics

The gastric cancer patients included in this study consisted of 103 men and 50 women. The median age at diagnosis was 69 years (range 35 to 78 years). Tumor locations included 104 (68%) in the antrum, 28 (18%) in the body, 5 (3%) in the cardia, and 16 (11%) in two or more locations. Histologically, 131 tumors (86%) were classified as tubular adenocarcinoma, 17 (11%) as signet ring cell carcinoma, 3 (2%) as mucinous adenocarcinoma, 1 as poorly differentiated neuroendocrine carcinoma and 1 as undifferentiated carcinoma. By Lauren classification, 84 tumors (55%) were classified as intestinal type and 69 (45%) as diffuse type. The mean tumor diameter was 4.8 cm (range 0.7 to 17.0 cm). Pathological T stage was T1 (pT1) for 46 tumors (30%), pT2 for 24 (16%), pT3 for 33 (22%), and pT4 for 50 (33%). Lymphovascular invasion and nodal involvement was detected in 104 (68%) and 96 (62%) patients, respectively. Nine (5%) had distant metastasis at initial diagnosis. The distribution of clinicopathological parameters is shown in **Table 1**.

Expression and correlation of PI3K isoforms

Of the PI3K isoforms, p110α and p110γ were expressed in the cytoplasm, p110β was expressed in the nucleus, and p110δ was expressed in the cell membrane (**Figure 1**). Of the 153 tumors, positivity was seen in 112 (73%) for p110α, 149 (97%) for p110β, 139 (91%) for p110γ and 148

Table 2. Correlation between PI3K isoforms

	p110β	p110γ	p110δ
p110α	R=-0.127	R=0.460	R=-0.358
	P=0.118	P<0.001	P<0.001
p110β		R=-0.096	R=0.231
		P=0.237	P=0.004
p110γ			R=-0.243
			P=0.002

R, Spearman's correlation coefficient.

(97%) for p110δ with variable staining intensity and extent. Expression was moderate or strong in 78 (51%) for p110α, 137 (90%) for p110β, 108 (71%) for p110γ and 138 (90%) for p110δ. Median H-score was 70 (range 0 to 300) for p110α, 180 (range 0 to 300) for p110β, 120 (range 0 to 270) for p110γ and 160 (range 0 to 300) for p110δ. H-score distributions by PI3K isoform are in **Figure 2**.

For p110α, moderately positive correlation was seen with p110γ (correlation coefficient, R= 0.460; P<0.001) and moderately negative correlation was seen with p110δ (R=-0.358; P< 0.001). Correlation between p110α and p110β was weakly negative but not significant (R=-0.127; P=0.118). For p110β, a weakly positive correlation was seen with p110δ (R=0.231; P=0.004), but no correlation was observed with p110γ. A significant negative, weak correlation was seen between p110γ and p110δ (R=-0.243; P=0.002). Correlations among PI3K isoforms are in **Table 2**.

Correlation between PI3K isoforms and clinicopathological factors

Expression levels for PI3K isoforms were dichotomized by median H-score for the isoforms and compared with clinicopathological factors (**Table 1**). Of the isoforms, p110α was expressed more highly in gastric cancers with a diameter smaller than 4 cm than those with a diameter of 4 cm or larger (P=0.009). The expression level of p110α inversely correlated with pT (P<0.001), pN (P=0.001) and TNM stages (P<0.001). Tumors with lymphovascular invasion expressed less p110α than tumors without lymphovascular invasion (P=0.001). In contrast, p110β was expressed significantly higher in tumors with advanced pT stage (P=0.028) and lymphovascular invasion (P= 0.014). Expression of p110β correlated with higher pN stage, but this finding was not significant (P=0.079). Expression of p110γ and p110δ were not significantly associated with clinicopathological factors.

Survival analysis

The median follow-up period was 95 months (range 1 to 139 months). During follow-up, 67 (44%) of the 153 patients died, 20 (13%) developed recurrent disease and 10 (7%) were lost to follow-up. The p110α high-expression group had a significantly longer OS (P=0.007) and RFS (P=0.048) than the low-expression group, whereas the p110β high-expression group had a shorter OS (P=0.008) and RFS (P=0.058) (**Figure 3**). High p110γ expression was associated with longer RFS, but this finding was not significant (P=0.053). Expression of p110δ was not significantly correlated with OS or RFS. Of the measured clinicopathological variables, cardia location (P=0.001); larger tumor size (P<0.001); higher pT, pN or TNM stage (P< 0.001); and lymphovascular invasion (P=0.012) were significantly associated with shorter OS. In addition, diffuse type (P=0.002); larger tumor size (P=0.001); higher pT, pN and TNM stages (P<0.001) were associated with shorter RFS.

Multivariate Cox regression analysis including p110α, p110β and clinicopathological variables that were significantly associated with prognosis in univariate analysis showed that p110β, tumor size and TNM stage were independent prognostic factors for OS. Lauren classification and TNM stage were independent prognostic factors for RFS (**Table 3**).

