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# Clinicopathological characteristics of *RHOA* mutations in a Central European gastric cancer cohort

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## ABSTRACT

Genomically stable gastric cancers (GCs) are enriched for the diffuse phenotype and hotspot mutations of *RHOA*. Here we aimed to validate the occurrence, phenotype and clinicopathological characteristics of *RHOA* mutant GCs in an independent Central European GC cohort consisting of 415 patients. The *RHOA* genotype (exon 2 and 3) was correlated with various genotypic, phenotypic and clinicopathological patient characteristics. Sixteen (3.9%) tumours had a *RHOA* mutation including four hitherto unreported mutations, that is, p.G17Efs\*24, p.V24F, p.T37A and p.L69R. *RHOA* mutation was more prevalent in women (5.4% vs 2.8%), distal GCs (4.5% vs 2.4%), in poorly differentiated GCs (G3/G4; 4.8% vs 1.1%), T1/T2 tumours (6.2% vs 3.1%) and lacked distant metastases. Nine *RHOA* mutant GCs had a diffuse, four an intestinal, two an unclassified and one a mixed Laurén phenotype. *KRAS* and *RHOA* mutations were mutually exclusive. A single case showed both a *RHOA* and a *PIK3CA* mutation. No significant difference was found in the overall survival between *RHOA* mutant and wildtype GCs. Our study confirms the occurrence and clinicopathological characteristics of *RHOA* hotspot mutations in an independent patient cohort. However, we found no evidence for a prognostic or growth advantageous effect of *RHOA* mutations in GC.

## INTRODUCTION

Gastric cancer (GC) is the second leading cause of cancer-related deaths in men and women.<sup>1 2</sup> Most patients are diagnosed with advanced stage disease with lymph node metastases already present, leading to a poor prognosis.<sup>3 4</sup> Treatment options in patients with advanced GC are limited, but perioperative, adjuvant and palliative chemotherapy improves progression-free and overall survival.<sup>5–7</sup> Evidence is accumulating that patient prognosis and treatment response does not only depend on tumour stage but also on specific genotypic and phenotypic tumour characteristics. The advancements of targeted therapy provide compelling evidence that cancers of the same anatomic origin, for example, lung or colon, show great variability in their response rates to chemotherapies, necessitating a more in-depth phenotypic/genotypic classification before treatment. Recently, whole-genome sequencing and comprehensive molecular profiling of GC found subtype-specific genetic and epigenetic alterations with unique mutational signatures.<sup>8 9</sup> A molecular classification of GC was proposed, which categorises four subtypes: Epstein–Barr virus (EBV)-positive, microsatellite instable (MSI),

chromosomal instable and genomically stable GCs.<sup>8</sup> Genomically stable GCs are enriched for the diffuse phenotype and hotspot mutations of *RHOA*.<sup>9 10</sup> Rho GTPases are small GTP/GDP-binding proteins that are found in all eukaryotes<sup>11</sup> and act as molecular switches by cycling from GTP-bound active to GDP-bound inactive state. Cycling is controlled by guanine nucleotide-exchange factors, the intrinsic GTPase activity, GTPase activating proteins and guanine nucleotide-dissociation inhibitors.<sup>12</sup> Rho GTPases play fundamental roles in cell migration, adhesion, cell survival, cell division, gene expression and vesicle trafficking,<sup>13</sup> and hence, tumour cell biology.<sup>14</sup> Interestingly, Rho/Rho-kinase inhibitors have been explored as putative therapeutic targets in diverse diseases.<sup>15</sup> In this study, we aimed to validate independently the prevalence and clinicopathological characteristics of *RHOA* mutant GCs in a Central European patient cohort.

## MATERIALS AND METHODS

### Study population

From our archive, we retrieved patients who had undergone total or partial gastrectomy for adenocarcinomas of the stomach or esophagogastric junction. The patient characteristics are summarised in table 1. Date of patient death was obtained from the *Epidemiological Cancer Registry* of the state of Schleswig-Holstein, Germany. Follow-up data were retrieved from hospital records and general practitioners.

### Study inclusion and exclusion criteria

Patients were included when (1) histology confirmed an adenocarcinoma and (2) the date of death or survival data were available. Patients were excluded when (1) histology identified a tumour type other than adenocarcinoma, (2) patients had previously undergone a resection of a Billroth-II stomach with cancer in the gastric remnant, (3) date of patient death or survival data had not been recorded and (4) perioperative chemotherapy was administered.

