Inverse Association between Raf Kinase Inhibitory Protein and Signal Transducers and Activators of Transcription 3 Expression in Gastric Adenocarcinoma Patients: Implications for Clinical Outcome

Devasis Chatterjee,1,3 Edmond Sabo,2,3 Rosemarie Tavares,2 and Murray B. Resnick2,3

Abstract

Purpose: Raf Kinase Inhibitory Protein (RKIP) plays a pivotal role in cancer by regulating apoptosis induced by chemotherapeutic agents, or immune-mediated stimuli and is a metastasis suppressor protein. The signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is frequently activated in gastric adenocarcinomas, thereby promoting tumor growth. We examined the expression patterns of RKIP and STAT3 with regard to human gastric cancer, predicting that elevated RKIP status may favor clinical outcome.

Experimental Design: Tissue microarrays were created from samples from 143 patients with gastric adenocarcinomas. The microarrays were immunohistochemically stained for RKIP and STAT3, and the intensity and extent of the staining was semi-quantitatively scored.

Results: In intestinal-type gastric adenocarcinomas, RKIP and STAT3 expression were inversely associated. Cytoplasmic RKIP expression directly correlated with patient survival. Nuclear STAT3 expression inversely correlated with survival. In the diffuse tumor type, no significant correlation was found between RKIP and patient outcome. In the intestinal-type gastric adenocarcinoma, multivariate analysis adjusted for treatment types revealed RKIP and tumor stage to be significant independent predictors of survival. In the diffuse tumor type, stage was the only significant predictor of survival.

Conclusion: These results indicate the predictive and protective role of cytoplasmic RKIP expression in gastric adenocarcinoma of the intestinal subtype. In contrast, nuclear STAT3 expression is associated with poor patient prognosis in the intestinal subtype. Significantly, we show an inverse association between RKIP and STAT3 and a positive correlation between RKIP and patient survival.

Gastric cancer is one of the most frequently diagnosed malignancies (1, 2) especially in East Asian countries such as China, Japan, and Korea (3). Advances in diagnosis have allowed excellent long-term survival results for early-detected gastric cancer, but the prognosis of advanced gastric cancer still remains poor (4). In the US, ~24,000 cases of gastric cancer are diagnosed annually (5), and there are 12,700 estimated deaths each year (3).

The most characterized gastric carcinogenesis sequence begins with inflammatory changes in the stomach lining, leading to chronic atrophic gastritis and intestinal metaplasia ending in gastric carcinoma (6). However, the precise molecular mechanism of this multistep progression remains largely unknown. Multiple cumulative series of structural and functional genetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules, growth factors/receptors, and genetic instability of other genetic foci are implicated in the development and progression of the multistep gastric carcinogenesis (7–10). Studies have indicated that gastric cancers may be related to genetic alterations in several genes, such as p53, APC, E-cadherin, β-catenin, TGF-β, c-met, and trefoil factor 1 (11, 12).

Constitutive activation of signal transducers and activators of transcription (STAT) proteins has been associated with a number of different malignancies, including gastric cancer (13). STAT proteins are latent cytoplasmic transcription factors that, upon activation, translocate to the nucleus and bind to specific regulatory elements that control gene expression. STAT3 protein constitutive activation and nuclear localization is associated with cancer cell proliferation and metastasis (13). Dysregulated STAT3 activation has been linked to the development and progression of gastric adenocarcinoma via induction of vascular endothelial growth factor overexpression leading to an elevated angiogenic phenotype (13). Unlike in normal cells and tissues, constitutively activated STATs are detected in a wide variety of human tumors. Thus, an aberrant activation of STATs,
Table 1. Clinicopathologic characteristics of 143 gastric carcinoma patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Age at surgery (y)</td>
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</tr>
<tr>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>31-96</td>
</tr>
<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
<td>75</td>
</tr>
<tr>
<td>Female</td>
<td>68</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
</tr>
<tr>
<td>Diffuse*</td>
<td>80</td>
</tr>
<tr>
<td>Intestinal</td>
<td>63</td>
</tr>
<tr>
<td>Diffuse</td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>60</td>
</tr>
<tr>
<td>Dead gastric cancer</td>
<td>57</td>
</tr>
<tr>
<td>Dead unrelated</td>
<td>7</td>
</tr>
<tr>
<td>Information Unavailable</td>
<td>19</td>
</tr>
</tbody>
</table>

*Diffuse subtype includes mixed diffuse and intestinal subtype.

