Absence of RKIP expression is an independent prognostic biomarker for gastric cancer patients

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Abstract. Gastric cancer is a leading cause of cancer-related mortality, and the presence of lymph node metastasis an important prognostic factor. Downregulation of RKIP has been associated with tumor progression and metastasis in several types of neoplasms, being currently categorized as a metastasis suppressor gene. Our aim was to determine the expression levels of RKIP in gastric tissues and to evaluate its impact in the clinical outcome of gastric carcinoma patients. RKIP expression levels were studied by immunohistochemistry in a series of gastric tissues. Overall, we analysed 222 non-neoplastic gastric tissues, 152 primary tumors and 42 lymph node metastasis samples. We observed that RKIP was highly expressed in ~83% of non-neoplastic tissues (including normal tissue and metaplasia), was lost in ~56% of primary tumors and in ~90% of lymph node metastasis samples. Loss of RKIP expression was significantly associated with several markers of poor clinical outcome, including the presence of lymph node metastasis. Furthermore, the absence of RKIP protein constitutes an independent prognostic marker for these patients. In conclusion, RKIP expression is significantly lost during gastric carcinoma progression being almost absent in lymph node metastasis samples. Of note, we showed that the absence of RKIP expression is associated with poor outcome features of gastric cancer patients, this being also an independent prognostic marker.

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Introduction

The incidence and mortality of gastric cancer have declined steadily over the past several decades. Nonetheless, gastric cancer remains a major public health issue being the fourth most common cancer and the second leading cause of cancer death worldwide (1). Gastric neoplasia is largely composed of adenocarcinomas, accounting for over 95% of cases (2). Pathologically, gastric adenorcarcinomas are most widely classified using Lauren and the World Health Organisation (WHO) classification system. In the Lauren classification, gastric cancers are stratified into intestinal, diffuse, and mixed types, whereas the WHO classification categorizes cancers according to features of histopathological differentiation, namely papillary, tubular, mucinous, and signet ring cell types (3). Genetically, gastric cancer is generally considered to result from accumulation of genetic alterations involving a variety of oncogenes and tumor suppressor genes, however, concrete genetic sequences in the development of gastric cancer still remain to be clarified (3,4).

Raf kinase inhibitor protein (RKIP; also known as PEBP, for phosphatidylethanolamine-binding protein) is a widely expressed and highly conserved protein (5-7), which was firstly identified as a MAP kinase pathway inhibitor by modulating the function of Raf-1 (8,9). Currently, is known that RKIP also suppresses the activation of the nuclear factor κB (NF-κB) (10) and the regulator of G-protein coupled receptors (GRK-2) (11), and may be involved in regulation of the cell cycle (12). Thus, RKIP mediates important cellular mechanisms, including cell differentiation, cell cycle, apoptosis and cell migration, and is deregulated in several human disorders (13).

In cancer, RKIP is considered to be a signal transduction modulator and a metastasis suppressor (14), and downregulated in several human tumors, mainly in highly metastatic carcinomas (15-27). Noteworthy, RKIP has been shown to be a prognostic marker in prostate cancer, colorectal carcinomas, gliomas and GISTs (25,27-30).

Concerning RKIP expression in gastric carcinomas, there are three, yet contradictory studies (31-33). Thus, in the present

Table I. Frequency of RKIP expression in gastric tissues.

Gastric tissues	N	RKIP Expression		p (vs. Normal)	p (vs. Primary tumor)
		Positive (%)	Negative (%)		
Normal	163	138 (84.7)	25 (15.3)	-	<0.001
Metaplasia	59	47 (79.7)	12 (20.3)	0.377	<0.001
Primary tumor	152	67 (44.1)	85 (55.9)	<0.001	-
Lymph node metastasis	42	4 (9.5)	38 (90.5)	<0.001	<0.001

p, pearson value from χ^2 test; vs., versus.

work, using a large and clinically well-characterized series of gastric carcinomas, we aimed first to evaluate the frequency of RKIP expression not only in tumor but also in normal and metastatic gastric tissues. Secondly, we aimed to determine whether RKIP expression could be used to predict clinical outcome of patients with gastric carcinomas.

