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Role of Raf Kinase Inhibitor Protein in *Helicobacter pylori*-Mediated Signaling in Gastric Cancer

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Abstract

Helicobacter pylori is a helical bacterium that colonizes the stomach in over half of the world's population. Infection with this bacterium has been linked to peptic ulcer disease and gastric cancer. The bacterium has been shown to affect regulatory pathways in its host cells through specific virulence factors that control gene expression. Infection with *H. pylori* increases levels of phosphorylation of Raf kinase inhibitor protein (pRKIP) in gastric adenocarcinoma (AGS) cells *in vitro* and *in vivo*. We investigated the role of *H. pylori* in the phosphorylation of RKIP as a possible mechanism to downregulate pro-survival signals in gastric adenocarcinoma. pRKIP induces RKIP transcriptional activity, which serves to induce apoptosis of damaged cells to prevent further tumorigenesis. Infection of wild type and RKIP knockout mice with *H. pylori* for 2 months further confirmed roles of RKIP and pRKIP in the prevention of gastric cancer progression. Loss of RKIP in AGS cells results in increased expression of the Cag A virulence factor after *H. pylori* infection and RKIP overexpression inhibits *H. pylori*-mediated STAT3 phosphorylation and STAT3 and NF- κ B transcriptional activity. We examined the role of mTOR (mammalian target of rapamycin) after *H. pylori* infection on the phosphorylation of RKIP. Cells treated with rapamycin, an inhibitor of mTOR, displayed less expression of pRKIP after *H. pylori* infection. Microarray antibody analysis was conducted on wild-type and RKIP-knockdown AGS cells and showed that in the absence of RKIP, there was increased expression of pro-tumorigenic proteins such as EGFR, Raf-1, and MAPKs. Although further work is needed to confirm the interaction of RKIP and mTOR in AGS cells as a result of *H. pylori* infection, we hypothesize that *H. pylori*-mediated induction of pro-survival signaling in gastric epithelial cells induces a feedback response through the activation of RKIP. The phosphorylated, or active, form of RKIP is important in protecting gastric epithelial cells from tumorigenesis after *H. pylori* infection.

Keywords

Cag A; gastric cancer; *H. pylori*; RKIP; STAT3

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I. INTRODUCTION

Despite its slowly decreasing incidence, gastric cancer remains the third most lethal neoplasm in North America.¹⁻³ In the United States, 24,000 diagnosed cases and 14,000 deaths are reported annually.³ Gastric adenocarcinoma usually develops asymptotically.³ By the time it is diagnosed, in 85% of the cases the tumor has already metastasized to the lymph nodes.³ Gastric adenocarcinoma can be classified into two pathologic categories depending on the growth pattern of the tumors. Diffuse tumors are non-glandular and deeply invasive into the stomach wall.³ This type of cancer, which occurs more frequently in younger patients, is generally influenced by hereditary rather than environmental factors. Intestinal gastric adenocarcinoma can be observed due to its expanding, rather than infiltrating, growth pattern.³ The tumor's name derives from its ability to form glands similar in morphology to the glands found in intestinal mucosa.³ Unlike diffuse tumors, intestinal-type cancers are usually the result of chronic atrophic gastritis.³

Environmental, rather than hereditary, factors have been implicated in the development of 90% of all gastric cancers³ indicating that the cancer can be prevented by identifying and eradicating those factors. Case studies have shown that the children of migrants from high-risk areas have the same incidence rate of gastric cancer as the natives in their new environment.³ Other environmental and social risk factors include a diet rich in salty or pickled foods, habitual alcohol consumption, tobacco, and obesity.³ However, the highest identifiable risk factor remains gastric infection with the bacteria *Helicobacter pylori*; 60% of gastric cancers are attributed to the bacterium.⁴ In 1994, the World Health Organization classified the bacterium as a Class I carcinogen due to its proven link to gastric cancer.²

Several biochemical and physiological factors may be contributing to the carcinogenic nature of the bacterium. The inflammatory response is one mechanism by which the bacterium contributes to tumorigenesis in gastric epithelia.¹ In transgenic mice, *H. pylori* was found to increase I-1 β (interleukin-1-beta) expression, resulting in dysplasia.¹ Furthermore, chronic cell turnover in response to infection increases the number of tumorigenic mutations in epithelial cells.¹ In addition to responses secondary to infection, *H. pylori* directly injects carcinogenic virulence factors into the host cells, including the protein Cag A, the product of the cytotoxin-associated gene A.¹ This protein alters the cytoskeleton of host cells, giving rise to cell scattering.¹ Furthermore, many studies have identified possible downstream pro-tumorigenic and anti-apoptotic signals that are activated in the host cell, including the downregulation of the apoptosis regulator p53.⁵

