Oncology

Analysis of *PIK3CA* mutations and PI3K pathway proteins in advanced gastric cancer

Short Title: PI3K pathway in advanced gastric cancer

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Abstract

Background: Although surgery and chemotherapy has extended advanced gastric cancer patient survival, some patients still experience relapse and metastasis. We postulated that PI3K pathway proteins could be prognostic biomarkers for the advanced gastric cancer patients.

Methods: A retrospective cohort of 160 advanced gastric cancer patients receiving potentially curative surgery with/without chemotherapy was investigated for *PIK3CA* mutation and PI3K pathway protein level in the context of overall survival (OS) and relapse-free survival (RFS).

Results: Thirteen patients (13/111 11.7%) had *PIK3CA* mutations in codon 545 whereas one patient (1/94 1.1%) had a mutation in *PIK3CA* codon 1047. PI3K pathway protein immunohistochemistry demonstrated that phosphorylated AKT positive (p-AKT (+)) patients in the surgery-only group had a good prognosis in terms of OS and RFS. No significant association between *PIK3CA* mutations and PI3K pathway protein level was seen.

Conclusions: This study revealed that: (i) *PIK3CA* hotspot mutations occurred with low frequency in gastric cancer; (ii) *PIK3CA* hotspot mutations were not directly associated

with PI3K pathway activation; and (iii) p-AKT (+) may be a biomarker for better outcomes for gastric cancer patients undergoing gastrectomy.

Keywords: biomarker; gastric cancer; p-AKT; *PIK3CA*; PI3K pathway

1. Introduction

Recent advancements in multidisciplinary treatments for gastric cancer have substantially prolonged the survival of gastric cancer patients. Surgery and peri- or post-operative therapy continue to be the mainstay treatments for these patients [1-3]. In Japan, gastrectomy with curative intent that includes D2 or more extensive lymph node dissection followed by S-1, an oral fluoropyrimidine (i.e., a precursor of 5-FU), monotherapy has been well-established as the standard therapy for Stage II/III advanced gastric cancer; however, approximately 30-40% of patients who underwent surgery and S-1 chemotherapy relapsed within 5 years of surgery [4]. Therefore, identification of gastric cancer biomarkers by gene expression and immunohistochemistry to predict treatment sensitivity has been of great interest [5, 6].

The phosphatidylinositol 3-kinase (PI3K) pathway that is partially represented by PI3K, AKT, mammalian target of rapamycin (mTOR), and tensin homologue deleted on chromosome ten (PTEN) proteins is essential for cell proliferation, differentiation, and metabolic control. PI3Ks are lipid kinases that phosphorylate PIP_2 (phosphatidylinositol 4, 5-bisphosphate) to generate PIP_3 (phosphatidylinositol 3, 4, 5-triphosphate), which in turn activates AKT and downstream effectors, including mTOR [7, 8]. The tumor suppressor PTEN negatively regulates this process by dephosphorylating PIP_3 [9]. Meanwhile, the PI3K regulatory subunit p85α stabilizes

the kinase activity of the p110α catalytic subunit via a helical domain interaction [10, 11]. The gene encoding the p110α catalytic subunit, *PIK3CA,* is frequently or infrequently mutated in a wide range of human cancers such as those from breast, colon and the stomach [12-15]. Most *PIK3CA* mutations occur in exon 9 and exon 20, which code for the helical and kinase domains, respectively [12]. Within these exons, recurrent (i.e., hotspot) mutations, including E545K and H1047R, have been reported for several tumor types [13-15], and these mutations may induce oncogenic effects [16, 17]. Although limited in *KRAS* wild type tumors, these hotspot mutations are associated with resistance to EGFR-blocking therapies [18] and a poor prognosis in colorectal cancer patients who underwent surgery with curative intent [19].

Here we investigated whether *PIK3CA* hotspot mutations and PI3K pathway protein level are associated with the prognosis of advanced gastric cancer patients who received curative gastrectomy with/without adjuvant S-1 chemotherapy. We aimed to clarify the association between prognosis and: (i) *PIK3CA* hotspot mutations; (ii) PI3K pathway-related protein levels; and (iii) *PIK3CA* hotspot mutations and phospho-PI3K (p-PI3K) levels.

2. Methods

2.1. Patients

This retrospective cohort included 160 patients with Stage IB/II/III gastric cancer (surgery only (Surgery) group, $n = 115$; and surgery + S-1 (S-1) group, $n = 45$) who underwent R0 resection at the Iwate Medical University Hospital before November 2009. Stage IB was used to validate candidate biomarkers and protein analyses. All patients were categorized according to the Union for International Cancer Control (UICC) TNM Classification of Malignant Tumours (7^{th} edition) [20]. The Iwate Medical University Ethics Committee approved this study (approval number H26-142, HGH26-22).

2.2. Direct sequencing of PCR products

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor samples using a WaxFree™ DNA kit (TrimGen, Sparks, MD, USA) after malignant lesion microdissection. PCR amplification was performed for hotspot point mutations at *PIK3CA* codons 545 and 1047. MKN1 (E545K) and HCT116 (H1047R) were used as mutated (MT) controls, and GSS served as a wild type (WT) control. Cell line integrity was confirmed by short tandem repeat (STR) analysis [21]. *PIK3CA* is on chromosome 3,

but shares 98% homology with an approximately 7.0 kbp region on chromosome 22q11.2 [22]. Since codon 545 is located in this highly homologous region, the codon 545 PCR primer was designed to avoid amplification of this pseudogene. Two nucleotides, located 23 and 24 bp downstream from the 3′ end of codon 545, are unique to *PIK3CA* [22, 23]. As such, the codon 545 primer was specific for the 3′ end of the reverse primer to prevent annealing with the homologous sequence. PCR reactions contained 2 x Emeraldamp® MAX PCR Master Mix (Takara Bio Inc., Otsu, Japan) (15 μl), primers (0.6 μl each), and template DNA (25 ng) in 30 μl. Amplification was carried out in a TaKaRa PCR Thermal Cycler Dice™ Version III TP600 (Takara Bio, Inc.) for 40 cycles of 10 seconds at 98 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C. Primer sequences for codon 545 were 5'-GGGAAAATGACAAAGAACAGCTC-3' (sense) and 5'-TCCATTTTAGCACTTACCTGTGAC-3' (antisense), and codon 1047 primers were 5'-CTAGCCTTAGATAAAACTGAGCAAG-3' (sense) and 5'-AGAGTTATTAACAGTGCA GTGTGGA-3' (antisense).

