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Increased RhoA Activity Predicts Worse Overall Survival in Patients Undergoing Surgical Resection for Lauren Diffuse-Type Gastric Adenocarcinoma

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Abstract

Background—Several studies have reported a high rate of *RHOA* mutations in the Lauren diffuse-type gastric adenocarcinoma (GA) but not in intestinal-type GA. The aim of this study was to determine if RhoA activity is prognostic for overall survival (OS) in patients with resectable GA.

Methods—Retrospective review was performed on a prospective database of GA patients who underwent potentially curative resection between 2003 and 2012 at a single institution. Tissue microarrays were constructed from surgical specimens and analyzed for phosphorylated RhoA, a marker of inactive RhoA signaling. OS was estimated by the Kaplan-Meier method, and multivariate analysis was performed by Cox proportional hazards regression modeling.

Results—One -hundred thirty-six patients with diffuse-type GA and 129 patients with intestinal-type GA were examined. Compared to intestinal-type GA, diffuse-type GA tumors were significantly associated with increased tumor size and advanced TNM stage. In patients with diffuse-type GA, high RhoA activity was associated with significantly worse OS when compared to low RhoA activity (5-year OS 52.5 % vs. 81.0 %, $p = 0.017$). This difference in OS was not observed in patients with intestinal-type GA (5-year OS 83.9 % vs. 81.6 %, $p = 0.766$). On multivariate analysis of diffuse-type GA patients, high RhoA activity was an independent negative prognostic factor for OS (HR 2.38, 95% CI 1.07–5.28).

Conclusions—Increased RhoA activity is predictive of worse OS in patients with diffuse-type GA who undergo potentially curative surgical resection. Along with findings from genomic studies, these results suggest RhoA may be a novel therapeutic target in diffuse-type GA.

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INTRODUCTION

Gastric cancer is the fifth most common malignancy worldwide with nearly one million new cases every year.¹ Adenocarcinomas arising from the gastric epithelium represent an overwhelming majority (more than 90 %) of gastric cancer cases.² The global distribution of gastric cancer varies across geographical regions, with the highest disease burden occurring in East Asian countries. The disease continues to carry a poor prognosis, accounting for over 700,000 deaths per year.^{1,3} The majority of patients with gastric cancer present with advanced or metastatic disease.⁴ The response rate to multi-agent chemotherapy is 50 % or greater; however, nearly all patients develop chemotherapy resistance, and median survival is extended to only 9–11 months.^{5,6}

In 1965, Lauren described two histological types of gastric adenocarcinomas (GA) – intestinal and diffuse – which have come to define two distinct entities with differing clinical profiles.^{7,8} The intestinal type exhibits components of glandular, solid, or intestinal architecture, with various degrees of differentiation, while the diffuse type consists of poorly cohesive cells that tend to infiltrate the gastric wall.⁹ The intestinal type has a male preponderance, is more commonly diagnosed in older patients, and is associated with environmental exposures such as *Helicobacter pylori* infection.⁷ The diffuse type is generally diagnosed in younger patients, occurs more commonly in female patients, and is associated in rare cases with familial occurrence due to germline mutation in the *CDH1* gene encoding the E-cadherin cell adhesion protein.⁷ A small proportion of GA remains characterized as unclassified or mixed-type tumors.

Surprisingly, a high rate of *RHOA* mutations was identified in diffuse gastric cancer by The Cancer Genome Atlas (TCGA).¹⁰ The TCGA network also found additional fusions in GTPase-activating proteins (GAPs), which regulate RhoA activity. Other studies confirmed recurrent *RHOA* mutations in 14.3–25.3 % of diffuse-type GA.^{10–12} These mutations were generally not found in intestinal-type GA. RhoA is a small GTPase protein that plays a fundamental role in actin-myosin-dependent cell contractility and cellular motility.^{13,14} Inhibition of the RhoA pathway has been explored as a putative therapeutic target in a variety of malignancies, but there is a relative dearth of studies evaluating the role of RhoA in diffuse gastric cancer.^{13,15}

The aim of this study was to determine if tumor RhoA activity is prognostic for survival in patients undergoing potentially curative surgical resection for gastric adenocarcinoma.

