Int J Clin Exp Pathol 2020;13(5):995-1007
www.ijcep.com /ISSN:1936-2625/IJCEP0107621

Original Article

Clinical significance of PI3K/Akt/mTOR signaling in gastric carcinoma

Eun-Jung Jung[1], Ja Hee Suh[1], Woo Ho Kim[2], Hee Sung Kim[3]

[1]Department of Pathology, National Medical Center, 245 Euljiro, Jung-gu 04564, Republic of Korea; [2]Department of Pathology, Seoul National University College of Medicine, 101, Daehak-Ro, Jongno-Gu, Seoul 03080, Republic of Korea; [3]Department of Pathology, Chung-Ang University College of Medicine, 84 Heukseok-Ro Dongjak-Gu Seoul 06973, Republic of Korea

Received January 9, 2020; Accepted February 7, 2020; Epub May 1, 2020; Published May 15, 2020

Abstract: The mTOR signaling pathway has been linked to various cancers, but the contribution of alterations in this pathway to clinicopathological characteristics have not been established in gastric cancer. To investigate *PIK3CA* mutations and the expression of proteins in the PI3K/Akt/mTOR signaling pathway in sporadic gastric cancer. We analyzed *PIK3CA* mutation and microsatellite instability as well as immunohistochemical expressions of p-Akt, PTEN, p-mTOR, p-4EBP1, p-S6, p-p70S6, and eIF4E in 368 FFPE (formalin-fixed paraffin embedded) tissue from patients with sporadic gastric cancer. Associations between expression and clinicopathologic parameters and patient survival were evaluated. We found *PIK3CA* mutations in 4 of 173 cases (2.3%). In immunohistochemical analyses, we detected positive p-Akt expression in 22.0% of cases (81/368), negative PTEN expression in 21.5% of cases (79/368), positive p-mTOR expression in 68.6% of cases (243/354), positive p-4EBP1 expression in 58.2% of cases (202/347), positive p-S6 expression in 42.7% of cases (148/347), positive p-p70S6 expression in 51.1% of cases (179/350), and positive eIF4E expression in 78.3% of cases (275/351). In a clinicopathologic analysis, intestinal type was significantly associated with positive p-4EBP1 expression ($P < 0.001$). In a Kaplan-Meier survival analysis, PTEN loss ($P = 0.002$) and pS6 positivity ($P = 0.043$) are significantly associated with reduced overall survival (OS). PTEN loss ($P = 0.001$), pS6 positivity ($P = 0.009$), and eIF4E positivity ($P = 0.003$) are significantly associated with reduced disease free survival (DFS) (disease free survival). In Cox regression multivariate analysis, PTEN loss was an independent factor of reduced time. Alterations of mTOR pathway protein expression are associated with reduced survival in gastric cancer. Significance was noted in the association of pS6 positivity and eIF4E positivity e with reduced survival in univariate analysis and the association of PTEN loss and reduced DFS in univariate analysis as well as multivariate analysis for DFS.

Keywords: mTOR, PI3K, mutation, immunohistochemistry, survival, therapeutic target

Introduction

Gastric cancer is the fourth most common cancer and the second most common cause of cancer deaths worldwide [1]. *PIK3CA* encodes the p110-alpha subunit of phosphoinositide-3-kinase (PI3K). It is a key oncogene, with a high frequency of somatic mutations in several types of human cancer [2, 3]. PI3K is part of a family of Ser-Thr kinases that interact with phosphatidylinositol bisphosphate (4,5-PIP2) to produce phosphatidylinositol trisphosphate (3,4,5-PIP3), a second messenger with several functions. PIP3 mainly binds to the pleckstrin homology domain of a number of target molecules, leading to their activation or modulation. One of the best characterized targets of PI3K lipid products is the protein kinase Akt. PI3K/Akt activation is involved in the regulation of several cellular functions, including cell survival, growth, angiogenesis, apoptosis, and protein translation, and thereby contributes to the development of cancer [3, 4].

PIK3CA includes 20 exons, and more than 75% of mutations in this gene are found in two hotspots in exons 9 and 20, within the helical and kinase domains, respectively [5]. The most common variants (E542K, E545K, and H1047R) are associated with increased lipid kinase

activity and are oncogenic in cell culture and in vivo [6]. Mutations in the two hotspots have different functional consequences [7] and mutation rates are associated with specific cancer types or clinical features [8, 9].

Mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase that mediates nutrient-dependent intracellular signaling related to cell growth, proliferation, and differentiation. mTOR promotes translation initiation by the phosphorylation of two targets, ribosomal p70S6 kinase (S6K1) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) [10-12]. mTOR exists as two distinct functional complexes known as mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin, a specific inhibitor of mTOR, whereas mTORC2 is resistant to rapamycin [13]. mTORC1 regulates the activity of the translational machinery by modulating eIF4E binding protein 1 (4EBP1) activity and S6 kinase (p70S6 K) through direct phosphorylation. 4EBP1 dimerizes with eukaryotic initiation factor 4E (eIF4E), blocking the formation of the initiation complex. When 4EBP1 is phosphorylated, eIF4E is released and translation can begin [14].

Several preclinical studies have detected the dysregulation of mTOR activity in gastric cancer cell models, suggesting that mTOR is a potential therapeutic target. Mutations in upstream regulators of the mTOR signaling pathway, epithelial growth factor receptor (EGFR), PI3K (phosphoinositide-3-kinase) [15], and PTEN [16], have been observed in patient-derived gastric tumor samples. In addition, preclinical studies have provided evidence for mTOR activation in gastric cancer cells and tumors; in particular, patient-derived gastric cancer samples express phosphorylated mTOR [17]. Phosphorylated mTOR is positively correlated with tumor progression and poor survival in patients with gastric cancer. However, few studies have assessed correlations between mTOR expression in human cancers and either clinicopathological features or outcomes [18]. mTOR pathway-related protein expression levels are higher in intestinal-type gastric cancer than in diffuse-type [19].