2.3. Allele-specific quantitative real-time PCR

We performed allele-specific quantitative real-time PCR (qPCR) to validate the possible mutations found by direct sequencing [24]. qPCR reactions included 10 μl LightCycler®

480 Probe Master (Roche Applied Science, Penzberg, Germany), 1 µl of 10 x primer and hydrolysis probe solutions, 30 ng template DNA and 3 µl sterile water (20 µl total volume) in a Light Cycler® Nano (Roche Diagnostics, Risch-Rotkreuz, Switzerland) with 10 minutes at 95 °C, and 45 cycles of 10 seconds at 95 °C, 30 seconds at 60 °C, and 15 seconds at 72 °C. Primer sequences were the same as for direct sequencing. Probe sequences for WT (E545E) were HEX-CTCTGAAATCACTGAGCA GG-BHQ and FAM-TCTCTGAAATCACTAAGCAGG-BHQ for MT (E545K). The H1047R primer/probe was purchased from Bio-Rad (Hercules, CA, USA).

2.4. E545K digital PCR

The same set of primers and probes was used for E545K qPCR. Reactions included template genomic DNA (3 µl of 10ng/µl), 10 µl QuantStudio™3D Digital PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µl each of primer and probe. This mixture was applied to a ProFlex™System chip and analyzed with the following program: 10 minutes at 96 °C, 39 cycles of 2 minutes at 60 °C and 30 seconds at 98 °C, and finally 2 minutes at 60 °C. Absolute quantification was conducted using a QuantStudio™ 3D Digital PCR system and analyzed with QuantStudio™ 3D AnalysisSuite™ Cloud Software (Thermo Fisher Scientific) [25].

2.5. Ultra deep sequencing for E545K

To confirm mutations with low allele frequency, a sequencing library was made from 5 samples with an insert size of the PCR product (111 bp) according to the protocol provided by Illumina (San Diego, CA, USA). The libraries were then sequenced using MiSeq2000 with approximately 111 bp paired-reads that represent the PCR product. Mapping and SNP/INDEL analysis was then performed to detect the *PIK3CA* codon 545 mutation [26].

2.6. Immunohistochemistry

Immunohistochemistry was performed on tissue microarrays made from tumor-rich areas taken from samples embedded in paraffin blocks [27]. Primary antibodies were incubated with the indicated dilution ratio: Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199), 1:100; Phospho-AKT (Ser473), 1:75; Phospho-mTOR (Ser2448) (49F9), 1:75; and PTEN (138G6), 1:450 (Cell Signaling Technology, Inc., Tokyo, Japan). Phospho-PI3K and phosphor-AKT antibodies are polyclonal. After antigen retrieval (EDTA buffer pH 9 for 30 min at 95 °C), samples were incubated with primary antibody for 60 minutes at room temperature. Peroxidase-labeled anti-rabbit secondary antibody

(Histofine® Simple Stain MAX PO, Nichirei Biosciences, Inc., Tokyo, Japan) was then applied for 30 minutes at room temperature. Diaminobenzidine (DAB) was used for colorimetric detection. When anti p-AKT antibody was the primary antibody, samples after antigen retrieval were incubated overnight at 4 °C, followed by a 15 min incubation with peroxidase-labeled anti-rabbit secondary antibody at room temperature. Colorimetric detection was performed using the DAKO's catalyzed signal amplification (CSA) II Biotin-free Tyramide Signal Amplification System (Agilent Technologies, Santa Clara, CA, USA). Samples were deemed to have positive staining when more than 5% of cancer cells were stained. Three investigators independently scored the staining.

2.7. Statistical analysis

The distributions of overall survival (OS) and relapse-free survival (RFS) time were estimated using the Kaplan-Meier method. A log-rank test and Cox proportional hazards model was used to compare survival distributions and hazards ratio, respectively, of the two subgroups based on therapeutic or diagnostic parameters. Interaction *P* values were calculated with the Likelihood ratio test. Statistical analysis was done with JMP, version 11 (SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Patient population

Study patient characteristics are listed in Table 1. Patients with stage II/III disease (n = 125) were primarily used for survival analysis. The 5-year overall survival (OS) rate for the S-1 and Surgery groups was 75.0% and 66.2%, respectively (Log-rank test, P = 0.26; HR (Hazards Ratio), 1.47; 95%CI (Confidence Interval), 0.76 to 2.99). The 5-year relapse-free survival (RFS) rate was 68.4% for the S-1 group and 62.5% for the Surgery group (Log-rank test, P = 0.23; HR, 1.48; 95%CI, 0.79 to 2.93) (Fig. 1). We considered these results were comparable to those from the ACTS-GC trial [4]. Stage IB patients were also analyzed to further validate candidate biomarkers.

3.2. *PIK3CA* hotspot mutations

PIK3CA PCR products were obtained from 111 and 94 samples for codon 545 and codon 1047, respectively. With Sanger sequencing, suspected but consistent sequence histograms for codon 545 were obtained for 22 of 111 samples; this result may be due to the low allele frequency of this mutation [28, 29] (Fig. S1). Of those samples with good DNA quality, three were validated by ultra-deep sequencing whose total coverage was

1,418,873 to 1,494,814 bp per amplicon with a mutant allele coverage of 22,613 to 71,026 bp (Table S1). All three samples had the same mutations in codon 545. Because genomic DNA from one sample ran short, the remaining 18 samples were validated for the E545K mutation by digital PCR (dPCR). The dPCR was successful for 16 of 18 samples (88.9%) and we detected the E545K mutation in 10 (62.5%) of these samples (Table S2). Therefore, 13/19 (68.4%) of informative cases with suspected mutation by Sanger sequencing were confirmed by independent methods. Meanwhile, one mutation in codon 1047 was detected by Sanger sequencing and allele-specific qPCR with an estimated allele frequency of 5-10%. Overall, 13 patients (11.7%, 13/111) had E545K mutations, and one patient (1.1%, 1/94) had an H1047R mutation.

3.3 PI3K pathway protein levels and prognosis

Samples used for immunohistochemistry were selected to have sufficient quality for tissue microarray sections from Stage II/III gastric cancer patients who underwent a gastrectomy with curative intent (Fig. 2). Several PI3K pathway proteins, including p-PI3K (p85(Tyr458)/p55(Tyr199)), p-AKT (Ser473), p-mTOR, and PTEN, were examined as potential prognostic markers.

