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Original Article

RAI3 is overexpressed in gastric adenocarcinoma but unrelated to prognosis

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Abstract: Purpose: Retinoic acid-induced gene 3 (*RAI3*) has been associated with tumorigeneses in several cancer types. To clarify the clinical significance of RAI3 expression in premalignant and malignant gastric epithelium, RAI3 protein expression was assessed by immunohistochemistry on tissue microarrays (TMAs) containing 140 gastric dysplasia and 230 GC samples. Findings: RAI3 protein expression was predominantly localized in the cell membrane and was detectable in low intensities in most of the benign gastric tissue samples. RAI3 expression was found in increased intensities in premalignant and malignant epithelium relative to non-malignant gastric epithelium ($P < 0.0001$). High RAI3 expression was found in 66.2% of interpretable gastric adenocarcinomas and was associated with advanced pathological tumor stage ($P = 0.0014$) and positive lymph node status ($P = 0.0137$) but was unrelated to overall survival of patients ($P = 0.3743$). Conclusion: The deregulation of RAI3 in premalignant and gastric epithelium suggests a relevant role of RAI3 during gastric carcinogenesis. Additionally, RAI3 overexpression defines a subset of GCs with aggressive tumor features. However, since RAI3 expression was not associated with clinical outcome of patients, RAI3 cannot be considered as a prognostic biomarker in patients with GCs.

Keywords: RAI3, tissue microarray, gastric dysplasia, gastric adenocarcinoma, immunohistochemistry

Introduction

Gastric cancer (GC) is one of the most common malignancies and a leading cause of cancer-related deaths worldwide [1]. Surgical treatment remains the primary curative treatment for GC, but the overall 5-year survival rate remains poor [1]. Since GC is a heterogeneous disease, novel therapeutic targets as well as prognostic markers are urgently needed.

The retinoic acid-induced gene 3 (RAI3), also known as Retinoic acid-induced gene 1 (*RAIG1*) or G protein-coupled receptor, class C, group 5, member A (*GPRC5A*) belongs to the family of G-protein coupled receptors (GPCRs) and is characterized by an extracellular ligand-binding domain, a transmembrane domain, and an internal C-terminal domain [2]. When an agonist binds to the extracellular portion of the receptor, the intracellular C-terminus interacts with G-proteins which in turn activate several downstream effectors such as adenyl cyclases, phospholipases, phosphodiesterases, and ion channels [3]. Physiologically, GPCRs activate numerous signal transduction cascades and thus play a pivotal role in the regulation of many physiological processes such as cell growth and differentiation [4]. Dysregulation of RAI3 has been reported in several malignancies [5]. However, its functional role might vary depending on tumor type. RAI3 acts as a tumor suppressor in some cancers, whereas in others RAI3 acts as an oncogene [5]. For example, RAI3 plays tumor-suppressive roles in lung [6-10] and head and neck cancers [11] and oncogenic roles in pancreatic [12-14] and colorectal [15] cancers. In breast cancer, the biological function of RAI3 has been controversially discussed. In one study *GPRC5A-gene* knockout resulted in reduced cell growth in breast cancer cell lines [16], and in another study GPRC5A inhibited cell proliferation, migration and invasion [17]. Additionally, RAI3 expression has been suggested as a prognostic marker in a variety of malignancies, including pancreatic

