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High levels of RAI3 expression is linked to shortened survival in esophageal cancer patients

Alexander Tarek El Gammal, Nathaniel Melling, Matthias Reeh, Florian Gebauer, Oliver Mann, Daniel Perez, Maximillian Bockhorn, Kai Bachmann, Jakob Robert Izbicki, Katharina Grupp*

General, Visceral and Thoracic Surgery Department and Clinic, University Medical Center Hamburg-Eppendorf, Germany

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ABSTRACT

Expression of the retinoic acid-induced protein 3 (RAI3) has been suggested to predict clinical outcome in a variety of malignancies. However, its role in esophageal cancers remains unclear. Immunohistochemical RAI3 staining was analyzed on tissue microarrays containing 359 esophageal adenocarcinomas (EAC) and 254 esophageal squamous cell carcinomas (ESCC). RAI3 immunostaining was typically absent or weakly detectable in the membranes in benign esophageal tissues. RAI3 staining was higher in malignant than in benign esophagus epithelium. High-levels of RAI3 staining were found in 79.2% of interpretable EACs and 55.9% of ESCCs. In EACs, strong RAI3 staining was associated with advanced pathological tumor stage ($p < .0001$), high UICC stage ($p < .0001$), high tumor grade ($p = .0133$), and positive lymph nodal status ($p = .0002$). Additionally, high RAI3 staining predicted shortened overall survival of EAC and ESCC patients ($p = .0298$ and $p = .0227$). RAI3 overexpression is associated with poor prognosis in esophageal cancers. We propose that RAI3 overexpression might play a biologically relevant role of RAI3 in esophageal cancers.

1. Introduction

Esophageal cancer is one of the most aggressive cancers and the sixth leading cause of cancer death worldwide (Jemal et al., 2011). Currently, the prediction of prognosis after curative surgery is based on the internationally accepted TNM classification system. Due to its limited ability to stratify esophageal patient prognosis, novel prognostic biomarkers that may enable better prediction of the tumor behavior in individual esophageal cancer patients are urgently needed.

The retinoic acid-inducible protein 3 (RAI3), also known as G-protein coupled receptor family C, member 5, group A (GPRC5A) (Cheng, 1998) consists of an extracellular ligand-binding domain, a transmembrane domain, and an internal C-terminal domain (Li et al., 2005). The intracellular C-terminus interacts with G-proteins that bind guanidine-nucleotides and can activate downstream effectors such as adenyl cyclases, phospholipases, phosphodiesterases and ion channels if agonists bind to the extracellular ligand-binding domain (McCudden et al., 2005). Physiologically GPRCs activate numerous signal transduction cascades and play a pivotal role in the regulation of many physiological processes, including cell growth and differentiation (Shore and Reggio, 2015).

In some cancers, RAI3 has been suggested as tumor suppressor whereas in others it has been suggested as oncogene (Zhou and Rigoutsos, 2014). For example in lung cancer, RAI3 has been described as a tumor suppressor (Tao et al., 2007) (Chen et al., 2010) (Zhong et al., 2015) (Xu et al., 2005) (Jin et al., 2017) since its overexpression has been described to reduce cell growth in cancer cell line (Xu et al., 2005) and *GPRC5A*-gene knockout upregulated tumor cell viability and reduced tumor cell apoptosis (Jin et al., 2017) and developed spontaneous lung tumors in mice (Tao et al., 2007). In head and neck squamous cell carcinoma, overexpression of GPRC5A was associated with inhibited growth in cells, indicating a tumor suppressive function in this tumor entity (Liu et al., 2017). Besides these tumor suppressive functions of RAI3, GPRC5A transfection resulted in increased colony formation in pancreatic cancer cell line (Wu et al., 2005) (Zhou et al., 2016) and suppression of GPRC5a resulted in decreased cell growth, proliferation and migration in pancreatic cancer cell lines (Jahny et al., 2017). In breast cancer, the biological functions of RAI3 have been discussed controversial. In one study, *GPRC5A*- gene knockout resulted in reduced cell growth in breast cancer cell lines (Nagahata et al.,

* Corresponding author.
E-mail addresses: a.el-gammal@uke.de (A.T. El Gammal), n.melling@uke.de (N. Melling), m.reeh@uke.de (M. Reeh), f.gebauer@uke.de (F. Gebauer), o.mann@uke.de (O. Mann), d.perez@uke.de (D. Perez), m.bockhorn@uke.de (M. Bockhorn), k.bachmann@uke.de (K. Bachmann), izbicki@uke.de (J.R. Izbicki), k.grupp@uke.de (K. Grupp).