In the S-1 group, the 5-year OS of p-AKT (+) was 74.0% and p-AKT (-) was 69.2%

(Log-rank test, P= 0.85), whereas the 5-year RFS of p-AKT (+) was 61.2% and p-AKT (-) was 69.2% (Log-rank test, P= 0.80) (Fig. 3A, B). In the Surgery group, the 5-year OS of p-AKT (+) (77.4%) was higher than that of p-AKT (-) (51.4%) (Log-rank test, P= 0.03; HR, 2.19; 95%CI 1.06 to 4.77), as was the RFS (p-AKT (+), 75.0%, and p-AKT (-), 54.1%; Log-rank test, P= 0.01; HR, 2.45; 95%CI 1.20 to 5.28) (Fig. 3C, D).

In the S-1 group, both the 5-year OS and RFS of PTEN (+) were higher than that for PTEN (-) (OS: 76.3% vs. 66.7%; Log-rank test, P= 0.54; HR, 1.42; 95%CI 0.42-4.47; and RFS: 67.3% vs. 60.0%; Log-rank test, P= 0.30; HR, 1.76; 95%CI 0.57-5.31) (Fig. 3E, F). Similar differences in the the 5-year OS and RFS of PTEN (+) and PTEN (-) were also seen for the Surgery group (OS: 71.7% vs. 60.5%; Log-rank test, P = 0.16; HR, 1.68; 95%CI 0.81-3.58; and RFS: 71.7% and 55.3%; Log-rank test, P= 0.07; HR, 1.90; 95%CI 0.94 -4.00) (Fig. 3G, H). Both the 5-year OS and RFS of PTEN (+) patients showed similar survival curves up to 2 years post-operation, and separated thereafter. Although statistical differences have not been confirmed, PTEN status may be associated with a latent effect on patient survival.

Levels of other PI3K pathway proteins, including p-mTOR and p-PI3K, showed no differences between the treatment groups (Fig. S2, S3). Patients with stage IB disease without adjuvant chemotherapy also had no difference in prognosis in terms of PI3K

pathway protein levels.

3.4. *PIK3CA* mutations and PI3K phosphorylation

Samples of all stages were compared. The low observed mutant allele frequency could indicate tumor heterogeneity [30] and thus may not be directly associated with the pathological scoring of p-PI3K. Among our 13 samples with the codon 545 mutation, only one was p-PI3K positive. The only sample with the codon 1047 mutation was p-PI3K negative. Hence, *PIK3CA* hotspot mutations likely do not give rise to PI3K phosphorylation in most tumor cells in these gastric cancer cases.

3.5. Subgroup analysis

OS and RFS in eligible patients were analyzed according to age, sex, histologic type, disease stage, PI3K signaling protein level, and *PIK3CA* genotype. In all parameter groups, both p-AKT (+) and PTEN (+) were a better prognostic factor for survival. There was no interaction between protein levels and any of these factors (Fig. 4, Fig. 5).

4. Discussion

Detection of mutations with low allele frequency is a challenging task in sequencing human tumor genomes. For *PIK3CA* hotspots, low allele frequency mutation (less than 10%) in human tumors was previously reported [29, 30]. In terms of anti-cancer drug-resistance acquisition, *PIK3CA* mutations with low allele frequency may be associated with minor subclones [31-33]. Following principles of Darwinian evolution, advantageous phenotypes are selected, and disadvantageous phenotypes are eliminated to promote tumor survival [34]. Cells with *PIK3CA* hotspot mutations could be present as minor clones before antitumor drugs are given, and then become the majority under selection pressure brought by antitumor drugs [35]. As such, these minor clones can play a critical role in resistance to antitumor drugs and acquisition of metastatic potential [36, 37]. We identified *PIK3CA* mutations in surgical specimens with an allele frequency of 1.6 to 4.8%. Although *PIK3CA* mutations did not appear to have a strong effect on prognosis, the significance of minor clones in the context of cancer recurrence in adjuvant chemotherapy should nonetheless be carefully investigated.

We found that p-AKT (+) had diagnostic power for favorable prognosis in the Surgery group but not the S-1 group. The malignant features of p-AKT (-) tumors may be indicated by the favorable outcome of p-AKT (+) tumors from a wide range of origins, including gastric cancer [38-40]. Our results suggest that p-AKT (-) tumors are more

malignant than p-AKT (+) but are rescued by the adjuvant chemotherapy. We also identified PTEN as another prognostic marker for gastric cancer. In the Surgery group, OS/RFS of PTEN (-) was similar to PTEN (+) during the first two post-operative years, but thereafter the OS/RFS of PTEN (-) was lower than PTEN (+). Indeed, our results are consistent with several reports showing that PTEN loss is associated with poor prognosis in pancreatic, prostate, ovarian, and gastric cancers [41-46]. Importantly, our results indicated that the OS/RFS of PTEN (-) might be associated with the relative resistance of S-1 that results in a consistently lower survival rate. The PTEN-dependent mechanism affected by S-1 chemotherapy is unclear, but the distinct pattern of PI3K pathway protein expression in gastric cancer suggests that PI3K inhibitors could be useful for treating 5-FU-resistant gastric cancer.

PIK3CA oncogenic mutation is thought to be a major cause of PI3K activation [47]. However, in contrast to *in vitro* studies, in clinical samples *PIK3CA* mutations appear to make only limited contributions to PI3K activation [48-50]. Of the 14 *PIK3CA* hotspot mutations we identified, only one (7.1%) case was p-PI3K (+) while most (22/23 95.7%) p-PI3K (+) cases had no *PIK3CA* mutations. Although the E545K and H1047R amino acid substitutions may affect the p110α structure to influence phosphoryl group binding [51], our observations indicate that the *PIK3CA* mutations may not be

sufficient indicators of PI3K pathway activation. Therefore, in practice, a comprehensive examination of PI3K pathway activation at a post-translational level would be needed when using PI3K pathway inhibitors. The clinical significance of *PIK3CA* mutations in PI3K pathway activation would require further investigation.