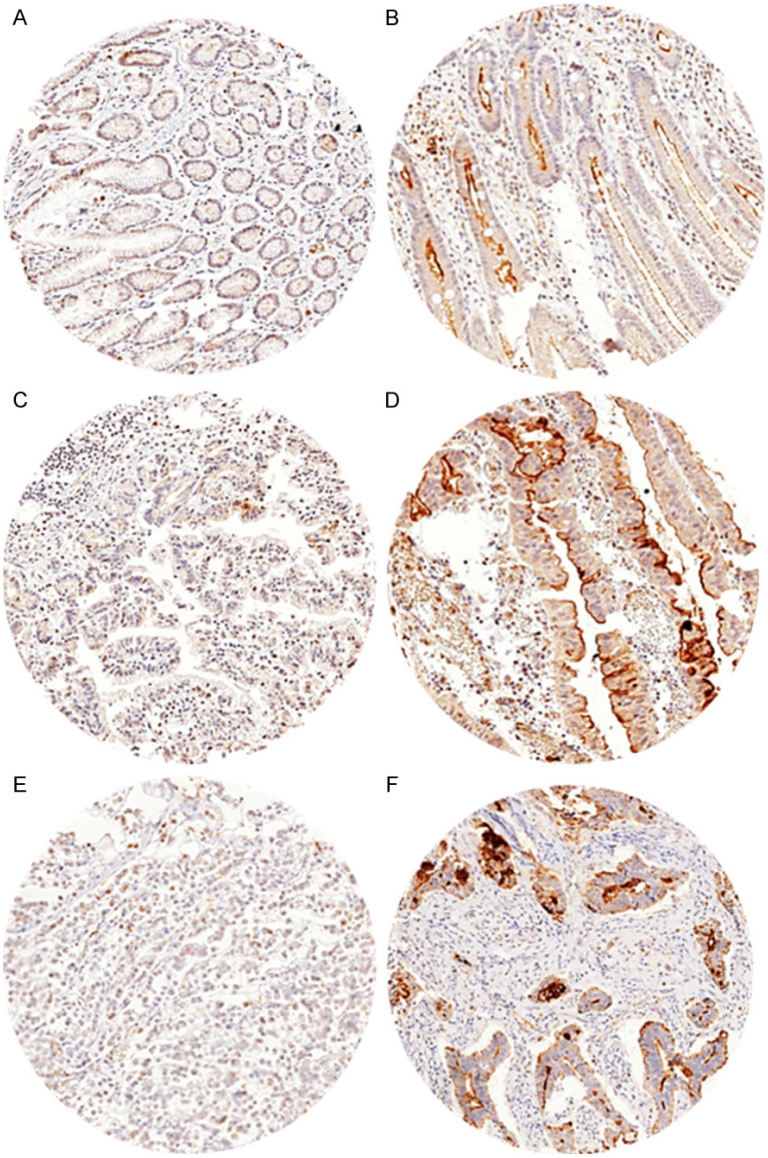

Figure 1. Expression of RAI3 in tissue microarrays of non-malignant, premalignant and malignant gastric epithelium. Low and high RAI3 immunostaining in benign gastric tissues (A, B), gastric dysplasias (C, D), and gastric adenocarcinomas (E, F).

[14], colon [18, 19], gastric [20, 21], oral squamous cell [22], and hepatocellular [23] cancers.

For GC, RAI3 expression has been suggested to be upregulated at both mRNA and protein levels and has been suggested as a biomarker for GC [20, 21]. Functional studies on RAI3 in GC suggested that RAI3 might be useful as a therapeutic target [24]. To further expand our knowledge on the clinical relevance of RAI3 expression in gastric dysplasias and cancers, we analysed a series of 317 benign gastric, 140 gastric dysplasia, and 230 GC tissue samples with follow up data on a set of TMAs. Here, we demonstrate that RAI3 expression is increased in premalignant and gastric epithelium. Additionally, RAI3 overexpression defined a subset of GCs with aggressive tumor features. However, since RAI3 expression was not associated with clinical outcome of patients, RAI3 cannot be considered as a prognostic biomarker in GC patients.

Material and methods

Patients and follow-up

To clarify the clinical significance of RAI3 expression in premalignant and malignant gastric epithelium, RAI3 protein expression was assessed by immunohistochemistry on TMAs containing 140 gastric dysplasia and 230 GC tissue samples. Tissue samples were available from 382 patients undergoing either endoscopic treatment at the Department of Interdisciplinary Endoscopy or surgery at the Department of General, Visceral and Thoracic Surgery at the University Medical Center Hamburg-Eppendorf between 1994 and 2006. Written informed consent for the use of resected samples was obtained from all patients and approval was obtained from the Ethics Committee of the Chamber of Physicians in Hamburg, Germany. Follow-up data were available of 143 patients with a median follow-up of 24.3 ± 26.8 months (range 1 to 145 months).

TMA and immunohistochemistry

The TMA manufacturing process was described earlier in detail [25]. In short, one 0.6 mm core was taken from a representative tissue block