In this study, we studied correlations between PI3K/Akt/mTOR signaling pathway expression in gastric cancer tissues and clinicopathological features and survival to determine the value of mTOR as a prognostic marker. We evaluated PI3K mutations, microsatellite instability (MSI), and mTOR pathway protein expression in gastric carcinoma.

Materials and methods

Patients and tissue samples

Formalin-fixed and paraffin-embedded (FFPE) gastric tumor samples were collected from 450 patients who underwent gastrectomy in 2004 at Seoul National University Hospital. Clinicopathological data, such as patient age and gender, histological tumor type, Lauren's classification, and evidence for lymphatic invasion were obtained by reviewing the medical charts and pathological records.

Ethical statement

All human specimens were obtained during therapeutic surgery or endoscopic resection. The retrospective study was performed using pathology specimens after diagnosis, and all samples were anonymized before use in this study. The participants did not provide written consent to participate in this study. The Institutional Review Board of Seoul National University Hospital approved of this retrospective study under the condition of anonymity (H-1706-105-860).

Mutation analysis

DNA was extracted from manually microdissected paraffin-embedded normal and tumor tissues, as described previously [20]. Mononucleotide microsatellites BAT25 and BAT26 (located in the introns of the *MSH2* and *KIT* genes, respectively) were examined by PCR amplification using fluorescent dye-labeled primers, as described previously [21]. PCR amplification and sequencing of *PIK3CA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide) exons 9 and 20 were performed as described previously [22].

Using the ABI PRISM 3100 Genetic Analyzer, *PI3KCA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide) mutations were detected using following primers: Exon10_Foward, TGACAAAGAACAGCTCAAAGCA; Exon10_Reverse, TGCTGAGATCAGCCAAATTCA; Exon 21_Foward, AATGATGCTTGGCTCTGGAA; Exon 21_Reverse, CCAATCCATTTTTGTTGTCCA.

Table 1. List of primary antibodies used in the study

Primary antibody	Catalog No.	Source	Dilution	Monoclonal or polyclonal
pAkt	2118-1	Epitomics	1:30	monoclonal
pAkt (Ser473)	3787	Cell Signaling	1:40	monoclonal
pmTOR (Ser2448) (49F9)	2976	Cell Signaling	1:100	monoclonal
PTEN (c-term)	1539-1	Epitomics	1:80	monoclonal
p4EBP1	9455	Cell Signaling	1:100	polyclonal
pS6 ribosomal protein	2215	Cell Signaling	1:100	polyclonal
Phospho-p70 S6 Kinase (Thr389)	9205	Cell Signaling	1:40	polyclonal
eIF4E	9742	Cell Signaling	1:80	polyclonal
HIF-1alpha (Ab655)	610958	BD Biosciences	1:30	monoclonal
VEGF	SC-7269	Santa Cruz	1:1000	monoclonal

Tissue microarray preparation

Tissues obtained from patients were fixed in 10% buffered formalin and embedded in paraffin. After screening the available samples from each patient, a paraffin block that was well-fixed and contained a representative tumor section was selected. A single tissue column (2.0 mm in diameter) was obtained from each selected paraffin block and samples were arranged separately in new 60-hole recipient paraffin blocks using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea).

Immunohistochemistry

Tissue microarray slides were cut at 4 μm, deparaffinized, and incubated in a dry oven at 60°C for 1 h. Slides were dewaxed and hydrated three times at 72°C for 3 min in alcohol. Slides were subjected to antigen retrieval using Epitope retrieval solution 2 (pH 9.0) at 100°C for 20 min. The slides were subsequently incubated in a peroxidase block for 5 min, primary antibody for 15 min, post-primary antibody for 8 min, polymer for 8 min, DAB substrate for 10 min, and hematoxylin for counterstaining for 1 min. The primary antibodies used in this study are listed in **Table 1**.

Interpretation of immunohistochemical staining

p-Akt, PTEN, p-4BP1, and p-p70S6 were detected in the cytoplasm, and p-mTOR and p-S6 were detected in the cytoplasm and cell membrane of gastric cancer cells. Immunoreactivity for various antibodies was evaluated using a previously described scoring system [17]. The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%) according to the percentage of cancer cells with cytoplasmic staining. Staining intensities were scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). Final staining score were calculated by summing intensity and extent scores; final staining scores of > 2 were considered positive.

Statistical analysis

Survival rates were calculated using the Kaplan-Meier method and groups were compared using the log rank test. Kaplan-Meier curves were generated using overall survival data. Cox regression analysis was used for multivariate analysis. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). Statistical review of the study was performed by a biomedical statistician.

Results

PI3KCA mutations and MSI in gastric cancer

PI3KCA mutations were detected in 4 out of 173 cases (1.1%) and MSI was detected in 30 out of 338 cases (8.2%) (**Figure 1**). The clinicopathologic significance of the two genetic alterations is tabulated in **Table 2**.

Immunohistochemistry

The frequency of p-Akt positivity was 22.0% (81/368), PTEN loss was 21.5% (79/368), p-mTOR positivity was 68.6% (243/354), p-4E-BP1 positivity was 58.2% (202/347), p-S6 positivity was 42.7% (148/347), p-p70S6 positivity