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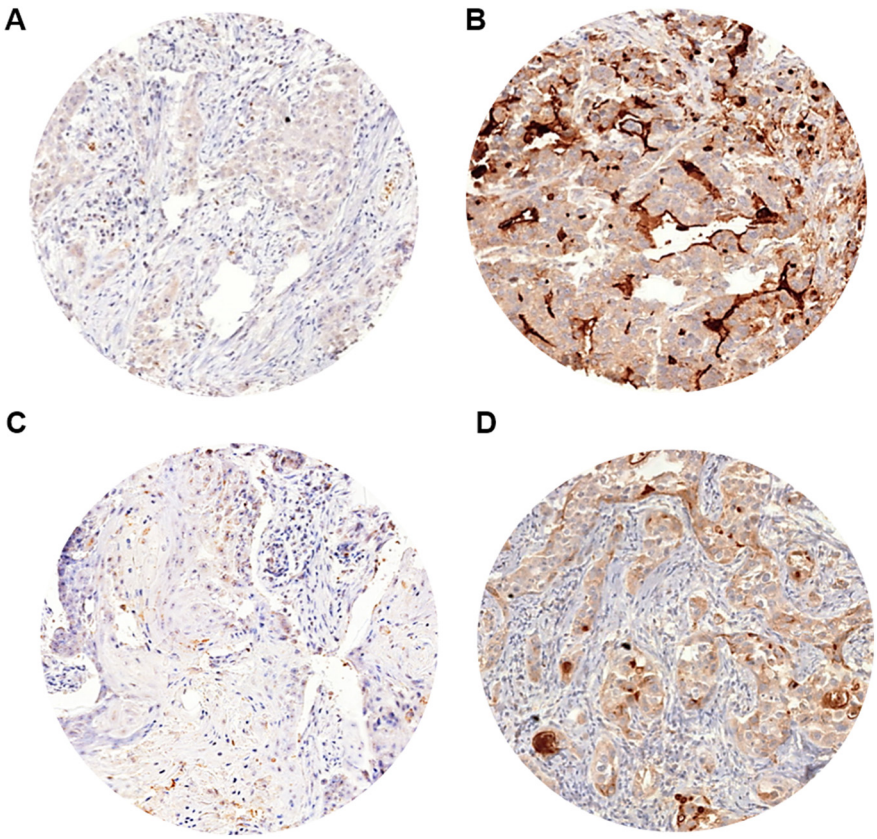

Fig. 1. Sample immunohistochemical images of RAI3 staining. Images of low and high RAi3 expression in EACs (A and B) and ESCCs (C and D).

2005), and in another study GPRC5A inhibited cell proliferation, migration and invasion *in vitro* (Yang et al., 2016). Additionally, an oncogenic role of GPRC5A has been suggested in colorectal cancer (Zhang et al., 2017).

Moreover, RAI3 expression has been suggested as prognostic marker in several cancers, including pancreatic (Jahny et al., 2017), colon (Kume et al., 2014) (Zougman et al., 2013), gastric (Cheng et al., 2012) (Liu et al., 2016), oral squamous cell (Liu et al., 2013) and hepatocellular (Zheng et al., 2014) cancers. To analyze its role in esophageal cancers, RAI3 expression was analyzed on tissue microarrays containing 359 EAC and 254 ESCC samples. Here, we demonstrate that increased RAI3 expression signifies a subset of esophageal cancer patients with poor clinical outcome. The prognostic value of RAI3 overexpression suggests a biologically relevant role of RAI3 in esophageal cancers.