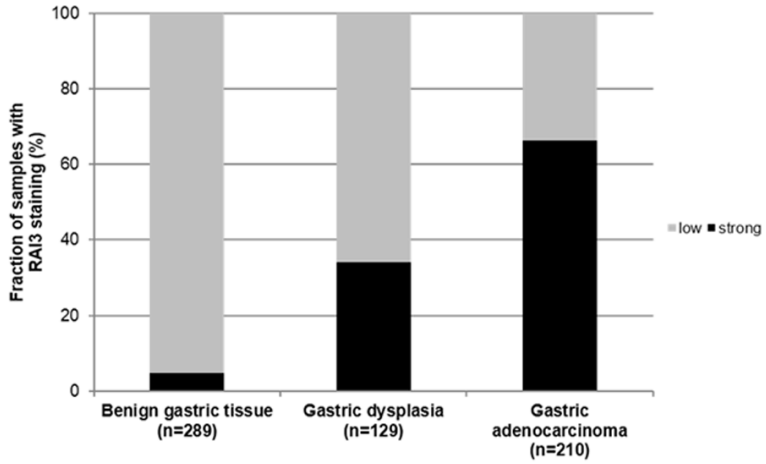

Figure 2. The contribution of RAI3 expression intensities in benign gastric epithelium, gastric dysplasia, and gastric adenocarcinoma. RAI3 expression was increased in premalignant and malignant as compared to benign gastric tissue ($P < 0.0001$).

from each patient. The tissues were distributed among 2 TMA blocks. The TMA contained 687 gastric tissue samples, including 317 normal gastric tissue, 140 gastric dysplasia (10 low-grade, 77 high-grade, 53 intramucosal cancers), and 230 primary gastric tumor samples. Freshly cut TMA sections were analyzed on one day and in one experiment. Primary antibody specific for RAI3 (polyclonal rabbit, NB100-310; Novus Biological; at 1/450 dilution) was applied at 37°C for 60 minutes. Visualization of the primary antibody was performed with the EnVision Kit (Dako, Glostrup, Denmark). RAI3 staining was analyzed by one person (KG) experienced in immunohistochemisty. Assessment of immunostaining was semiquantitatively assessed in two categories: low and high immunostaining.

Statistical analysis

For statistical analysis, the JMP 9.0 software (SAS Institute Inc., NC, USA) was used. Contingency tables were calculated to study association between protein expression of RAI3 and clinico-pathological variable, and the Chi-square (Likelihood) test was used to find significant relationships. Kaplan Meier curves were generated for overall, recurrence-free and metastasis-free survival. The log-Rank test was applied to test the significance of differences between stratified survival functions. Cox proportional hazards regression analysis was performed to test the statistical independence and significance between pathological, molecular, and clinical variables.

Results

Technical aspects

A total of 628 of 687 (91.4%) arrayed tissue samples were successfully analyzed for IHC. Analysis failed either because of lack of tissue spots in the tissue microarray section or absence of unequivocal cancer cells.

Expression of RAI3 in gastric tissues

RAI3 expression was predominantly localized in the membranes of the cells and was accompanied by lower levels of RAI expression in the cytoplasm of the cells. **Figure 1** shows representative immunostainings of RAI3.

In benign gastric mucosa, RAI3 immunostaining was detectable in low intensities in majority of interpretable spots (95.2%). RAI3 expression was found in increased intensities in premalignant and malignant relative to non-malignant gastric epithelium ($P < 0.0001$). High-levels of RAI3 expression were found in 34.1% of interpretable gastric dysplasias and 66.2% of cancers. The contribution of RAI3 immunostaining in non-malignant, premalignant and malignant gastric tissue is shown in **Figure 2**.

Clinical impact of RAI3 expression

High RAI3 expression was significantly associated with advanced pathological tumor stage ($P = 0.0014$) and positive lymph node metastasis ($P = 0.0137$) (**Table 1**).

However, the statistically analysis showed that there was no significant association between RAI3 expression and overall survival ($P = 0.3743$), recurrence-free survival ($P = 0.5673$), and metastasis-free survival ($P = 0.6718$) of gastric adenocarcinoma patients (**Figure 3**).

Discussion

Our data demonstrate that increased RAI3 expression defines a subset of GCs with aggressive tumor features. However, RAI3 expression was unrelated to prognosis of GC patients.

Table 1. RAI3 expression and tumor phenotype

	Analyzable (n)	RAI3 low (%)	RAI3 high (%)	P
All cancers	210	33.81	66.19	
Sex				
Male	139	30.22	69.78	
Female	70	40	60	0.1599
Age				
< 40	4	75	25	
40-50	15	20	80	
51-60	41	39.02	60.98	
61-70	69	23.19	76.81	
> 70	76	40.79	59.21	0.0399
UICC stage				
I	33	36.36	63.64	
II	32	40.63	59.38	
III	98	28.57	71.43	
IV	47	38.3	61.7	0.4953
pT category				
pT1	30	60	40	
pT2	110	34.55	65.45	
pT3	47	17.02	82.98	
pT4	21	28.57	71.43	0.0014
G category				
G1	3	33.33	66.67	
G2	60	30	70	
G3	144	34.72	65.28	0.8067
pN category				
N0	60	46.67	53.33	
N+	147	28.57	71.43	0.0137
M category				
M0	138	31.16	68.84	
M+	24	50	50	0.0785