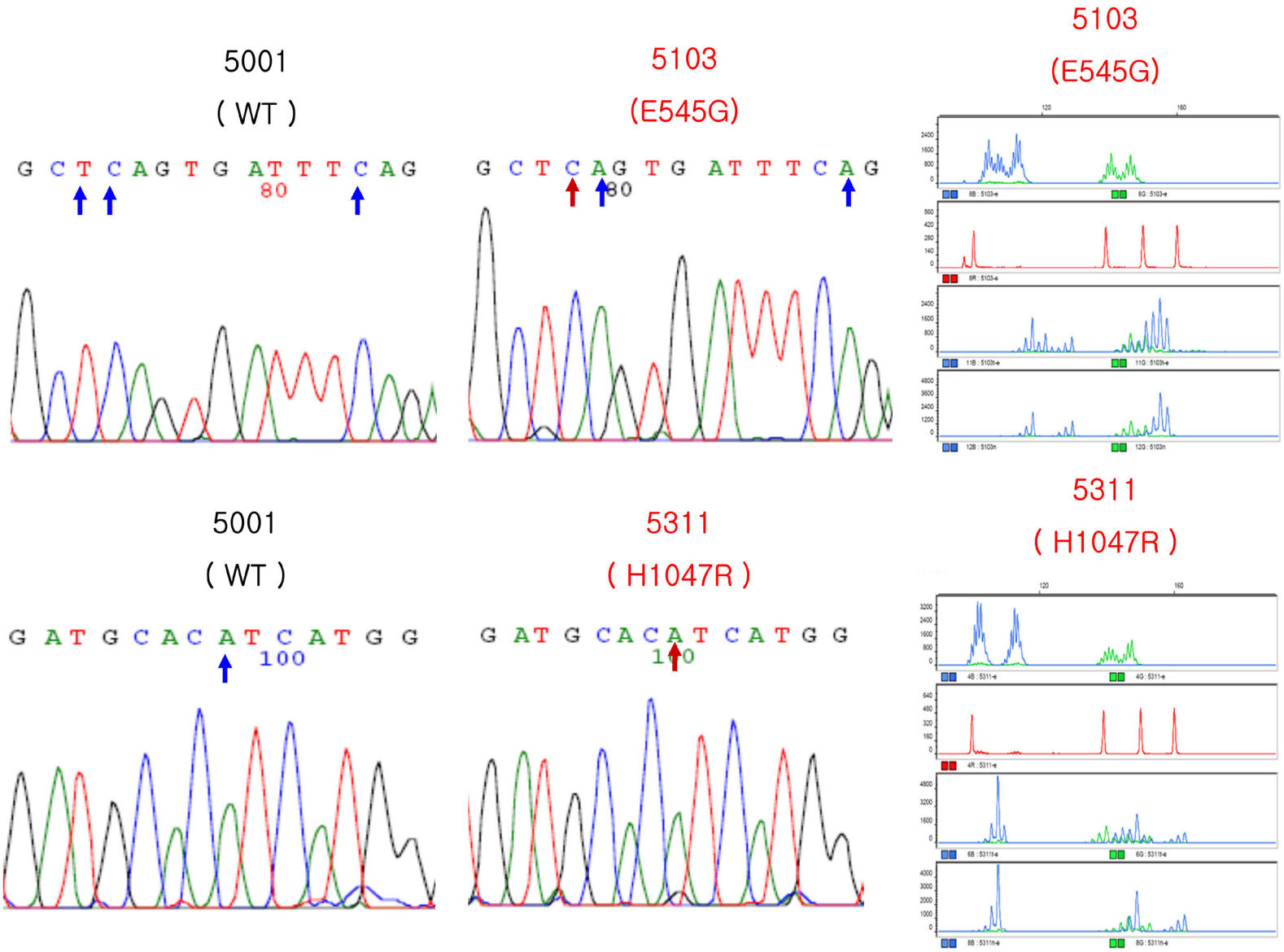

Figure 1. Sequencing results for the detection of PIK3CA mutations in gastric cancer tissues. Upper left, PI3KCA gene exon 10 wild type; upper middle, exon 10 E545G mutation, upper right, microsatellite instability unstable. Lowe left, PI3KCA gene exon 21 wild type; lower middle, exon 21 H1047R mutation, lower right, microsatellite instability unstable.

Table 2. Correlations between clinicopathological factors and proteins upstream of the mTOR pathway in sporadic gastric cancers

	PIK3CA					pAkt					PTEN					pmTOR				
	No mutation		Mutation		P	Negative		Positive		P	Negative		Positive		P	Negative		Positive		P
	n	%	n	%		n	%	n	%		n	%	n	%		n	%	n	%	
Sex																				
Male	120	71%	1	25%	0.047	208	72%	62	77%	0.464	63	80%	207	72%	0.148	177	73%	83	75%	0.702
Female	49	29%	3	75%		79	28%	19	23%		16	20%	82	28%		66	27%	28	25%	
Age																				
< 60 years	82	49%	0	0%	0.055	145	51%	41	51%	0.988	27	34%	159	55%	0.001	127	52%	50	45%	0.208
≥ 60 years	87	51%	4	100%		142	49%	40	49%		52	66%	130	45%		116	48%	61	55%	
Lauren type																				
Intestinal	69	41%	1	25%	0.524	115	40%	47	58%	0.004	46	58%	116	40%	0.004	97	40%	59	53%	0.02
Diffuse	100	59%	3	75%		172	60%	34	42%		33	42%	173	60%		146	60%	52	47%	
Lymph node																				
No metastasis	69	41%	2	50%	0.712	134	47%	55	68%	0.001	25	32%	164	57%	< 0.001	126	52%	54	49%	0.576
Metastasis	100	59%	2	50%		153	53%	26	32%		54	68%	125	43%		117	48%	57	51%	
Lymphatic invasion																				
Not identified	55	33%	1	25%	0.75	135	47%	47	58%	0.081	19	24%	163	56%	< 0.001	120	49%	52	47%	0.658
Present	114	67%	3	75%		152	53%	34	42%		60	76%	126	44%		123	51%	59	53%	
Perineural invasion																				
Not identified	75	44%	4	100%	0.027	158	55%	63	78%	< 0.001	41	52%	180	62%	0.095	146	60%	66	59%	0.912
Present	94	56%	0	0%		129	45%	18	22%		38	48%	109	38%		97	40%	45	41%	
Stromal reaction																				
Not identified	116	69%	4	100%	0.613	214	75%	60	74%	0.834	61	77%	213	74%	0.904	181	74%	80	72%	0.875
Lymphoid	17	10%	0	0%		23	8%	7	9%		5	6%	25	9%		19	8%	11	10%	
Desmoplasia	28	17%	0	0%		37	13%	12	15%		10	13%	39	13%		32	13%	16	14%	
Neutrophilic	8	5%	0	0%		13	5%	2	2%		3	4%	12	4%		11	5%	4	4%	
MSI																				
Stable	155	92%	0	0%	< 0.001	260	91%	78	96%	0.098	71	90%	267	92%	0.469	221	91%	103	93%	0.563
Unstable	14	8%	4	100%		27	9%	3	4%		8	10%	22	8%		22	9%	8	7%	