† American Joint Committee on Cancer stage.

especially STAT3, is often associated with cell survival, proliferation, and transformation (14, 15). Constitutive activation of STAT3 seems to have an important role in the survival of gastric cancer cells by suppressing apoptosis and enhancing cell proliferation (16). Supporting this hypothesis, several reports indicate that constitutively activated STAT3 is a target for antitumor drug discovery (17).

As opposed to STAT3, the Raf kinase inhibitory protein (RKIP) is a member of the phosphatidylethanolamine-binding protein family (18) and is a negative regulator of the mitogen-activated protein kinase cascade initiated by Raf-1 (19, 20). RKIP is also considered to play a pivotal role in cancer, immune-mediated stimuli (21). Recent studies have shown that RKIP is a novel and clinically relevant metastasis suppressor gene of prostate (22), breast (23), colon (24, 25), and melanoma human tumor models (26).

In studies directed to investigate chemotherapy-triggered apoptosis, we observed that the levels of RKIP were robustly induced (21). Concomitant with the induction of RKIP, chemotherapy-triggered apoptosis resulted in the inhibition of STAT3 activation and expression (27). Therefore, we hypothesized that there may be an association between RKIP and STAT3 levels in tumors. In this study, we used tissue microarrays to correlate the degree of RKIP and STAT3 expression with patient survival in gastric cancer and further correlated their expression with patient outcome.

Materials and Methods

Patients and specimens. Archival cases of distal gastric cancer from 143 consecutive patients were collected between the years of 1992 and 2004 from the archives of the Department of Pathology at the Rhode Island Hospital. Proximal (cardia) cancers were excluded so as to minimize confounding variables, as cancers in this part are considered by many to be a distinct clinical entity compared with distal tumors. Stage was defined according to American Joint Committee on Cancer criteria (28). Recurrence and survival data were ascertained through the Rhode Island Tumor Registry and Rhode Island Hospital chart review. The Institutional Review Board at the Rhode Island Hospital approved this study. All tissue samples were formalin fixed and paraffin embedded. The corresponding H&E slides were reviewed for confirmation of diagnosis and adequacy of material by ES and MR. All the cases were classified using the Lauren system as intestinal or diffuse-type gastric adenocarcinoma. Tumors exhibiting a mixed intestinal and diffuse pattern were classified as diffuse.

Tissue microarray construction. Paraffin blocks containing areas consisting of pure invasive carcinoma were identified on corresponding H&E-stained sections as previously described (29). Areas of interest that represented nonneoplastic invasive adenocarcinoma were identified and marked on the source block. The source block was cored, and a 1-mm core was transferred to the recipient “master block” using the Beecher Tissue Microarrayer (Beecher Instruments). Three to six cores of tumor were arrayed per specimen. In addition, a core of normal adjacent gastric mucosa and areas of intestinal metaplasia were also sampled when present.

