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Exon 9 Mutation of *PIK3CA* Associated With Poor Survival in Patients With Epstein-Barr Virus-associated Gastric Cancer

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Abstract. *Background: Epstein-Barr virus (EBV)-associated gastric cancer (GC) is known to harbor a significant enrichment of of phosphatidylinositol 4, 5-biphosphate 3- kinase catalytic subunit alpha isoform (PIK3CA). Therefore, this study investigated the clinical relevance and prognostic role of PIK3CA mutations in patients with EBV-GC. Materials and Methods: After reviewing 1,318 consecutive cases of surgically resected GC, 120 patients were identified as EBV-positive using EBV-encoded RNA in situ hybridization. PIK3CA mutations were identified in formalin-fixed and paraffin-embedded surgical specimens from 112 patients with EBV-GC with available tumor tissue samples. Real-time polymerase chain reaction was used to evaluate hot-spot mutations of exons 1, 4, 7, 9, and 20 of PIK3CA. Results: Among the 112 patients, the frequency of PIK3CA mutations was 25.0% (n=28), and among the 28 patients harboring a PIK3CA mutation, most mutations were identified in exon 9 (n=21, 18.8%). The presence of PIK3CA mutation was also correlated with a higher T category (p<0.001) and N category (p<0.001), as well as the presence of perinueral invasion (p<0.001) and venous invasion (p<0.001). In a univariate analysis, PIK3CA mutation showed no association with overall survival (OS) (p=0.184) or disease-free survival (DFS) (p=0.150). Patients harboring exon 9 PIK3CA mutations exhibited a significantly shorter OS (p=0.023) and DFS (p=0.013) than the patients lacking an exon 9 PIK3CA mutation, yet without statistical significance in the multivariate analysis. Notably, exon 9 E542K mutation of PIK3CA was associated with the worst DFS (p=0.011). Conclusion: The current data show that PIK3CA mutations appear to play an important role in carcinogenesis and tumor aggressiveness in EBV-GC, and also support the concept that exon 9 mutation of PIK3CA is a prognostic indicator for predicting patient outcomes and a rationale for therapeutic targeting in EBV-GC.*

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Key Words: Gastric cancer, Epstein-Barr virus, *PIK3CA*, prognosis.

The Cancer Genome Atlas (TCGA) research group identified four genomic subtypes of gastric cancer (GC): Epstein-Barr

virus (EBV)$^+$ tumors, microsatellite-instable tumors (MSI), genomically stable tumors, and tumors with chromosomal instability (1). In particular, EBV$^+$ tumors comprised 9% of the TCGA gastric cancer samples and exhibited frequent mutations of phosphatidylinositol 4, 5-biphosphate 3-kinase (PI3K) catalytic subunit alpha isoform (*PIK3CA*), extreme DNA hypermethylation, and amplification of Janus kinase 2, programmed death-ligand 1 (PD-L1), and PD-L2 (1). It is also worth noting that non-silent *PIK3CA* mutations were found in 80% of the EBV-positive group. More recently, the Asian Cancer Research Group (ACRG) provided a molecular characterization of 300 GCs using various platforms, and proposed four distinct types: MSI, microsatellite stable/epithelial-to-mesenchymal transition (MSS/EMT), MSS/TP53$^+$, and MSS/TP53$^-$ (2). The ACRG found that EBV infection occurred in 6.5% of patients overall and more frequently in the MSS/TP53$^+$ subgroup, which had the second-best overall survival (OS) (3). In particular, the MSS/TP53$^+$ subtype showed a relatively higher prevalence of mutations in *PIK3CA* (2). Therefore, these findings point to the possibility of *PIK3CA* as a targetable biomarker in EBV-associated GC (EBV-GC), suggesting that *PIK3CA* activates several important cellular pathways that potentially play a role in patients with EBV-GC.

The PI3K-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) signaling pathway, which is involved in multiple processes such as cell survival, proliferation, differentiation, and tumorigenesis, is one of the most dysregulated pathways in human cancer (4, 5). Among these genomic alterations, abnormal activation and amplification of *PIK3CA* have been identified as playing a crucial role in the initiation and progress of cancerous tumors (6). PI3Ks are divided into three main classes, where class I includes two categories: catalytic and regulatory. Class I PI3Ks are heterodimers and consist of a p85 regulatory subunit and p110 catalytic subunit (6). Class I PI3Ks also interact with cell-surface receptors, leading to the phosphorylation of phosphatidylinositol 4, 5-biphosphate to generate phosphatidylinositol 3, 4, 5-triphosphate (5, 7), which is a second messenger that activates AKT kinases (4). *PIK3CA* is responsible for encoding the p110 subunit and can be up-regulated by activating molecular alterations in the PI3K catalytic subunit isoform. Interestingly, *PIK3CA* mutations are mostly hot-spot mutations located in exon 9 (E542K and E545K) and exon 20 (H1047R) in human cancer, while these mutations are more dispersed in EBV$^+$ tumors (1, 8).