METHODS

Patient population

Institutional Review Board approval was obtained from Seoul National University Bundang Hospital (SNUBH; Seongnam, South Korea). A retrospective review was performed of a prospectively maintained database of patients treated for GA between May 2003 and March 2012 at SNUBH. The subjects for this study were identified as patients with intestinal-type or diffuse-type GA who underwent radical gastrectomy or esophagogastrectomy with potentially curative intent for tumors arising in the stomach or the gastroesophageal junction

(GEJ), limited to Siewert type II or type III tumors.¹⁶ Patients with mixed-type GA tumors were excluded.

A cohort of 265 patients met these criteria. Informed consents for the use of tumor tissue in a research capacity had been obtained pre-operatively from all patients. A portion of each patient's tumor was fixed in 10 % buffered formalin for 24 hours and processed into 5µm paraffin-embedded sections. Clinicopathological data were collected from a prospective database for the 265 patients included in the study. The end of the follow-up period was March 5, 2012. The median follow-up for all patients was 38.8 months (range 1–96 months). Pathologic staging was determined using the 2010 7th edition American Joint Committee on Cancer (AJCC) staging system.¹⁷

Tissue microarray (TMA) and immunohistochemistry (IHC)

A representative core biopsy measuring 2 mm in diameter was obtained from each tumor paraffin block and deposited into a recipient TMA block using a precision tissue array instrument (Beecher Instruments, Inc., Sun Prairie, WI).¹⁸ IHC was performed with the VECTASTAIN Elite ABC Kit per the manufacturer's protocol (Vector Laboratories, Burlingame, CA). Anti-phosphorylated(Ser188)-RhoA antibody from Abcam (ab125275) was used. Phosphorylated RhoA (phospho-RhoA) is sequestered in the cytosol in an inactive state and serves as a marker of inactive RhoA signaling.¹⁹ TMA sections were deparaffinized by xylene, rehydrated through alcohol gradient, and subjected to antigen retrieval by citrate buffer (pH 6.0) and microwave heating. Endogenous peroxidase activity was blocked, and the sections were incubated overnight at 4 °C with the primary anti-phospho-RhoA antibody diluted 1:50 in CAS-Block blocking reagent (Invitrogen, Grand Island, NY). The staining was visualized using anti-IgG biotinylated secondary antibody, streptavidin-HRP (Vector Laboratories), and 3,3'-diaminobenzidine. The sections were counterstained by hematoxylin.

Stained cells were visualized with an inverted confocal microscope, and the images were processed in Imaris 7.6 (Bitplane, Concord, MA). Phospho-RhoA scores (0–300) were calculated by multiplying the staining intensity (ranging in integral values from 0 to 3) by the staining extent (0–100 %). Low RhoA activity was defined as above the median level of phospho-RhoA expression. High RhoA activity was defined as below the median level of phospho-RhoA expression.

Cell lines and cell lines studies

AGS is a Lauren intestinal-type gastric adenocarcinoma cell line, and MKN-45 is a Lauren diffuse-type gastric adenocarcinoma cell line. AGS and MKN-45 cells were obtained from the America Type Culture Collection (ATCC). Cell lysates were collected in RIPA buffer (Sigma) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics), and protein concentration was determined by the Bio-Rad Protein Assay. Anti-phosphorylated(Ser188)-RhoA antibody (Abcam ab125275, Cambridge, MA) was used to report phosphorylated RhoA (p-RhoA), and anti-RhoA (67B9) antibody (Cell Signaling #2117, Danvers, MA) was used to report total RhoA expression.

AGS and MKN-45 cell lysates were treated with lambda phosphatase (Santa Cruz Biotechnology sc-200312, Dallas, TX) to abolish immunoreactivity to phospho-dependent antibodies.

Silencing of RhoA was achieved via lentiviral transduction of human RhoA shRNA (sc-29471-V; Santa Cruz Biotechnology). A scramble shRNA control (SC-108080) was also used.

