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Research Paper

KRAS G12V Mutation is an Adverse Prognostic Factor of Chinese Gastric Cancer Patients

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Abstract

This study aims to investigate the molecular characteristics of Chinese gastric cancer patients. In our study, the *KRAS*, *BRAF*, and *PIK3CA* mutation status of 485 GC patients were analyzed by Sanger sequencing. Kaplan-Meier analysis was used to plot survival curves according to different genotypes. The results show that the frequency of *KRAS*, *BRAF* and *PIK3CA* mutations were 4.1%, 1.2% and 3.5%, respectively. *BRAF* mutations were significantly concentrated in stage III and IV gastric cancer (P=0.009). *KRAS* G12V mutation carriers have much shorter OS than other mutation carriers and wild-type group patients (P=0.013). In conclusion, only the *KRAS* G12V mutation has an adverse effect on patient survival.

Key words: *KRAS*; *BRAF*; *PIK3CA*; Mutation; Gastric cancer

Introduction

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death in the world (http://globocan.iarc.fr/Default.aspx) [1]. In China, the incidence of GC is much higher than in any other countries and it is the third most common cancer and the leading cause of death [2]. The geographical differences may partly reflect differences in population-specific genetic risk factors and the prevalence of Helicobacter pylori infection, which plays critical roles in GC pathogenesis [3, 4]. Surgery is the primary treatment for patients with early-stage GC. However, GC is often diagnosed at an advanced stage [5]. In most of the world, GC continues to pose a significant challenge for health care professionals.

In the present, management and prognosis of patients with GC are based entirely on the TNM staging system. However, TNM staging information is not enough for individual treatment and potential targeted therapy. Increased understanding of oncogenic mutations and cell signaling pathways led to the successful application of targeted therapies in various cancers.

The RAS/RAF/MEK and phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling pathway are key signals that are activated in the different tumors. These pathways are involved in the diverse cellular process, including cell growth, survival and motility [6-8]. Mutations in *KRAS*, *BRAF* and *PIK3CA* in the above pathways have been detected in various malignancies. Identification of these mutations in tumor has predictive value or prognostic value for clinical application. In colorectal cancer, anti-EGFR drugs should only be provided to patients with

wild-type *KRAS* [9]. *BRAF* mutation has an adverse prognosis in patients with metastatic colorectal cancer [10, 11]. In melanoma with *BRAF* mutation, vemurafenib therapy improves rates of overall and progression-free survival of melanoma patients [12]. Mutated *PIK3CA* in colorectal cancer patients indicated low dose aspirin use improves patient's survival [13, 14]. Several studies have partially analyzed *KRAS*, *BRAF* and *PIK3CA* mutation in GC patients [15-18], but the clinical implications of these mutations in GC patients are not addressed. Further investigation for these genetic alterations in GC is required.

In the present study, we analyzed the molecular characteristics of GC in Chinese patients. We accessed the status of *KRAS*, *BRAF* and *PIK3CA* mutations by using Sanger sequencing, and investigated the clinicopathological characteristics and prognostic role of gene mutations in GC patients.

Materials and Methods

Patients

The study retrospectively analyzed 485 GC patients who underwent surgical resection at the Sixth Affiliated Hospital of Sun Yat-sen University from December 2009 to May 2016. All patients underwent informed a consent process approved by the Institutional Review Board of the hospital. The criteria for patient inclusion were: (1) Aged 18-80 years; (2) Primary lesion was pathologically diagnosed as gastric carcinoma; (3) Clinical information, including follow-up data, was completed. The criteria for exclusion were: (1) With a history of other tumors or hematological malignancy; (2) Accompanied with severe infection, severe kidney dysfunction, or severe hepatic dysfunction; (3) Accepted preoperational chemotherapy, radiotherapy, targeted therapy, and immunotherapy. Formalin-fixed, paraffin-embedded tumor tissues were obtained. Clinical data was collected. The study protocol was approved by the Institute Research Medical Ethics Committee of Sun Yat-sen University. Overall survival (OS) was defined as the time from the beginning of surgical resection to death or last follow-up.