Discussion

Of the PI3K isoforms, p110α has been studied the most because it includes a cancer-specific mutation. Studies on expression of wild-typep110α and nonalpha protein isoforms are limited. In this study, we assessed differential protein expression of 4 PI3K isoforms and found that the isoforms had distinct clinicopathological associations with gastric cancer.

Mutational activation of p110α is suggested to contribute to cellular transformation, whereas wild-type p110α lacks oncogenic activity [16, 23]. Most studies have not observed significant associations between p110α expression and clinicopathological factors in different cancers

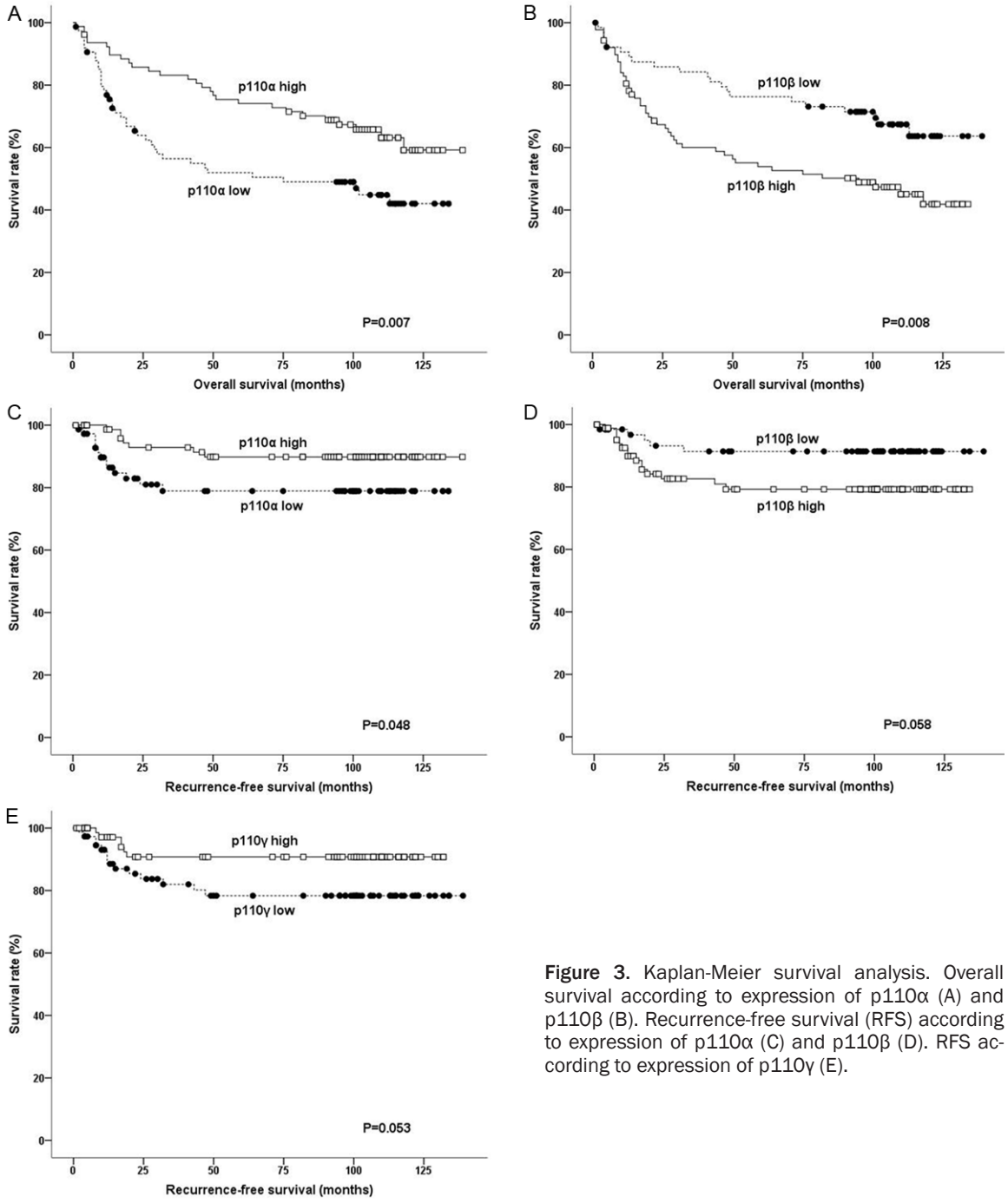

Figure 3. Kaplan-Meier survival analysis. Overall survival according to expression of p110α (A) and p110β (B). Recurrence-free survival (RFS) according to expression of p110α (C) and p110β (D). RFS according to expression of p110γ (E).