5. Conclusions

The low allele frequency of *PIK3CA* mutations may have a limited effect in Stage II/III gastric cancer. The present results show that the clinical significance of the PI3K pathway in gastric cancer is represented by both p-AKT and PTEN. Further studies will provide insight into the potential utility of PI3K pathway molecules in differential diagnoses and molecular targeting therapies as part of multi-disciplinary gastric cancer treatments.

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Disclosure

All authors declare no conflict of interest.

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Figure Legends

Fig. 1. Kaplan-Meier estimates. (A) OS and (B) RFS for gastric cancer patients with stage II/III disease. S-1, surgery + S-1 group; Surgery, surgery-only group; *P*, P value of Log-rank test; HR, hazards ratio; CI, confidence interval. Vertical dashed line indicates 5 years.

Fig. 2. Immunohistochemistry of PI3K pathway-related proteins.

(A) p-PI3K (-), negative; (B) p-AKT (-), negative; (C) p-mTOR (-), negative; (D) PTEN (-), negative; (E) p-PI3K (+), positive; (F) p-AKT (+), positive; (G) p-mTOR (+), positive; and (H) PTEN (+), positive. Scale bar, 100 μm.

Fig. 3. Kaplan-Meier survival curves stratified by p-AKT and PTEN levels.

(A) OS and (B) RFS of p-AKT (+) (n = 28) and p-AKT (-) (n = 13) in the S-1 group. (C) OS and (D) RFS of p-AKT (+) (n = 40) and p-AKT (-) (n = 37) in the Surgery group. (E) OS and (F) RFS of PTEN (+) (n = 26) and PTEN (-) (n = 15) in the S-1 group. (G) OS and (H) RFS of PTEN (+) (n = 39) and PTEN (-) (n = 38) in the Surgery group. S-1, surgery + S-1 group; Surgery, surgery-only group; P, P value of Log-rank test; HR, hazards ratio; CI, confidence interval. Vertical dashed line indicates 5 years.

Fig. 4. Subgroup analysis based on hazards ratios for OS/RFS and P values for the interaction between p-AKT level and baseline characteristics. HR, hazards ratio; CI, confidence interval; IHC, immunohistochemistry.

Fig. 5. Subgroup analysis based on hazards ratios for OS/RFS and P values for the interaction between PTEN level and baseline characteristics. HR, hazards ratio; CI,

confidence interval; IHC, immunohistochemistry.

Table 1. Patient characteristics

Characteristics	*N*(%)
All patients	160
Age (years), median	68.6
Gender	
Male	113 (70.6)
Female	47 (29.4)
Histologic type	
Differentiated	79 (49.4)
Undifferentiated	81 (50.6)
Stage (TMN7)	
IB	35 (21.9)
IIA	38 (23.8)
IIB	37 (23.1)
IIIA	18 (11.2)
IIIB	19 (11.9)
IIIC	13 (8.1)
Type of lymph node dissection	
D1	43 (27)
D2	112 (70)
D3	5 (3)
Modality	
Surgery (surgery only)	115 (71.9)
S-1 (surgery + S-1)	45 (28.1)
Lesions of relapse	
NA	123 (76.9)
Lymph nodes	7 (4.4)
Hematogenous	15 (9.4)
Peritoneum	13 (8.1)
Local	2 (1.2)

Figure1

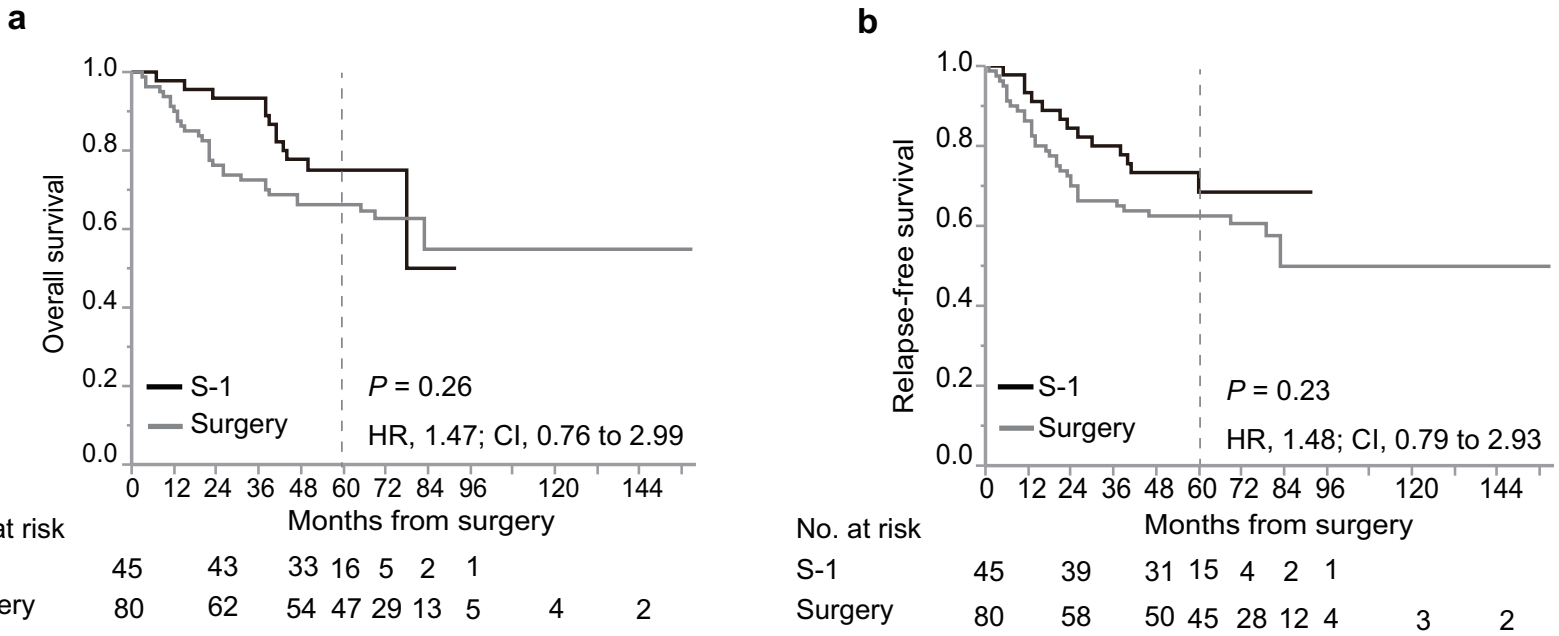

Fig.1.