Ten percent of hosts infected with the bacterium develop peptic ulcer disease and 1–2% develop noncardia gastric cancer.² Three important virulence factors have been identified in the bacterial genome: *cagA*, which codes for Cag A; *vacA* (vacuolating exotoxin), and *BabA* (blood group antigen binding adhesion).² The *cag* pathogenicity island (*cag* PAI) contains genes for cytotoxin-associated antigens, which encode the type 4 secretion system characteristic of the bacterium.² This system proceeds through an appendage on the bacterium that attaches to the host cell in order to efficiently deliver its virulent gene products, including Cag A, which is positively correlated to the development of atrophic gastritis, ulceration, and gastric adenocarcinoma in Western populations.² Several studies

have established a link between Cag A and bacterial carcinogenesis. One study found that Cag A deletion prevents gastric tumorigenesis in gerbils, while transgenic expression of Cag A in mice manifests into gastric epithelial hyperplasia and adenocarcinoma in the stomach and small intestine.²

Cag A alters the gene expression in the host cell through phosphorylation-dependent and phosphorylation-independent effects.² Once in the host, Cag A is phosphorylated in its 5 amino-acid repeat region EPIYA (Glu-Pro-Ile-Tyr-Ala).² Phosphorylated Cag A interacts with SRC homology (SH2) domain-containing host cell proteins, including tyrosine phosphatase SHP-2 and C-terminal SRC tyrosine kinase (CSK).² SHP-2 dephosphorylates FAK (focal adhesion kinase 1), downregulating FAK kinases, which in turn upregulates ERK (extracellular signal-regulated kinases) and MAPK (mitogen activated protein) kinases.² Through the activation of ERK and MAP kinases and its interaction with the adaptor protein CRK, Cag A contributes to cytoskeletal reorganization and cell elongation, giving rise to the “hummingbird” phenotype, characterized by cell scattering.² Through its activation of MAP/ERK kinases, Cag A also facilitates the progression of the cell cycle, further linking the protein to cellular transformation and, thus, gastric tumorigenesis.² Cag A also exerts phosphorylation-independent effects on the cell’s functions, including trans-differentiation of morphology and the recruitment of immune cells to trigger the inflammatory response.² Non-phosphorylated Cag A disrupts tight and adherent junctions, giving rise to an invasive phenotype.² Furthermore, its interaction with E-cadherin prevents the formation of the E-cadherin/ β -catenin complex.² The accumulation of β -catenin in the nucleus induces transcription of genes responsible for intestinal differentiation, which may be the cause of intestinal metaplasia in precancerous gastric cells.² Through its interaction with growth factor GRB2, the RAS/MEK/ERK pathway is activated, which further contributes to cell scattering and proliferation.² Finally, Cag A expression induces the recruitment of inflammatory cytokines and chemokines, including IL-8 (interleukin-8), which through the Ras/Raf pathway stimulates the activation of the anti-apoptotic protein NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells).² IL-8 expression has been identified in gastric mucosal biopsy samples of infected patients.⁶ Interleukin-8 recruits neutrophils to the site of infection, stimulating the inflammatory response.⁶ Cag A-positive *H. pylori* infection of AGS cells has been shown to induce the phosphorylation of Akt, or protein kinase B, an apoptotic inhibitor.⁷ Cag A-negative strains, on the other hand, fail to induce this response. Phosphorylation of Akt causes a simultaneous decrease in p21 and p27 mRNA levels in AGS cells.⁷ All of these events further support the carcinogenic role of Cag A within the host cell.

Since *H. pylori* can activate cell survival signals through the Raf/MAPK/ERK pathways, we investigated the role of an inhibitor of these pathways, RKIP. RKIP is a phosphatidylethanolamine-binding protein that acts as an inhibitor of the Raf-1-initiated protein kinase cascade, which is activated by mitogen activity during cell division.⁸ Furthermore, Raf-1 activates the ERK pathway via MEK-1 phosphorylation; ERK kinases control cell proliferation and cell differentiation.⁹ RKIP inhibits phosphorylation of MEK-1 by binding to both Raf-1 and MEK; RKIP can also bind to ERK, thereby inactivating it.⁹ RKIP inhibits cell survival by downregulating effectors in the Ras-Raf, NF- κ B, and GRK2 pathways.^{10,11} Furthermore, RKIP acts as a metastasis suppressor in colon, melanoma,