KRAS, *BRAF* and *PIK3CA* mutation analysis

Assessment of *KRAS*, *BRAF* and *PIK3CA* mutation was performed in the Molecular Diagnostic Laboratory of the Sixth Affiliated Hospital of Sun Yat-sen University, using an adequate quality-control procedure. All tissue samples were formalin-fixed paraffin-embedded and histologically confirmed. Genomic DNA from analyzed samples was extracted with Hipure FFPE DNA Kit (Cat No: D3126-02, Magen, China) according to the manufacturer's protocol. Exon 2 (codon 12 and 13) of *KRAS*, exon 9 (codon 542 and 545) and exon 20 (codon 1047) of *PIK3CA*, and exon 15 (codon 600) of *BRAF* were assessed.

The prior PCR amplification was performed on an ABI 9700 PCR system. Amplification was done in 20μL reaction contain 50-100ng of DNA template and 500nM primers, with the following program: 5min at 98°C for initial denaturation followed by 45cyclers of 25sec at 95°C, 25 sec at 58°C and 25 sec at 72°C, and a final extension at 72°C for 10 min. The primers were listed in Table 1. PCR products were purified, sequenced by using BigDye Terminator v3.1 Sequencing Standard Kit (Thermo Fisher Scientific, USA) with an ABI Prism 3500Dx genetic Analyzer (Applied Biosystems, Foster City, CA).

Table 1. Primers and conditions of Sanger sequencing.

Gene	Primers	Length (bp)	Conditions
KRAS			
Exon 2	F: ATGTTCTAATATAGTCACATTTTC	202	
	R: GTCCTGCACCAGTAATATGC		
BRAF			
Exon 15	F: TCATAATGCTTGCTCTGATAGGA	224	98°C 5min, (95°C 25sec, 58°C 25 sec, 72°C 25 sec) 45 cycles, 72°C 10min
	R: GGCCAAAAATTTAATCAGTGGA		
PIK3CA			
Exon 9	F: ATCCAGAGGGGAAAAATATG	194	
	R: TTAGCACTTACCTGTGACTC		
Exon 20	F: CGAAAGACCCTAGCCTTAGAT	215	
	R: GTCTTTGCCTGCTGAGAGTTATT		

Statistical analysis

Associations of *KRAS/BRAF/PIK3CA* mutation status with demographic and clinical characteristics were evaluated using continuous variables, categorical data analysis. Statistical analyses were performed with SPSS software (SPSS, Chicago, IL, USA). Statistical analysis for Kaplan-Meier survival curves for OS was performed using GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA). A two-sided probability value of less than 0.05 was considered to be statistically significant.

Results

Patient characteristics

Table 2 summarizes the clinicopathological characteristics of study subjects. Of these 485 patients, males were over twice females (68.0% vs. 32.0%). A majority of patients (79.1%) were older than 50 at diagnosis. Most patients (65.2%) had stage III or stage IV tumor. Nearly half of the tumors (41.4%) were located in the lower gastric.

KRAS, *BRAF* and *PIK3CA* mutations, and their correlations with patient characteristics

The mutation rate of *KRAS* was 4.1% (20 out of 485). Five different substitutions were detected,

including G13D (n=6), G12S (n=3), G12D (n=5), G12V (n=5) and G12A (n=1). Six *BRAF* V600E was detected, which was *KRAS* wild-type. The mutation rate of *PIK3CA* was 3.5% (17 out of 485). Among 17 patients, 10 carried mutations within exon 9 and 7 carried mutations within exon 20. Mutation types identified in exon 9 included E542K (n=5), E545K (n=4), Q546R (n=1), whereas H1047R accounted for all the mutations in exon 20. One patient was identified with concomitant *PIK3CA* mutation (E545K) and *KRAS* mutation (G13D).

Table 2. Clinicpathological characteristics of 485 GC patients.

Characteristics	Frequency %(n)
Gender	
Male	68.0 (330)
Female	32.0 (155)
Age, years	
<45	12.2 (59)
45-49	8.7 (42)
50-70	59.5 (289)
≥70	19.6 (95)
TNM stage	
I	14.0 (68)
II	20.8 (101)
III	40.0 (194)
IV	25.2 (122)
Tumor location	
Upper	30.3 (147)
Middle	18.8 (91)
Lower	41.4 (201)
Residual gastric or total gastric	9.5 (46)

The associations of patients' clinicopathological characteristics and *KRAS*, *BRAF*, *PIK3CA* gene mutations were summarized in Table 3. *BRAF* mutations were significantly concentrated in stage III and IV gastric cancer (P=0.009). The associations of patients' clinicopathological characteristics and different *KRAS* mutations were summarized in Table 4. *KRAS* G12V mutation was associated with female (P=0.038). *KRAS* G12D mutation was significantly correlated with tumor location (P=0.020) and lymph node status (P=0.045).