Materials and methods

Patients and tissue samples. For the present study, the cases were retrospectively selected from the University of São Paulo Gastric Cancer Database: 152 patients with gastric adenocarcinoma operated at the Stomach and Small Bowel Unit Hospital das Clinicas, University of São Paulo, School of Medicine, São Paulo, Brazil, between February 1993 and December 2002. In total 416 different gastric tissues were analysed, including 163 normal mucosa, 59 metaplastic mucosa, 152 primary tumors and 42 lymph node metastasis samples. The samples were retrieved from the files of the Department of Pathology, and organized in tissue microarrays (TMAs). To achieve representative sampling and minimize sample loss, each case was included in duplicate in the TMAs. Additionally, formalin-fixed and paraffin-embedded prostate carcinoma and GISTs samples were used as positive and negative controls for immunohistochemistry, as previously described (27).

Relevant patient clinical data available included patients age, gender, tumor size and location, WHO classification, Lauren's classification, TNM staging, depth of invasion, lymph node metastases and lymphatic, vascular and neural invasions, desmoplasia, inflammatory infiltrated and follow-up, as previously described (34). The inclusion criteria were: patients with primary gastric adenocarcinoma submitted to subtotal or total gastrectomy with D2 lymphadenectomy, submucosa layer invasion or deeper (T1b or higher), at least 25 lymph nodes retrieved per case, absence of distant metastasis (M0), and available follow-up data. All patients were treated according to a well-established surgical protocol following the Japanese Gastric Cancer Association rules.

The histological sections, obtained from original paraffin blocks and, after hematoxylin and eosin staining, were submitted to histological review by senior pathologist. Primary tumors were histologically classified according to World Health Organization (35) in tubular, intestinal, signet-cell and mucinous carcinomas. Lauren and Ming (36,37) classifications were also applied to primary tumors, stratifying lesions into diffuse or intestinal or infiltrating or expansive types, respectively, the latter derived from evaluation of the deepest tumor edge. Lymphatic, vascular or perineural invasion was assessed as non-detected or present. Pathological nodal status (pN) was determined histologically by counting the affected lymph nodes and classified as pN0, pN1, pN2 or pN3 according to AJCC TNM Staging system (38). Information on the tumor size as well its main location, classified as proximal or distal tumors, were obtained from original surgical pathology reports. Peri/intratumoral inflammatory infiltrate was semi-quantified as absent, mild/moderate and intense whereas desmoplasmatic stromal response as absent to mild or moderate to intense. Final pathological TNM stage was also assessed.

Immunohistochemistry analysis for RKIP. Tissue microarray (TMA) slides with 3 μm-thick sections were subjected to immunohistochemical analysis according to the streptavidin-biotin peroxidase complex system (UltraVision Large Volume Detection System Anti-Polyvalent, HRP; LabVision Corporation, CA, USA), as previously described (27,30,39). Briefly, deparaffinised and rehydrated slides were submitted to heat-induced antigen retrieval for 20 min at 98˚C with 10 mM citrate buffer (pH 6.0). After incubation with the primary antibody raised against RKIP (dilution 1:800; incubation 2 h at RT; Upstate Biotechnology, Lake Placid, NY, USA), the secondary biotinylated goat anti-polyvalent antibody was applied for 10 min followed by incubation with the streptavidin-peroxidase complex. The immune reaction was visualized by 3,3'-diamonobenzidine (DAB) as a chromogen. All sections were counterstained with Gill-2 haematoxylin. For negative controls, primary antibodies were omitted and also replaced by a universal negative control antibody (CEA, rabbit anti-human, Dako Corporation, Carpinteria, CA, USA). Prostate carcinoma and GISTs cases, previously analysed (27,30,39), were used as positive and negative controls. Sections were independently scored by two of the authors (K.S. and A.L.F.), following a semi-quantitative criterion: (-), 0% of immunoreactive cells; (+), <5% of immunoreactive cells; (++), 5-50% of immunoreactive cells; and (+++), >50% of immunoreactive cells. Samples with scores (-) and (+) were considered negative, and those with scores (++) and (+++) were considered positive.