[23-25]. Recently, Cui et al. [22] showed that p110α expression is associated with shorter OS in patients with diffuse large B-cell lymphoma. In our study, however, p110α expression was higher in gastric cancers with favorable prognostic markers, including smaller tumor size, early stage and no lymphovascular invasion, and was associated with significantly longer OS and RFS. Although these results are similar to some studies showing that the *PIK3CA* mutation is associated with improved outcomes in breast cancer [30-32], the results are unexpected because p110α is pivotal in the oncogenic PI3K/AKT signaling pathway. Emerging evidence indicates that activation of the PI3K/AKT signaling pathway leads to oncogene-induced senescence, maintenance of differentiation, or suppression of invasion and metastasis although the pathway is involved in the early stages of tumorigenesis [32-34].

Table 3. Multivariate analysis for overall and recurrence-free survival

Factor	Parameter	Overall survival		Recurrence-free survival	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Lauren classification	Diffuse Intestinal	1.18 (0.70-1.97)	0.542	2.97 (1.01-8.68)	0.047
Size (cm)	≥4 <4	2.06 (1.08-3.94)	0.028	2.58 (0.61-10.86)	0.195
TNM stage	III-IV I-II	3.17 (1.68-5.99)	<0.001	7.67 (1.74-33.76)	0.007
Lymphovascular invasion	Present Absent	1.32 (0.68-2.56)	0.413	2.38 (0.62-9.10)	0.206
p110α	Low High	1.42 (0.85-2.38)	0.182	2.03 (0.75-5.49)	0.165
p110β	High Low	1.91 (1.12-3.27)	0.018	2.46 (0.84-7.17)	0.099

CI, confidence interval.

These results could explain the unexpected tumor-suppressive effect of p110α and indicate that other mechanisms might be more important in tumor progression than activating mutations or p110α overexpression.

Overexpression of p110β is a possible mechanism for inducing cellular transformation and tumor progression in gastric cancer. Wild-type p110β has oncogenic potential despite the absence of a cancer-specific mutation [16, 25] and p110β is critical in the AKT-independent signaling pathway for growth of tumors driven by HER2 or PTEN alteration [25, 35, 36]. HER2 and PTEN aberrations are closely linked to the development and progression of gastric cancer [20, 37]. Taken together, these data imply that p110β could have an essential function in gastric cancer carcinogenesis. This hypothesis is supported by the results presented here that p110β expression correlated significantly with advanced pT stage and lymphovascular invasion, and was an independent factor for poor prognosis. Additional studies on the association between p110β and HER2 or PTEN in gastric cancer are warranted to elucidate the detailed mechanism of p110β.

Another notable finding about p110β was its nuclear expression, which contrasted with previous studies in which p110β showed cytoplasmic or membranous expression [22, 25]. Control of the subcellular localization of p110β and the influence on its functional activity is not fully understood. A recent in vitro cell study demonstrated that p110β regulates cell viability only when it is correctly localized to the nucleus and not when it is in cytoplasm [38]. This result is in accordance with results in this study using gastric cancer tissues. The disagreement with previous studies might be attributed to tissue specificity or use of different primary antibodies. Further investigations are needed to clarify the exact significance of p110β subcellular localization.

Studies have reported that p110γ and p110δ are mainly associated with hematologic malignancies [39, 40]. This study found no significant association between these isoforms and clinicopathological factors in gastric cancer although the isoforms were highly expressed. Correlations were observed among PI3K isoforms and could be used to classify PI3K isoforms into two groups. In one group, p110γ positively correlated with p110α and better prognosis, similar to p110α. In the other group, p110δ was positively correlated with p110β and negatively associated with the other isoforms. Thus, complex intracellular crosstalk might connect different PI3K isoforms and simultaneously regulate them. Molecular exploration and validation are required to verify this interconnection.

Pan-PI3K and isoform-specific inhibitors have been evaluated or are under evaluation in clinical trials [13, 18]. Based on our results, p110β appeared to be the most important isoform in the development and progression of gastric cancer. Therefore, p110β-specific inhibition might be more effective against gastric cancer

and have fewer side effects than other PI3K inhibitors. The influence of isoform selectivity on treatment index and the toxic effects of PI3K inhibitors in gastric cancer should be explored in future studies.

In summary, this study investigated the differential expression of 4 PI3K isoforms and showed significant correlations between their expression and clinicopathological factors and gastric cancer patient survival. While p110α correlated with favorable prognostic factors, p110β was significantly associated with advanced pT stage, lymphovascular invasion and poor prognosis. These differences between PI3K isoforms implied that isoform selectivity should be seriously considered when PI3K inhibitors are studied or adopted for gastric cancer treatment. This study supports the potential of PI3K isoforms, especially p110β, as prognostic biomarkers of gastric cancer. However, the study design was retrospective, included a relatively small number of cases, and lacked molecular validation. Large-scale, prospective studies with molecular validation are needed to verify the results.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Seo Hee Rha, Department of Pathology, Dong-A University College of Medicine, 26 Daesingongwon-ro, Seo-gu, Busan 602-715, South Korea. Tel: +82-51-240-2882; Fax: +82-51-243-7396; E-mail: shrha@dau.ac.kr

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