

## Original Article

# Expression of the phosphoinositide 3-kinase p110 $\delta$ isoform and its clinicopathological significance in gastric cancer

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**Abstract:** Protein p110 $\delta$  is an isoform of the catalytic subunit of class I phosphoinositide 3-kinases (PI3Ks). PI3Ks are involved in the regulation of cell survival, growth, proliferation, and migration, and have been implicated in the oncogenesis of hematologic malignancies. In this study, we evaluated the expression of p110 $\delta$  in gastric cancer (GC) and its association with various clinicopathological factors and patient survival. One hundred seventy-four GC cases, included in our previous tissue microarray blocks, were immunohistochemically stained for p110 $\delta$ . Of the 174 tumors, 158 (90.8%) were positive for p110 $\delta$ . The rate of p110 $\delta$  positive GCs was significantly higher in those with diffuse type ( $P=0.018$ ), larger tumor size ( $P=0.002$ ), lymphovascular invasion ( $P=0.012$ ), and advanced pT ( $P<0.001$ ), pN ( $P<0.001$ ), and TNM stages ( $P<0.001$ ). In addition, the p110 $\delta$ -positive group had poorer overall survival ( $P=0.002$ ) and recurrence-free survival ( $P=0.075$ ) than that of the negative group. However, multivariate analysis showed that p110 $\delta$  was not an independent prognostic factor. Our results suggest that p110 $\delta$  can play an important role in GC oncogenesis and is a promising therapeutic target in GC. Additional studies on the use of p110 $\delta$ -specific inhibitors, and the possibility of p110 $\delta$  immunostaining as a prognostic or predictive biomarker in solid cancers, including GC, are recommended.

**Keywords:** Gastric cancer, immunohistochemistry, PI3K, p110 $\delta$ , prognosis

## Introduction

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate the 3'-hydroxyl position of the inositol ring of phosphatidylinositol-4, 5-bisphosphate (PIP<sub>2</sub>) in response to extracellular stimuli and generate phosphatidylinositol-3, 4, 5-triphosphate (PIP<sub>3</sub>). PIP<sub>3</sub> is a critical second messenger, mediating diverse signaling cascades for cell growth, proliferation, and survival [1-3]. PI3Ks are abnormally activated by mutation or amplification of *PIK3CA*, a gene encoding p110 $\alpha$ , a catalytic subunit of PI3K, or by inactivation of phosphatase and tensin homolog (PTEN) protein, a negative regulator of PI3K signaling [4]. Aberrant

upregulation of PI3K signaling can promote the oncogenesis of various types of cancers [4-7].

The PI3Ks are grouped into three classes on the basis of their structural features and substrate specificity. Only class I PI3Ks have been linked to the oncogenesis of human cancers [6]. Class I PI3Ks are heterodimeric enzymes comprised of a catalytic subunit and a regulatory subunit. The catalytic subunits of class I PI3Ks are p110 $\alpha$ , p110 $\beta$ , p110 $\gamma$ , and p110 $\delta$  [6]. These four isoforms have non-redundant functions and different expression patterns in different cell types. While p110 $\alpha$  and p110 $\beta$  are ubiquitously expressed, p110 $\gamma$  and p110 $\delta$  are expressed largely in cells of hematopoietic

lineage [6, 8]. Thus, p110 $\delta$  expression has been implicated exclusively in hematologic malignancies [8-10].

Previous studies examining aberrant activation of PI3Ks in gastric cancer (GC) have focused on the amplification or mutation of *PIK3CA* or loss of function of PTEN [7, 11-14]; however, the expression of PI3K isoform proteins has not been investigated. In particular, there are no available data on p110 $\delta$  expression in solid tumors, including GC. Recently, we investigated differential expression of PI3K isoforms in GC and found that p110 $\alpha$  and p110 $\beta$  expression significantly correlated with various clinicopathological factors and patient survival [15]. The expression of p110 $\delta$  tended to be higher in tumors with lymph node metastasis or advanced stage than in those without lymph node metastasis or in the early stage, but this was not statistically significant.