Figure2

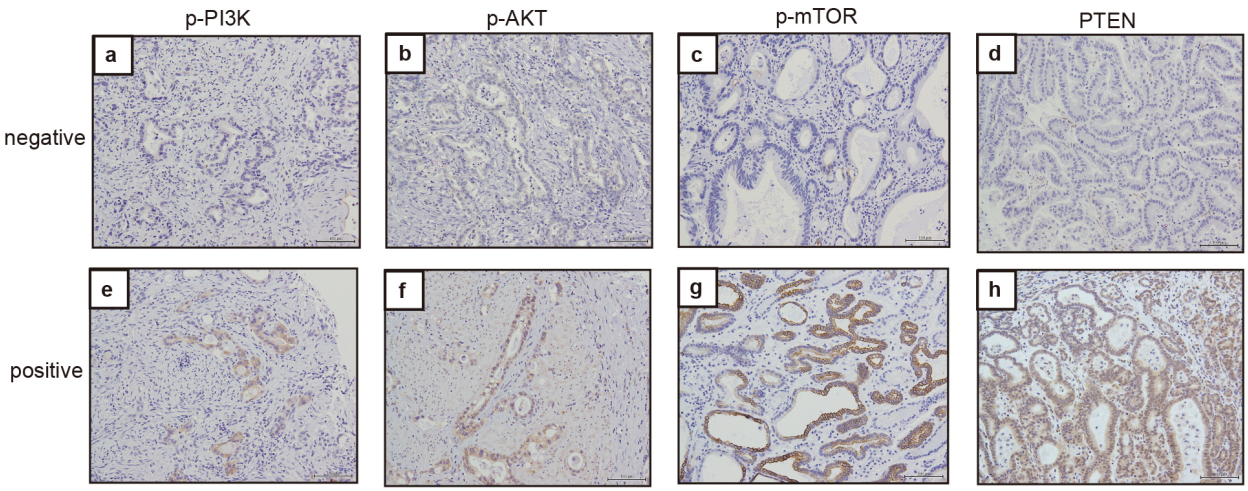

Fig. 2.

Figure3

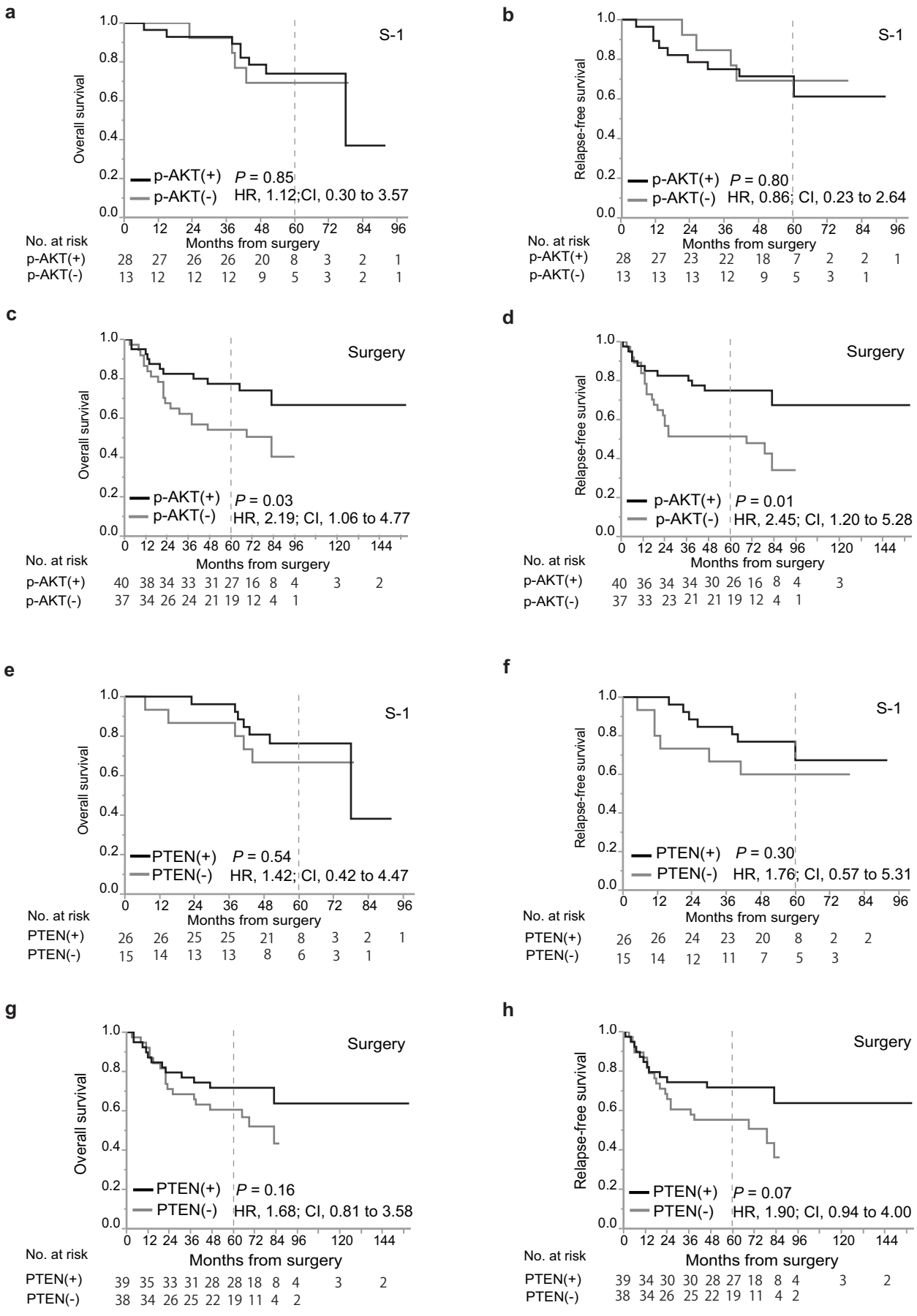

Fig. 3.