### Histology and TNM classification

Tissue samples had been fixed in formalin and embedded in paraffin (FFPE). Sections were stained with H&E. Tumours were classified according to Laurén.<sup>16</sup> Pathological tumour, node, metastases (pTNM) stage was determined according to the seventh edition of the Union for International Cancer Control UICC guidelines.<sup>17</sup> Tissue micro arrays were generated as described previously.<sup>18</sup>



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**Table 1** Clinicopathological patient characteristics of the gastric cancer cohort and correlation with *RHOA* genotype

Characteristic	Valid (n)	Total	<i>RHOA</i> wildtype (n (%))	<i>RHOA</i> mutation (n (%))	p Value	
Gender	415	Female	166 (40.0)	157 (94.6)	9 (5.4)	0.199*
		Male	249 (60.0)	242 (97.2)	7 (2.8)	
Age	402	≤68 years	198 (49.3)	190 (96.0)	8 (4.0)	1.000*
		>68 years	204 (50.7)	196 (96.1)	8 (3.9)	
Laurén phenotype	415	Intestinal	210 (50.6)	206 (98.1)	4 (1.9)	0.110*
		Diffuse	134 (32.3)	125 (93.3)	9 (6.7)	
		Mixed	27 (6.5)	27 (96.4)	1 (3.6)	
		Unclassified	41 (9.9)	41 (95.3)	2 (4.7)	
Mucin phenotype	365	Intestinal	102 (27.9)	99 (97.1)	3 (2.9)	0.753*
		Gastric	57 (15.6)	55 (96.5)	2 (3.5)	
		Mixed	146 (37.3)	139 (95.2)	7 (4.8)	
		Unclassified	60 (16.4)	59 (98.3)	1 (1.7)	
Localisation	415	Proximal	127 (30.6)	124 (97.6)	3 (2.4)	0.410*
		Distal	288 (69.4)	275 (95.5)	13 (4.5)	
T-category	415	T1a/b	47 (11.3)	44 (93.6)	3 (6.4)	0.086†
		T2	49 (11.8)	46 (93.9)	3 (6.1)	
		T3	169 (40.7)	162 (95.9)	7 (4.1)	
		T4a/b	150 (36.1)	147 (98.0)	3 (2.0)	
T-category (grouped)	415	T1/T2	96 (22.6)	90 (93.8)	6 (6.2)	0.221*
		T3/T4	319 (77.4)	309 (96.9)	10 (3.1)	
N-category	413	N0	116 (28.1)	111 (95.7)	5 (4.3)	0.786†
		N1	58 (14.0)	56 (96.6)	2 (3.4)	
		N2	69 (16.7)	66 (95.7)	3 (4.3)	
		N3/a/b	170 (41.2)	164 (96.5)	6 (3.5)	
L-category	403	L0	186 (46.2)	178 (95.7)	8 (4.3)	0.802*
		L1	217 (53.8)	209 (96.3)	8 (3.7)	
M-category	415	M0	343 (82.7)	327 (95.3)	16 (4.7)	0.086*
		M1	72 (17.3)	72 (100)	0 (0)	
V-category	402	V0	353 (87.8)	339 (96.0)	14 (4.0)	1.000*
		V1	49 (12.2)	47 (95.9)	2 (4.1)	
Stage (7th edn)	407	IA	35 (8.6)	33 (94.3)	2 (5.7)	0.127†
		IB	29 (7.1)	27 (93.1)	2 (6.9)	
		IIA	52 (12.8)	51 (98.1)	1 (1.9)	
		IIB	43 (10.6)	41 (95.3)	2 (4.7)	
		IIIA	46 (11.3)	42 (91.3)	4 (8.7)	
		IIIB	70 (17.2)	67 (95.7)	3 (4.3)	
		IIIC	60 (14.7)	58 (96.7)	2 (3.3)	
		IV	72 (17.7)	72 (100)	0 (0)	
LNR (median=0.214)	407	≤0.214	204 (50.1)	197 (96.6)	7 (3.4)	0.622*
		>0.214	203 (49.9)	194 (95.6)	9 (4.4)	
Tumour grade	405	G1/G2	91 (22.5)	90 (98.9)	1 (1.1)	0.136*
		G3/G4	314 (77.5)	299 (95.2)	15 (4.8)	
Resection margin	394	R0	346 (87.8)	335 (96.8)	11 (3.2)	0.664*
		R1/R2	48 (12.2)	46 (95.8)	2 (4.2)	
E-Cadherin‡	382	Negative	280 (73.3)	268 (95.7)	12 (4.3)	0.768*
		Positive	102 (26.7)	99 (97.1)	3 (2.9)	
β-Catenin‡	384	Negative	213 (55.5)	202 (94.8)	11 (5.2)	0.191*
		Positive	171 (44.5)	167 (97.7)	4 (2.3)	
Lysozyme‡	384	Negative	182 (47.4)	179 (98.4)	3 (1.6)	0.035*
		Positive	202 (52.6)	190 (94.1)	12 (5.9)	
Survival (months)	402	Events (Dead)	318 (79.1)	305 (95.9)	13 (4.1)	0.457§
		Alive	84 (20.9)	81 (96.4)	3 (3.6)	
		Median survival		14.6±1.1	11.6±2.4	
		95% CI		12.4 to 16.8	6.9 to 16.4	

\*Fisher's exact test.