Survival analysis

Kaplan–Meier survival analysis was performed to clarify the prognostic effect of these mutations on the GC patients. During the follow-up, 136 patients died. No significant differences were reported between patients with and without any mutation of *KRAS*, *BRAF*, and *PIK3CA* in the survival analysis (The median survival time in mutation group is 55 months, the median survival time of wild-type group does not reach, log-rank P=0.7858, Figure 1A). Individual *KRAS* mutation types were further examined. As shown in Figure 1E, *KRAS* G12V mutation carriers experienced much shorter OS than wild-type group patients (The median survival time in *KRAS* G12V mutation group is 18 months, the median survival time of other mutation group and wild-type group has not reached, log-rank P=0.0131). As shown in Figure 2, there was no significant difference in OS among the patients with different location of the tumor.

Table 3. Associations between gene mutations and clinicpathological characteristics of patients.

	KRAS		*P* -value	*PIK3CA*		*P* -value	*BRAF*		*P* -value
	Mutation	Wild type		Mutation	Wild type		Mutation	Wild type	
	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
Gender									
Male	12 (60.0)	318 (68.4)	0.432	12 (66.7)	318 (68.1)	1.000	3 (50.0)	327 (68.3)	0.341
Female	8 (40.0)	147 (31.6)		6 (33.3)	149 (31.9)		3 (50.0)	152 (31.7)	
Age, years									
<45	2 (10.0)	57 (12.3)	0.933	4 (22.2)	55 (11.8)	0.332	1 (16.7)	58 (12.1)	0.572
45-49	2 (10.0)	40 (8.6)		1 (5.6)	41 (8.8)		0	42 (8.8)	
50-70	13 (65.0)	276 (59.4)		10 (55.6)	279 (59.7)		5 (83.3)	284 (59.3)	
≥70	3 (15.0)	92 (19.7)		3 (16.6)	92 (19.7)		0	95 (19.8)	
TNM stage									
I	1(5.0)	67 (14.4)	0.525	3 (16.7)	65 (13.9)	0.179	0	68 (14.2)	0.009
II	7 (35.0)	94 (20.2)		6 (33.3)	95 (20.3)		0	101 (21.1)	
III	5 (25.0)	189 (40.6)		7 (38.9)	187 (40.0)		1 (16.7)	193 (40.3)	
IV	7 (35.0)	115 (24.8)		2 (11.1)	120 (25.8)		5 (83.3)	117 (24.4)	
Tumor location									
Upper	7 (35.0)	140 (30.1)	0.646	5 (27.8)	142 (30.4)	0.634	3 (50.0)	144 (30.1)	0.937
Middle	6 (30.0)	85 (18.3)		6 (33.3)	85 (18.2)		0	91 (19.0)	
Lower	3 (15.0)	198 (42.6)		6 (33.3)	195 (41.8)		1 (16.7)	200 (41.8)	
Residual gastric or total gastric	4 (20.0)	42 (9.0)		1 (5.6)	45 (9.6)		2 (33.3)	44 (9.1)	
Lymph node status									
pN0	10 (50.0)	138 (29.7)	0.139	22.2(4)	144 (30.8)	0.556	0	148 (30.9)	0.320
pN1	5 (25.0)	132 (28.4)		38.9(7)	130 (27.8)		3 (50.0)	134 (28.0)	
pN2	2 (10.0)	139 (29.9)		16.7(3)	138 (29.6)		2 (33.3)	139 (29.0)	
pN3a/b	3 (15.0)	56 (12.0)		22.2(4)	55 (11.8)		1 (16.7)	58 (12.1)	

Table 4. Associations between different mutations of *KRAS* and clinicpathological characteristics of patients.