Determination of the optimal interpretation method and cut-off value for immunostaining is crucial in immunohistochemical studies because they can affect the results. In this study, we reassessed the association between p110 $\delta$  expression and clinicopathological factors, including patient survival, in patients with GC by using an interpretation method and cut-off value different from those used in our previous study. Using this approach, we obtained new significant results.

### Materials and methods

#### *Patients and tissue samples*

This study included 174 patients with GC who underwent radical gastrectomy at Samsung Changwon Hospital, Changwon, South Korea, between January 2002 and December 2005. All pathological slides were reviewed and Lauren classification, location, depth of invasion, regional lymph node metastasis, and lymphovascular invasion were reevaluated. Other clinicopathological data such as age, sex, distant metastasis, and survival data were obtained from medical records. Pathological stage was re-determined according to the 7th edition of the American Joint Committee on Cancer TNM staging system [16]. No patients had undergone neoadjuvant chemotherapy. Follow-up data were included through December 2015 or until death or failure to follow-up.

This study was approved by the Institutional Review Board (2015-SCMC-051-00).

#### *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  $\mu$ m 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 the primary antibody for 60 minutes at 37°C. The primary antibody was against p110 $\delta$  (clone A-8, 1:100, Santa Cruz Biotechnology). An UltraView Universal DAB kit (Roche-Ventana) was used to detect primary antibody followed by counterstaining with hematoxylin according to manufacturer's recommendations (Roche-Ventana). Breast carcinoma was used as the positive control. The negative control used buffer instead of primary antibody.

Immunostained slides were evaluated by two independent pathologists (HWL and EHL) without clinicopathological information. Discrepant cases were discussed on a multihead microscope until agreement was reached. The extent of the staining was expressed as the percentage of tumor cells expressing p110 $\delta$  by 10% increments. The optimal cutoff of positivity was determined as the percentage producing the greatest prognostic significance. When the cut-off value was 40%, the lowest *P*-value for overall survival (OS) was obtained. Thus, cases were considered positive if 40% or more of tumor cells expressed p110 $\delta$ .

#### *Statistical analysis*

Statistical analyses were performed with SPSS Ver. 18 (SPSS Inc., Chicago, IL, USA). To evaluate correlations between expression of p110 $\delta$  and clinicopathological factors, we used Fisher's exact test for categorical variables or the Mann-Whitney test for ordinal variables. Influences of parameters on overall survival (OS) and recurrence-free survival (RFS) were analyzed by the Kaplan-Meier method and differ-

p110δ expression in gastric cancer

**Table 1.** Correlation of p110δ expression with clinicopathological factors in 174 patients with gastric cancer

Clinicopathological factors	p110δ expression, n (%)		P
	Negative	Positive	
Total	16 (100.0)	158 (100.0)	
Age (years)			0.286
<60	4 (25.0)	65 (41.1)	
≥60	12 (75.0)	93 (58.9)	
Sex			0.176
Male	13 (81.3)	99 (62.7)	
Female	3 (18.8)	59 (37.3)	
Location			0.455
Antrum	12 (75.0)	103 (65.2)	
Body	4 (25.0)	30 (19.0)	
Cardia	0 (0.0)	5 (3.2)	
Two or more portions	0 (0.0)	20 (12.7)	
Histological type			1.000
Tubular	14 (87.5)	131 (82.9)	
Signet ring cell	2 (12.5)	22 (13.9)	
Mucinous	0 (0.0)	3 (1.9)	
Others	0 (0.0)	2 (1.3)	
Lauren classification			0.018
Intestinal	13 (81.3)	78 (49.4)	
Diffuse	3 (18.8)	80 (50.6)	
Tumor size (cm)			0.002
<4	13 (81.3)	61 (38.9)	
≥4	3 (18.8)	96 (61.1)	
Pathological T stage			<0.001
pT1	14 (87.5)	43 (27.2)	
pT2	2 (12.5)	25 (15.8)	
pT3	0 (0.0)	36 (22.8)	
pT4	0 (0.0)	54 (34.2)	
Pathological N stage			<0.001
pN0	13 (81.3)	57 (36.1)	
pN1	3 (18.8)	40 (25.3)	
pN2	0 (0.0)	22 (13.9)	
pN3	0 (0.0)	39 (24.7)	
Distant metastasis			1.000
No metastasis	16 (100.0)	149 (64.3)	
Metastasis	0 (0.0)	9 (5.7)	
TNM stage			<0.001
I	14 (87.5)	51 (32.3)	
II	2 (12.5)	40 (25.3)	
III	0 (0.0)	58 (36.7)	
IV	0 (0.0)	9 (5.7)	
Lymphatic invasion			0.012
Negative	11 (68.7)	54 (34.2)	
Positive	5 (31.3)	104 (65.8)	