†Kendall's tau test.

‡Dichotomized at the median.

§Log-rank test.

### DNA sequence analysis

Genomic DNA was extracted from FFPE tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Tissue sections were manually microdissected prior to DNA isolation to enrich tumour cells (>80%). For mutational analysis of exon 2 and 3 of *RHOA*, a 282 and 215 bp fragment were amplified by PCR using the

primers *5'-caggaaacagctatgacAGCTCTAATTCTCTACATGCTCC-3'* (sense) and *5'-gtaaaacagcggcagtcCTATGACTTCTTGTCATTGC-3'* (antisense) for exon 2 and the primers *5'-gtaaaacagcggcagtcACTAGCTACACAGGCAGTGACAA-3'* (sense) and *5'-caggaaacagctatgacGTGGGGGGATTAAACC TTGCA-3'* (antisense) for exon 3 (universal *M13 sequencing primer* binding sites were added to the 5'-end of the PCR primers). PCR products were purified using the QIAquick 96

PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by dye terminator cycle sequencing (BigDye Terminator v1.1 Cycle Sequencing kit, Applied Biosystems, Darmstadt, Germany) with universal M13 primers. The sequencing products were purified using the DyeEx 96 Kit (Qiagen) and analysed on a Genetic Analyzer 3500 (Applied Biosystems).

### Assessment of phenotype, genotype and infectious status

The *KRAS*- (codon 12 and 13), *PIK3CA*- (exon 9 and 20) genotype, the mucine-, E-cadherin- and  $\beta$ -catenin-immunophenotype, as well as the *Helicobacter pylori*, *Epstein–Barr virus*, microsatellite and Her2/neu status were assessed as described in detail previously (see online supplementary materials and methods).<sup>18 19</sup>

### Statistical methods

Statistical analyses were performed using SPSS V20.0 (IBM Corporation). For continuous variables, cases were divided into two groups by splitting at the median value. Median overall survival was determined using the Kaplan–Meier method, and the log-rank test was used to determine significance. For comparison purposes, the median survival time, its SD and 95% CI were calculated. The significance of correlation between clinicopathological variables was tested using Fisher's exact test. For variables of ordinal scale (T-category, N-category, tumour stage), we applied Kendall's tau test instead. A  $p \leq 0.05$  was considered statistically significant. The  $p$  values are given unadjusted.

## RESULTS

### GC cohort

A total of 415 patients fulfilled all study criteria, including 249 (60.0%) men and 166 women (40.0%; [table 1](#)). The median patient age was 68 years. According to Laurén, an intestinal-type GC was found in 210 (50.6%), a diffuse type in 134 (32.3%), a mixed type in 27 (6.5%) and an unclassifiable type in 41 (9.9%) patients. According to the mucin-phenotype, 102 (27.9%) GCs were of the intestinal, 57 (15.6%) of the gastric, 146 (37.3%) of the mixed and 60 (16.4%) of the unclassified type ([table 1](#)). In total, 202 (52.6%) GCs were categorised as lysozyme-positive, and 280 (73.3%) as E-cadherin-negative and 213 (55.5%) as  $\beta$ -catenin-negative. Non-neoplastic mucosa was available from 351 patients and was screened for *H. pylori*. Fifty-three (15.1%) patients had a persistent infection with *H. pylori*. EBV-RNA was found in 15 (3.7% of 402 valid results) GCs.

### Prevalence of *RHOA* mutation

Sixteen (3.9%) tumours had an *RHOA* mutation in exon 2 (12 (75%); p.R5Q, p.G17E, p.G17Efs\*24, p.L22R, p.V24F, p.T37A and p.Y42C) or 3 (4 (25%); p.L57V and p.L69R) ([table 2](#)). Fourteen tumours had a single-point mutation and two harboured a deletion/insertion mutation. A transition (A>G or G>A) was found in nine cases, which was restricted to exon 2. A transversion (G>T or T>G) was present in five cases being more prevalent in exon 3 ([table 2](#)).

### Correlation of *RHOA* mutation with clinicopathological patient characteristics

*RHOA* mutation was more prevalent in women (5.4% vs 2.8%), distal GCs (4.5% vs 2.4%) and in poorly differentiated GCs (G3/G4; 4.8% vs 1.1%). Interestingly, *RHOA* mutant GCs usually showed a lower T-category. However, neither of these findings was statistically significant ([table 1](#)).