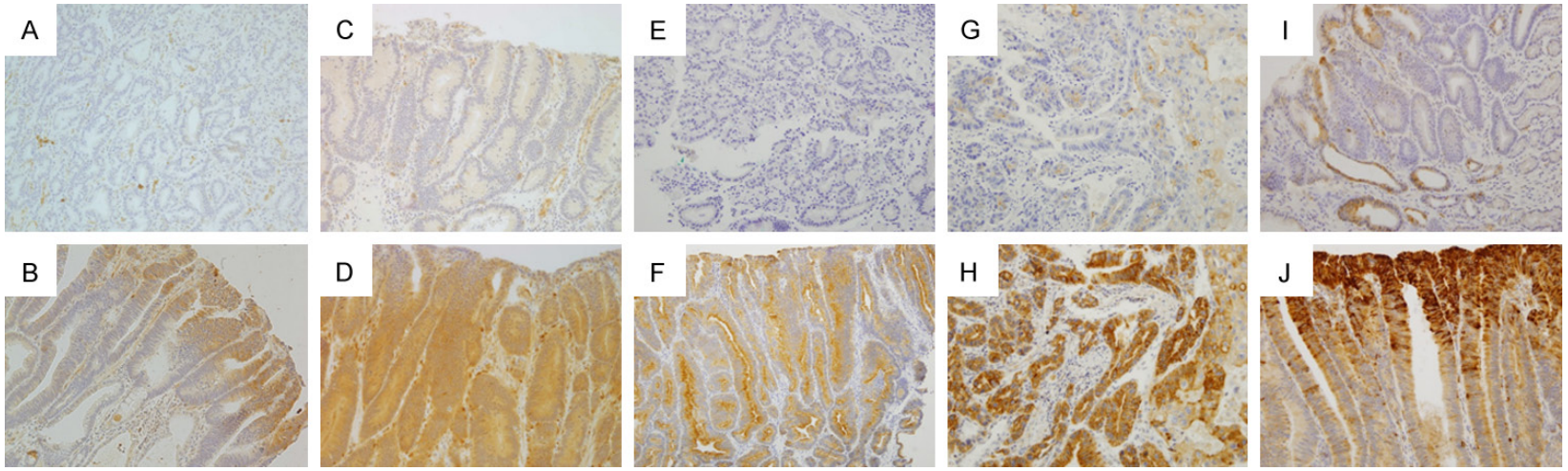

Figure 2. Immunohistochemical analysis of mTOR pathway proteins in gastric cancer tissues. A, B. p-Akt negative and positive; C, D. PTEN negative and positive; E, F. p-mTOR negative and positive; G, H. p-P70S6 negative and positive; I, J. p-S6 negative and positive.

was 51.1% (179/350), and eIF4E positivity was 78.3% (275/351) (**Figure 2**). The expression frequencies of mTOR pathway proteins were compared with clinicopathologic variables and MSI (**Table 2**). All 4 cases with PI3K mutation exhibited MSI. Gastric cancer with eIF4E positivity were nearly unstable. The intestinal-type Lauren classification was frequently associated with p-Akt positivity (P = 0.04) and p-mTOR positivity (P = 0.02) as upstream of mTOR pathway (**Table 2**). As mTOR pathway proteins, p-4EBP1 (P < 0.001), p-S6 (P = 0.01), p-p70S6 (P = 0.001), and eIF4E (P < 0.001) were significantly associated with intestinal-type Lauren classification (**Table 3**).

Correlations among mTOR pathway proteins

Spearman non-parametric correlations among mTOR pathway proteins were evaluated (**Table 4**). Strong correlations were detected between MSI and *PIK3CA* mutations (Rho = 0.451, P < 0.001) as well as pAkt positivity and pS6 positivity (Pho = 0.397, P < 0.001).

Survival analysis

Overall survival and disease-free survival in all TNM stage and subgroups of TNM stage 1 and TNM stage 2 or 3 were calculated, respectively (**Figure 3**). In all TNM stage group, PTEN loss (P = 0.002) and pS6 positivity (P = 0.043) are significantly associated with reduced OS; PTEN loss (P = 0.001), pS6 positivity (P = 0.009), and eIF4E positivity (P = 0.003) are significantly associated with reduced DFS. Gastric cancer with p-4EBP1 positivity showed a significantly reduced overall survival in TNM stage 1. Gastric cancer with eIF4E positivity was significantly associated with a reduced disease-free survival in TNM stage 2 or 3. MSI was associated with a reduced overall survival in TNM stage 1 (P = 0.033).

In the multivariate Cox regression analysis with forward conditional method was performed. Six parameters were input in the order of age, < 60 vs. ≥ 60 years; sex, male vs. female; TNM stage, stage 1 vs. stage 2 or 3, PTEN expression, preserved vs. loss; pS6 expression, negative vs. positive; eIF4E expression, negative vs. positive as covariates. This revealed that PTEN loss is an independent predictors of DFS time (P = 0.013, Hazard ratio = 1.905; 95.0% Confidence interval, 1.148-3.160) (**Table 5**).