Statistical analysis

Descriptive statistical analysis was performed by IBM SPSS Statistics software, 64-bit version 22.0.0 (IBM Corporation, Armonk, NY). The distribution of tumor- and treatment-specific factors was evaluated using Fisher's exact test or Pearson χ^2 test for categorical variables and Student's *t* test for continuous variables. Kaplan–Meier estimates were used to generate overall survival (OS) plots; the log-rank test was used to compare survival outcomes.²⁰ Cox proportional hazards regression modeling was used in univariate and multivariate survival analyses to examine the effect of RhoA activity while controlling for confounding variables, such as age and gender.²¹ A *p*-value of <0.05 was considered statistically significant.

RESULTS

The study examined 265 patients with adenocarcinoma arising from the stomach or GEJ (Siewert type II or III) who underwent potentially curative surgical resection at a single academic institution between May 2003 and March 2012. The study subjects were comprised of 129 intestinal-type GA tumors and 136 diffuse-type GA tumors.

Table 1 summarizes the the demographic and clinicopathologic characteristics of the study group stratified by Lauren histologic type.. The median age among all patients was 57 years (range, 25–87 years), and 162 patients (61.1 %) were male. Compared to the intestinal-type GA cohort, patients with diffuse-type GA were younger ($p = <0.001$), and a greater proportion of them were female (47.1 % vs. 30.3 %, $p = 0.005$). The majority of intestinal-type GA tumors were located in the antrum (55.0 %) whereas diffuse-type GA tumors were more evenly distributed within the stomach. Diffuse-type GA tumors were larger (median size 5.0 cm vs. 3.5 cm, $p = <0.001$) and associated with more advanced AJCC T status, N status, and TNM stage ($p = <0.001$). A greater proportion of diffuse-type GA tumors had lymphatic invasion ($p = 0.008$), vascular invasion ($p = 0.010$), and perineural invasion ($p = <0.001$).

Immunohistochemical analysis of the tumor samples was performed to determine RhoA activity. No reliable antibody exists to detect activated RhoA in human tissues. An antibody for phosphorylated RhoA (phospho-RhoA) was used. RhoA cycles between a GDP-bound inactive form and a GTP-bound active form (Fig 1a). Protein kinase A (PKA)-mediated phosphorylation at serine-188 inactivates RhoA by preventing the dissociation of RhoA from Rho-GDP-dissociation inhibitor (RhoGDI), a well-known chaperone protein that sequesters RhoA in the cytosol in an inactive state.^{19,22–24} Thus, phospho-RhoA is inversely associated with RhoA activity. Phospho-RhoA staining was predominantly localized to the

cytosol on immunohistochemistry analysis (Fig. 1b). To confirm that our phospho-RhoA antibody was specific for phospho-RhoA, we treated lysates from two gastric cancer cell lines, AGS and MKN-45, with lambda phosphatase and confirmed that this antibody did not detect any phospho-RhoA after phosphatase treatment (Suppl Fig. 1a). We also transduced AGS and MKN-45 cells with RhoA shRNA or control scrambled shRNA and confirmed loss of phospho-RhoA detection following RhoA shRNA treatment (Suppl. Fig. 1b)

Table 2 outlines the demographic and clinical characteristics further stratified by tumor RhoA activity. The proportion of patients with high RhoA activity was greater in diffuse-type tumors than in intestinal-type tumors (54.4 % vs. 45.7 %, $p = 0.158$). When comparing patients who exhibited low vs. high RhoA activity, there was no significant difference in percentage of family history of gastric cancer or the extent of gastric resection performed. Within the intestinal-type GA cohort, a greater proportion of patients with high RhoA activity received laparoscopic operations compared to patients with low RhoA activity (66.1 % vs. 42.9 %, $p = 0.013$). This difference was not seen in the diffuse-type GA cohort (36.5 % vs. 32.3 %, $p = 0.606$). A single patient died within the first 30 post-operative days; a patient in the intestinal-type GA cohort developed gastric perforation leading to septic shock and eventual cardiac arrest on post-operative day 30. This patient was not included in the survival analyses.