ences were 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.

**Results**

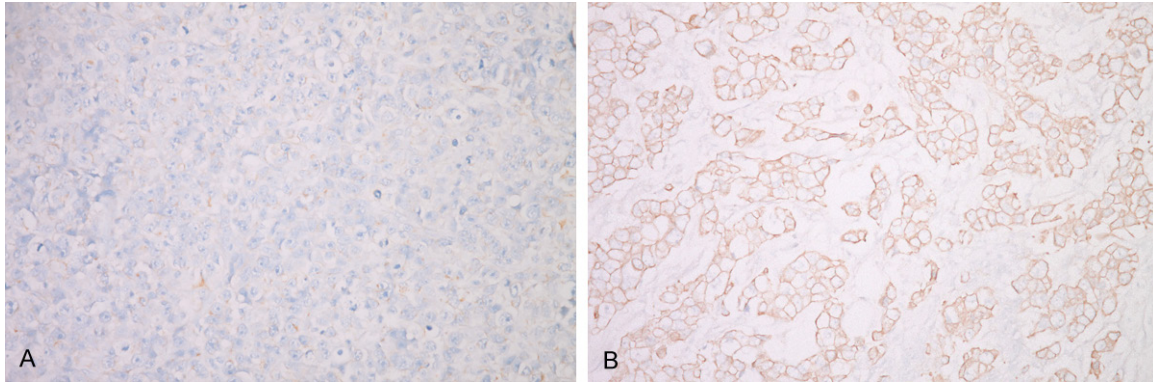
*Clinicopathological characteristics*

The patients with GC included in this study consisted of 112 men and 62 women. The median age at diagnosis was 63 years (range 32 to 78 years). Tumor locations included 115 (66.1%) in the antrum, 34 (19.5%) in the body, 5 (2.9%) in the cardia, and 20 (11.5%) in two or more locations. Histologically, 145 tumors (83.3%) were classified as tubular adenocarcinoma, 24 (13.8%) as signet ring cell carcinoma, 3 (1.7%) as mucinous adenocarcinoma, 1 as poorly differentiated neuroendocrine carcinoma, and 1 as undifferentiated carcinoma. By Lauren classification, 91 tumors (52.3%) were classified as intestinal type and 83 (47.7%) 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 57 tumors (32.8%), pT2 for 27 (15.5%), pT3 for 36 (20.7%), and pT4 for 54 (31.0%). Lymphovascular invasion and nodal involvement were detected in 109 (62.6%) and 104 (59.8%) patients, respectively. Nine (5.2%) had distant metastasis at initial diagnosis. These clinicopathological characteristics are summarized in **Table 1**.