	KRAS G12V mutation status		*P*-value	*KRAS* G12S mutation status		*P*-value	*KRAS* G12D mutation status		*P*-value	*KRAS* G12A mutation status		*P*-value	*KRAS* G13D mutation status		*P*-value
	Mutation n (%)	Wild type n (%)		Mutation n (%)	Wild type n (%)		Mutation n (%)	Wild type n (%)		Mutation n (%)	Wild type n (%)		Mutation n (%)	Wild type n (%)	
Gender															
Male	1 (20.0)	329 (68.5)	0.038	3 (100)	327 (67.8)	0.555	3 (60.0)	327 (68.1)	1.000	0	330 (68.2)	0.320	5 (83.3)	325 (67.8)	0.670
Female	4 (80.0)	151 (31.5)		0	155 (32.2)		2 (40.0)	153 (31.9)		1 (100.0)	154 (31.8)		1 (16.7)	154 (32.2)	
Age, years															
<45	1 (20.0)	58 (12.1)	1.000	0	59 (12.2)	0.272	0	59 (12.3)	0.300	0	59 (12.2)	1.000	1 (16.7)	58 (12.1)	1.000
45-49	0	42 (8.8)		1 (33.3)	41 (8.5)		0	42 (8.8)		0	42 (8.7)		1 (16.7)	41 (8.6)	
50-70	3 (60.0)	286 (59.6)		1 (33.3)	288 (59.8)		5(100)	284 (59.2)		1 (100.0)	288 (59.5)		3 (50.0)	286 (59.7)	
>70	1 (20.0)	94 (19.5)		1 (33.3)	94 (19.5)		0	95 (19.7)		0	95 (19.6)		1 (16.7)	94 (19.6)	
TNM stage															
I	1 (20.0)	67 (14.0)	0.928	0	68 (14.1)	0.385	0	68 (14.2)	0.065	0	68 (14.0)	1.000	0	68 (14.2)	0.303
II	1 (20.0)	100 (20.8)		0	101 (21.0)		3 (60.0)	98 (20.4)		0	101 (20.9)		3 (50.0)	98 (20.5)	
III	1 (20.0)	193 (40.2)		1 (33.3)	193 (40.0)		0	194 (40.4)		1 (100.0)	193 (39.9)		2 (33.3)	192 (40.1)	
IV	2 (40.0)	120 (25.0)		2 (66.7)	120 (24.9)		2 (40.0)	120 (25.0)		0	122 (25.2)		1 (16.7)	121 (25.3)	
Tumor location															
Upper	2 (40.0)	145 (30.2)	1.000	1 (33.3)	146 (30.3)	0.322	1 (20.0)	146 (30.4)	0.020	1 (100.0)	146 (30.2)	0.586	2 (33.3)	145 (30.3)	0.557
Middle	1 (20.0)	90 (18.8)		1 (33.3)	90 (18.7)		2 (40.0)	89 (18.5)		0	91 (18.8)		2 (33.3)	89 (18.6)	
Lower	2 (40.0)	199 (41.5)		0	201 (41.7)		0	201 (41.9)		0	201 (41.5)		1 (16.7)	200 (41.8)	
Residual gastric or total gastric	0	46 (9.5)		1 (33.3)	45 (9.3)		2 (40.0)	44 (9.2)		0	46 (9.5)		1 (16.7)	42 (9.4)	
Lymph node status															
pN0	3 (60.0)	145 (30.2)	0.562	0	148 (30.7)	0.144	4 (80.0)	144 (30.0)	0.045	0	148 (30.6)	.122	3 (50.0)	145 (30.3)	0.664
pN1	1 (20.0)	136 (28.3)		2 (66.7)	135 (28.0)		0	137 (28.5)		0	137 (28.3)		2 (33.3)	135 (28.2)	
pN2	1 (20.0)	140 (29.2)		0	141 (29.3)		0	141 (29.4)		1 (100.0)	141 (29.1)		1 (16.7)	140 (29.2)	
pN3a/b	0	59 (12.3)		1 (33.3)	58 (2.0)		1 (20.0)	58 (12.1)		0	58 (12.0)		0	59 (12.3)	

Whereas, *PIK3CA* mutation was associated with a trend towards longer OS in upper (The median survival time in wild-type group is 50 months, the median survival time of *PIK3CA* mutation group and other mutation group have not reached, log-rank *P*=0.5402, Figure 3A), middle (The median survival time in other mutation group is 18 months, median survival time of *PIK3CA* mutation group and wild-type group have not reached, log-rank *P*=0.3722, Figure 3B) and lower (The median survival time in other mutation group is 34 months, median survival time of *PIK3CA* mutation group and wild-type group does not reach, log-rank *P*=0.5889, Figure 3C) gastric tumors.

Discussion

Of these 485 GC patients in the study, males were over twice females. The mutations in *KRAS* exon2 (codons 12, 13), *BRAF* codon 600 and *PIK3CA* exon 9/20 (codons 542, 545 and 1047) were not frequent in Chinese GC patients.