To date, several studies have provided potential evidence that *PIK3CA* mutations contribute to determining the prognosis for patients with solid tumors (5, 9, 10). However, the significance of *PIK3CA* mutations in GC remains unclear. A recent study reported that PI3K-AKT-mTOR pathway activation negatively affected survival outcomes (11). In contrast, Harada *et al*. reported no correlation between *PIK3CA* mutations and prognosis in patients with GC (12). Another study also confirmed no relationship between *PIK3CA* mutations and clinical outcomes (13). However, in a recent meta-analysis of 2, 481 patients in 11 trials using pooled estimates for OS, Li *et al*. evaluated the relationship between *PIK3CA* expression and GC prognosis and found no correlation between *PIK3CA* gene mutation and OS, yet significant association with poor tumor differentiation (14). In the case of EBV-GC, Böger *et al*. reported that the *PIK3CA* status had no significant effect on OS or tumor-specific survival, suggesting an intratumoral heterogeneity of *PIK3CA* mutations (15). Moreover, significant differences in *PIK3CA* mutation frequencies have been described between Asian and Caucasian patient populations with GC (16). Thus, while *PIK3CA* would seem to play an important role in the prognosis of GC, very few studies have reported on the clinical implications and prognostic effects of these mutations in patients with EBV-GC. Accordingly, this study attempted to identify the prognostic significance of *PIK3CA* mutations in a large number of patients with EBV-GC patients and the relationship of these mutations to clinicopathological parameters associated with prognosis.

Patients and Methods

Patients. After reviewing 1,318 consecutive cases of surgically resected gastric cancer at the Kyungpook National University Chilgok Hospital (KNUCH) between January 2011 and November 2014, 120 patients were identified as EBV$^+$ using EBV-encoded RNA *in situ* hybridization. As this independent cohort was also used in several of our previous studies, detailed information, including patient selection and demographics, has already been reported (17, 18). Among the 120 patients, eight were excluded due to insufficient or unavailable material (n=6) and technical issues (n=2). The related medical files and pathological reports were all reviewed to identify the clinical and demographic characteristics. The tumor staging was conducted in accordance with the seventh edition of the American Joint Committee on Cancer Staging Manual for Stomach Cancer (19). The histological classification was divided into two groups according to the 2010 WHO classification of digestive systems: Gastric carcinoma with lymphoid stroma (GCLS) and non-GCLS (20). All the cases in this study were previously classified into three histological subtypes based on the pattern of the host cellular immune response to the tumor cells: Typical lymphoepithelioma-like carcinoma, carcinoma with Crohn's disease-like lymphoid reaction, and conventional adenocarcinoma (17). Similarly, the percentage of intratumoral tumor-infiltrating lymphocytes (TILs) and stromal TILs had also been previously determined by interpreting full sections of hematoxylin and eosin-stained slides (17), while the expression of PD-L1 had been determined using immunohistochemistry (18). The present study was approved by the Institutional Review Board of KNUCH and exempted from the need for informed consent (IRB no.: KNUMC 2016-05-012). Furthermore, since part of the investigation evaluated the prognostic influence of biological markers, this study followed the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria (21).

Real-time polymerase chain (PCR) reaction for PIK3CA mutation. Tumors areas with least ≥50% tumor cells were marked on hematoxylin and eosin-stained slides by a pathologist (ANS). One or two serial sections (5 μm-thick) from each representative formalin-fixed paraffin-embedded tumor block were cut and manually macro-dissected. The blades were cleaned or replaced after sectioning and dissectioning each paraffin block to prevent carryover contamination. These dissected and deparaffinized sections were then used for genomic DNA extraction using a Cobas® DNA Sample Preparation Kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA) following the manufacturer's protocols. The quantity and quality of the isolated genomic DNA were measured using a NanoDrop™ UV spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

The *PIK3CA* mutations were identified using the Cobas® PIK3CA Mutation Test kit (Roche) based on a real-time PCR procedure of the Cobas 4800 system (Roche) according to the manufacturer's instructions and as described previously (22, 23). This test kit is designed to detect R88Q in exon 1, N345K in exon 4, C420R in exon 7, E542K, E545X (E545A, E545D*, E545G, and E545K), Q546X (Q546E, Q546K, Q546L, and Q546R) in exon 9, and M1043I†, H1047X (H1047L, H1047R, and H1047Y), and G1049R in exon 20 of *PIK3CA*. The target DNA was then amplified and detected on a Cobas z 480 analyzer (Roche). To ensure a valid interpretation of the amplified curves, the Cobas 4800 system uses a specific and tested algorithm for an automatic and standardized analysis of the specific kinetic of each individual curve. No operator-mediated evaluation or interpretation was needed nor was possible (23).