Statistical analysis. Either the χ^2 test or Fisher's exact test (when required) was done to determine the correlation between

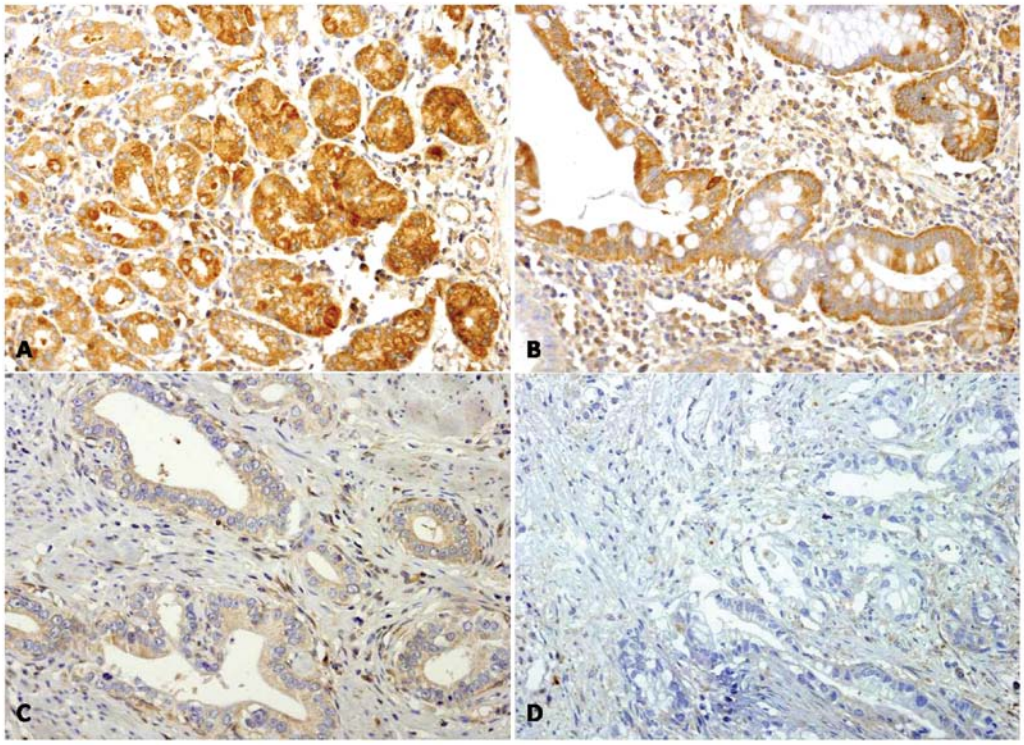

Figure 1. Immunohistochemistry analysis of RKIP in gastric samples. (A) Strong expression in normal gastric mucosa (x200). (B) Strong expression in gastric metaplasia tissue (x200). (C) Weak expression in gastric primary carcinoma (x200). (D) Negative expression in gastric lymph node metastasis tissue (x200).

RKIP expression status and clinicopathologic features. Survival curves were estimated using the Kaplan-Meier product-limit method, and the significance of the differences between survival curves was determined using a log-rank test. Multivariate survival analyses were done using the Cox proportional hazards model. Results were considered to be statistically significant for $p<0.05$. All statistical analyses were conducted using SPSS 16.0 statistical software program.

Results

Patient data. We have available tissue in 152 patients, 114 with advanced and 38 early gastric cancer. The mean age was 60.89 (range: 26-87 years). The majority of patients had tumors located at the distal part of the stomach. The mean tumor size was 3.92 (range: 0.8-20 cm). There was a predominance of the Lauren intestinal type. All patients were submitted to D2 lymphadenectomy with a mean number of retrieved lymph nodes of 43.7 (range: 25-113). The mean follow-up was 62.3 months (range: 6-76 months), and 13.4% was lost to follow-up.