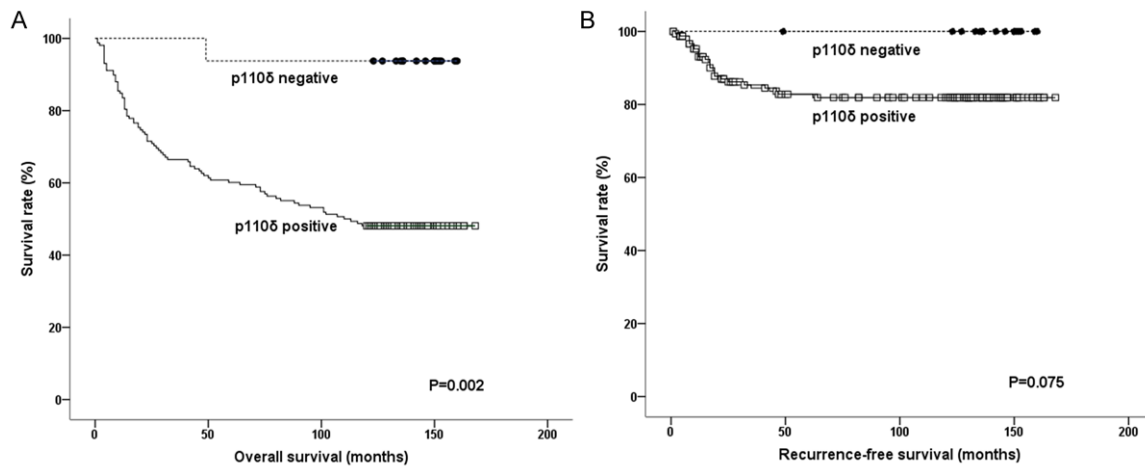
*Correlation between p110δ expression and clinicopathological factors*

In most cases, p110δ was moderately to strongly expressed in the cell membrane (**Figure 1**). The median extent of p110δ expression was 80% (range 0 to 100%). As described above, cases were considered positive when 40% or more of tumor cells expressed p110δ. Of the 174 tumors, 158 (90.8%) were positive. The positive rate of p110δ was significantly higher in GCs with diffuse type (P=0.018), larger tumor size (P=0.002),

## p110δ expression in gastric cancer



**Figure 1.** Immunohistochemical staining for p110δ in gastric cancer: negative (A) and positive (B).



**Figure 2.** Survival curves for gastric cancer according to p110δ expression: overall survival (A) and recurrence-free survival (B).

lymphovascular invasion ( $P=0.012$ ), advanced pT ( $P<0.001$ ), pN ( $P<0.001$ ) and TNM stages ( $P<0.001$ ). These results are summarized in **Table 1**.

### Survival analysis

The median follow-up period was 122.5 months (range 1 to 168 months). During follow-up, 83 (47.7%) of the 174 patients died and 24 (13.8%) developed recurrent disease. The p110δ-positive group had a worse OS ( $P=0.002$ ) and RFS ( $P=0.075$ ) than the negative group (**Figure 2**). Of the measured clinicopathological factors, cardia location ( $P=0.017$ ); diffuse type ( $P=0.038$ ); larger tumor size ( $P<0.001$ ); higher pT, pN, or TNM stage ( $P<0.001$ ); and lymphovascular invasion ( $P=0.002$ ) were significantly associated with shorter OS. In addition, diffuse type ( $P<0.001$ ); 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 p110δ and clinicopathological factors significantly associated with poor prognosis in univariate analysis showed that only TNM stage was an independent prognostic factor for OS. p110δ expression had an increased risk of worse OS, but the association was not statistically significant (**Table 2**).

### Discussion

GC is the fifth most common cancer worldwide, with almost one million new cases annually, and is the third leading cause of cancer death, with more than 700,000 deaths annually [17]. Although traditional treatments, including curative surgery and perioperative chemotherapy,

## p110δ expression in gastric cancer

**Table 2.** Multivariate analysis for overall survival

Factor	Parameter	Overall survival	
		Hazard ratio (95% CI)	P
Lauren classification	Diffuse	1.061 (0.676-1.666)	0.796
	Intestinal		
Tumor size	≥4 cm	1.601 (0.899-2.851)	0.110
	<4 cm		
Lymphovascular invasion	Positive	0.978 (0.568-1.681)	0.935
	Negative		
Stage	III-IV	3.614 (2.058-6.348)	<0.001
	I-II		
p110δ expression	Positive	5.421 (0.734-40.053)	0.098
	Negative		

have improved the survival rate of patients with resectable GC, many GC cases are either diagnosed at an inoperable advanced stage or are subject to recur after prior curative surgery, and they have a poor prognosis. For patients with advanced or recurrent disease, fluoropyrimidin-based chemotherapy has been widely used and results in prolonged median OS. However, the duration of survival is usually less than 12 months [18]. These limitations of current chemotherapy treatments suggest a need for novel targeting agents that can provide better outcomes for patients with advanced GC. Currently, several molecular targeting agents, including PI3K inhibitors, are undergoing clinical trials for their efficacy in patients with GC [19-22].