Statistical analyses. The statistical analyses were all evaluated using SSPS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). All associations with clinicopathological characteristics were evaluated using Fisher's exact test or Chi-squared test. Disease-free survival (DFS) was measured from the time of surgery to initial tumor relapse (local recurrence or distant) or death as a result of any cause. OS was calculated from the time of surgery to death or the last follow-up date. The time-to event outcomes (DFS and OS) were calculated using the Kaplan–Meier method with curves compared using a log-rank test. The hazard ratios (HRs) and their 95% confidence intervals (CIs) were evaluated using a Cox proportional hazard model. A multivariate Cox regression analysis was conducted using backward stepwise selection by including variables of $p<0.05$ in the univariate analysis. Hazard ratios (HRs) and their 95% confidence intervals (CIs) were estimated for each factor. All p-values were two-sided and statistical significance was set at $p<0.05$.

Results

Patient characteristics. A total of 112 patients with EBV-GC were analyzed for PIK3CA hot-spot mutation. As summarized in Table I, the majority of patients were male (81.3%), with early pathological stage tumors (60.7% stage I and 22.3% stage II), and the median age was 62 years. For 68 of the patients (60.7%), the percentage of stromal TILs was ≥25%, while for 56 patients (50.0%), the percentage of itratumoraI TILs was ≥27.5%.

PIK3CA mutations. Overall, 28 patients (25.0%) were identified with *PIK3CA* mutations using real-time PCR of formalin-fixed paraffin-embedded tissues. The detailed features of these 28 cases are summarized in Table II. Exon 9 *PIK3CA* mutations were detected in 21 (18.8%) patients (exon 9 alone in 20 patients: E545X in 12 patients, E542K in seven, and E542K and E545X in one), where one patient exhibited *PIK3CA* mutations

Table I. *Patient characteristic (n=112)*.

Variable	Value
Age, years	
Median (range)	62 (32.0-80.0)
Gender, n (%)	
Male	91 (81.3)
Female	21 (18.8)
Lauren classification, n (%)	
Intestinal	22 (19.6)
Diffuse	79 (70.5)
Mixed	11 (9.8)
Tumor depth, n (%)	
T1	61 (54.5)
T2	12 (10.7)
T3	19 (17.0)
T4	20 (17.9)
Lymph node metastasis, n (%)	
N0	80 (71.4)
N1	13 (11.6)
N2	8 (7.1)
N3	11 (9.8)
pTNM stage, n (%)	
I	68 (60.7)
II	25 (22.3)
III	18 (16.1)
IV	1 (0.9)
TILs, n (%)	
Stromal (cut-off=25%)	
Negative	44 (39.3)
Positive	68 (60.7)
Intratumoral (cut-off=27.5%)	
Negative	56 (50.0)
Positive	56 (50.0)
PD-L1, n (%)	
Stromal*	
Negative	47 (42.0)
Positive	65 (58.0)
Intratumoral*	
Negative	57 (50.9)
Positive	55 (49.1)
WHO classification, n (%)	
Non-GCLS	57 (50.9)
GCLS	55 (49.1)
Histological subclassification, n (%)	
LELC	33 (29.5)
CLR	48 (42.9)
CA	31 (27.7)

CA: Conventional adenocarcinoma; CLR: Crohn's disease-like lymphoid reaction; GCLS: gastric carcinoma with lymphoid stroma; LELC: lymphoepithelioma-like carcinoma; PD-L1: programmed death ligand-1; TILs: tumour-infiltrating lymphocytes; WHO: World Health Organization. *Positive=2+/3+ intensity in ≥1% area.

Table II. *Summary of cases with phosphatidylinositol 4, 5-biphosphate 3- kinase catalytic subunit alpha isoform (PIK3CA) mutation of Epstein-Barr virus-associated gastric cancer. In all cases, hot-spot for PIK3CA mutations in formalin-fixed paraffin-embedded tumors were identified by real-time polymerase chain reaction.*