Discussion

We performed a comprehensive analysis of PI3K/Akt/mTOR signaling in gastric carcinoma tissues. We detected a *PIK3CA* mutation rate of 1%, and *PIK3CA* mutations were only detected in MSI gastric cancer cases. This rate is lower than previously reported rates of 4.3% to 25% [5]. *PIK3CA* mutations are rare, but their amplification is very common in gastric carcinoma; therefore, amplification could be a major mechanism underlying the activation of the PI3K/Akt pathway in this type of malignancy. [23] *PIK3CA* mutations tend to occur as isolated events, e.g., mutations involved in mismatch repair deficiency in gastric carcinoma [24]. Consistent with previous findings, all 4 cases involving a *PIK3CA* mutation showed MSI.

The activation of mTOR has been observed in patient-derived gastric cancer cells and tumors [25, 26] and activated mTOR pathway proteins have prognostic value for lymph node metastasis [27]. In addition, the prognostic roles of mTOR and p-mTOR expression have been studied extensively in other types of cancers [12, 25, 28-34]. In experimental cancer models, elevated eIF4E function selectively and disproportionately increases the translation of weak mRNAs and mRNAs involved in growth and survival in malignancies [14]. The inhibition of eIF4E may be an effective therapeutic approach for many different tumor types [14].

The PI3K/Akt/mTOR pathway is negatively regulated by the tumor suppressors phosphatase and tensin homologue (PTEN) and tuberous sclerosis proteins 1 and 2 (TSC1-2). The loss of PTEN and TSC1-2 leads to increased p-Akt, p-mTOR, S6K1, and 4EBP1 expression [29]. It is suggested that phosphate and tensin homolog (PTEN) may play an important role in regulation of infiltration and metastasis of gastric cancer, and PTEN gene might be a prognostic biomarker of gastric cancer [16, 35]. Reduced expression of PTEN in gastric cancer points to another mechanism apart from PTEN mutation that may be involved in the pathogenesis of gastric cancer [36]. In contrast, PTEN mutations of gene have been observed frequently in various neoplasms, including glioblastoma, melanoma, prostate cancer and breast cancer [35], aberrant promoter methylation has been

Table 3. Correlations between clinicopathologic factors and mTOR pathway proteins in sporadic gastric cancers

	p4EBP1					pS6					pp70S6					eIF4E				
	Negative		Positive		P	Negative		Positive		P	Negative		Positive		P	Negative		Positive		P
	n	%	n	%		n	%	n	%		n	%	n	%		n	%	n	%	
Sex																				
Male	99	68%	156	77%	0.062	146	73%	108	73%	0.935	125	73%	132	74%	0.892	55	72%	202	73%	0.850
Female	46	32%	46	23%		53	27%	40	27%		46	27%	47	26%		21	28%	73	27%	
Age																				
< 60 years	80	55%	93	46%	0.093	93	47%	80	54%	0.177	89	52%	87	49%	0.520	38	50%	138	50%	0.978
≥ 60 years	65	45%	109	54%		106	53%	68	46%		82	48%	92	51%		38	50%	137	50%	
Lauren type																				
Intestinal	42	29%	109	54%	< 0.001	71	36%	79	53%	0.001	59	35%	93	52%	0.001	17	22%	135	49%	< 0.001
Diffuse	103	71%	93	46%		128	64%	69	47%		112	65%	86	48%		59	78%	140	51%	
Lymph node																				
No metastasis	74	51%	101	50%	0.849	87	44%	89	60%	0.002	87	51%	91	51%	0.994	31	41%	148	54%	0.044
Metastasis	71	49%	101	50%		112	56%	59	40%		84	49%	88	49%		45	59%	127	46%	
Lymphatic invasion																				
Not identified	78	54%	89	44%	0.073	84	42%	82	55%	0.015	89	52%	79	44%	0.139	29	38%	141	51%	0.043
Present	67	46%	113	56%		115	58%	66	45%		82	48%	100	56%		47	62%	134	49%	
Perineural invasion																				
Not identified	86	59%	120	59%	0.986	102	51%	104	70%	< 0.001	103	60%	104	58%	0.685	36	47%	173	63%	0.015
Present	59	41%	82	41%		97	49%	44	30%		68	40%	75	42%		40	53%	102	37%	
Stromal reaction																				
Not identified	107	74%	148	73%	0.158	140	70%	115	78%	0.367	128	75%	130	73%	0.908	56	74%	203	74%	0.042
Lymphoid	7	5%	22	11%		19	10%	10	7%		14	8%	15	8%		2	3%	27	10%	
Desmoplasia	24	17%	24	12%		29	15%	19	13%		23	13%	25	14%		16	21%	32	12%	
Neurophilic	7	5%	8	4%		11	6%	4	3%		6	4%	9	5%		2	3%	13	5%	
MSI																				
Stable	131	90%	186	92%	0.571	184	92%	133	90%	0.394	162	95%	158	88%	0.031	75	99%	246	89%	0.011
Unstable	14	10%	16	8%		15	8%	15	10%		9	5%	21	12%		1	1%	29	11%	

Table 4. Spearman non-parametric correlation analysis of relationships between mTOR signaling pathway alterations