KRAS is a downstream effector of EGFR. Activating mutation of *KRAS* is thought to stimulate the RAS/RAF/MEK/signaling pathway independent of EGFR activation. We found the overall *KRAS* mutation rate was 4.1%, which were close to most of the previous studies in China and Japan (4% - 4.9% in Japan and 4.5% in China) [15, 16, 18]. In patients and animal models, the malign function of *KRAS* G12V mutation has been identified in other tumors, such as colorectal cancer, lung cancer, and pancreatic cancer by multiple techniques [16, 19-25]. As to gastric cancer, we found *KRAS* G12V is a poor prognostic marker, which has not been reported before. Moreover, our cohort is the biggest one in the related reports [15, 26], which can more effectively reflect the southern China GC patients' molecular profile. Also, we found tumor locations were not associated with the overall survival of GC patients or with mutations of *KRAS, BRAF,* and *PIK3CA*. *KRAS* is a key biomarker for predicting response to anti-EGFR therapy in colorectal cancer[11, 27] .Some phase III trials on addition of cetuximab or panitumumab to system chemotherapy reported that in advanced GC or oesophagogastric cancer, the anti-EGFR antibodies provided no additional benefit[28, 29]. Thus, more evidence is needed to elucidate *KRAS* mutations' predictive value to GC.

BRAF is a downstream effector of *KRAS*, its prognostic value for colorectal cancer is wildly accepted [10, 11]. In GC, the frequency of *BRAF* mutation is very low. Large-scale trials are needed to test its clinical value in GC.

PIK3CA, encodes the p110 catalytic submit of PI3K, frequently mutated in some human tumors [30]. Exon 9 and exon 20 are two *PIK3CA* mutational hotspots that affected the helical and catalytic protein domains, respectively. The PI3K/AKT/mTOR pathway has a close association with the RAS/RAF/MEK/signaling pathway, that active RAS can interactive catalytic submit of PI3K and lead its activation in the regulation of cellular functions [31].