Figure4

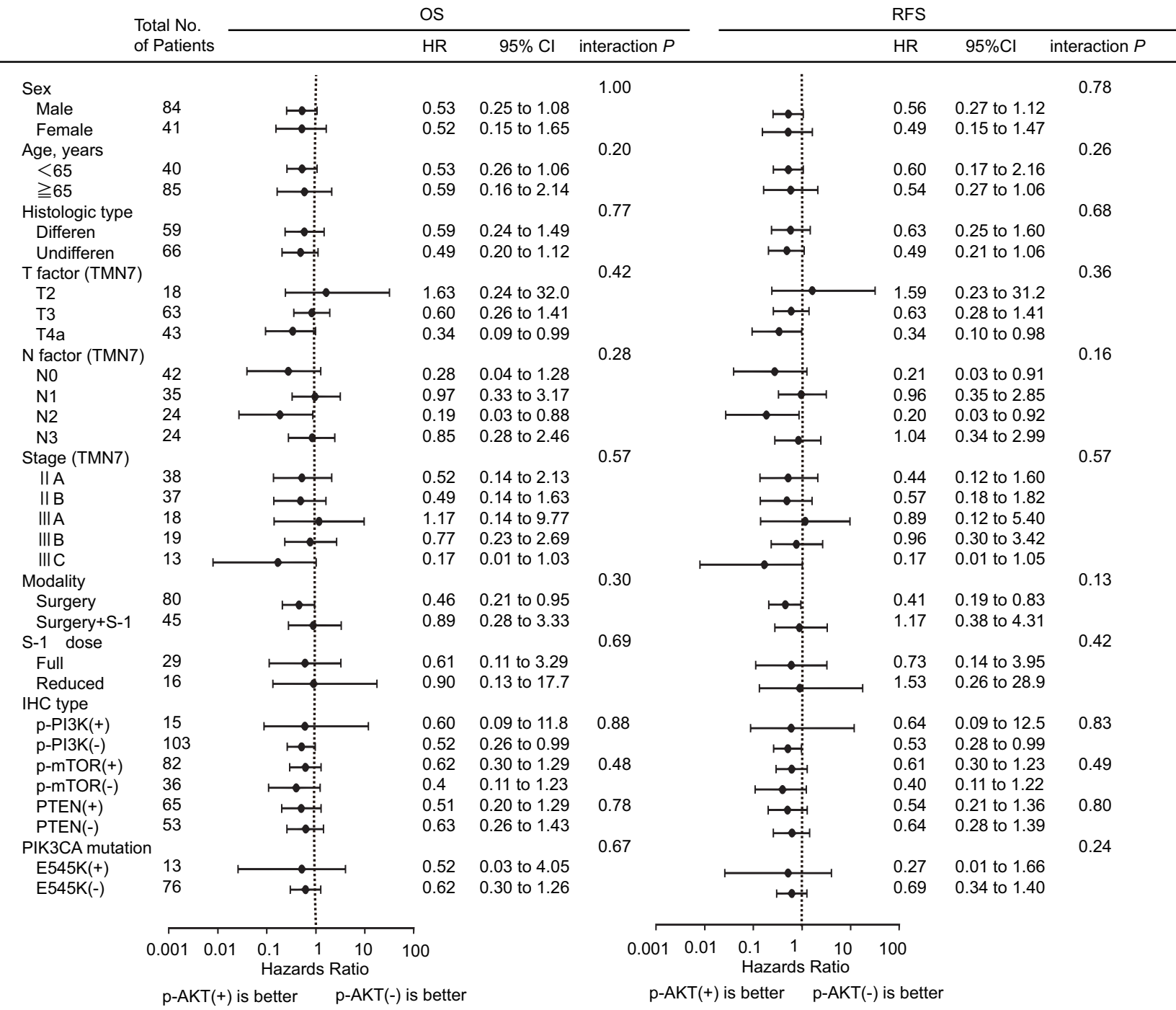

Fig. 4.