Case	Age, years	Gender	Hot-spot for *PIK3CA* mutation					T-Category	N-Category	Stage
			Exon 1	Exon 4	Exon 7	Exon 9	Exon 20			
1	59	F	wt	wt	wt	E542K	wt	T1b	N0	IA
2	74	M	wt	wt	wt	E545X	wt	T4a	N3	IIIC
3	72	M	wt	wt	wt	E542K E545X	wt	T2	N2	IIB
4	70	M	R88Q	wt	wt	wt	wt	T3	N1	IIB
5	51	F	wt	wt	wt	E545X	wt	T3	N3	IIIB
6	74	M	wt	wt	wt	E545X	wt	T3	N0	IIA
7	50	M	wt	wt	wt	E545X	wt	T4a	N1	IIIA
8	63	F	wt	wt	wt	E545X	wt	T1b	N0	IA
9	73	F	wt	wt	wt	E542K	wt	T3	N1	IIB
10	74	M	wt	wt	wt	E545X	wt	T4a	N3	IIIC
11	63	M	wt	wt	wt	E542K	wt	T4a	N3	IIIC
12	46	M	wt	wt	wt	wt	H1047X	T1b	N0	IA
13	61	M	wt	wt	wt	E545X	wt	T4a	N3	IIIC
14	53	M	wt	wt	wt	E545X	wt	T4a	N2	IIIB
15	62	M	wt	wt	wt	E542K	wt	T4a	N3	IIIC
16	66	F	wt	wt	wt	E542K	wt	T1b	N0	IA
17	65	M	wt	wt	wt	E545X	wt	T4a	N2	IIIB
18	52	M	wt	N345K	wt	wt	wt	T1b	N0	IA
19	71	M	wt	wt	wt	E542K	wt	T4a	N3	IIIC
20	48	F	wt	wt	C420R	wt	wt	T3	N1	IIB
21	45	F	wt	wt	wt	wt	H1047X	T4a	N2	IIIB
22	62	M	wt	wt	wt	E545X	wt	T3	N1	IIB
23	46	M	wt	wt	wt	E545X	wt	T1b	N0	IA
24	77	M	wt	wt	C420R	wt	H1047X	T1b	N0	IA
25	55	M	wt	wt	wt	wt	H1047X	T3	N0	IIA
26	65	M	wt	wt	wt	E542K	wt	T4a	N3	IIIC
27	73	M	wt	wt	wt	E545X	H1047X	T3	N0	IIA
28	56	M	wt	wt	wt	E545X	wt	T1b	N0	IA

wt: Wild-type.

in both exon 9 and exon 20 (E545X and H1047X). As shown in Table III, *PIK3CA* mutation in tumors was significantly associated with a higher T-stage ($p<0.001$), lymph node metastasis ($p<0.001$), higher stage ($p<0.001$), perineural invasion ($p<0.001$), venous invasion ($p=0.004$), and histological subtype of conventional adenocarcinoma ($p=0.003$). In particular, exon 9 PIK3CA mutation was significantly associated with a higher T-stage ($p<0.001$), lymph node metastasis ($p<0.001$), higher stage ($p<0.001$), lymphatic invasion ($p=0.020$), perineural invasion ($p<0.001$), venous invasion ($p=0.001$), and conventional adenocarcinoma ($p=0.028$).

Survival outcomes. For this analysis (June 2018), the median follow-up period for OS and DFS was 57.9 months (range=6.0 to 97.3 months) and 57.5 (range=4.3 to 87.3 months), respectively. During this period, 13 patients (11.6%) experienced recurrence and 19 (17.0%) died. In the univariate analysis, the DFS rate and OS rate did not differ significantly between the patients with *PIK3CA* mutations and *PIK3CA* wild-type (DFS rate: 71.4% *vs*. 83.3%, $p=0.150$, Figure 1A; OS rate: 75.0% *vs*. 85.7%, $p=0.184$, Figure 1B). The patients were categorized into two groups: those with *PIK3CA* helical domain (exon 9) or kinase domain (exon 20) mutations and those lacking such mutations. The DFS and OS rates for the group with exon 9 or exon 20 mutations were lower than those for the group lacking exon 9 or exon 20 mutations, yet without statistical significance (DFS: 76.67% *vs*. 82.3%, $p=0.063$; OS: 81.3% *vs*. 83.8%, $p=0.087$). In contrast, when analyzing the relationship between survival and exon 9 mutation, the group harboring exon 9 mutations had significantly lower DFS and OS rates when compared with the group lacking exon 9 mutations (DFS: 76.4 *vs*. 82.6, $p=0.013$, Figure 2A; OS: 79.8 *vs*. 83.9, $p=0.023$, Figure 2B). In a Cox proportional hazard model adjusted for pTNM stage, lymphatic invasion, venous invasion, perineural invasion, and stromal TILs, exon 9

Table III. *Clinicopathological characteristics according to phosphatidylinositol 4, 5-biphosphate 3- kinase catalytic subunit alpha isoform (PIK3CA) mutation*.