The pathologic characteristics stratified by Lauren classification and tumor RhoA activity are also shown in Table 2. For intestinal-type GA tumors, high RhoA activity was significantly associated with earlier AJCC T status ($p = 0.002$) and TNM stage ($p = 0.005$). Conversely, high RhoA activity was associated with more advanced AJCC T status in diffuse-type GA tumors ($p = 0.046$). Within the intestinal-type GA cohort, low RhoA activity was significantly associated with less lymphatic invasion ($p = 0.009$), but this association was not observed in the diffuse-type GA cohort.

In examining survival outcomes in all patients, the estimated 5-year OS rate was 73.9 %. When stratified by level of RhoA activity, i.e., high vs. low, there was a significant difference in OS (Fig. 2a). Five-year survival in high RhoA-activity patients was 63.6 % compared to 81.6 % in low RhoA-activity patients ($p = 0.041$). Neither patient group had reached median survival. When diffuse-type GA was examined separately, patients with high RhoA-activity tumors had significantly worse OS than patients with low RhoA-activity tumors (52.5 % vs. 81.0 %, $p = 0.017$) (Fig 2b). In the intestinal-type GA cohort, this difference in OS was not observed (Fig 2c); 5-year survival was 83.9 % and 81.6 % for the high RhoA-activity patients and the low RhoA-activity patients, respectively ($p = 0.766$).

Univariate and multivariate analyses of clinicopathological factors possibly associated with OS in diffuse-type GA were performed (Table 3). The following factors were significant negative prognostic factors on univariate analysis: tumor size, diffuse/multifocal tumor location, advanced AJCC T & N status, overall TNM stage, lymphatic invasion, vascular invasion, perineural invasion, positive resection margin, and high RhoA activity. Multivariate analysis was performed with the above significant factors, controlling for age and gender as possible confounding variables. High RhoA activity remained an independent negative prognostic factor (HR 2.38, 95% CI 1.07–5.28, $p = 0.033$). Advanced AJCC T status and

positive resection margin also remained significant factors. Kaplan-Meier overall survival curves were also generated for each TNM stage I-IV stratified by RhoA activity level, and high RhoA activity was still significantly associated with worse overall survival (Suppl. Fig. 2).

DISCUSSION

In this study, we examined RhoA activity in patients undergoing surgical resection for GA. High RhoA activity, as measured inversely by expression of phospho-RhoA, predicted worse survival outcomes only in patients with diffuse-type GA. We confirmed the specificity of our phospho-RhoA antibody (Fig. 1b and Suppl. Fig. 1a, b). No significant difference in OS was observed in intestinal-type GA tumor samples. High RhoA activity was also an independent predictor of worse overall survival in patients with diffuse GA.

Contemporary studies of next generation sequencing and comprehensive molecular profiling have demonstrated distinct molecular subtypes within gastric cancer.^{10–12,25–27} The Cancer Genome Atlas (TCGA) Research Network proposed a molecular classification for gastric cancer into four subtypes: Epstein-Barr virus (EBV)-positive, microsatellite instability (MSI), genomically stable (GS), and chromosomal instability (CIN).¹⁰ Notably, the GS subtype contained the vast majority of diffuse-type gastric carcinomas as well as hotspot mutations in *RHOA*.

The consequences of *RHOA* mutation in gastric cancer have not been fully elucidated. There are discordant findings from the genome analyses suggesting *RHOA* mutation could lead to a biologic gain of function or loss of function.^{11,12,28} Kakiuchi et al. performed small interfering RNA (siRNA)-mediated knockdown and rescue experiments which demonstrated that *RHOA* mutations resulted in strong growth-promoting effects in diffuse-type GA.¹² Conversely, Wang et al. demonstrated that most *RHOA* mutations clustered at sequences encoding established functional domains, and functional assays confirmed that these mutations translated to defective RhoA signaling, thereby promoting evasion of anoikis (programmed cell death induced by inadequate or inappropriate cell-matrix interactions).¹¹ Escape from anoikis is a key step in the carcinogenesis of diffuse-type GA since loss of E-cadherin leaves the tumor cell without the cadherin-mediated compensatory mechanism that promotes anchorage-independent growth.²⁹