Figure5

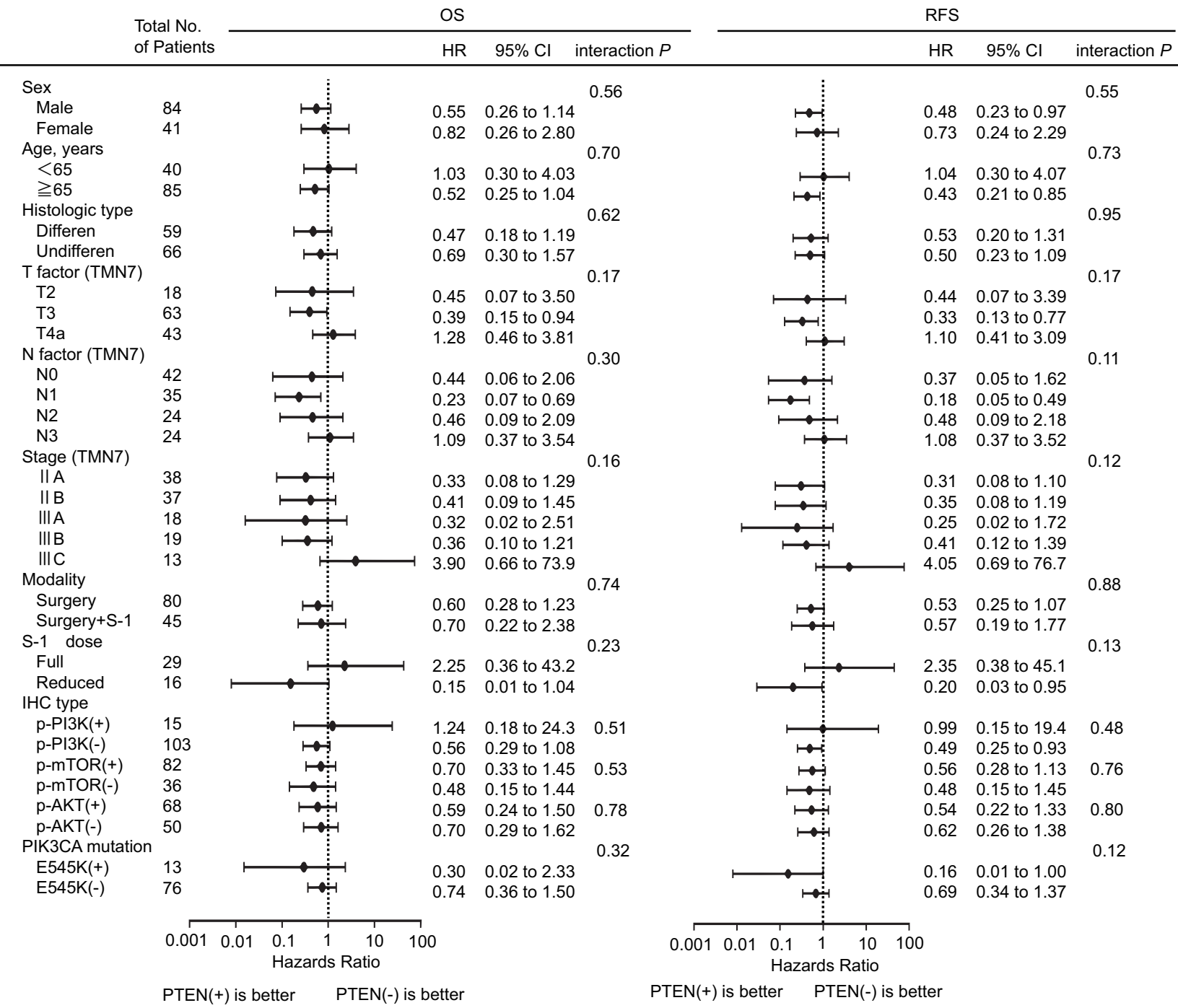

Fig. 5.

SI

Click here to download Supplementary File (video, audio, movie): JSR_SI_sn_10012016.doc

Supplementary material

Table S1. Next generation sequencing of codon 545

	Reference Position	Type	Reference	Allele	Variant Count	Coverage	Frequency (%)
MKN1*	1633	SNV	G	A	1,257,031	1,925,310	65.30
HA20**	1633	SNV	G	A	22,613	1,482,130	1.53
HD7**	1633	SNV	G	A	38,275	1,418,873	2.70
HE2**	1633	SNV	G	A	71,026	1,494,814	4.75

*Cell line for positive control; **Patient tumor samples

Table S2. Validation of E545K status

Assay	Sample ID	Copies/µl (WT)	Copies/µl (MT)	Allele frequency (%)
E545K	HA1	17.29	0.15	0.88
E545K	HA6	3.04	0	0
E545K	HA7	49.00	0	0
E545K	HA8	13.11	1.37	9.49
E545K	HB7	NA	NA	NA
E545K	HB8	6.97	0.16	2.17
E545K	HC3	56.97	0.08	0.14
E545K	HC4	65.62	0.56	0.84
E545K	HD3	5.70	0	0
E545K	HD8	87.55	0.32	0.37
E545K	HE1	81.81	0.23	0.28
E545K	HE3	45.32	0.23	0.51
E545K	HE5	47.19	1.57	3.22
E545K	HF2	NA	NA	NA
E545K	HA12	4.91	0.08	1.61
E545K	HA18	1.30	0	0
E545K	HA25	5.27	0	0
E545K	HB28	14.88	0	0

WT, wild type; MT, mutant type; NA, not applicable.

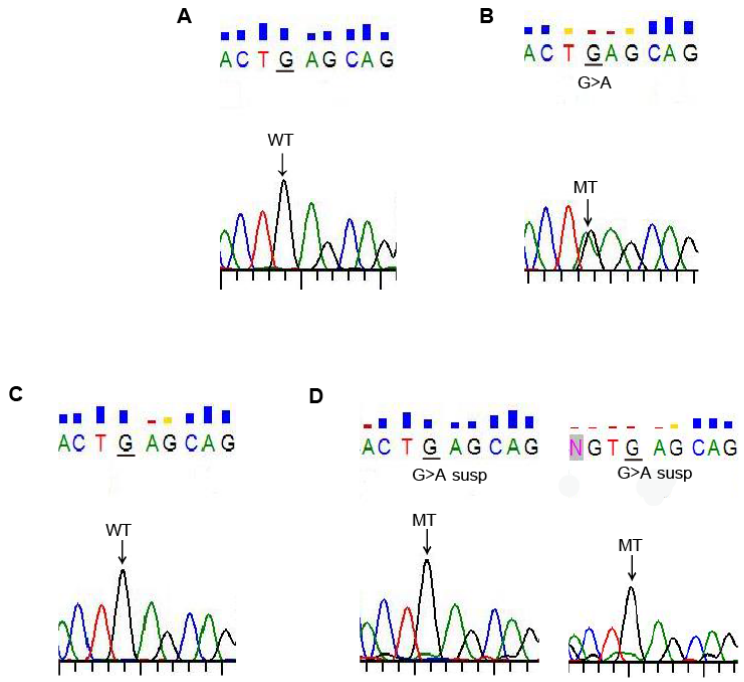

Fig. S1. Direct sequencing histogram of codon 545.

(A) The sense strand of the WT control (GSS) and (B) MT control (MKN1).

(C) A gastric cancer patient sample considered to be WT. (D) Two gastric cancer patient samples suspected to have the MT allele. The arrows indicate c.1633 G>A substitution at codon 545.