breast, and prostate cancers.¹²⁻¹⁵ In addition, RKIP is necessary for chemotherapy-induced apoptosis in breast and prostate cancer cells.¹⁶ RKIP activates mitotic checkpoints and cell-cycle arrest by binding to Raf-1, which in turn suppresses the MAPK pathway that controls mitotic spindle assembly.¹⁷ Phosphorylation of RKIP at the S153 site by PKC (protein kinase C) has been suggested to induce the release of Raf-1 from its binding pocket on RKIP, thereby activating the MAPK pathway.¹⁸ The phosphorylation of RKIP by *H. pylori* is required for *H. pylori*-mediated apoptosis in AGS gastric cancer cells.¹⁹ We investigated the role of *H. pylori* in the phosphorylation of RKIP as a possible mechanism by which downstream pro-survival signals are activated in gastric cancer epithelial cells.

In intestinal type gastric carcinoma, there has been observed an inverse expression of STAT3 and RKIP, with RKIP expression serving as a positive indicator of patient survival.⁸ STAT3, or signal transducer and activator of transcription 3, is associated with cell survival, proliferation, and transformation.⁸ NF- κ B, a downstream protein of STAT3 activation, is constitutively expressed in gastric carcinoma, suggesting that *H. pylori* infection increases NF- κ B expression through STAT3 activation. Although RKIP expression was associated with a better prognosis in gastric cancer patients in one study, cells undergoing intestinal metaplasia also showed positive RKIP staining.⁸ This could be an attempt by the host cell to induce RKIP-mediated apoptosis to prevent further tumorigenesis.⁸

Rapamycin is an antibiotic and chemotherapeutic drug that inhibits the activation of mTOR (mammalian target of rapamycin), which induces cellular growth and proliferation²⁰ and is a Ser/Thr kinase.²¹ Rapamycin only suppresses one type of mTOR complex known as raptor-mTOR (TORC1); rictor-mTOR (TORC2) is rapamycin resistant.²⁰ By binding to the FKB12 protein, rapamycin forms a drug-receptor complex that interrupts mTOR kinase activity, although the direct mechanism of inhibition is still unclear.¹⁹ The downstream effectors of mTOR, S6 kinase 1 (S6K1) and 4E-BP1 proteins, control mRNA translation to induce cell growth¹⁹; their activation proceeds via phosphorylation by mTOR.^{21,22} Rictor-mTOR, on the other hand, activates Akt/PKB signaling, a component of the insulin/P13K signaling pathway that controls cell survival and proliferation.²⁰

Growth factors, amino acids, glucose, energy status, and stresses such as DNA damage act upstream to activate mTOR.²⁰ Akt signaling reduces the AMP/ATP ratio, which activates raptor-mTOR to raise it.²⁰ Interestingly, rictor-mTOR acts upstream of Akt, which indicates that it could serve as a regulator of raptor-mTOR.²⁰ Cells that lack tumor suppressors such as PTEN, p53, and NF1 exhibit hyperactive Akt signaling, and therefore, active raptor-mTOR signaling.²⁰ These cancer cells are sensitive to treatment with rapamycin to halt cell proliferation, which is clinically favorable.²⁰ In poorly vascularized tumors, raptor-mTOR activity is low due to the absence of nutrients.²⁰ The downstream effector of mTOR, S6K1, has been shown to provide negative feedback to the insulin/P13K/Akt pathway.²⁰ Hypothetically, this could create a clinically unfavorable response to overtreatment with rapamycin.²⁰ Overtreatment would downregulate mTOR, which would in turn downregulate S6K1, removing the negative feedback to Akt, which would stimulate cell survival signaling.²⁰

The kinase mTOR's role in tumor growth has been attributed both to its activation by phosphorylation and location within the cell.²² A clinical study found that cytoplasmic p-mTOR levels are positively correlated to deeper tumor invasion, higher tumor stage, lower survival rate, and a higher relapse rate.²² Nuclear p-mTOR showed the opposite result among gastric cancer patients, suggesting that mTOR-mediated tumorigenesis proceeds by localization of active mTOR in the cytoplasm.²² In gastric cancer cells, Akt is an activator of mTOR.²¹ However, no clear link between upregulated, phosphorylated Akt has been established.²² One study observed that p-Akt suppressed p53 activity⁵ and another found p-Akt present in preneoplastic lesions of bronchioles.²² In contrast, several studies found no link between expression and pathological factors or survival rates.²² Nevertheless, normal gastric mucosa does not show p-mTOR or p-Akt expression,²² indicating that the activation and dysregulation of the pathway plays a role in tumorigenesis.