According to Laurén, nine (56%) *RHOA* mutant GCs had a diffuse, four (25%) an intestinal, two (13%) an unclassified and one (6%) a mixed phenotype ([figure 1](#)). *RHOA* mutant GCs

were most commonly lysozyme-immunopositive (80%), and E-cadherin-immunonegative (80%) and  $\beta$ -catenin- (73%) immunonegative. *KRAS* and *RHOA* mutations were mutually exclusive. A single case showed both a *RHOA* and a *PIK3CA* mutation. MSI and EBV were found each in a single case with *RHOA* mutation.

No significant difference was found in the overall survival between *RHOA* mutant and wildtype GCs ([figure 2](#)).

## DISCUSSION

Hitherto, oncological treatment of malignant epithelial tumours largely depended on their anatomical origin. With the advancements of targeted therapy, it became increasingly evident that cancers of the same anatomical origin show great variability in their response rates to chemotherapies, necessitating a more in-depth phenotypic/genotypic classification prior to treatment. While this has led to major improvements in few cancer types, it is still in its infancies in GC. Except for *HER2* status, no other molecular (ie, diagnostic, prognostic or predictive) classifier has reached clinical practice despite evidence that response to chemotherapy may depend on genotypical/phenotypical characteristics of GC: using gene expression profiling, Tan *et al*<sup>20</sup> identified intrinsic subtypes of GC (intestinal vs diffuse type) that respond differently to 5-fluouracil, cisplatin and oxaliplatin. Recently, whole-genome sequencing and comprehensive molecular profiling proposed four molecular subtypes of GC,<sup>8</sup> of which the genomically stable subtype was specifically enriched for the diffuse phenotype and hotspot mutations of *RHOA*.<sup>9 10</sup>

Here we aimed to validate the occurrence, phenotype and clinicopathological characteristics of *RHOA* mutant GCs in an independent Central European GC cohort. Sixteen (3.9%) tumours of our cohort had a *RHOA* mutation. The prevalences reported hitherto ranged from 6%<sup>8 10</sup> to 25.3%<sup>9</sup> and largely depend on the composition of the cohorts, being higher in cohorts with a greater number of diffuse-type GCs. As yet, our study comprises the only single-centre GC cohort tested for *RHOA* mutation and the largest GC patient series: *The Cancer Genome Atlas Research Network*<sup>8</sup> studied 295 patients, Wang *et al*<sup>10</sup> 100 patients and Kakiuchi *et al*<sup>9</sup> 87 patients. Five mutations of our cohort had been described previously, that is, p.R5Q, p.G17E, p.L22R, p.Y42C, p.L57 and p.L69R.<sup>8–10</sup> However, we also found mutations hitherto unreported in GC including a deletion-insertion mutation in exon 2, that is, p.G17Efs\*24, p.V24F and p.T37A ([table 2](#)). The mutations affect the functional domains of *RHOA*, such as the GTP-binding sites and the effector binding region and may have different effects.<sup>8–10</sup> p.Tyr42Cys and p.Gly17Glu were considered as *gain-of-function* mutations, which may provide strong growth advantage in diffuse-type GC progression.<sup>9</sup> To the contrary, Wang *et al*<sup>10</sup> provided evidence that p.Tyr42Cys and p.Leu57Val lead to defective signalling, promoting escape from anoikis. In order to support any of these contentions, we correlated the presence of *RHOA* mutations with various clinicopathological patient characteristics. In accordance with previous observations, *RHOA* mutation was more prevalent in women, distal GCs and in poorly differentiated GCs.<sup>10</sup> Interestingly, *RHOA* mutant GCs usually showed a lower T-category and no distant metastases, seemingly contradicting a growth advantageous effect. However, neither of these findings was significant ([table 1](#)) and larger patient cohorts may be needed to demonstrate a putative effect on local tumour growth or tumour spread.