Immunohistochemistry. Immunohistochemistry for each antigen was done on 5-µm-thick paraffin sections of each gastric cancer tissue microarray sample described above. The microarrays were immunohistochemically stained for RKIP (polyclonal rabbit, 1:100; Upstate Biotechnology) and STAT3 (polyclonal rabbit; 1:150; Santa Cruz Biotechnology, Inc.) using the Ventana Discovery automated system (Ventana Medical Systems, Inc.). It has been our experience that the pY705 STAT3 antibody does not stain resection specimens very well or consistently (all of our cases are resections). We have found that pY705 STAT3 antibody is more effective with biopsy specimens. STAT3 is not localized to the nucleus unless it is dimerized, which requires tyrosine and serine phosphorylation and lysine K685 acetylation (30). Therefore, the localization of STAT3 to the nucleus can be detected with a full-length STAT3 antibody. Brieﬂy, after deparaffinization, antigen retrieval was done in 10-mmol/L citrate buffer using a microwave/pressure cooker. Protease pretreatment at 37°C for 5 min was done before blocking. After H2O2 and serum blocking, slides were incubated with the primary antibody at room temperature overnight. The secondary antibody was a goat anti-rabbit IgG biotin (1:500; Vector Laboratories). Detection was done with the 3,3′-diaminobenzidine kit (Vector Laboratories). Slides were counterstained with hematoxylin, dehydrated, cleared, and mounted. Positive controls consisted of multitumor and normal tissue microarrays generated in our department. Negative controls included replacement of the primary antibody with nonreacting antibodies of the same species.

Quantitative immunohistochemical analysis. The nuclear as well as the cytoplasmic staining patterns were separately quantified, for both RKIP and STAT3, using a semiquantitative system for evaluation and grading of the immunostaining pattern, successfully applied by others (31). The staining intensity was scored into four grades: 0 for complete absence of the staining, 1 for weak staining, 2 for moderate, and 3 for strong staining. The extent of the positively stained cells was also scored into four grades: 0 for a completely negative staining, 1 for <10%, 2 for 10% to 50%, and 3 for tumors with 50% or greater cells staining positive. The final scores were derived from multiplication of the extent by the intensity. For the statistical analysis, scores were further grouped in two categories as follows: negative (final scores, <4) and positive (final scores, ≥4). At least three cores were scored per case. All sections were scored independently by MR and ES and were blinded to the clinicopathologic features or clinical outcome.

Statistical analysis. Statistical analyses were done using the SPSS version 10 statistical program. Kaplan Meier survival curves were

4 M.B. Resnick, unpublished results.
constructed and the Log-rank or the Breslow tests were used as needed for the univariate comparison between RKIP and STAT3 expression categories. Cox’s multivariate test applied in a stepwise forward method was used to adjust for potentially confounding variables (e.g., stage and type of therapy) and to evaluate the role of RKIP and STAT3 as independent predictors of patient prognosis. The Fisher’s exact test was used to evaluate the association between the RKIP and STAT3 expression values in the gastric tumors. Two-tailed P values of 0.05 or less were considered to be statistically significant.

Results

Clinicopathologic features. The mean age of the patients at initial surgery was 71.1 years (range, 31-96 years); 75 men and 68 women were included in this study. The mean duration of follow-up was 34 months (range, 12-180 months). Based on the Lauren Classification System, 63 cases were of the intestinal type (moderately/well-differentiated or low grade) and 80 were diffuse or mixed types (poorly differentiated or high grade). All of the tumors originated from the antral/pyloric and body/fundic regions. The American Joint Committee on Cancer tumor stage distribution and vital status of the patients are shown in Table 1. In the intestinal type, 17 patients received chemotherapy, whereas 43 patients did not; 12 patients received radiotherapy and 48 patients did not. In the diffuse type, 27 patients received chemotherapy, whereas 53 patients did not; 17 patients received radiotherapy and 63 did not.

Expression of RKIP and STAT3 in nonneoplastic gastric mucosa. Table 2 shows the distribution of RKIP and STAT3 staining scores in the foveolar, glandular, and intestinal metaplasia epithelial cells in a number of nonneoplastic evaluated cores. The RKIP nuclear scores were positive in 0 of 74, 2 of 74, and 12 of 51 of the foveolar, glandular, and metaplastic cells, respectively. The RKIP cytoplasmic scores were positive in 4 of 74, 25 of 74, and 25 of 51 of the foveolar, glandular, and metaplastic cells, respectively. The STAT3 nuclear scores were positive in 0 of 62, 3 of 62, and 8 of 51 of the foveolar, glandular, and metaplastic cells, respectively, in these control cores. The STAT3 cytoplasmic scores were positive in 0 of 62, 14 of 62, and 18 of 51 of the foveolar, glandular, and metaplastic cells, respectively.