Biological RhoA overexpression has been identified in a number of other malignancies (including colon, liver, and skin) and has been linked to tumor progression and metastatic potentiation.^{30–33} However, the role of RhoA in gastric cancer tumorigenesis is poorly understood. Pan et al. evaluated mRNA levels of the seven main members of the Rho GTPase family, including RhoA, in 53 gastric carcinoma tumor samples as well as seven gastric cancer cell lines.³⁴ Increased RhoA expression was significantly associated with advanced TNM stage and poorly differentiated histology. Su et al. performed tissue array analysis of 189 gastric carcinoma tumor samples.¹⁵ Expression of RhoA was significantly associated with depth of tumor invasion, and a greater proportion of tumor tissue exhibited RhoA overexpression than normal gastric mucosa.

There are several limitations to this study. First, there are 265 patients in this study but a larger number of patients would have allowed for more definitive associations and conclusions. Second, all the patients were from one institution and these results require validation in other patient cohorts. Third, some patients were excluded and thus selection bias may exist. For example, patients receiving neoadjuvant treatment were excluded because such treatment can affect phospho-RhoA expression. Lastly, there are no correlations made in this study between RhoA activity and response to therapy for patients who developed recurrence and receive treatments in the metastatic setting, but current studies are underway to address this issue.

In summary, the advent of next generation sequencing has expanded our knowledge of gastric carcinoma by identifying subtype-specific genetic alterations with distinct mutational signatures. Recurrent *RHOA* mutations in diffuse-type gastric carcinoma identify the RhoA signaling pathway as a putative therapeutic target. In this study, increased RhoA activity independently predicted worse overall survival in patients who underwent potentially curative surgical resection for diffuse-type gastric cancer. These findings may inform future studies evaluating RhoA as an important molecular biomarker for gastric cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SYNOPSIS

RHOA is frequently mutated in Lauren diffuse-type gastric adenocarcinoma. In patients undergoing surgical resection for this disease, increased RhoA activity within the tumor is an independent predictor of worse overall survival. Thus, the RhoA pathway is a promising therapeutic target.

FIG. 1.

(a) Schematic overview of the RhoA signaling pathway. RhoA and its downstream effector Rho-associated protein kinase (ROCK) have key regulatory roles in cell cycle regulation, actin-myosin-dependent cell contractility, cell motility, and cell proliferation. RhoA cycles between a GDP-bound inactive form and a GTP-bound active form. This process is mediated by guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP). Protein kinase A (PKA) phosphorylation of active Rho-GTP induces a tight association with Rho-GDP-dissociation inhibitor (GDI), thereby sequestering RhoA in the cytosol in an inactive state. (b) Immunohistochemistry analysis of phosphorylated(Ser188)-RhoA (phospho-RhoA) in Lauren intestinal-type and diffuse-type gastric adenocarcinoma tumor samples. Tissue incubated without primary antibody served as a negative control. Representative fields corresponding to variable staining intensities are shown. Phospho-RhoA staining was predominantly localized to the cytosol. Scale bars represent 20 μm.

FIG. 2. Kaplan-Meier overall survival curves stratified by RhoA activity level in **(a)** all patients with gastric adenocarcinoma (GA), **(b)** patients with intestinal-type GA, and **(c)** patients with diffuse-type GA.

Table 1

Demographic and Clinicopathologic Characteristics in Gastric Adenocarcinoma Patients Stratified by Lauren Classification