II. RKIP-STAT3 AXIS IN GASTRIC CANCER

In previous studies, RKIP and STAT3 showed an inverse expression in intestinal gastric cancer tumors.⁸ This relationship is expected since RKIP is an inhibitor of dysregulated cell proliferation through the Raf/MAPK pathways. STAT3, on the other hand, is activated in tumorigenesis and activates NF- κ B, which is constitutively active in tumor cells and promotes DNA transcription.⁸ Therefore, we expected to see an inhibitory relationship between RKIP and STAT3 in AGS cells. The RKIP-STAT3 axis in AGS cells was followed by quantitative analysis of relative transcriptional activity through the luciferase assay. STAT3 is a transcription factor that is associated with cell survival, proliferation, and transformation⁸ to enhance tumorigenesis in cancers, including gastric carcinoma.² We observed an increase in STAT3 transcriptional activity and pY705 phosphorylation after *H. pylori* infection (Fig. 1). Interestingly, STAT3 transfection is able to inhibit some of this activation, perhaps by inactivating RKIP before infection, suggesting a feedback mechanism (data not shown). Transfection RKIP is able to inhibit STAT3 transcription levels and pY705 phosphorylation. Because it is still unclear if pRKIP enhances or suppresses the apoptotic effects of RKIP, it is hard to determine if STAT3-mediated inhibition of RKIP transcriptional activity occurs due to an induction or downregulation of phosphorylation of RKIP. More likely, pRKIP enhances the transcription of RKIP, and thereby its apoptotic effects.

NF- κ B, a downstream effector of STAT3 activation, is constitutively expressed in gastric carcinoma,⁸ suggesting that *H. pylori* infection increases NF- κ B expression through STAT3 activation. Since there is an inverse relationship between STAT3 and RKIP expression in gastric carcinoma, one would expect *H. pylori* to activate NF- κ B in order to promote cell survival. *H. pylori* infection did result in the increase of NF- κ B transcriptional activation (Fig. 1). This effect again was abrogated by RKIP overexpression.

III. *H. PYLORI* AND mTOR PATHWAY REGULATE RKIP

We examined the role of mTOR pathway in the regulation and phosphorylation¹⁹ of RKIP after *H. pylori* infection. Rapamycin is able to block phosphorylation of RKIP in AGS cells, it diminishes pRKIP expression in the presence of *H. pylori* after half an hour of infection and totally abolishes expression after treatment for three hours (Fig. 2). This evidence

suggests that *H. pylori*-mediated phosphorylation of RKIP in AGS cells proceeds through the mTOR pathway. RKIP expression returns to constitutive levels in the presence of rapamycin after 3 h. This could be due to a delay in time in the transcription of RKIP in response to infection and rapamycin-mediated suppression of the mTOR pathway.

If phosphorylation of RKIP does indeed proceed through the mTOR pathway, which is hyperactive in cancers, this presents support of a complex feedback mechanism in the AGS cells in response to *H. pylori* infection. A tumorigenic pathway is phosphorylating RKIP, which may be further activating RKIP transcriptional activity to induce apoptosis of the cell before further damage can be induced. However, there is still the possibility that the phosphorylated form of RKIP is downregulating or inactivating the Raf/MAPK-inhibitory properties of RKIP. In this case, the tumorigenic properties of *H. pylori* would actually be enhanced by mTOR activity. Longer experiments should be explored to deduce whether the phosphorylated form of RKIP induces transcriptional activity of RKIP, and thereby apoptosis of unhealthy cells.

The same treatment conditions were tested for pRKIP expression in the course of 2 h. *H. pylori*-mediated phosphorylation of RKIP increases from 1 h to 2 h of infection (Fig. 3). Rapamycin is able to reduce *H. pylori*-mediated induction of pRKIP. Both trends are stronger with time. To normalize for differences in actin expression, pRKIP density, which was measured by pixel volume in expression bands by Image Lab Software (Bio-Rad), was divided by actin density to generate relative pRKIP expression measures (Fig. 4). These experiments showed that *H. pylori*-mediated phosphorylation of RKIP increases with time. Rapamycin inhibition of *H. pylori*-mediated pRKIP expression also increases with time, where the data was normalized for differences in actin expression.