Variable		*PIK3CA* mutation, n (%)			*PIK3CA* mutation, n (%)		
	Total	Wild-type	Mutant	*p*-Value	Harboring exon 9		*p*-Value
		N=84	N=28		No (N=91)	Yes (N=21)	
Age							
<62 Years	53 (47.3)	41 (48.8)	12 (42.9)	0.665	46 (50.5)	7 (33.3)	0.225
≥62 Years	59 (52.7)	43 (51.2)	16 (57.1)		45 (49.5)	14 (66.7)	
Gender							
Male	91 (81.3)	70 (83.3)	21 (75.0)	0.402	75 (82.4)	16 (76.2)	0.540
Female	21 (18.8)	14 (16.7)	7 (25.0)		16 (17.6)	5 (23.8)	
Lauren classification							
Intestinal	22 (19.6)	19 (22.6)	3 (10.7)		20 (22.0)	2 (9.5)	
Diffuse	79 (70.5)	57 (67.9)	22 (78.6)	0.445	62 (68.1)	17 (81.0)	0.420
Mixed	11 (9.8)	8 (9.5)	3 (10.7)		9 (9.9)	2 (9.5)	
Tumor depth							
T1/2	73 (65.2)	64 (76.2)	9 (32.1)	<0.001	67 (73.6)	6 (28.6)	<0.001
T3/4	39 (34.8)	20 (23.8)	19 (67.9)		24 (26.4)	15 (71.4)	
Lymph node metastasis							
Absent	80 (71.4)	69 (82.1)	11 (39.3)	<0.001	73 (80.2)	7 (33.3)	<0.001
Present	32 (28.6)	15 (17.9)	17 (60.7)		18 (19.8)	14 (66.7)	
Pathological stage							
I & II	93 (83.0)	77 (91.7)	16 (57.1)	<0.001	83 (91.2)	10 (47.6)	<0.001
III & IV	19 (17.0)	7 (8.3)	12 (42.9)		8 (8.8)	11 (52.4)	
Lymphatic invasion							
Absent	74 (66.1)	59 (70.2)	15 (53.6)	0.166	65 (71.4)	9 (42.9)	0.020
Present	38 (33.9)	25 (29.8)	13 (46.4)		26 (28.6)	12 (57.1)	
Perineural invasion							
Absent	72 (64.3)	63 (75.0)	9 (32.1)	<0.001	67 (73.6)	5 (23.8)	<0.001
Present	40 (35.7)	21 (25.0)	19 (67.9)		24 (26.4)	16 (76.2)	
Venous invasion							
Absent	106 (94.6)	83 (98.8)	23 (82.1)	0.004	90 (98.9)	16 (76.2)	0.001
Present	6 (5.4)	1 (1.2)	5 (17.9)		1 (1.1)	5 (23.8)	
TILs, n (%)							
Stromal (cut-off=25%)							
Negative	44 (39.3)	30 (35.7)	14 (50.0)	0.264	34 (37.4)	10 (47.6)	0.460
Positive	68 (60.7)	54 (64.3)	14 (50.0)		57 (62.6)	11 (52.4)	
Intratumoral (cut-off=27.5%)							
Negative	56 (50.0)	40 (47.6)	16 (57.1)	0.513	45 (49.5)	11 (52.4)	>0.99
Positive	56 (50.0)	44 (52.4)	12 (42.9)		46 (50.5)	10 (47.6)	
PD-L1, n (%)							
Stromal*							
Negative	47 (42.0)	35 (41.7)	12 (42.9)	>0.99	39 (42.9)	8 (38.1)	0.808
Positive	65 (58.0)	49 (58.3)	16 (57.1)		52 (57.1)	13 (61.9)	
Intratumoral*							
Negative	57 (50.9)	45 (53.6)	12 (42.9)	0.386	47 (51.6)	10 (47.6)	0.811
Positive	55 (49.1)	39 (46.4)	16 (57.1)		44 (48.4)	11 (52.4)	
WHO classification							
Non-GCLS	57 (50.9)	39 (46.4)	18 (64.3)	0.128	43 (47.3)	14 (66.7)	0.147
GCLS	55 (49.1)	45 (53.6)	10 (35.7)		48 (52.7)	7 (33.3)	
Histological subclassification							
LELC	33 (29.5)	28 (33.3)	5 (17.9)		29 (31.9)	4 (19.0)	
CLR	48 (42.9)	40 (47.6)	8 (28.6)	0.003	42 (46.2)	6 (28.6)	0.028
CA	31 (27.7)	16 (19.0)	15 (53.6)		20 (22.0)	11 (52.4)	

CA: Conventional adenocarcinoma; CLR: Crohn's disease-like lymphoid reaction; GCLS: gastric carcinoma with lymphoid stroma; LELC: lymphoepithelioma-like carcinoma; PD-L1: programmed death ligand-1; TILs: tumour-infiltrating lymphocytes; WHO: World Health Organization. *Positive=2+/3+ intensity in ≥1% area.