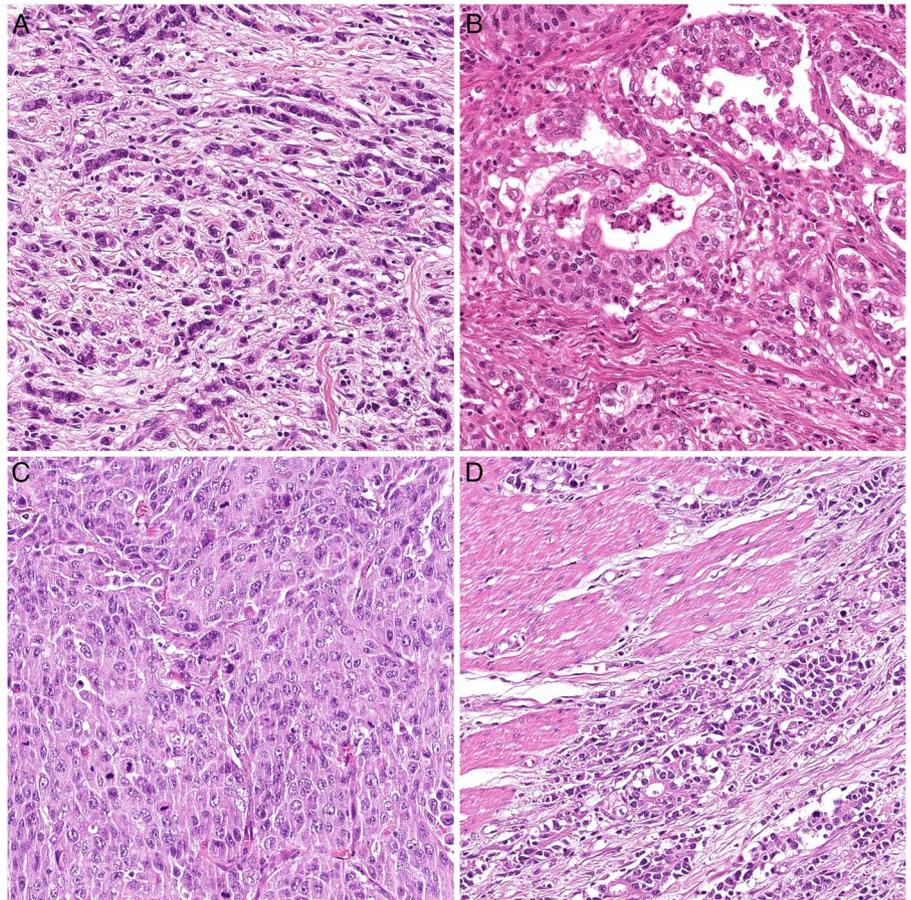
Previously, Tan *et al*<sup>20</sup> described 171 genes, which separate intestinal-type from diffuse-type GC. Lysozyme was among the genes highly significantly differentially expressed and was

**Table 2** Clinicopathological characteristics and genotype of the *RHOA* mutant gastric cancers

Case number	Gender	Age at diagnosis	Tumour localisation	Laurén phenotype	T-category	N-category	M-category	UICC stage	Tumour grade	<i>KRAS</i> codon 12 and 13	<i>PIK3CA</i> exon 9	<i>PIK3CA</i> exon 20	MSI status	EBV status	<i>Helicobacter pylori</i> status	<i>RHOA</i> mutation	<i>RHOA</i> exon	<i>RHOA</i> substitution mutation	Functional region—interaction with
1	F	44	Distal	Diffuse	T1b	N0	M0	IA	G2	WT	WT	WT	MSS	–	mv	c.125A>G, p.Y42C	2	Transition	Effector domain
2	F	38	Distal	Diffuse	T3	N1	M0	IIIA	G3	WT	WT	WT	MSS	–	–	c.169T>G, p.L57V	3	Transversion	PKN/PRK1
3	F	66	Distal	Diffuse	T1a	N0	M0	IA	G3	WT	WT	WT	MSS	–	–	c.169T>G, p.L57V	3	Transversion	PKN/PRK1
4	F	80	Distal	Diffuse	T4a	N2	M0	IIIB	G3	WT	WT	WT	MSS	–	–	c.65_66delinsGT, p.L22R	2	Deletion/Insertion	–
5	F	68	Distal	Diffuse	T4a	N3a	M0	IIIC	G3	WT	WT	WT	MSS	–	mv	c.206T>G, p.L69R#	3	Transversion	–
6	F	69	Proximal	Diffuse	T2	N0	M0	IB	G3	WT	WT	WT	MSS	–	–	c.125A>G, p.Y42C	2	Transition	Effector domain
7	M	90	Proximal	Diffuse	T3	N3	M0	IIIB	G3	WT	WT	WT	MSS	–	–	c.50G>A, p.G17E	2	Transition	GTP
8	M	68	Proximal	Diffuse	T3	N2	M0	IIIA	G3	WT	WT	WT	MSS	–	–	c.14G>A, p.R5Q	2	Transition	–
9	M	62	Distal	Diffuse	T3	N2	M0	IIIA	G3	WT	WT	WT	MSS	–	mv	c.109A>G, p.T37A#	2	Transition	Effector domain
10	F	72	Distal	Intestinal	T3	N3a	M0	IIIB	G3	WT	WT	WT	MSS	–	–	c.125A>G, p.Y42C	2	Transition	Effector domain
11	M	84	Distal	Intestinal	T2	N0	M0	IB	G3	WT	WT	WT	MSS	–	–	c.125A>G, p.Y42C	2	Transition	Effector domain
12	M	85	Distal	Intestinal	T2	N3a	M0	IIIA	G3	WT	WT	WT	MSS	–	–	c.70G>T, p.V24F	2	Transversion	–
13	M	66	Distal	Intestinal	T3	N0	M0	IIA	G3	WT	WT	WT	MSI	–	+	c.50delinsAA, p.G17Efs*24#	2	Deletion/Insertion	GTP
14	F	80	Distal	Unclassified	T3	N1	M0	IIB	G3	WT	WT	WT	MSS	–	–	c.169T>G, p.L57V	3	Transversion	PKN/PRK1
15	F	58	Distal	Unclassified	T1b	N3b	M0	IIB	G3	WT	WT	WT	MSS	–	–	c.125A>G, p.Y42C	2	Transition	Effector domain
16	M	59	Distal	Mixed	T4b	N3a	M0	IIIC	G3	WT	MUT	WT	MSS	+	+	c.14G>A, p.R5Q	2	Transition	–