The present study shows that RAI3 expression increased from non-malignant to premalignant and further increased from premalignant to malignant gastric tissue. Our result is consistent with earlier studies describing an upregulation of RAI3 at both mRNA and protein levels in GCs [20, 21]. Additionally, we demonstrated that RAI3 expression is increased in pre-malignant gastric lesions. Thus, it can be speculated that RAI3 dysregulation might be an early event during gastric tumorigenesis. In our study, high RAI3 expression was detected in 4.8% of non-neoplastic, 34.1% of premalignant, and 66.2% of malignant tissue samples. In our study, the rate of positive RAI3 protein expression is somewhat higher than in the study of Cheng *et al.* [20] reporting a positive RAI3 expression in 25% (7/28) of cancerous samples. Possible explanations for these discrepant results potentially include differences in experimental procedures. Our data better fit to the results of the quantitative reverse transcription-PCR (qRT-PCR) experiments of Cheng *et al.* [20] describing an up-regulation of RAI3 in 71.4% of GCs and to the analysis of Liu *et al.* [21] describing a positive RAI3 protein expression in 56.6% of GCs.

Earlier studies on RAI3 expression status obtained by IHC and qRT-PCR described that RAI3 expression is increased in breast [16, 26], colon [19], and hepatocellular cancers [23] and decreased in lung [6] and oral squamous cell carcinomas [22]. Therefore, it can be assumed that the status of RAI3 expression may largely depend on the cell type and the molecular context.

The upregulation of RAI3 expression in gastric dysplasia and cancers suggests that RAI3 overexpression might have clinical value in gastric cancer tumorigenesis. This assumption is underlined by our finding that increased RAI3 expression defined a subset of GCs with aggressive tumor features. Our results are in general in line with the study of Liu *et al.* [21]. In detail, Liu *et al.* [21] suggested that RAI3 overexpression is linked to aggressive tumor features such as larger tumor-sizes, diffuse type (Lauren classification), deeper serosal invasion, and lymph node metastasis.

Functional studies on RAI3 described tumor-suppressive as well as oncogenic roles of RAI3 in dependence of the cancer cell type. For example, RAI3 has been suggested to play tumor-suppressive functions in lung cancer, since *GPRC5A* knockout mice develop spontaneous lung tumors [6]. In addition, *GPRC5A* knockout resulted in cell transformation, enhanced cell survival and inflammation by activation of STAT3-regulated cell survival genes