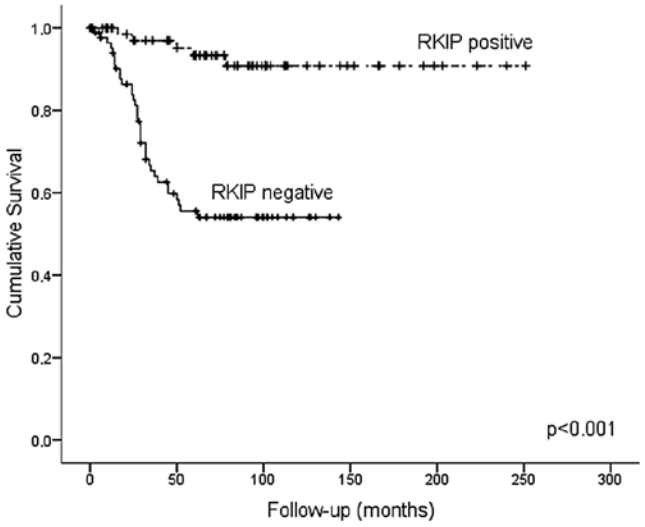

Figure 2. Overall survival according to RKIP expression in gastric carcinomas (n=126). Cumulative survival is significantly lower in cases with RKIP absence of expression ($p<0.001$).

RKIP expression in gastric tissues. Four hundred and sixteen gastric tissue samples were studied for RKIP expression by immunohistochemistry. RKIP staining was always present in the cytoplasm of the cells and, according to the immunohistochemistry score, we found RKIP-positive expression in 84.7% (138/163) of normal gastric tissue, in 79.7% (47/59) of gastric mucosa metaplasia, in 44.1% (67/152) of gastric primary tumors, and in 9.5% (4/42) of gastric lymph node metastasis (Table I and Fig. 1). There are no statistically significant differences between RKIP expression in normal and metaplasias ($p=0.377$). However, a statistically significant ($p<0.001$) decrease of RKIP expression was found in primary tumors, compared to normal and metaplasia tissues, and in lymph-node metastasis, when compared to normal, metaplasia and primary tumors (Table I). Additionally, in 35 patients we had normal, tumor and metastatic tissues available. We found that RKIP was positive in non-neoplastic tissues in ~83% of the patients, but was absent ~69% of the primary tumors and completely absent in metastatic tissues (data not shown).

Table II. Associations between RKIP expression and clinicopathologic features in gastric cancer patients.

Parameter	N	RKIP expression		p
		Negative (%)	Positive (%)	
Gender				
Female	48	27(56.2)	21 (43.8)	0.917
Male	103	57 (55.3)	46 (44.7)	
Depth of invasion				
Early	38	6 (15.8)	32 (84.2)	<0.001
Advanced	114	79 (69.3)	35 (30.7)	
Age (years)				
<61	68	33 (48.5)	35 (61.4)	0.112
≥61	83	51 (51.5)	32 (38.6)	
Tumor size (cm)				
<4	52	19 (36.5)	33 (63.5)	<0.001
≥4	98	65 (66.3)	33 (33.7)	
Tumor location				
Proximal	16	9 (56.2)	7 (43.8)	0.974
Distal	129	72 (55.8)	57 (44.2)	
WHO classification				
Intestinal adenocarcinoma	14	1 (7.1)	13 (92.9)	0.030
Tubular adenocarcinoma	66	46 (69.7)	20 (30.3)	
Mucinous adenocarcinoma	14	9 (64.3)	5 (35.7)	
Signet-ring cell carcinoma	36	23 (63.9)	13 (36.1)	
pT				
Muscular propria/subserosa	111	76 (68.5)	35 (31.5)	<0.001
Submucosa	38	6 (15.8)	32 (84.2)	
Lauren				
Intestinal	99	55 (55.6)	44 (44.4)	0.855
Diffuse	49	28 (57.1)	21 (42.9)	
Lymphatic Invasion				
Absent	84	39(46.4)	45 (53.6)	0.009
Present	68	46 (67.6)	22 (32.4)	
Vascular Invasion				
Absent	131	69 (52.7)	62 (47.3)	0.044
Present	21	16 (76.2)	5 (23.8)	
Perineural invasion				
Absent	86	40 (46.5)	46 (53.5)	0.008
Present	66	45 (67.2)	21 (31.8)	
Inflammatory infiltrated				
Absent/mild	118	62 (52.5)	56 (47.5)	0.233
Moderated/accentuated	31	20 (64.5)	11 (35.5)	
Desmoplasia				
Absent/discrete	84	42(50.0)	42 (50.0)	0.081
Moderated/accentuated	67	43 (64.2)	24 (35.8)	
TNM				
IA	37	6 (16.2)	31 (83.8)	<0.001
IB	34	24 (70.6)	10 (29.4)	
II	52	34 (65.4)	18 (34.6)	
IIIA+IV	27	20 (74.1)	7 (25.9)	
Lymph node metastasis				
pN0	72	29 (40.3)	43 (59.7)	0.004
pN1	55	37 (67.3)	18 (32.7)	
pN2	23	17 (73.9)	6 (26.1)	

N, number of cases; cm, centimeters; SD, standard deviation.