#, hitherto unreported mutations; –, negative; +, positive; EBV, Epstein–Barr virus; GTP, guanosine triphosphate; MSI, microsatellite instable; MSS, microsatellite stable; mv, missing value; PKN/PRK1, protein kinase N1; UICC, Union for International Cancer Control; WT, wildtype.

**Figure 1** Histomorphology of gastric cancers with *RHOA* mutation. *RHOA* mutant gastric cancers were most commonly of diffuse type according to Laurén (A). Intestinal (B), unclassified (C) and mixed (D) phenotype were found in a minority of the cases. H&E; original magnification 200-fold.

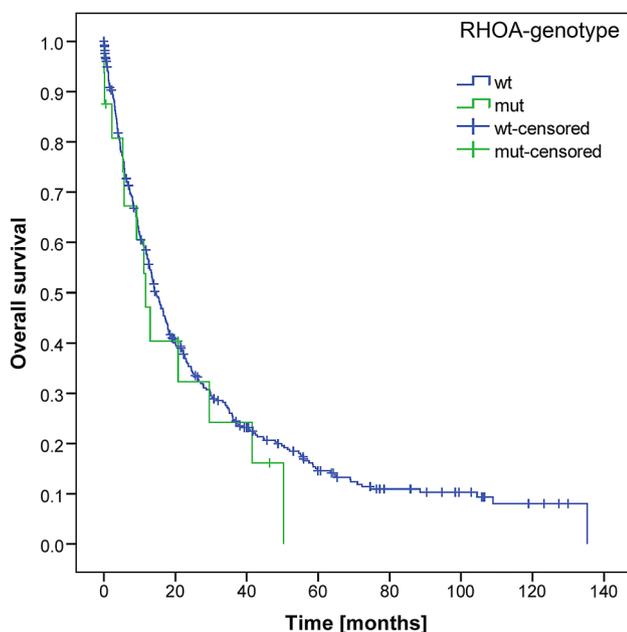


selected by us as another putative immunohistochemical marker between intestinal-type and diffuse-type GC.<sup>20</sup> While lysozyme expression correlated significantly with *RHOA* genotype, immunostaining, in general, was unsuitable to detect *RHOA* mutant GCs as only a small proportion of E-cadherin-immunonegative

$\beta$ -catenin-immunonegative and lysozyme-immunopositive GCs harboured *RHOA* mutations (<6%; table 1).

Rho GTPases have been associated with the alterations of *P53*, *KRAS* and *APC*, as well as *KRAS*/*PIK3CA*-signalling in colon cancer, lung cancer and liver carcinogenesis.<sup>14 21–23</sup> Therefore, we were interested to compare the *RHOA* genotype with other genetic alterations found previously in our cohort<sup>18</sup>: *KRAS* and *RHOA* mutations were mutually exclusive, lending support to the hypothesis that the *KRAS*–*RHOA* axis may be a putative signalling axis in GC as it has recently been shown in non-small cell lung cancer.<sup>22</sup> A single case showed both an *RHOA* and a *PIK3CA* mutation. However, the overall prevalences of *KRAS*, *PIK3CA* and *RHOA* mutations are low in our GC cohort (each below 5%) and no firm conclusions can be drawn.