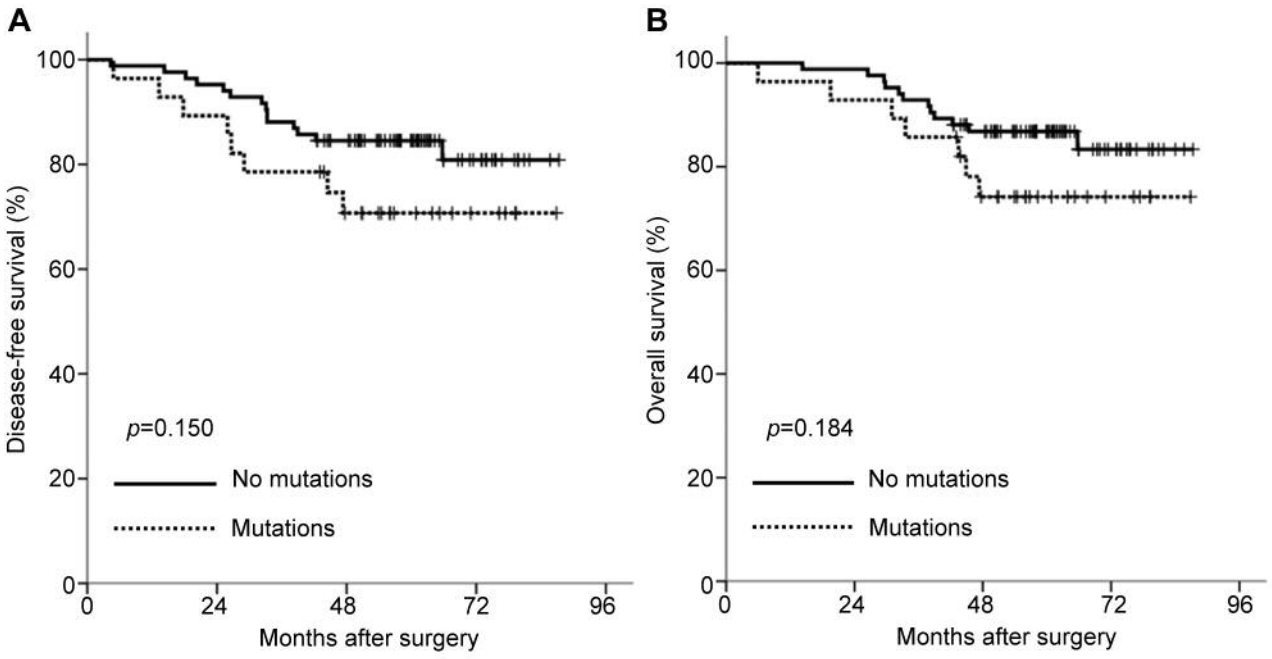

Figure 1. *Kaplan–Meier survival curves for disease-free (A) and overall (B) survival stratified according to phosphatidylinositol 4, 5-biphosphate 3- kinase catalytic subunit alpha isoform (PIK3CA) status.*

PIK3CA mutation failed to show any independent correlation with OS or DFS (OS: p=0.442; DFS: p=0.726), whereas venous invasion (OS: HR=6.464, 95% CI=2.150 to 19.432, p=0.001; DFS: HR=4.404, 95% CI=1.459 to 13.289, p=0.009) and perineural invasion (OS: HR=5.942, 95% CI=1.862 to 18.963, p=0.003; DFS: HR=3.253, 95% CI=1.153 to 9.174, p=0.026) were both highlighted as independent prognostic factors for OS and DFS. Notably, exon 9 E542K mutation of *PIK3CA* was associated with the worst DFS (p=0.011) (Figure 3).

Relationship between PIK3CA mutation and immune-related factors. As shown in Table III, the *PIK3CA* mutation showed no relationship with the immune-related factors, such as TILs, and PD-L1 (all p>0.05). Moreover, among patients with *PIK3CA* mutations, intratumoral TIL positivity and intratumoral PD-L1 positivity showed no association with DFS and OS in the survival analysis.

Discussion

This study evaluated the association of *PIK3CA* mutation with clinicopathological characteristics and their influence on survival in patients with EBV-GC. The results showed that *PIK3CA* mutations were positively associated with T category, N category, stage, perineural invasion, and venous invasion. Consistent with other studies of GC (14, 15, 22, 24, 25), the current data also showed no association between *PIK3CA* mutation and prognosis. However, a novel finding in this study was that patients with exon 9 *PIK3CA* mutation exhibited lower DFS and OS rates than those without exon 9 *PIK3CA* mutation, although no independent correlation was demonstrated.

The recent guideline for GC described the EBV status of a tumor as a potential biomarker for personalized treatment strategies (26). Moreover, EBV-GC has a higher expression of PD-L1 and prevalence of *PIK3CA* mutation compared to EBV-negative GC. Since *PIK3CA* regulates the PIK3-AKT-mTOR pathway, it has been suggested that *PIK3CA* alterations might be a predictor for therapy with mTOR inhibitors and an interesting therapeutic target (27). Several AKT, PI3K, and mTOR inhibitors, such as MK-2206, AZD5363, GDC-0068, PX-866, BYL719, MLN1117, and everolimus, have already been studied in clinical trials, thereby focusing interest on the precise involvement of *PIK3CA* alterations in GC (27). The frequency of *PIK3CA* mutations, as determined by the current real-time PCR for hot-spots, was 25.0% of the patients with EBV-GC, comparable with 32% reported in a recent study of Central European patient cohort (15). The frequency of *PIK3CA* mutations seems to differ according to ethnicity based on a study by Jia *et al.*, who also completely excluded the possibility of differences due to the detection methods (28). Jia *et al.* demonstrated that the rates of somatic mutations for