2. Material and methods

2.1. Patients and TMA construction

A TMA was constructed from cancer tissues from 359 EAC and 254 ESCC patients who underwent surgery at the University Medical Center Hamburg-Eppendorf. Follow-up data were available of 359 EAC and 254 ESCC patients with a median follow-up of 17.3 and 12.2 months (range: 0 to 208 and 0 to 191 months). All esophageal specimens were analyzed according to a standard procedure, including complete embedding of the entire esophagus for histological analysis. The TMA manufacturing process was described earlier in detail (Mirlacher and Simon, 2010). In short, one 0.6 mm core was taken from a representative tissue block from each patient. The tissues were distributed among 2 TMA blocks. For internal controls, each TMA block also contained various control tissues, including normal esophageal tissue. The study was approved by the Ethics commission Hamburg and conducted in accordance with the Declaration of Helsinki. Informed consent has not been collected specifically for the patient samples included in this study. Usage of routinely archived formalin fixed leftover patient tissue samples for research purposes by the attending physician is approved by local laws and does not require written consent (HmbKHG, §12,1).

2.2. Immunohistochemistry and staining evaluation

Freshly cut TMA sections were immunostained in one day and in one experiment. Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 min in an autoclave at 121 °C in pH 7.8 Tris-EDTA-Citrate buffer. Primary antibody specific for RAI3 (polyclonal rabbit, NB100-310; Novus Biological) was applied at a dilution of 1:450 according to the manufacturer's directions. RAI3 staining was analyzed by one person (KG) experienced in immunohistochemisty. Bound antibody was then visualized using the EnVision Kit (Dako, Glostrup, Denmark). RAI3 staining was evaluated according to the following scoring system: The staining intensity (0, 1+, 2+, and 3+) and the fraction of positive tumor cells were recorded for each tissue spot. A score was built from these two parameters according to the following established score, as previously described (Grupp et al., 2013a) (Grupp et al., 2013b) (Minner et al., 2011). Negative scores had absence of RAI3 staining, weak scores had staining intensity of 1+ in ≤70% of tumor cells or staining intensity of 2+ in ≤30% of tumor cells; moderate scores had staining intensity of 1+ in ≥70% of tumor cells, staining intensity of 2+ in > 30% but in ≤70% of tumor cells or staining intensity of 3+ in ≤30% of tumor cells; strong scores had staining intensity of 2+ in > 70% of tumor cells or staining intensity of 3+ in > 30% of tumor cells. The final score summarized both scores: low staining score included negative and weak staining and high staining score included moderate and strong staining.

Table 1
Clinico-pathological parameters relative to RAI3 IHC results in EACs.

Parameter	Immunostaining			
	Evaluable (n)	Low (%)	High (%)	p value
Tumors	284	20.78	79.23	
Age group				
< 65 years	94	21.28	78.72	0.8836
> 65 years	190	20.53	79.47	
Sex				
Male	240	20.42	79.58	0.7307
Female	44	22.73	77.27	
Tumor stage				
pT1	59	38.98	61.02	< 0.0001
pT2	31	29.03	70.97	
pT3	171	14.62	85.38	
pT4	20	0	100	
UICC stage				
I	57	45.61	54.39	< 0.0001
II	35	20	80	
III	168	13.1	86.9	
IV	22	13.64	86.36	
Grading				
G1	16	100	0	0.0133
G2	110	43.75	56.25	
G3	148	21.82	78.18	
G4	6	15.54	84.46	
R status				
R0	205	22.93	77.07	0.2123
R1	72	13.89	86.11	
R2	3	33.33	66.67	
pN category				
N0	85	36.47	63.53	0.0002
N1	48	22.92	77.08	
N2	71	12.68	87.32	
N3	77	10.39	89.61	
M status				
M0	262	21.37	78.63	0.3684
M1	22	13.64	86.36	

Table 2
Clinico-pathological parameters relative to RAI3 IHC results in ESCCs.