Table III. Correlations between clinicopathologic features and overall survival in gastric cancer patients.

		Univariate analysis		Multivariate analysis	
Parameter	N	(months ± SD)	p[a]	Hazard ratio (95% CI)	p[b]
Gender					
Female	40	211.6±13.5	0.018	1.00	
Male	85	145.6±10.6		2.95 (1.25-6.98)	0.014
Tumor size (cm)					
<4	74	203.1±10.9	0.002	1.00	
≥4	50	100.7±10.1		2.35 (0.88-6.24)	0.086
WHO classification					
Intestinal adenocarcinoma	11	173.3±17.3	0.008		
Tubular adenocarcinoma	59	125.9±8.4			
Mucinous adenocarcinoma	11	84.2±15.3			
Signet-ring cell carcinoma	24	55.54±7.3			
Lauren					
Intestinal	81	193.3±11.1	0.019	1.00	
Diffuse	39	139.5±17.2		1.56 (0.74-3.28)	0.244
Lymph node metastasis					
pN0	59	231.4±8.3	<0.001	1.00	
pN1	47	106.2±10.3		4.05 (1.45-11.27)	0.007
pN2	15	48.1±10.6		6.63 (2.08-21.14)	0.001
Lymphatic invasion					
Absent	70	206.9±10.9	0.002		
Present	50	103.0±9.9			
Vascular invasion					
Absent	105	189.1±9.9	0.009	1.00	
Present	14	85.8±18.9		1.01 (0.49-2.10)	0.974
Perineural invasion					
Absent	68	222.7±9.3	<0.001	1.00	
Present	52	82.8±7.9		1.26 (0.52-3.06)	0.605
RKIP expression					
Positive	58	230.2±8.8		1.00	
Negative	68	84.5±7.1	<0.001	4.53 (1.52-13.53)	0.007

[a]Log-rank test; [b]multivariate Cox proportional of Hazards model; SD, standard deviation; CI, confidence interval.

RKIP expression and correlation with clinical data. The correlations between RKIP expression and clinicopathologic features are summarized in Table II. We found that RKIP is differently expressed between the different WHO histological types (p=0.03), being highly expressed in intestinal type, and lost in tubular, mucinous and signet-ring cell carcinomas. At variance, no statistically differences were observed among the Lauren subtypes. RKIP is significantly lost in advanced gastric cancer when compared with early tumors (p<0.001). Additionally, absence of RKIP expression was statistically associated with tumors with higher tumor size, with higher TNM stage, with the presence of vascular, lymphatic and neuronal invasion and with the presence of lymph node metastasis (Table II).

Correlations with patients survival. We found that additionally to the above mentioned clinical factors, the absence of RKIP expression is also significantly (p<0.001) associated with poor overall survival in gastric carcinoma patients (Table III and Fig. 2). To evaluate whether RKIP expression is an independent prognostic factor, we carried out a multivariate Cox regression analysis and found that absence of RKIP expression is independently associated with patients poor survival with a 4.53 hazard ratio (Table III). Additionally, male gender, higher tumor size and presence of lymph node metastasis were also found to be independent prognostic factors in our series of gastric carcinomas (Table III).

Discussion

Gastric carcinoma is still the fourth most common cancer and the second leading cause of cancer-related death in the world (1). Although excellent long-term survival results for early-

detected gastric cancer exist, prognosis of advanced gastric cancer still remains poor (40). Prognosis of gastric carcinoma patients depends on several pathological and genetic variables, such as TNM grading and p53, MUC1, and E-cadherin (41-44). However, patient outcome is difficult to predict using classic histological and molecular classifications. Therefore, additional markers are required to identify patients with risk to metastasize and with poor prognosis.