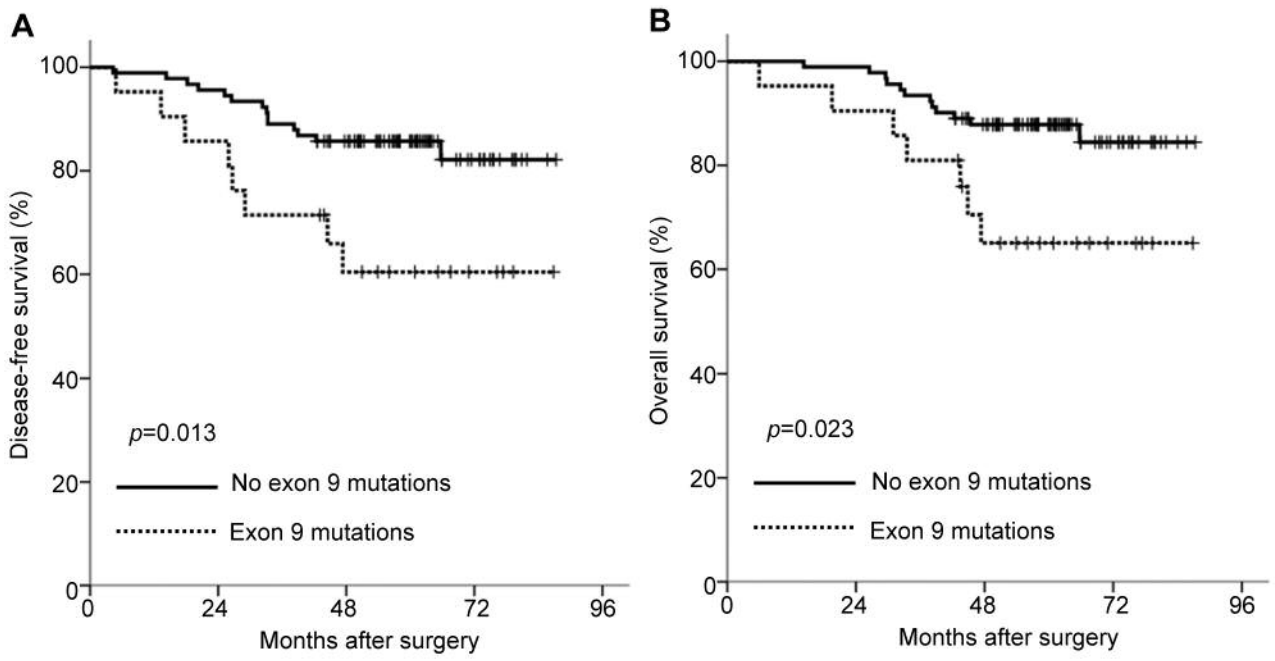

Figure 2. *Kaplan–Meier survival curves for disease-free (A) and overall (B) survival stratified according to presence or not of exon 9 phosphatidylinositol 4, 5-biphosphate 3- kinase catalytic subunit alpha isoform (PIK3CA) mutation.*

five driver genes [adenomatous polyposis coli (*APC*), AT-rich interactive domain-containing protein 1A (*ARID1A*), histone-lysine *N*-methyltransferase 2A (*KMT2A*), *PIK3CA*, and phosphatase and tensin homolog (*PTEN*)] showed significant differences between Asian and Caucasian patients with GC (28). The mean frequencies of these five genes were higher in Caucasians compared to Asian patients with GC. In particular, PIK3CA mutations were detected in only 9.6% of Asian patients with GC but in 18.5% of Caucasian patients with GC. Furthermore, among Asian countries, South Korean patients with GC exhibited the highest frequencies of alteration of *APC, ARID1A, KMT2A, PIK3CA*, and GLI family zinc finger 3 (*GLI3*) in their study. Moreover, Kim *et al*. reported a 12.5-13.2% frequency of *PIK3CA* mutations in South Korean patients with GC when using the same detection method as used in the current study (22).