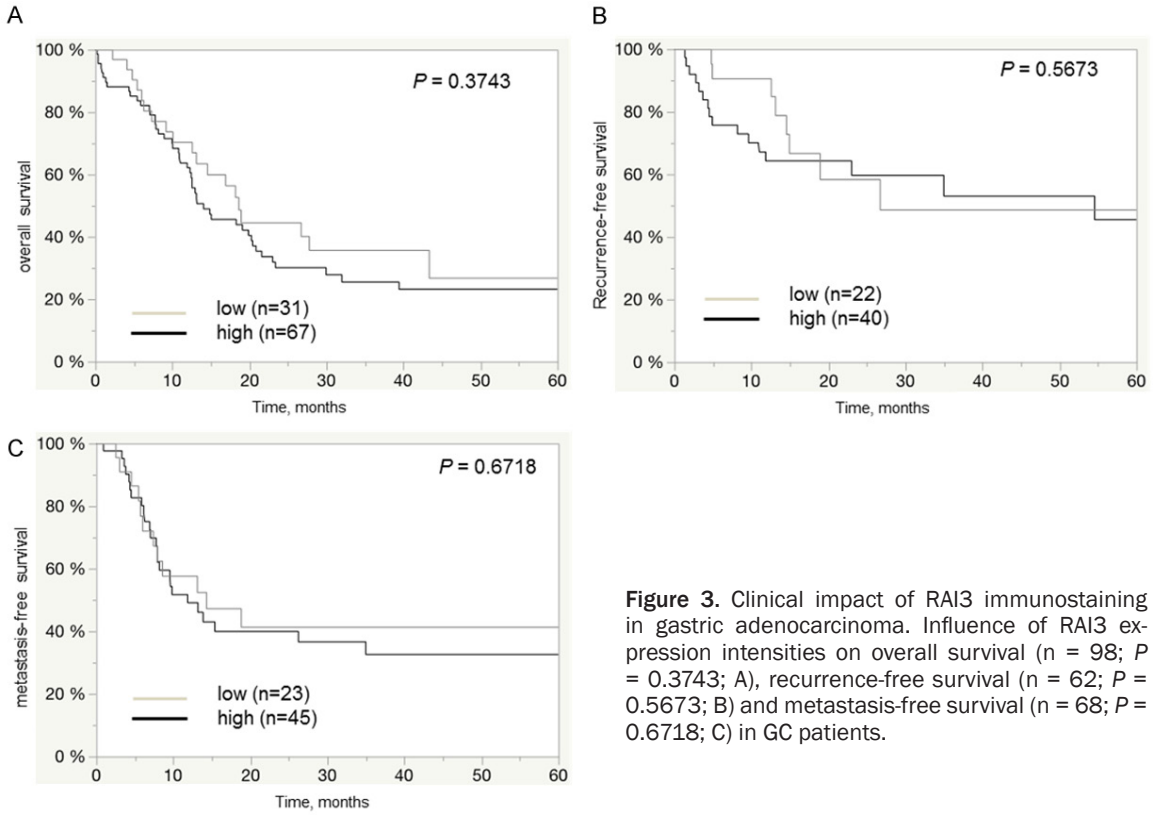

Figure 3. Clinical impact of RAI3 immunostaining in gastric adenocarcinoma. Influence of RAI3 expression intensities on overall survival (n = 98; *P* = 0.3743; A), recurrence-free survival (n = 62; *P* = 0.5673; B) and metastasis-free survival (n = 68; *P* = 0.6718; C) in GC patients.

and NF-kB signaling pathway [27-30]. Contrary to these tumor suppressive functions, other studies suggested that RAI3 might play oncogenic roles. RAI3 has been described as a growth-promoting gene, since ectopic expression of RAI3 in 293 cells promoted cell growth and cell lines expressing mutant p53 were characterized by elevated RAI3 expression [12].

Importantly, RAI3 was unrelated to clinical outcome of patients with GC. Earlier studies analyzing the prognostic impact of RAI3 expression in cancers described divergent results. While some studies described an association between RAI3 overexpression and clinical outcome in hepatocellular carcinoma [23] and colon cancers [19], others found no significant association between RAI3 expression and prognosis in breast cancer patients [26]. Previously, Liu et al. [21] analyzed a cohort of 106 GC patients and suggested that the subgroup of positive RAI3 expressing tumors were linked to shortened overall survival of patients. However, we were not able to identify a correlation between clinical outcome and RAI3 expression in our study including 230 primary gastric tumor samples.

GPCRs which are a large family of signaling receptors and of protein targets for approved drugs [31] are rarely targeted for cancer treatment, except for certain endocrine and hormone-responsive tumors although GPCRs signaling pathways play important roles in regulating cellular functions integral to the hallmarks of cancer (e.g., growth/proliferation, metabolism, death/apoptosis, ion and nutrient transport, and migration [32-34]. Thus, it can be speculated that highly expressed GPCRs in cancer cells may contribute to the malignant phenotype, serve as prognostic markers, and may be useful as a therapeutic target. In accordance with this suggestion Shrestha *et al.* [24] described that RAI3 might be also an effective therapeutic target in gastric cancer therapy. In detail, Shrestha *et al.* [24] showed that in an integrated microRNA-mRNA analysis that miR-204 inhibits cell proliferation in gastric cancer by targeting GPRC5A.

In summary, our study shows that RAI3 expression was linked to a subset of cancers with

aggressive tumor phenotype but was unrelated to prognosis. Thus, our study excludes RAI3 expression as a prognostic biomarker in GC. However, it can be assumed that RAI3 might be useful as a therapeutic target in a subset of high RAI3 expression GCs due to its membranous localization.

Conclusions

The deregulation of RAI3 in premalignant and gastric epithelium suggests a relevant role of RAI3 during gastric carcinogenesis. Additionally, RAI3 overexpression defines a subset of GCs with aggressive tumor features. However, since RAI3 expression was not associated with clinical outcome of patients, RAI3 cannot serve as a prognostic biomarker in GCs.

Acknowledgements

The authors declare full consent for publication. Written informed consent for the use of resected samples was obtained from all patients and approval was obtained from the Ethics Committee of the Chamber of Physicians in Hamburg, Germany.

Disclosure of conflict of interest

None.

Abbreviations

RAI3, Retinoic acid-induced gene 3; IHC, immunohistochemistry; TMA, tissue microarray; GC, gastric cancer; RAIG1, Retinoic acid-induced gene 1; GPRC5A, G protein-coupled receptor, class C, group 5, member A.

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