Parameter	Immunostaining			
	Evaluable (n)	Low (%)	High (%)	p value
Tumors	236	44.07	55.93	
Age group				
< 65 years	91	47.25	52.75	0.4624
> 65 years	144	42.36	57.64	
Sex				
Male	170	44.71	55.29	0.822
Female	65	43.08	56.92	
Tumor stage				
pT1	45	46.67	53.33	0.3107
pT2	45	51.11	48.89	
pT3	130	43.08	56.92	
pT4	16	25	75	
UICC stage				
I	60	55	45	0.1225
II	62	41.94	58.06	
III	103	41.75	58.25	
IV	10	20	80	
Grading				
G1	3	100	0	0.0021
G2	149	36.91	63.09	
G3	83	55.42	44.58	
G4	0	0	0	
R status				
R0	174	47.13	52.87	0.2956
R1	49	34.69	65.31	
R2	11	45.45	54.55	
pN category				
N0	110	47.27	52.73	0.5996
N1	53	43.4	56.6	
N2	42	35.71	64.29	
N3	29	48.28	51.72	
M status				
M0	226	45.13	54.87	0.1596
M1	9	22.22	77.78	

2.3. Statistical analysis

Statistical calculations were performed with JPM 9 software (SAS Institute Inc., NC, USA). Contingency tables and the chi^2-test were performed to search for associations between molecular parameters and tumor phenotype. Survival curves were calculated according to Kaplan-Meier. The Log-Rank test was applied to detect significant survival differences between groups. Cox proportional hazards regression analysis was performed to test the statistical independence and significance between pathological, molecular and clinical variables. Logistic regression was used to quantify the area under receiver-operator curve (ROC).

3. Results

3.1. RAI3 expression in benign and malignant esophageal tissue

RAI3 expression could be evaluated in 79.1% (284/359) of EAC and 92.9% (236/254) of ESCC samples. The reasons for non-informative cases included lack of tissue samples or absence of unequivocal cancer tissue in individual TMA samples. RAI3 staining was typically absent or weakly detectable in the membranes in normal esophagus epithelium cells. RAI3 expression was detectable in increased intensities in malignant relative to benign tissue. High RAI3 immunostaining was seen in 79.2% of analyzable EACs and 55.9% of ESCCs. Sample immunohistochemical images are shown in Fig. 1.

3.2. Relationship of RAI3 IHC with p53 IHC

TP53 is involved in DNA repair and cell cycle arrest, and is the most common mutation found in cancers, including esophageal squamous cell carcinoma (Toh et al., 2010) (Mandard et al., 2000) (Hollstein et al., 1991) and esophageal adenocarcinoma (Gregson et al., 2016) (Ross-Innes et al., 2015) (Stachler et al., 2015) (Weaver et al., 2014). Moreover, it has been suggested that p53 interacts with the promoter of *RAI3* and repressed its expression at the onset of apoptosis in breast tumor cells (Wu et al., 2005). Thus, next we analyzed whether RAI3 expression might be linked to p53 IHC in both subset of esophageal cancer histology (unpublished data). Interestingly, our data show that RAI3 expression levels were significantly linked to p53 IHC in the subset of esophageal adenocarcinoma (p = .0075) but not in the subset of esophageal squamous cell carcinoma (p = .8609).

3.3. Associations of RAI3 expression in primary tumors with clinicopathological parameters

The associations of RAI3 immunostaining results with esophageal cancer phenotype are summarized in Tables 1 and 2. In EACs, increased RAI3 staining was associated with advanced pathological tumor stage (p < .0001), high UICC stage (p < .0001), high tumor grade (p = .0133), positive lymph nodal status (P = .0002), and shortened overall survival of patients (p = .0263). In ESCCs, increased RAI3 staining was only significantly linked to high tumor grade (p = .0021).