As previously reported (1), *PIK3CA* mutations are known to be more dispersed in EBV-GC in contrast to localization in exon 20 (kinase domain) in EBV-negative GC. This study also found *PIK3CA* mutations in various exons, such as exon 1, 4, 7, 9, and 20, although the most common mutations were E545X and E542K in exon 9 (21/28, 75%). Accumulating evidence indicates that mutations affecting hot-spots located in exons 9 and 20 have functionally different and independent mechanisms (29, 30). Zhao *et al*. suggested that the gain of function induced by helical domain mutations requires interaction with RAS-GTP, while kinase domain mutations are highly dependent on interaction with p85, despite the absence of RAS-GTP binding (29, 30). In particular, Barbareschi *et al*. reported that *PIK3CA* mutations in exon 9 were the strongest independent prognostic factor for a worse DFS and OS, whereas *PIK3CA* mutations in exon 20 were related to a favorable prognosis (31). For GC, Polom *et al*. showed completely different outcomes for those with *PIK3CA* mutations in exon 9 and exon 20, with the former being more unfavorable (32). They also reported no difference in survival between those with mutant and those with wild-type *PIK3CA* (32). Meanwhile, in 35 patients with GCLS, Hissong *et al*. noted that EBV^+/mismatch repair (MMR)-proficient tumors showed *PIK3CA* mutations affecting the kinase domain (E542K), whereas EBV^-/MMR-deficient tumors displayed helical domain mutations (H1047R and R899C) (33).

As already known, EBV-GC has a tendency to be intimately accompanied by numerous TILs and a dense peritumoral lymphoid response, and exhibit enhanced PD-L1 expression. For GCs, Kim *et al*. showed that *PIK3CA*-mutant tumors were significantly related to increased lymphocytic or neutrophilic infiltration in the cancer stroma (22). These findings may come from the histological characteristics of EBV-GC and GC with high MSI. Actually, *PIK3CA* mutations are frequently detected in EBV-GC and GC with high MSI (1). Regarding the true relationship with the immune system, this study found no association between the

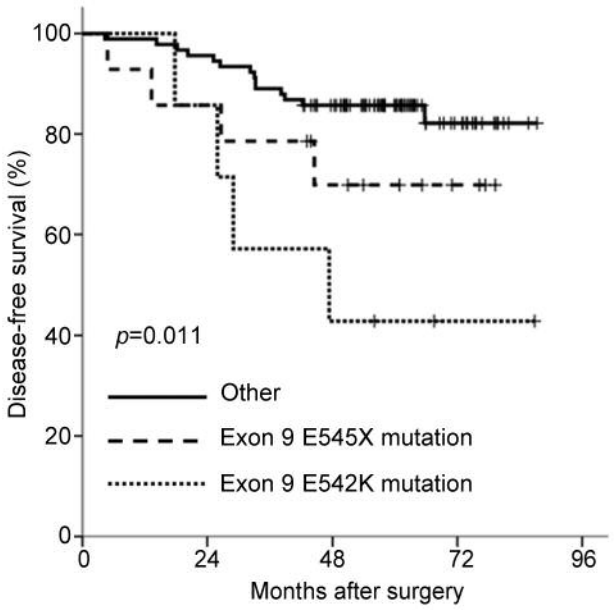

Figure 3. *Kaplan-Meier survival curves for disease-free survival of patients according to the presence of exon 9 E542K mutation of phosphatidylinositol 4, 5-biphosphate 3- kinase catalytic subunit alpha isoform (PIK3CA).*

PIK3CA mutations and TILs or PD-L1 in the patients with EBV-GC. Nonetheless, a comprehensive analysis of these association is still needed to enhance treatment strategies for patients with EBV-GC.

Several limitations of this study should be taken into account, particularly stemming from its retrospective nature. The intratumoral heterogeneity of the *PIK3CA* mutations was not evaluated in different tumor areas to compare the immunohistochemistry and real-time PCR results. Notwithstanding, as far as we are aware, this is the largest published single-institution study of East Asians with EBV-GC and demonstrates its negative prognostic impact. Thus, further large-cohort studies on the clinical significance of *PIK3CA* in Western patients with EBV-GC are necessary to validate the current results.

In summary, *PIK3CA* mutations were found to occur in 25.0% of East Asian patients with EBV-GC, where most of these mutations (75.0%) were detected in exon 9. *PIK3CA* mutations harbored in exon 9 were also strongly related to clinicopathological parameters associated with an unfavorable prognosis. Although exon 9 *PIK3CA* mutation was not identified as an independently robust prognostic factor, they might play an important role in carcinogenesis and tumor aggressiveness in EBV-GC. Consequently, these findings support the theory that exon 9 *PIK3CA* mutation is a prognostic indicator for predicting patient outcome and a rationale for therapeutic targeting in EBV-GC.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

Authors' Contributions

B.W. Kang, J.G. Kim, and A.N. Seo designed the study, revised the drafts, and reviewed the final article. A.N. Seo performed the experiments. A.N. Seo and B.W. Kang contributed to the statistical analysis and wrote the article. H. I. Bae, O. K. Kwon, K. B. Park, S. S. Lee, H. Y. Chung, W. Yu, and S. W. Jeon collected the clinical data. All Authors approved the final version and agreed to be accountable for all aspects of the work.

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