The whole-genome sequencing and comprehensive molecular profiling classified EBV-positive and MSI GCs as distinct molecular subtypes. Interestingly, MSI and EBV were found each in a single case with *RHOA* mutation. Similar findings were made by others: a minority of *RHOA*-mutant GCs can be MSI or EBV-positive.<sup>8–10</sup> Thus, molecular subtypes may overlap in a single patient. However, the vast majority of *RHOA* mutant GCs was microsatellite stable (93%) and EBV-negative (94%), confirming previous findings.<sup>8–10</sup> No significant difference was found in the overall survival between *RHOA* mutant and wild-type GCs (figure 2), again raising doubt about a significant effect on tumour progression. In this respect, it is interesting to note that 17 patients of our cohort, commonly showing an intestinal-type GC, carried a *KRAS* mutation.<sup>18</sup> Thus, *RHOA* and *KRAS* mutation are not only mutually exclusive but are linked also to different phenotypes. In summary, our study



**Figure 2** Patients' survival. Kaplan–Meier curves depicting patients' survival according to *RHOA* genotype. mut, mutant; wt, wildtype.

confirms the occurrence and clinicopathological characteristics of *RHOA* hotspot mutations in an independent Central European patient cohort. Currently, we cannot confirm the prognostic or growth advantageous effect of *RHOA* mutations in GC and further studies into this topic are warranted.

### Take home messages

- ▶ *RHOA* hotspot mutations are rare and diverse in gastric cancer.
- ▶ *RHOA* mutations are more prevalent in women, distal and poorly differentiated gastric cancers.
- ▶ No significant difference was found in the overall survival between *RHOA* mutant and wildtype gastric cancers.
- ▶ *RHOA*- and *KRAS* mutations are mutually exclusive. *RHOA* mutations may occur occasionally in microsatellite instable or Epstein-Barr-virus-positive gastric cancers.

**Handling editor** Cheok Soon Lee

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German abstract: Genomisch stabile Magenkarzinome sind häufig vom diffusen Phänotyp und weisen *hotspot*-Mutationen im *RHOA*-Gen auf. An einer unabhängigen, 415 Patienten umfassenden mitteleuropäischen Magenkarzinomkohorte untersuchten wir die Prävalenz, den histologischen Phänotyp und die klinisch-pathologischen Charakteristika *RHOA*-mutierter Magenkarzinome. Sechzehn (3.9%) Karzinome wiesen eine *RHOA*-Mutation auf. Darunter befanden sich vier bislang unbekannte Mutationen: p.G17Efs\*24, p.V24F, p.T37A und p.L69R. *RHOA*-Mutationen fanden sich häufiger bei Frauen (5.4% vs. 2.8%), in distalen Magenkarzinomen (4.5% vs. 2.4%), in gering differenzierten Tumoren (G3/G4; 4.8% vs. 1.1%), T1/2-Tumoren (6.2% vs. 3.1%) und wiesen keine Fernmetastasen auf. Neun *RHOA*-mutierte Magenkarzinome waren vom diffusen, vier vom intestinalen, zwei vom nichtklassifizierbaren und einer vom gemischten Typ nach Laurén. *KRAS*- und *RHOA*-Mutationen schlossen einander aus. Ein einziger Fall wies eine *RHOA*- und eine *PIK3CA*-Mutation auf. Es fand sich kein signifikanter Unterschied im Gesamtüberleben zwischen *RHOA*-mutierten und Wildtyp-Karzinomen. Unsere Studie bestätigt das Vorkommen und die klinisch-pathologischen Besonderheiten der *RHOA*-Mutationen beim Magenkarzinom in einer unabhängigen Kohorte. Wir fanden keine Hinweise auf einen prognostischen oder wachstumsfördernden Effekt der *RHOA*-Mutation beim Magenkarzinom.

## Supplementary Materials and Methods

### Immunohistochemistry

Immunostaining was done with monoclonal antibodies directed against mucin 1 (clone MA695; dilution 1:100), mucin 2 (clone Ccp58; both Novocastra, Leica Microsystems GmbH, Wetzlar, Germany; 1:100), mucin 5 (clone 45M1; Thermo Scientific, Schwerte, Germany; 1:100), mucin 6 (clone CLH5; 1:100), CD10 (clone 56C6; both Novocastra; 1:10), E-cadherin (clone SPM471; ZYTOMED Systems GmbH, Berlin, Germany; 1:400),  $\beta$ -catenin (clone Cat-5H10; Life Technologies GmbH, Darmstadt, Germany; 1:300), and a polyclonal antibody directed against lysozyme (1:3000; DAKO, Glostrup, Denmark).