Staining pattern distribution of RKIP and STAT3 in gastric cancer. The staining pattern for RKIP in the tumor cells was cytoplasmic in all but two diffuse-type tumors showing a mixed cytoplasmic and nuclear pattern. Representative examples of
RKIP staining in both intestinal and diffuse types of gastric cancer are shown in Fig. 1A-D. The STAT3 staining pattern was mixed, cytoplasmic and nuclear, in both intestinal and diffuse tumor types (Fig. 2A-D).

We examined cytoplasmic and nuclear staining patterns for RKIP and STAT3 in intestinal and diffuse tumor types (Table 3). In diffuse tumor type, nuclear RKIP staining was positive in 4 of 80 cases (5%). The cytoplasmic RKIP staining was positive in 20 of 80 cases (25%). In the intestinal tumor type, RKIP nuclear staining was positive in 3 of 63 cases (4.7%). RKIP cytoplasmic staining was positive for 22 of 63 cases (35%). STAT3 nuclear (28 of 80) was positive in 28 of 80 cases (35%), and STAT3 cytoplasmic was positive in 17 of 80 cases (21%). In the intestinal tumor–type STAT3 nuclear staining, 12 of 63 cases (19%) were positive, whereas for cytoplasmic staining, 20 of 63 cases (32%) was positive.

**RKIP and STAT3 coexpression patterns.** In the intestinal type, 17.5% (11 of 63) tumors that exhibited positive STAT3 expression were negative for RKIP, whereas 36.5% (23 of 63) tumors with negative STAT3 expression were positive for RKIP. Only 1 of 63 cases (1.5%) exhibited a positive staining pattern for both STAT3 and RKIP markers. In 41% (26 of 63) of cases, both RKIP and STAT3 were negative (Fig. 3A-B). An example of the discordant staining for RKIP and STAT3 in the intestinal tumor type is shown in Fig. 3A-B.

Statistically, these findings clearly indicated the existence of a significantly inverse association between STAT3 and RKIP expression ($P = 0.02$; odds ratio, 0.11; Fisher’s exact test). In contrast, the diffuse tumor type showed no significant

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**Table 3.** Distributions of RKIP and STAT3 IHC expression in both tumor types

<table>
<thead>
<tr>
<th>Marker and staining score</th>
<th>Intestinal tumor type</th>
<th>Diffuse tumor type</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKIP nuclear</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (score, ≥4)</td>
<td>3</td>
<td>4</td>
<td>0.94</td>
</tr>
<tr>
<td>Negative (score, &lt;4)</td>
<td>60</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>RKIP cytoplasmic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (score, ≥4)</td>
<td>22</td>
<td>20</td>
<td>0.19</td>
</tr>
<tr>
<td>Negative (score, &lt;4)</td>
<td>41</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>STAT3 nuclear</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (score, ≥4)</td>
<td>12</td>
<td>28</td>
<td>0.034</td>
</tr>
<tr>
<td>Negative (score, &lt;4)</td>
<td>51</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>STAT3 cytoplasmic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (score, ≥4)</td>
<td>20</td>
<td>17</td>
<td>0.15</td>
</tr>
<tr>
<td>Negative (score, &lt;4)</td>
<td>43</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>
RKIP disrupts the Raf-1-MEK1/2-ERK1/2 and NF-
involved in growth and differentiation signaling regulation. 

is a highly species-conserved ubiquitously expressed protein 
(19, 20). Although several tissue microarray studies have 
been done on RKIP and clinical outcome, our study is the first 
to evaluate the association between RKIP and STAT3 and 
clinical outcome.