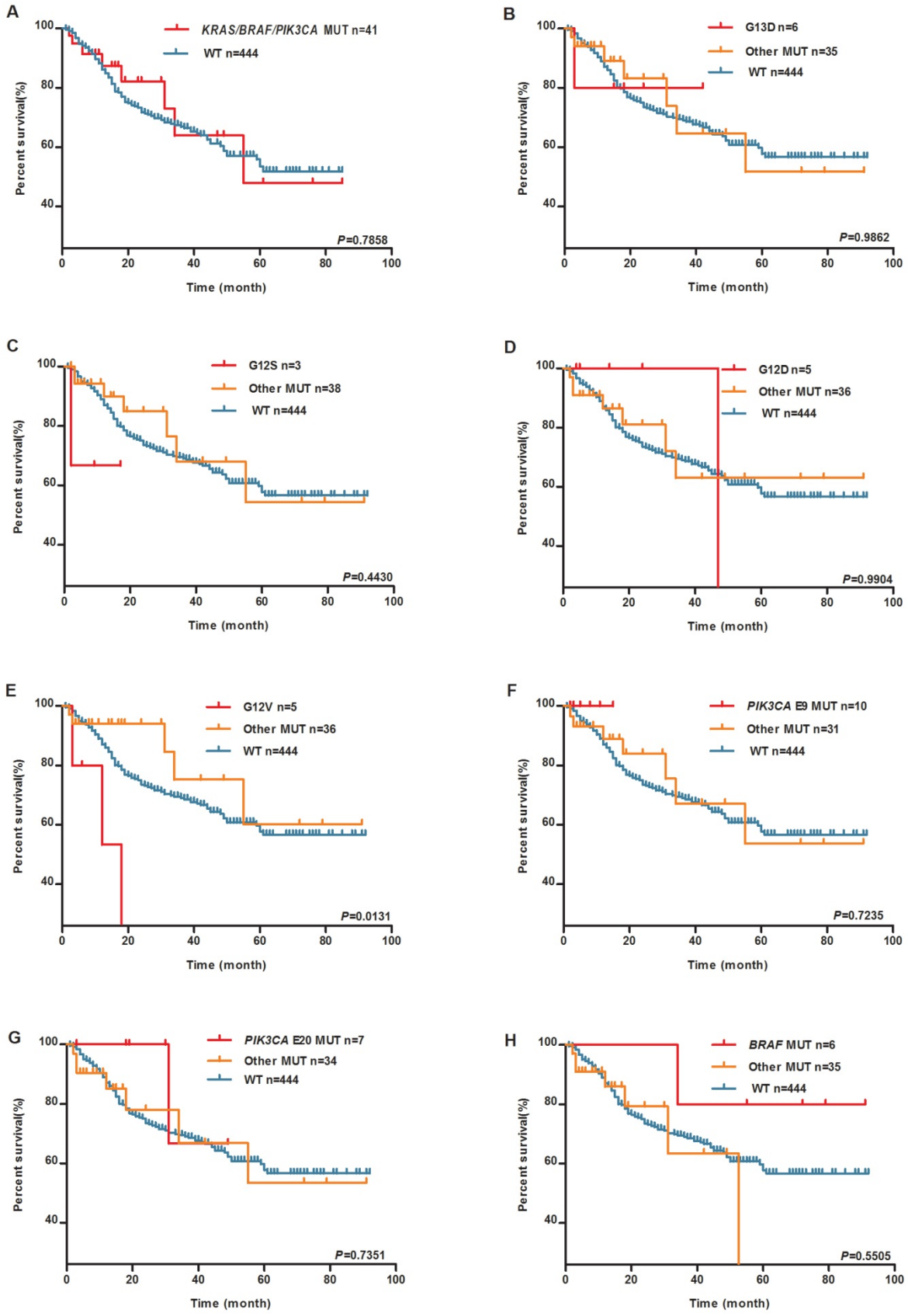

Figure 1. *KRAS* G12V is associated with worse patient survival. Kaplan–Meier plots of overall survival (OS) for GC patients by tumor *KRAS*, *BRAF* and *PIK3CA* mutations. (A) *KRAS*/*BRAF*/*PIK3CA* mutation and WT groups. (B) *KRAS* G13D mutation, (C) *KRAS* G12S mutation, (D) *KRAS* G12D mutation, (E) *KRAS* G12V mutation, (F) *PIK3CA* exon9 mutation, (G) *PIK3CA* exon20 mutation, (H) *BRAF* V600E mutation, other mutation and WT groups.

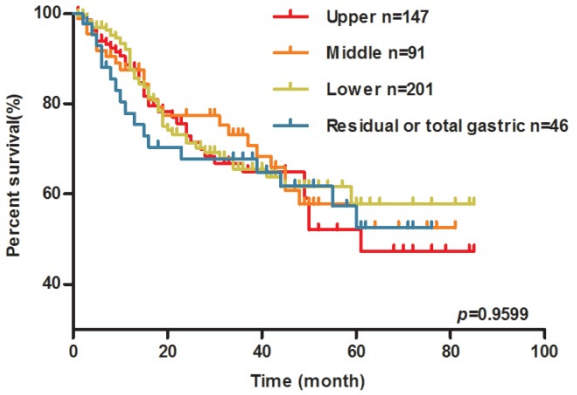

Figure 2. Tumor location has no effect on the OS of GC patients. Kaplan–Meier plots of overall survival (OS) for GC patients by tumor location (upper group/middle group / lower group and residual or total gastric group).

Previous studies indicated that the mutation rate of *PIK3CA* in GC was 3.8% to 12% [15, 16, 32-35]. In our analysis, 3.5% of Chinese GC patients had *PIK3CA* mutations. It has been reported that *PIK3CA* mutation was frequently concomitant with *KRAS* or *BRAF* mutations in a wide variety of tumors especially in colorectal cancer [30, 36-38]. In GC, simultaneous mutations in *PIK3CA* and *KRAS* were observed in rare cases [16, 33]. In our study, only one patient had concomitant *PIK3CA* mutation (E545K) and *KRAS* mutation (G13D). Among the 17 *PIK3CA* mutation carries, only one patient died whose tumor located in the residual gastric. The other *PIK3CA* mutation carriers may have longer OS than wild-type group patients.

To validate our main conclusion, we analyzed clinical and somatic mutations data of the TCGA database on LinkedOmics website (http://www.linkedomics.org). However, the result showed that there is no significant association between *KRAS* G12V mutation and patients' survival (P=0.87, Supplementary Figure 1) [39]. The deviation between our cohort and TCGA cohort may be due to the small sample of *KRAS* G12V mutated patients in the database and the genetic background difference between Asian people and western people. Further validation will be carried out in our center.