Antigen retrieval was done with ER1 (citrate buffer Bond pH 6.0; mucin 1, CD10) or ER2 (EDTA-buffer Bond pH 8.9; mucin 2, mucin 6, E-cadherin,  $\beta$ -catenin) according to the manufacturer's instructions and using the autostainer Bond™ Max System (Leica Microsystems GmbH, Wetzlar, Germany). The immunoreaction was visualized with either the Bond™ Polymer Refine Detection Kit (DS 9800; brown labelling) or the Bond™ Polymer Refine Red Detection Kit (DS 9390; red labelling; both Novocastra). Epstein-Barr-Virus encoded RNA was detected using the EBER-probe (Novocastra) and the Bond Refine Detection system according to the manufacturer's instructions (Leica Microsystems GmbH).

### Evaluation of Immunostaining

Immunostaining of the TMAs was evaluated by applying an immunoreactivity scoring system (IRS). Briefly, category A documented the intensity of immunostaining as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). Category B documented the percentage of immunoreactive cells as 0 (no immunoreactive cells),

1 (few scattered immunoreactive cells, less than <1%), 2 (1-10%), 3 (11-50%), 4 (51-80%), and 5 (>80%). The addition of category A and B resulted in an IRS ranging from 0 to 8 for each individual case. For statistical analyses, the IRS was split at the median into negative and positive cases.

### Mutational analysis

Mutational analyses of codons 12 and 13 of the *KRAS* and *NRAS* genes, codon 600 of the *BRAF* gene and mutational hotspots in exons 9 and 20 of the *PIK3CA* gene were performed by pyrosequencing on a PyroMark Q24 instrument (QIAGEN, Hilden, Germany). Fragments of the different genes were amplified by PCR with following primers: *KRAS* (codon 12/13), forward 5'-GGCCTGCTGAAAATGACTGAA-3' and reverse 5'-TTAGCTGTATCGTCAAGGCACTCT-3';<sup>1</sup> *NRAS* (codon 12/13), forward 5'-CTTGCTGGTGTGAAATGACTG-3' and reverse 5'-TTCTGGATTAGCTGGATTGTCAGT-3'; *BRAF* (codon 600), forward 5'-TGAAGACCTCACAGTAAAAATAGG-3' and reverse 5'-TCCAGACAACACTGTTCAAACACTGAT-3'; *PIK3CA* exon 9, forward 5'-AACAGCTCAAAGCAATTTCTACACG-3' and reverse 5'-ACCTGTGACTCCATAGAAAATCTTT-3'; and *PIK3CA* exon 20, forward 5'-CAAGAGGCTTTGGAGTATTTCA-3' and reverse 5'-CAATCCATTTTTGTTGTCCA-3'.<sup>2</sup> The resulting PCR products were analyzed by agarose electrophoresis and sequenced using following sequencing primers: *KRAS* (codon 12/13), 5'-TGTGGTAGTTGGAGCT-3'; *NRAS* (codon 12/13), 5'-GTGGTGGTTGGAGCA-3'; *BRAF* (codon 600), 5'-GTAAAAATAGGTGATTTTGG-3'; *PIK3CA* exon 9, 5'-TAGAAAATCTTTCTCCTGCT-3' and 5'-TTCTCCTGCTCAGTGATTT-3'; and *PIK3CA* exon 20, 5'-GTTGTCCAGCCACCA-3'.

### Microsatellite Instability Assay

Microsatellite instability (MSI) was determined by comparison of the allelic profiles of the mononucleotide repeat markers BAT-25, BAT-26, NR-21, NR-24, and NR-27 in tumour and corresponding normal tissue.<sup>3</sup> All markers were coamplified in a pentaplex PCR assay with the QIAGEN Multiplex PCR Master Mix (QIAGEN, Hilden, Germany) following the manufacturer's recommendations for amplification of microsatellite loci. The amplified loci were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Samples were judged as microsatellite unstable (MSI-H) when the tumour showed instability in at least two of the five (40%) microsatellites analyzed.

### External quality assurance

The *KRAS* mutational assay, the microsatellite instability assay and the immunohistochemical evaluation of DNA-mismatch repair proteins (MSH2, MSH6, MLH1 and PMS2) were certified successfully by the quality assurance program of the German Society of Pathology and the *Bundesverband Deutscher Pathologen e.V.*

### Detection of *Helicobacter pylori* by Histology and Polymerase Chain Reaction

Infection with *H. pylori* was evaluated histologically using the modified Giemsa-staining and PCR.<sup>4</sup> *H. pylori*-specific DNA sequences were detected by a PCR-based assay targeting the 16S rRNA gene of *H. pylori*.<sup>5</sup> In short, a 109 bp fragment of the 16S rRNA gene was amplified with the primers Hp1 (5'-CTGGAGAGACTAAGCCCTCC-3') and Hp2 (5'-ATTACTGACGCTGATTGTGC-3') following the PCR parameters described by Chisholm et al.<sup>5</sup> Amplification products were assessed by agarose gel electrophoresis.

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