3.4. Impact of RAI3 staining on overall survival of patients

Follow-up data was available for 284 adenocarcinoma patients and 235 squamous cell carcinoma patients with successfully analyzed RAI3 staining. As demonstrated in Fig. 2, high RAI3 immunostaining was linked to shortened overall survival of patients with EAC (p = .0298)

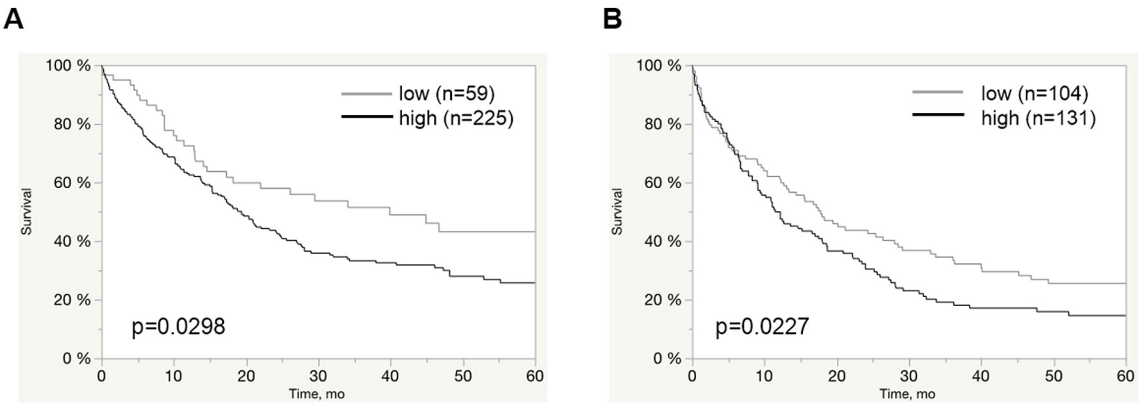

Fig. 2. Prognostic impact of RAI3 expression in esophageal cancers. Relationship of RAI3 immunostaining intensity with overall survival in EACs (p = .0298; A) and ESCCs (p = .0227; B).

and ESCC (p = .0227).

3.5. Multivariate analysis

Multivariate analysis including tumor stage, UICC stage as well as RAI3 IHC demonstrated independent prognostic value for tumor stage and UICC stage in EACs (p = .0191 and p < .0001) and in EACCs (p = .0004 and p = .0063), but not for RAI3 IHC (EACs: p = .693 and EACCs p = .167).

4. Discussion

The present study shows that increased RAI3 expression is associated with shortened survival in esophageal cancer patients. The prognostic value of RAI3 overexpression suggests a biologically relevant role of RAI3 in esophageal cancers.

This is the first study analyzing immunohistochemical RAI3 protein expression in esophageal cancers. RAI3 staining was typically absent or weakly in the membranes in normal esophagus epithelia and was detectable in increased intensities in malignant relative to benign esophageal tissue. Increased RAI3 immunoreactivity was found in 79.2% of interpretable EACs and 55.9% of ESCCs. Previous studies have suggested that dysregulation of RAI3 expression is linked to with several malignancies, including increased RAI3 expression in pancreatic (Jahny et al., 2017), colorectal (Kume et al., 2014) (Zougman et al., 2013), gastric cancer (Cheng et al., 2012) (Liu et al., 2016), hepatocellular (Zheng et al., 2014), and breast cancer (Jörissen et al., 2009) or decreased RAI3 expression in oral squamous cell (Liu et al., 2013), and non-small cell lung (Fujimoto et al., 2012). Theses discrepancy of RAI3 expression intensities in malignant relative to corresponding benign tissue might be due to tissue-specific interactions of RAI3 with tumor-associated pathways in dependence of the cell type.

The molecular mechanism underlying the overexpression of RAI3 in esophageal cancers remains unknown. However, since RAI3 was overexpressed in esophageal cancers, it can be speculated that GPRC5A is transcriptionally activated by alterations of proteins involved in esophageal carcinogenesis. For example, *GPRC5A* gene locus contains a p53 consensus DNA binding sequences in the promoter region (Wu et al., 2005) and TP53 mutations occur in about 56% of EACs and 47% of ESCCs (Abedi-Ardekani and Hainaut, 2014). Previous studies have demonstrated that overexpression of wild-type p53 represses RAI3 expression and that PT53 mutated cell lines result in an upregulation of *GPRC5A* mRNA in breast tumor cells (Wu et al., 2005). Moreover, knockdown of p53 resulted in a decrease in GPRC5A expression, inhibited apoptosis and increased viability in lung cancer cells (Jin et al., 2017).