As to the tumor stage of diagnosed GC patients, we found only 14% of patients were diagnosed at stage I. The overwhelming majority of patients were diagnosed at advanced stage. Considering 8.7% of the GC patient were aged between 45 years to 49 years, we recommend that individuals of 45 years age and older should be screened for gastric cancer.

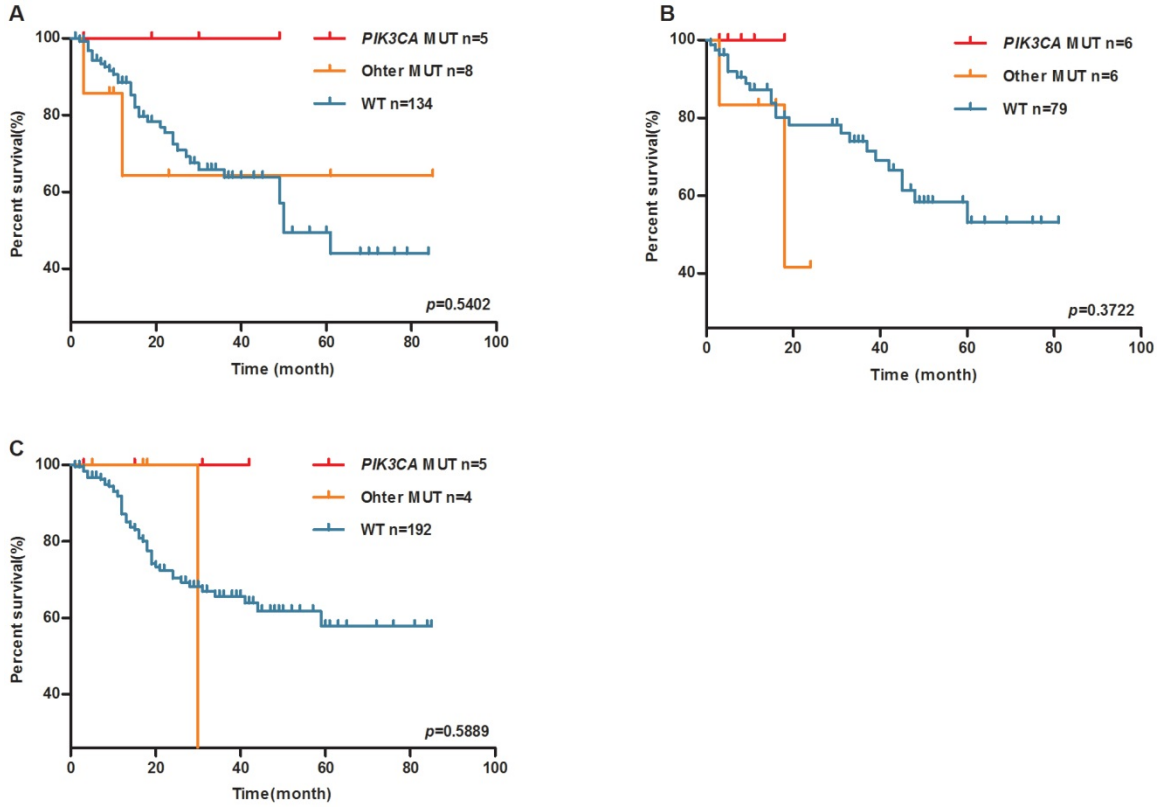

Figure 3. *PIK3CA* mutation may be a favorable prognosis marker of the OS of upper, middle and lower GC patients. Kaplan–Meier plots of overall survival (OS) for GC patients by *PIK3CA* mutation (A) upper GC patients, (B) middle GC patients, (C) lower GC patients.

There are several limitations to the studies. First, the small sample size was the major limitation. We could not adequately evaluate the prognostic impact of each gene mutation on GC patients with this small cohort. Second, the study population was collected from a single center. The genetic variations we observed may mainly reflect the signatures of southern China. Insufficient event outcome was the third limitation. More significant finding needs longer follow-up time in the future.

Overall, we found the frequency of *KRAS*, *BRAF* and *PIK3CA* mutations in GC patients were 4.1%, 1.2%, and 3.5%, respectively. Meanwhile, we found *KRAS* G12V is an adverse prognostic factor for gastric cancer patients.

Supplementary Material

Supplementary figure.
http://www.jcancer.org/v10p0821s1.pdf

Acknowledgments

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Compliance with ethical standards

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Competing Interests

The authors have declared that no competing interest exists.

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