In drug-curable malignancies, apoptosis is a prominent 
mechanism associated with the induction of tumor remission 
(33). Apoptotic cell death triggered by the activation of caspase 
proteases is regulated by numerous chemotherapeutic com-
ounds (33). A number of cell death pathways converge on the 
activation of the caspase cascade and are initiated with cell 
surface death receptors and their ligands (CD95 and TNFR; 
ref. 34). Conversely, activation of other cell surface receptors 
can lead to the activation of cell survival pathways (Raf/MEK/ 
ERK and Janus-activated kinase/STAT). Clearly, the balance 
between apoptotic and survival pathways will predict the 
response of malignant cells to chemotherapeutic compounds. 
The induction of RKIP expression results in apoptosis in human 
cancer models (21), indicating that RKIP is an important 
determinant of apoptosis sensitivity. In nonneoplastic gastric 
tissue, the staining for nuclear and cytoplasmic RKIP was 
predominantly negative (Table 2). However, ~25% of cases 
stained positive for nuclear and 50% of cases stained positive 
for cytoplasmic RKIP in epithelium with intestinal metaplasia 
(Table 2). A sequence of histomorphologic changes leads to 
gastric cancer. The molecular changes that gradually occur in 
the intracellular signaling pathways are reflected in the 
development of a morphologic sequence of changes in the 
gastric mucosa, ranging from chronic atrophic gastritis through 
testinal metaplasia to different grades of dysplasia that 
eventually may transform into gastric cancer (35, 36). We 
speculate that positive nuclear and cytoplasmic RKIP staining 
in epithelium with intestinal metaplasia may indicate that some of 
these cells undergo RKIP-directed apoptosis in an attempt to 
inhibit further development of the metaplasia-dysplasia-
carcinoma sequence.

**RKIP expression in other malignancies.** Our results are in 
agreement with several studies that have shown that RKIP is a 
tumor suppressor protein. Fu et al. (37) showed that the low 
levels of RKIP mRNA and protein are correlated with the 
metastatic potential of human C4-2B prostate cancer cells when 
compared with parental nonmetastatic LNCaP cells. Moreover, 
overexpression of RKIP in C4-2B cells decreased cell invasion 
in vitro and progression of lung metastases in vivo. In addition,
increased levels of RKIP were associated with decreased vascular invasion in the primary tumor with no effect on primary tumor growth in mice (37). In human breast cancer, RKIP is a metastasis suppressor protein whose expression must be downregulated for metastases to develop. Hagan et al. (23) found that RKIP expression is reduced in lymph node metastases of 103 breast cancer patients. Their results support the conclusion that RKIP is a metastasis suppressor protein, as its expression is consistently lost in lymph node metastases but not in primary tumors. In malignant melanoma cells, loss of RKIP also correlates with enhanced invasion and progression of the disease (29). These results together with the study in prostate cancer (37) suggest that RKIP does not affect the tumorigenic properties of these cells but may represent a clinically significant suppressor of metastasis by decreasing vascular invasion (37).

Similar results have been obtained in a study of metastatic colorectal cancer (24, 25) where RKIP expression was downregulated in lymph node metastases. The authors have also reported RKIP expression to inversely correlate with tumor recurrence and directly correlate with survival in these colon cancer patients. These correlation were maintained after adjusting for stage and for other variables, in a multivariate analysis. Loss of cytoplasmic RKIP has also been associated with distant metastasis, vascular invasion, and poor prognosis in mismatch repair proficient and deficient colorectal cancer patients (25). Thus, loss of RKIP expression may be considered to be a marker of tumor progression. Our results in the intestinal type of gastric adenocarcinoma have shown that a positive cytoplasmic RKIP expression (found in 33% of the cases; Table 3), significantly and directly correlated with patient survival (Fig. 4A). Paradoxically, this result was not observed in the diffuse gastric adenocarcinoma type that is a less differentiated and more aggressive tumor than the intestinal type of gastric adenocarcinoma. We speculate that distinct molecular pathways (postranslational modifications) may differently regulate the activation and expression of RKIP in these two tumor types. Alternatively, proteins regulated by STAT3 may also be differentially expressed in the intestinal or diffuse tumor type, leading to the inhibition of RKIP function. Protein factors that RKIP regulates to induce apoptosis are also unknown and hypothetically could be mutated or silenced, in the diffuse gastric tumor type. We are currently examining the molecular and genetic pathways linked to RKIP and STAT3 regulation via gene chip microarrays to better understand the differences in the biological behavior between these two tumor types.