		PIK3CA	pAkt	PTEN	pmTOR	p4EBP1	pS6	pp70S6	eIF4E	Hif1 alpha	VEGF	MSI
PIK3CA	Rho	1.000	0.018	0.006	0.069	-0.031	0.049	-0.008	0.080	-0.083	-0.007	0.451
	Significance		0.812	0.942	0.378	0.693	0.532	0.922	0.307	0.315	0.930	< 0.001
	N	173	173	173	167	166	165	164	166	149	168	173
pAkt	Rho	0.018	1.000	0.022	0.204	0.136	0.397	0.065	0.122	0.246	0.057	-0.086
	Significance	0.812		0.671	0.000	0.011	0.000	0.227	0.022	0.000	0.293	0.098
	N	173	368	368	354	347	347	350	351	310	348	368
PTEN	Rho	0.006	0.022	1.000	-0.032	-0.093	0.002	0.124	0.034	0.114	-0.142	-0.038
	Significance	0.942	0.671		0.546	0.083	0.971	0.020	0.531	0.044	0.008	0.471
	N	173	368	368	354	347	347	350	351	310	348	368
pmTOR	Rho	0.069	0.204	-0.032	1.000	0.200	0.189	0.129	0.016	0.036	0.091	-0.031
	Significance	0.378	< 0.001	0.546		< 0.001	< 0.001	0.016	0.759	0.530	0.098	0.564
	N	167	354	354	354	347	347	348	350	308	334	354
p4EBP1	Rho	-0.031	0.136	-0.093	0.200	1.000	0.240	0.163	0.183	0.065	0.143	-0.030
	Significance	0.693	0.011	0.083	< 0.001		< 0.001	0.002	0.001	0.263	0.010	0.572
	N	166	347	347	347	347	342	343	344	301	327	347
pS6	Rho	0.049	0.397	0.002	0.189	0.240	1.000	0.039	0.240	0.117	0.065	0.046
	Significance	0.532	< 0.001	0.971	< 0.001	< 0.001		0.464	< 0.001	0.041	0.239	0.396
	N	165	347	347	347	342	347	346	347	306	329	347
pp70S6	Rho	-0.008	0.065	0.124	0.129	0.163	0.039	1.000	0.150	0.194	0.191	0.116
	Significance	0.922	0.227	0.020	0.016	0.002	0.464		0.005	0.001	< 0.001	0.031
	N	164	350	350	348	343	346	350	347	306	331	350
eIF4E	Rho	0.080	0.122	0.034	0.016	0.183	0.240	0.150	1.000	0.197	0.050	0.136
	Significance	0.307	0.022	0.531	0.759	0.001	0.000	0.005		0.001	0.360	0.011
	N	166	351	351	350	344	347	347	351	307	332	351
Hif1 alpha	Rho	-0.083	0.246	0.114	0.036	0.065	0.117	0.194	0.197	1.000	0.045	-0.052
	Significance	0.315	0.000	0.044	0.530	0.263	0.041	0.001	0.001		0.445	0.359

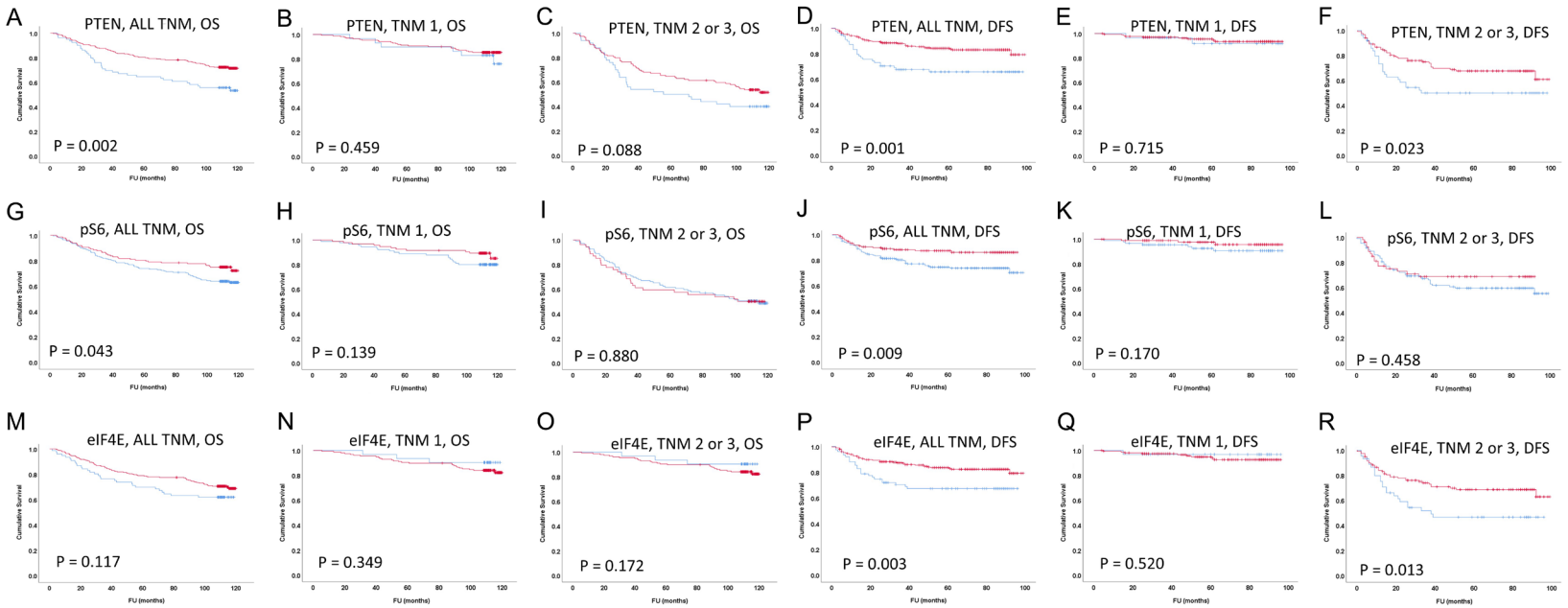

Figure 3. Kaplan-Meier survival analyses of patients with gastric cancer according to mTOR signaling molecule alterations. Red line, gastric cancer patients with positive expression of protein; blue line, gastric cancer patients with negative expression of protein.

Table 5. Multivariate Cox analysis of overall survival and recurrence-free survival time patients with gastric cancer

Variables	Classification	N	Overall Survival				Recurrence Free Survival			
			Significance	Hazard Ratio	95.0% Confidence Interval		Significance	Hazard Ratio	95.0% Confidence Interval	
					Lower limit	Upper limit			Lower limit	Upper limit
Age	< 60	173								
	≥ 60	174	< 0.001	2.514	1.673	3.778	Not Available	Not Available	Not Available	Not Available
Sex	Female	93								
	Male	254	0.246	0.780	0.512	1.187	0.042	0.588	0.352	0.981
TNM	Stage 1	184								
	Stage 2 or 3	163	< 0.001	4.023	2.600	6.223	< 0.001	7.066	3.568	13.993
PTEN	Preserved	274								
	Loss	73	0.170	1.343	0.882	2.044	0.013	1.905	1.148	3.160
pS6	Negative	199								
	Positive	148	0.625	0.905	0.608	1.349	0.181	0.694	0.406	1.185

suggested as a potential mechanism of PTEN inactivation in gastric cancer [37]. Our results showed that PTEN loss is an independent predictive marker for DFS time.

mTOR is an evolutionarily conserved member of the phosphoinositide kinase-related kinase family, whose activity is regulated by phosphorylation in response to insulin or muscle activity [38]. p-mTOR overexpression is related to clinicopathological factors and p-mTOR appears to be a more sensitive biomarker than total mTOR for the prediction of patient survival [33].