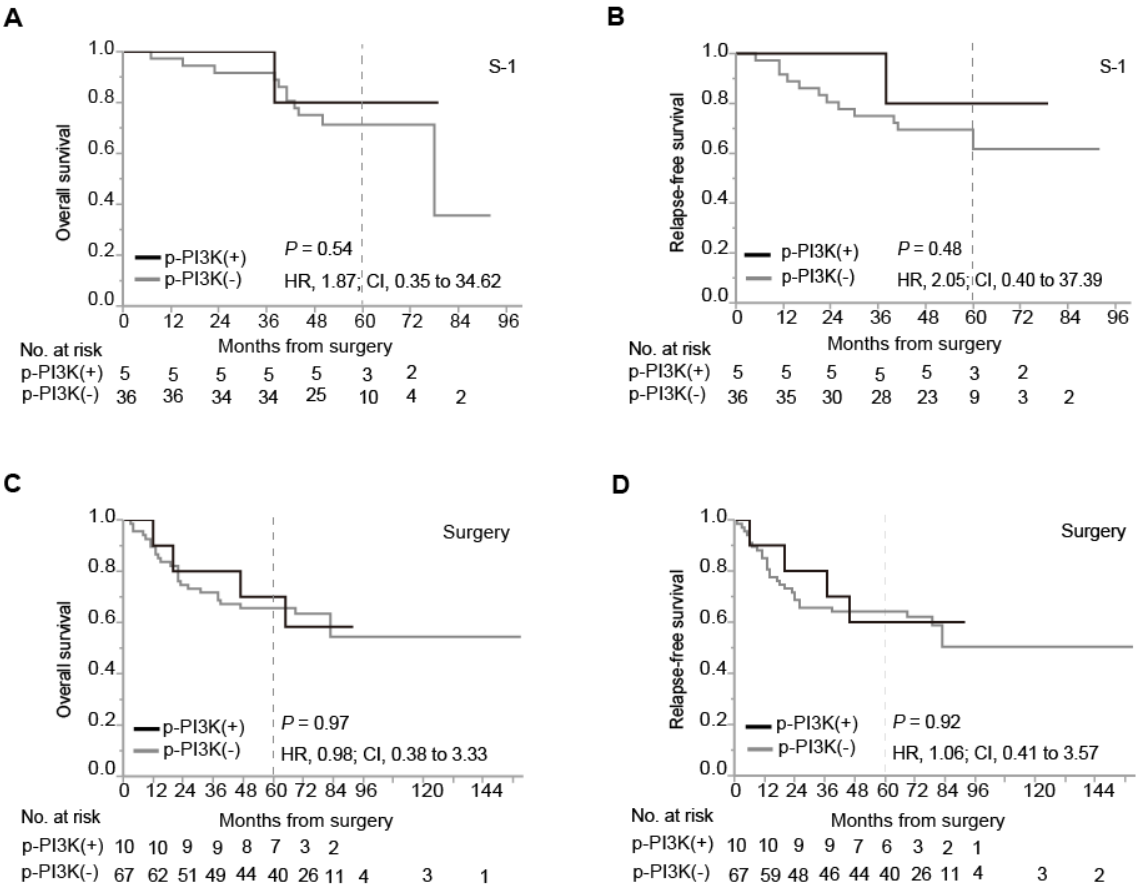

Fig. S2. Kaplan-Meier survival curves stratified by p-PI3K level.

(A) OS and (B) RFS of p-PI3K (+) (n = 5) and p-PI3K (-) (n = 36) in the S-1 group. (C) OS and (D) RFS of p-PI3K (+) (n = 10) and p-PI3K (-) (n = 67) in the Surgery group.

Dashed vertical line indicates 5 years. P, P value of Log-rank test; HR, hazards ratio; and CI, 95% confidence interval.

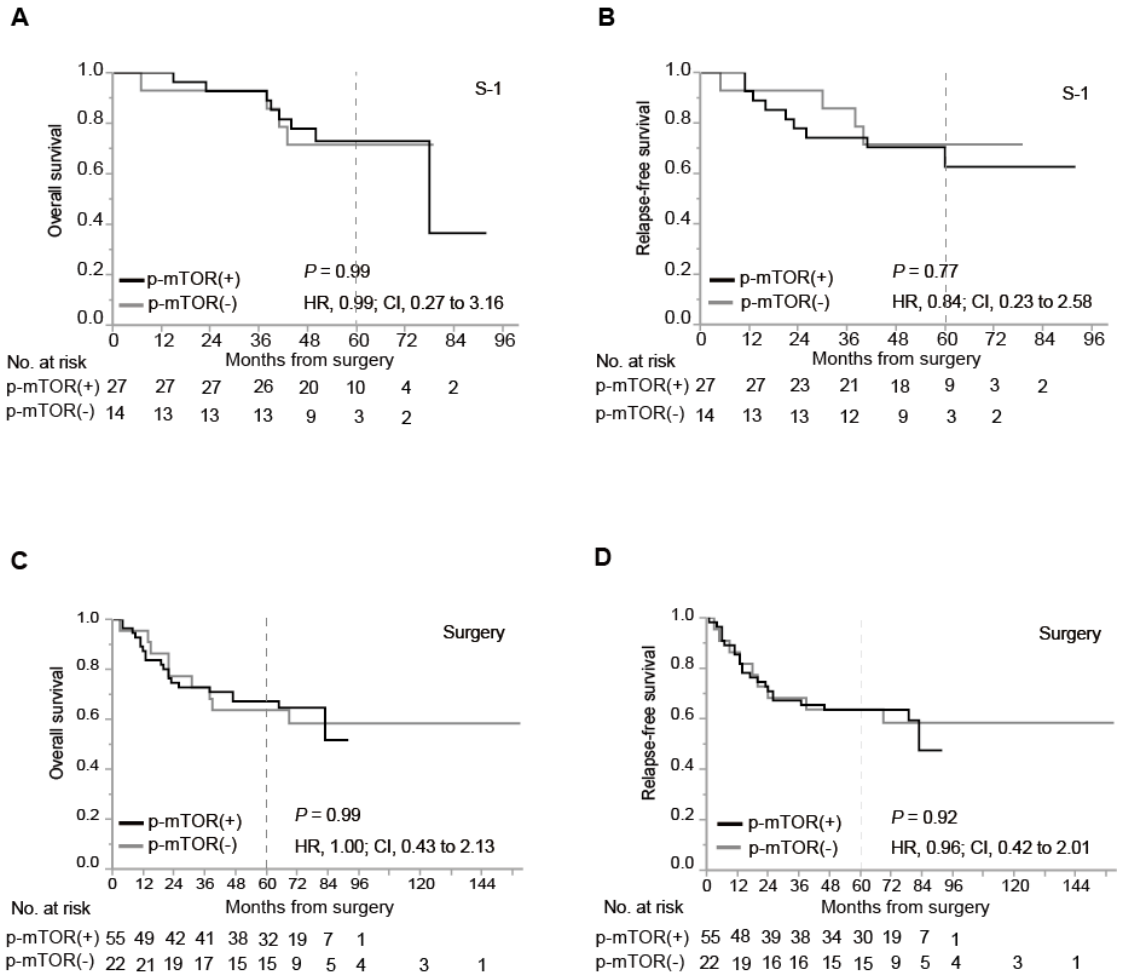

Fig. S3. Kaplan-Meier survival curves stratified by p-mTOR level.

(A) OS and (B) RFS of p-mTOR (+) (n = 27) and p-mTOR (-) (n = 14) in the S-1 group. (C) OS and (D) RFS of p-mTOR (+) (n = 55) and p-mTOR (-) (n = 22) in the Surgery group. Dashed vertical line indicates 5 years. P, P value of Log-rank test; HR, hazards ratio; and CI, 95% confidence interval.