**Potential mechanism of action.** The acquisition of resistance to conventional therapies such as radiation and chemotherapeutic drugs remains a major obstacle in the successful treatment of gastric cancer patients. In this regard, one of the major determinants of apoptosis sensitivity in gastric cancer and other malignancies is the transcription factor, NF-κB. Constitutive expression and activation of NF-κB has been implicated in decreasing apoptosis in gastric cancer cell lines and tumors (38). Inhibition of NF-κB activity reduces chemoresistance to 5-fluorouracil in human stomach cancer cell lines (39). A major target of NF-κB transcriptional activation in human cancer cells is the prosurvival cytokine interleukin-6 (IL-6; ref. 40). Aberrant and/or constitutive expression of IL-6 and elevated levels has been implicated in the progression of gastric cancer as well as acquired resistance to chemotherapy (41). Serum IL-6 levels correlate with disease status of gastric cancer and has been proposed to represent a new tumor marker for monitoring treatment and response of gastric cancer patients (42). IL-6 activates the Janus-activated kinase and STAT signaling pathways for cell proliferation and survival (16). STAT3 is constitutively activated in gastric cancer cell lines and tumors (42–44) and may enhance gastric tumor progression.

**Table 4. Multivariate analysis of survival in the intestinal tumor type (Cox’s test)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SE</th>
<th>P</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>0.5036</td>
<td>0.1732</td>
<td>0.0036</td>
<td>1.6547</td>
</tr>
<tr>
<td>RKIP</td>
<td>-1.2156</td>
<td>0.5567</td>
<td>0.0290</td>
<td>0.2965</td>
</tr>
</tbody>
</table>
by affecting the expression of various genes related to cell survival, the cell cycle, invasion, and angiogenesis (45–48). The inhibition of constitutive STAT3 activation induces apoptosis in association with a reduction of survivin expression in gastric carcinoma cell lines (16). Gong et al. (13) showed that abnormally activated STAT3 expression represents a potential risk factor for poor prognosis and directly contributes to gastric cancer angiogenesis and patient survival. Thus, our findings are in agreement and show that increased nuclear STAT3 expression is correlated with poor prognosis (Fig. 4B), a result that is consistent with the oncogenic properties of this protein. We have extended our analysis and show for the first time that there is an inverse association between RKIP and STAT3 and gastric patient survival.

Dysregulation of apoptosis will allow metastatic cell survival and confer resistance to chemotherapeutic drugs. STAT3 proteins, which are activated via posttranslational modifications by the IL-6 family of cytokines, prevent apoptosis through the regulation of antiapoptotic proteins such as Bcl-2 and survivin. We have shown that in human cell culture models, RKIP blocks IL-6–mediated STAT3 phosphorylation and activation (49, 50). Strict regulation of STAT3 activation is imperative for preventing tumorigenesis and maintaining normal cell growth; STAT3 destabilization and transcriptional inactivation are crucial early events required to prevent neoplastic transformation of cells. STAT3 plays a critical role in normal cell migration or cancer cell metastasis. However, the common pathways of carcinogenesis and the subsequent progression of gastric cancer remained to be elucidated. The ability to manipulate RKIP expression represents a novel and important therapeutic approach for the treatment of gastric cancer. Further studies need to be done to determine if the RKIP-STAT3 axis can be used as a chemotherapeutic target to improve clinical outcome.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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