There are two types of PI3K inhibitors: pan-PI3K inhibitors, which target all of the class I PI3K isoforms, and isoform-specific PI3K inhibitors. To date, pan-PI3K inhibitors and p110α-specific inhibitors have been evaluated in GC patients [21, 22]. The significant toxicity associated with pan-PI3K or p110α-specific inhibitors may be due to the ubiquitous expression and essential functions of p110α and limits the tolerability of these agents. Such limitations have not been observed for p110δ-specific inhibitors [10, 23]. Therefore, p110δ-specific inhibitors may have more potential for cancer therapy than broad spectrum or p110α-specific inhibitors.

Our study showed that p110δ was highly expressed in GCs and significantly associated with well-established poor prognostic factors, such as diffuse type, larger tumor size, lymphovascular invasion, and advanced pT, pN and TNM stages. The p110δ-positive group had a

worse OS and RFS than the negative group. These results suggest that p110δ can play an important role in the oncogenesis of GC and be a promising therapeutic target for GC. To date, isoform-specific inhibition of p110δ has been studied exclusively in hematologic malignancies [8-10]. Based on our results, inhibition of p110δ needs to be applied to solid cancers, including GC.

Interestingly, p110δ was notably expressed in the cellular membrane. Activated receptor tyrosine kinases (RTKs) increase the affinity of p110δ for binding to membranes and PIP<sub>2</sub> [24]. Thus, the localization of p110δ at the membrane reflects the activated status of RTKs and their downstream signaling pathways. This is consistent with our results indicating that the membranous expression of p110δ was associated with tumor growth, invasion, and survival.

Our previous study, with almost the exact same cohort of GC patients, was unable to elucidate a significant correlation between p110δ expression and clinicopathological factors or patient survival, even though GCs with advanced stage or nodal metastasis had a trend toward higher p110δ expression. In that study, we used H-score, calculated by multiplying staining intensity and extent and ranged from 0 to 300, to assess immunostaining results. The median H-score was used as the cutoff value for dividing the subjects into low and high expression groups. Here, we used an alternative interpretation method for immunostaining and the optimal cutoff for positivity, and obtained different results. These differences highlight the importance of using an appropriate interpretation method and positive cutoff value for immunostaining. Further investigations should be performed to determine the most objective and accurate methodology for the assessment of p110δ immunostaining.

To our knowledge, this is the first study to show that p110δ expression significantly correlates with various clinicopathological factors and patient survival in solid cancer. Based on our results, p110δ could have a critical role in the tumorigenesis of solid cancers as well as hema-

tologic malignancies. Additionally, isoform-specific inhibition of p110 $\delta$  could be a novel and attractive therapeutic strategy in cancer treatment as it has fewer side effects than pan-PI3K or p110 $\alpha$ -specific inhibition. Therefore, further studies on the use of p110 $\delta$ -specific inhibitors and the possibility of p110 $\delta$  immunostaining as a prognostic or predictive biomarker in solid cancers, including GC, are warranted.

Here, we have shown that p110 $\delta$  was highly expressed in GCs, and showed membranous localization. p110 $\delta$  expression was significantly associated with poor prognostic factors of GCs, as well as shorter patient survival. However, further studies with a larger number of cases and a standardized interpretation method for p110 $\delta$  immunostaining are needed to verify our results.

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#### Disclosure of conflict of interest

None.

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## p110 $\delta$ expression in gastric cancer

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