All tumors (n=265)			
Lauren classification			
	Intestinal (n=129)	Diffuse (n=136)	p value
	n or median (% or range)	n or median (% or range)	
Age (y)	65 (37–87)	57 (25–79)	<0.001
Gender			0.005
Male	90 (69.7)	72 (52.9)	
Female	39 (30.3)	64 (47.1)	
Family history of gastric cancer	14 (10.9)	14 (10.3)	0.681
Unknown	3 (2.3)	1 (0.7)	
Resection			0.033
Distal/subtotal gastrectomy	109 (84.5)	97 (71.3)	
Proximal/esophagogastrectomy	1 (0.8)	1 (0.7)	
Total gastrectomy	19 (14.7)	38 (27.9)	
Laparoscopic operation	69 (53.5)	47 (34.6)	0.001
30-day mortality	1 (0.8)	0 (0)	0.487
Tumor size (cm)	3.5 (0.8–11.0)	5.0 (1.4–20.0)	<0.001
Tumor location			0.008
GE junction/cardia	18 (14.0)	23 (16.9)	
Body/fundus	40 (31.0)	55 (40.5)	
Antrum	71 (55.0)	52 (38.2)	
Diffuse/multifocal	0 (0)	6 (4.4)	
Lymphatic invasion	53 (41.1)	78 (57.4)	0.008
Vascular invasion	8 (6.2)	22 (16.2)	0.010
Perineural invasion	26 (20.2)	68 (50.0)	<0.001
T status			<0.001
T1	76 (58.9)	50 (36.8)	
T2	19 (14.7)	10 (7.4)	
T3	21 (16.3)	24 (17.6)	
T4	13 (10.1)	52 (38.2)	
N status			<0.001
N0	82 (63.6)	53 (39.0)	
N1	17 (13.2)	20 (14.7)	
N2	16 (12.4)	18 (13.2)	
N3	14 (10.8)	45 (33.1)	
TNM stage			<0.001
I	82 (63.6)	51 (37.5)	
II	21 (16.3)	20 (14.7)	
III	24 (18.6)	55 (40.4)	

All tumors (<i>n</i> =265)			
Lauren classification			
	Intestinal (<i>n</i> =129)	Diffuse (<i>n</i> =136)	<i>p</i> value
	<i>n</i> or median (% or range)	<i>n</i> or median (% or range)	
IV	2 (1.5)	10 (7.4)	0.958
Resection margin			
R0	127 (98.4)	134 (98.5)	
R1	2 (1.6)	2 (1.5)	

Significant *p* values in **bold**.

Table 2

Demographic and Clinicopathologic Characteristics in Gastric Adenocarcinoma Patients Stratified by Lauren Classification and RhoA Activity

	Intestinal-type tumors (n=129)			Diffuse-type tumors (n=136)		
	RhoA activity		p value	RhoA activity		p value
	Low (n=70) n or median (% or range)	High (n=59) n or median (% or range)		Low (n=62) n or median (% or range)	High (n=74) n or median (% or range)	
Age (y)	65 (40–87)	64 (37–85)	0.514	67 (29–77)	58 (25–79)	0.238
Gender			0.950			0.685
Male	49 (70.0)	41 (69.5)		34 (54.8)	38 (51.4)	
Female	21 (30.0)	18 (30.5)		28 (45.2)	36 (48.6)	
Family history of gastric cancer	10 (14.3)	4 (6.8)	0.164	8 (12.9)	6 (8.1)	0.374
Unknown	2 (2.9)	1 (1.7)		0 (0)	1 (1.4)	
Resection			0.270			0.561
Distal/subtotal gastrectomy	62 (88.6)	47 (79.7)		46 (74.2)	51 (68.9)	
Proximal/esophagogastrectomy	0 (0)	1 (1.7)		0 (0)	1 (1.4)	
Total gastrectomy	8 (11.4)	11 (18.6)		16 (25.8)	22 (29.7)	
Laparoscopic operation	30 (42.9)	39 (66.1)	0.013	20 (32.3)	27 (36.5)	0.606
30-day mortality	1 (1.4)	0 (0)	1.000	0 (0)	0 (0)	--
Tumor size (cm)	4.0 (0.8–10.0)	3.2 (1.5–11.0)	0.109	5.0 (1.4–20.0)	5.0 (1.6–20.0)	0.480
Tumor location			0.355			0.754
GE junction/cardia	7 (10.0)	11 (18.7)		11 (17.8)	12 (16.2)	
Body/fundus	22 (31.4)	18 (30.5)		22 (35.5)	33 (44.6)	
Antrum	41 (58.6)	30 (50.8)		26 (41.9)	26 (35.1)	
Diffuse/multifocal	0 (0)	0 (0)		3 (4.8)	3 (4.1)	
Lymphatic invasion	36 (51.4)	17 (28.8)	0.009	37 (59.7)	41 (55.4)	0.616
Vascular invasion	6 (8.6)	2 (3.4)	0.288	7 (11.3)	15 (20.3)	0.157
Perineural invasion	15 (21.4)	11 (18.7)	0.695	29 (46.8)	39 (52.7)	0.491
T status			0.002			0.046
T1	31 (44.3)	45 (76.3)		23 (37.1)	27 (36.5)	
T2	16 (22.9)	3 (5.1)		8 (12.9)	2 (2.7)	
T3	14 (20.0)	7 (11.8)		13 (21.0)	11 (14.9)	