In our study, high RAI3 immunostaining was associated with poor prognosis of patients with EACs and ESCCs. This assumption is consistent with numerous previous studies implicating tumor relevant functional consequences of RAI3 dysregulation. In some cancers RAI3 has been described as a tumor suppressor, whereas in others it has been suggested as an oncogene. For example, tumor suppressive functions of RAI3 have been suggested in lung (Tao et al., 2007) (Chen et al., 2010) (Zhong et al., 2015) (Xu et al., 2005) (Jin et al., 2017) since its overexpression resulted in reduced cell growth in lung cancer cell line (Xu et al., 2005) and *GPRC5A*-gene knockout upregulated tumor cell viability and reduced tumor cell apoptosis (Jin et al., 2017) and developed spontaneous lung tumors in mice (Tao et al., 2007). In head and neck squamous cell carcinoma, overexpression of GPRC5A was associated with inhibited growth in cells, indicating a tumor suppressive function in this tumor entity (Liu et al., 2017). Besides these tumor suppressive functions of RAI3, GPRC5A transfection resulted in increased colony formation in pancreatic cancer cell line (Wu et al., 2005) (Zhou et al., 2016) and suppression of GPRC5a resulted in decreased cell growth, proliferation and migration in pancreatic cancer cell lines (Jahny et al., 2017). In breast cancer, the biological functions of RAI3 have discussed controversial. In one study, *GPRC5A*- gene knockout resulted in reduced cell growth in breast cancer cell lines (Nagahata et al., 2005), and in another study GPRC5A inhibited cell proliferation, migration and invasion *in vitro* (Yang et al., 2016). Additionally, an oncogenic role of GPRC5A has been suggested in colorectal cancer (Zhang et al., 2017). The present study demonstrates that RAI3 overexpression is linked to poor prognosis in esophageal cancers. Thus, it can be assumed that RAI3 plays rather an oncogenic role than a tumor-suppressive role in esophageal tumorigenesis.

Deregulation of the p53 tumor suppressor pathway occurs as a common event in most if not all types of human cancers. *TP53* is involved in DNA repair and cell cycle arrest, and is the most common mutation found in cancers, including esophageal squamous cell carcinoma (Toh et al., 2010) (Mandard et al., 2000) (Hollstein et al., 1991) and esophageal adenocarcinoma (Gregson et al., 2016) (Ross-Innes et al., 2015) (Stachler et al., 2015) (Weaver et al., 2014). The biochemical activity of p53 that is most closely linked to tumor suppression is its function as a transcription factor. The transcriptional targets of p53 form a network involved in DNA repair, cell cycle regulation, differentiation, development, and apoptosis (Vogelstein et al., 2000). Studies on RAI3 suggested that RAI3 is a novel p53 transcriptional target gene (Wu et al., 2005). Our data show that that RAI3 expression levels were significantly linked to p53 IHC in esophageal adenocarcinoma but not in the subset of esophageal squamous cell carcinoma.

In summary, the present study shows that increased RAI3 expression is associated with shortened overall survival of patients with esophageal cancers. Our findings highlight the potentially important role of RAI3 in esophageal cancer biology.

5. Conclusions

Increased RAI3 immunostaining signifies a subset of esophageal cancer patients with poor clinical outcome. The prognostic relevance of RAI3 overexpression suggests a biologically relevant role of RAI3 in esophageal cancers.

Disclosures

The authors have no potential conflict of interest to disclose.

Ethics approval and consent to participate

The local ethical committee of Hamburg approved this study. Informed consent was obtained from all patients before inclusion in the study.

Consent for publication

Not applicable.

Availability of data and material

Please contact author for data requests.

Competing interests

The authors declare that they have no competing interests.

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