Rapamycin, a specific inhibitor of mTOR, is widely used as an immunosuppressant in organ transplantation and inhibits cell proliferation in a wide range of human tumors [11, 34, 39, 40]. The inhibition of mTOR results in the inhibition of the phosphorylation of downstream proteins, including 4E-BP1 and S6K1, which ultimately results in growth arrest in the G1 phase of the cell cycle. A comprehensive understanding of the PI3K/Akt/mTOR pathway could improve our understanding of the mechanism underlying tumor development and could lead to the development of a targeted anticancer therapy for gastric carcinoma. Appropriate clinical trials that incorporate predictive biomarkers need to be developed for personalized therapy.

In conclusion, the expression of mTOR pathway proteins is frequently altered in intestinal-type gastric cancer, and PTEN loss, p4EBP1 and eIF3E are associated with poor survival in patients with gastric cancer.

Acknowledgements

Ms. Hye Jung Lee and Seung Hee Jung provided help with tissue array construction and immunohistochemical staining. The present study was supported by a National Research Foundation of Korea grant funded by the Korean government (grant no. 2014R1A1A2057925).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hee Sung Kim, Department of Pathology, Chung-Ang University College of Medicine, 84 Heukseok-Ro Dongjak-Gu Seoul 06973, Republic of Korea. Tel: +82-26299-3169; Fax: +82-2-6293-5630; E-mail: kim1967@cau.ac.kr

References

[1] Sitarz R, Skierucha M, Mielko J, Offerhaus GJA, Maciejewski R and Polkowski WP. Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manag Res 2018; 10: 239-248.

[2] Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B and Velculescu VE. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004; 304: 554.

[3] Fruman DA, Meyers RE and Cantley LC. Phosphoinositide kinases. Annu Rev Biochem 1998; 67: 481-507.

[4] Cantley LC. The phosphoinositide 3-kinase pathway. Science 2002; 296: 1655-1657.

[5] Barbi S, Cataldo I, De Manzoni G, Bersani S, Lamba S, Mattuzzi S, Bardelli A and Scarpa A. The analysis of PIK3CA mutations in gastric carcinoma and metanalysis of literature suggest that exon-selectivity is a signature of cancer type. J Exp Clin Cancer Res 2010; 29: 32.

[6] Bader AG, Kang S and Vogt PK. Cancer-specific mutations in PIK3CA are oncogenic in vivo. Proc Natl Acad Sci U S A 2006; 103: 1475-1479.

[7] Zhao L and Vogt PK. Helical domain and kinase domain mutations in p110alpha of phosphatidylinositol 3-kinase induce gain of function by different mechanisms. Proc Natl Acad Sci U S A 2008; 105: 2652-2657.

[8] Barbareschi M, Buttitta F, Felicioni L, Cotrupi S, Barassi F, Del Grammastro M, Ferro A, Dalla Palma P, Galligioni E and Marchetti A. Different prognostic roles of mutations in the helical and kinase domains of the PIK3CA gene in breast carcinomas. Clin Cancer Res 2007; 13: 6064-6069.

[9] Benvenuti S, Frattini M, Arena S, Zanon C, Cappelletti V, Coradini D, Daidone MG, Pilotti S, Pierotti MA and Bardelli A. PIK3CA cancer mutations display gender and tissue specificity patterns. Hum Mutat 2008; 29: 284-288.

[10] Schmelzle T and Hall MN. TOR, a central controller of cell growth. Cell 2000; 103: 253-262.

[11] Petroulakis E, Mamane Y, Le Bacquer O, Shahbazian D and Sonenberg N. mTOR signaling: implications for cancer and anticancer therapy. Br J Cancer 2005; 94: 195-199.

[12] Murayama T, Inokuchi M, Takagi Y, Yamada H, Kojima K, Kumagai J, Kawano T and Sugihara K. Relation between outcomes and localisation of p-mTOR expression in gastric cancer. Br J Cancer 2009; 100: 782-788.

[13] Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A and Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is