	Intestinal-type tumors (n=129)				Diffuse-type tumors (n=136)			
	RhoA activity		p value	n or median (% or range)	RhoA activity		p value	n or median (% or range)
	Low (n=70) n or median (% or range)	High (n=59) n or median (% or range)			Low (n=62) n or median (% or range)	High (n=74) n or median (% or range)		
T4	9 (12.8)	4 (6.8)			18 (29.0)	34 (45.9)		
N status			0.433					0.519
N0	40 (57.2)	42 (71.2)			26 (41.9)	27 (36.5)		
N1	11 (15.7)	6 (10.2)			11 (17.7)	9 (12.2)		
N2	10 (14.3)	6 (10.2)			6 (9.7)	12 (16.2)		
N3	9 (12.8)	5 (8.4)			19 (30.6)	26 (35.1)		
TNM stage			0.005					0.389
I	37 (52.9)	45 (76.3)			24 (38.7)	27 (36.5)		
II	18 (25.7)	3 (5.1)			12 (19.4)	8 (10.8)		
III	13 (18.6)	11 (18.6)			23 (37.1)	32 (43.2)		
IV	2 (2.8)	0 (0)			3 (4.8)	7 (9.5)		
Resection margin			0.903					0.900
R0	69 (98.6)	58 (98.3)			61 (98.4)	73 (98.6)		
R1	1 (1.4)	1 (1.7)			1 (1.6)	1 (1.4)		

Significant *p* values in **bold**.

Table 3

Univariate and Multivariate Analyses of Clinicopathological Factors in Lauren Diffuse-Type Gastric Adenocarcinoma Patients

Characteristic	Hazard ratio (95% CI)	p value	Multivariate analysis	Hazard ratio (95% CI)	p value
Univariate analysis					
Age	1.01 (0.98 – 1.04)	0.402	Age	1.03 (0.99 – 1.06)	0.116
Gender: Male	1.34 (0.66 – 2.70)	0.412	Gender: Male	1.00 (0.44 – 2.27)	0.992
Tumor size	1.20 (1.12 – 1.28)	<0.001	Tumor size	1.11 (1.02 – 1.26)	0.019
Tumor location					
Antrum	Ref				
Proximal/Body	1.00 (0.46 – 2.16)	0.998			
Diffuse/Multifocal	4.07 (1.40–11.85)	0.010	Tumor location: Diffuse/multifocal	0.90 (0.23 – 3.61)	0.884
AJCC T status					
pT1-T2	Ref				
pT3-T4	27.67 (3.77 – 203.01)	0.001	AJCC T status: pT3-T4	8.61 (1.01 – 73.52)	0.049
AJCC N status					
pN0	Ref				
pN1-N3	10.16 (2.42–42.57)	0.002	AJCC N status: pN1-N3	1.58 (0.31 – 7.98)	0.579
Lymphatic invasion	5.87 (2.06 – 16.76)	0.001	Lymphatic invasion	2.59 (0.74 – 9.10)	0.137
Vascular invasion	3.43 (1.65 – 7.16)	0.001	Vascular invasion	0.88 (0.35 – 2.23)	0.788
Perineural invasion	6.41 (2.46 – 16.68)	<0.001	Perineural invasion	1.58 (0.53 – 4.70)	0.414
High RhoA activity	2.47 (1.14 – 5.36)	0.022	High RhoA activity	2.44 (1.08 – 5.53)	0.032

Significant factors in **bold**.