Initial *in vitro* studies, showed that cell lines derived from metastatic prostate cancer displayed decreased levels of RKIP as compared with primary tumor cell lines, leading to the suggestion of RKIP as a metastasis suppressor (18). Subsequent studies showed that overexpression of RKIP in prostate and melanoma cell lines suppresses metastasis by decreasing vascular invasion (18,20). Previous studies have described low levels of RKIP in other metastatic tumors, such as breast and colorectal carcinoma (23,45), as well as in many other primary tumors, including GISTs (27), insulinoma (22), hepatocarcinoma (24), ovarian carcinoma (26), merckel cell carcinoma (16) and thyroid carcinoma (15), cutaneous squamous cell carcinoma (46) and nasopharyngeal carcinoma (17). Furthermore, loss of cytoplasmic RKIP has also been associated with poor prognosis in prostate, colorectal, GISTs and glial tumors (25,27-30).

In gastric tumors, previous studies concerning RKIP expression are contradictory. Chatterjee and collaborators reported that in non-neoplastic gastric tissue RKIP cytoplasmic staining was predominantly negative, and in tumor tissues only 29% (42/143) of cases stained positive (31). In contrast, Wang and collaborators described that RKIP is present in ~88% (35/40) of non-neoplastic tissues, in 52% (39/75) of the primary tumors and only in 19% (5/26) of lymph node metastasis (32). More recently, RKIP was shown to be present in 38% (21/55) of gastric cancer tumor tissues (33). In the present study, we showed that RKIP is highly expressed in ~83% (185/222) of non-neoplastic tissues (normal and metaplastic gastric mucosa), but is significantly lost in 55% (93/168) of primary tumors and almost absent in gastric lymph node metastasis with only ~10% (4/42) of the sample staining positive, being in accordance to that described by Wang *et al* (32) in a smaller series. Our results suggested that RKIP could have an important role in normal gastric mucosa and downregulation to gastric cancer progression and metastatic mechanisms. To support this hypothesis, we also found that absence of RKIP protein is statistically associated with the presence of lymph node metastasis, which fits well with described for gastric and other epithelial tumors (18,23,33,45). Additionally, the absence of RKIP expression is significantly associated with clinical features that were associated with poor prognosis in these patients (i.e., advanced tumors, higher tumor size, WHO classification, muscular propria/subserosa invasion, higher TNM stage, and lymphatic, neural and vascular invasion). Significantly, we found that RKIP negativity is an independent prognostic marker of worse prognosis in gastric cancer patients. Despite the reported absence of RKIP expression in non-neoplastic gastric tissue, Chatterjee *et al* also found that the absence of RKIP expression was associated with poor prognosis, but only in intestinal type of gastric cancer (31).

Despite the importance of RKIP as a metastasis and prognostic marker in human cancer, the mechanisms of RKIP downregulation remains to be unraveled (12). Some studies have investigated the methylation status of RKIP promoter in colorectal cancer as a possible mechanism, however, the results are discrepant (12,45,47). In GISTs loss of RKIP expression was not associated with gene promoter methylation (27). Of note, in GISTs, the absence of RKIP was predominant (4 out of 6) in tumors with a gastric location (27). Further studies are needed to evaluate the possible mechanisms of RKIP downregulation in gastric cancer.

Due to the pivot role of RKIP in tumor progression and in metastasis, its re-activation can constitute an attractive therapeutic strategy. In non-Hodgkin's lymphoma cell lines it was shown that treatment with Rituximab induced RKIP upregulation, with further sensitization to chemotherapeutic induced apoptosis (48). Other studies reported that RKIP can be induced by nitric oxide or the proteasome inhibitor NPI-0052, via NF-κB inhibition (49-51).

We herein reported the frequency of RKIP expression in a large and clinically well-characterized series of different gastric tissue samples. We showed that RKIP expression is lost during gastric tumor progression, been practically absent in lymph node metastasis. Importantly, we observed that the RKIP loss is associated with other clinical characteristic of tumor aggressiveness and constitutes an independent biomarker of poor prognosis in gastric cancer patients.

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