rapamycin insensitive. Nat Cell Biol 2004; 6: 1122-1128.
[14] Graff JR, Konicek BW, Carter JH and Marcusson EG. Targeting the eukaryotic translation initiation factor 4E for cancer therapy. Cancer Res 2008; 68: 631-634.
[15] Corso G, Velho S, Paredes J, Pedrazzani C, Martins D, Milanezi F, Pascale V, Vindigni C, Pinheiro H, Leite M, Marrelli D, Sousa S, Carneiro F, Oliveira C, Roviello F and Seruca R. Oncogenic mutations in gastric cancer with microsatellite instability. Eur J Cancer 2011; 47: 443-451.
[16] Wen YG, Wang Q, Zhou CZ, Qiu GQ, Peng ZH andTang HM. Mutation analysis of tumor suppressor gene PTEN in patients with gastric carcinomas and its impact on PI3K/AKT pathway. Oncol Rep 2010; 24: 89-95.
[17] Lang SA, Gaumann A, Koehl GE, Seidel U, Bataille F, Klein D, Ellis LM, Bolder U, Hofstaedter F, Schlitt HJ, Geissler EK and Stoeltzing O. Mammalian target of rapamycin is activated in human gastric cancer and serves as a target for therapy in an experimental model. Int J Cancer 2007; 120: 1803-1810.
[18] Zhou X. Activation of the akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. Clin Cancer Res 2004; 10: 6779-6788.
[19] Xiao L, Wang YC, Li WS and Du Y. The role of mTOR and phospho-p70S6K in pathogenesis and progression of gastric carcinomas: an immunohistochemical study on tissue microarray. J Exp Clin Cancer Res 2009; 28: 152.
[20] de Manzoni G, Tomezzoli A, Di Leo A, Moore PS, Talamini G and Scarpa A. Clinical significance of mutator phenotype and chromosome 17p and 18q allelic loss in gastric cancer. Br J Surg 2001; 88: 419-425.
[21] Moore PS, Zamboni G, Brighenti A, Lissandrini D, Antonello D, Capelli P, Rigaud G, Falconi M and Scarpa A. Molecular characterization of pancreatic serous microcystic adenomas: evidence for a tumor suppressor gene on chromosome 10q. Am J Pathol 2001; 158: 317-321.
[22] Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, Gambacorta M, Siena S and Bardelli A. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. Lancet Oncol 2005; 6: 279-286.
[23] Shi J, Yao D, Liu W, Wang N, Lv H, Zhang G, Ji M, Xu L, He N, Shi B and Hou P. Highly frequent PIK3CA amplification is associated with poor prognosis in gastric cancer. BMC Cancer 2012; 12: 50.
[24] Velho S, Oliveira C, Ferreira A, Ferreira AC, Suriano G, Schwartz S Jr, Duval A, Carneiro F, Machado JC, Hamelin R and Seruca R. The prevalence of PIK3CA mutations in gastric and colon cancer. Eur J Cancer 2005; 41: 1649-1654.
[25] Al-Batran SE, Ducreux M and Ohtsu A. mTOR as a therapeutic target in patients with gastric cancer. Int J Cancer 2012; 130: 491-496.
[26] Matsuoka T and Yashiro M. The role of PI3K/Akt/mTOR signaling in gastric carcinoma. Cancers (Basel) 2014; 6: 1441-1463.
[27] An JY, Kim KM, Choi MG, Noh JH, Sohn TS, Bae JM and Kim S. Prognostic role of p-mTOR expression in cancer tissues and metastatic lymph nodes in pT2b gastric cancer. Int J Cancer 2010; 126: 2904-2913.
[28] Ying J, Xu Q, Liu B, Zhang G, Chen L and Pan H. The expression of the PI3K/AKT/mTOR pathway in gastric cancer and its role in gastric cancer prognosis. Onco Targets Ther 2015; 8: 2427-2433.
[29] Prins MJ, Verhage RJ, Ruurda JP, ten Kate FJ and van Hillegersberg R. Over-expression of phosphorylated mammalian target of rapamycin is associated with poor survival in oesophageal adenocarcinoma: a tissue microarray study. J Clin Pathol 2013; 66: 224-228.
[30] Byeon SJ, Han N, Choi J, Kim MA and Kim WH. Prognostic implication of TSC1 and mTOR expression in gastric carcinoma. J Surg Oncol 2014; 109: 812-817.
[31] Fukamachi H, Kim SK, Koh J, Lee HS, Sasaki Y, Yamashita K, Nishikawaji T, Shimada S, Akiyama Y, Byeon SJ, Bae DH, Okuno K, Nakagawa M, Tanioka T, Inokuchi M, Kawachi H, Tsuchiya K, Kojima K, Tokino T, Eishi Y, Kim YS, Kim WH, Yuasa Y and Tanaka S. A subset of diffuse-type gastric cancer is susceptible to mTOR inhibitors and checkpoint inhibitors. J Exp Clin Cancer Res 2019; 38: 127.
[32] Chaux A, Schultz L, Albadine R, Hicks J, Kim JJ, Allaf ME, Carducci MA, Rodriguez R, Hammers HJ, Argani P, Reuter VE and Netto GJ. Immunoexpression status and prognostic value of mammalian target of rapamycin and hypoxia-induced pathway members in papillary cell renal cell carcinomas. Hum Pathol 2012; 43: 2129-2137.
[33] Yu G, Wang J, Chen Y, Wang X, Pan J, Li G, Jia Z, Li Q, Yao JC and Xie K. Overexpression of phosphorylated mammalian target of rapamycin predicts lymph node metastasis and prognosis of chinese patients with gastric cancer. Clin Cancer Res 2009; 15: 1821-1829.
[34] Iwenofu OH, Lackman RD, Staddon AP, Goodwin DG, Haupt HM and Brooks JS. Phospho-S6 ribosomal protein: a potential new predictive sarcoma marker for targeted mTOR therapy. Mod Pathol 2007; 21: 231-237.
[35] Oki E, Kakeji Y, Baba H, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, Yamamoto M, Ikebe M

and Maehara Y. Impact of loss of heterozygosity of encoding phosphate and tensin homolog on the prognosis of gastric cancer. J Gastroenterol Hepatol 2006; 21: 814-818.

[36] Fei G, Ebert MP, Mawrin C, Leodolter A, Schmidt N, Dietzmann K and Malfertheiner P. Reduced PTEN expression in gastric cancer and in the gastric mucosa of gastric cancer relatives. Eur J Gastroenterol Hepatol 2002; 14: 297-303.

[37] Kang YH, Lee HS and Kim WH. Promoter methylation and silencing of PTEN in gastric carcinoma. Lab Invest 2002; 82: 285-291.

[38] Chiang GG and Abraham RT. Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. J Biol Chem 2005; 280: 25485-25490.

[39] Dowling RJ, Topisirovic I, Fonseca BD and Sonenberg N. Dissecting the role of mTOR: lessons from mTOR inhibitors. Biochim Biophys Acta 2010; 1804: 433-439.

[40] Alqurashi N, Gopalan V, Smith RA and Lam AK. Clinical impacts of mammalian target of rapamycin expression in human colorectal cancers. Human Pathology 2013; 44: 2089-96.