IV. *H. PYLORI* INFECTION REGULATES THE MAPK PATHWAY

ERK 1/2 (extracellular signal-regulated kinases), or MAP kinases, are downstream effectors of the Ras/Raf pathways that dysregulate cellular proliferation.²³ mTOR has been suggested to be a cross-activator of ERK.²³ *H. pylori* infection slightly increases pERK expression (Fig. 5), while rapamycin reduces pERK expression after 1.5 h of treatment (infection occurs 1 h after treatment), but does not do so after 3 h. *H. pylori* is able to reverse rapamycin-mediated downregulation of pERK after half an hour. Interestingly, ERK expression is reduced after 3 h of infection in the presence of rapamycin. This might occur due to earlier downregulation of activated (phosphorylated) ERK by rapamycin, which may reduce transcriptional activity of ERK.²⁴ The subsequent increase in pERK expression may be due to the cell's feedback mechanism to restore ERK transcription. Another possibility includes rapamycin's undesirable role in suppressing feedback regulators of the mTOR pathway. Since rapamycin treatment reduces pRKIP expression, and thereby RKIP transcriptional activity according to our hypothesis, Raf-1 becomes overactive and induces more pERK expression.²⁵

Rapamycin blocks the expression of mTOR after 4 h of treatment (Fig. 5). *H. pylori* was not able to restore mTOR expression in the presence of rapamycin. Since *H. pylori*-mediated phosphorylation of RKIP is blocked by rapamycin before the mTOR kinase is

downregulated, one can deduce that rapamycin's activity against RKIP phosphorylation occurs upstream from mTOR. Exploring mTOR's upstream regulators' activity on the phosphorylation of RKIP may elucidate the direct link between rapamycin and pRKIP downregulation.

V. EXPLORING CANDIDATE PROTEIN EXPRESSION AFTER KINEXUS MICROARRAY ANALYSIS: *H. PYLORI* AND RAPAMYCIN ALTER EXPRESSION OF SEVERAL KEY PROTEINS INVOLVED IN TUMORIGENESIS

The Kinex protein microarray can identify changes in the expression of candidate proteins under different treatment conditions and backgrounds. To explore candidate proteins altered in expression by *H. pylori* and rapamycin, cellular lysates generated under different treatment conditions (Table 1) were analyzed by the Kinex antibody microarray (Kinexus) using 540 pan-specific antibodies according to the company's protocol.²⁶

Comparison Z-scores were generated showing significant differences in protein expression between samples. Z-scores were defined as a transformation of the spot intensity corrected for the background "as a unit of standard deviation (SD) from the normalized mean of zero" (Kinexus Order Report). A Z-ratio is obtained by dividing Z-score differences between samples by the standard deviation of all the differences for the comparison. Protein expression differences were ranked by Z-ratios (± 1.00), where a positive number indicates an increase in expression or phosphorylation from the control sample.

Several key proteins of interest to us after micro-array analysis were tested for expression through Western blotting under various treatment conditions: 200 versus 400 multiplicity of infection (MOI) *H. pylori* infection, treatment with rapamycin, and rapamycin treatment 1 h prior to *H. pylori* infection at 200 or 400 MOI (Fig. 6). Four key proteins (EGFR, p53, Raf, and VEGF) were altered by the treatment conditions.

An interesting trend in the Kinexus array was that EGFR (phospho site: Y1110) expression increased in all RKIP knockdown samples when compared to the wild-type AGS cells. The greatest increase was observed in knockdown samples infected with *H. pylori* and treated with rapamycin (Z-ratio = 11.22). EGFR (epidermal growth factor receptor), a cell surface receptor controlling epidermal growth, has been found overexpressed in certain cancers.²⁷ In the treatment conditions described in Fig. 6, *H. pylori* infection at 200 MOI significantly increases EGFR expression, which rapamycin is not able to reverse. At infection of 400 MOI, EGFR expression appears to disappear, but reappears in the rapamycin-*H. pylori* treated sample. It is possible that *H. pylori*-mediated phosphorylation of RKIP serves to inactivate RKIP's activity. If so, RKIP serves to downregulate EGFR activity, which is supported by the results of the Kinexus microarray showing increased EGFR expression in the absence of RKIP.

p53 is a tumor suppressor that regulates the cell cycle. According to the Kinexus microarray data, p53 expression (phospho site: S392) shows the greatest increase in wild-type samples

treated with both rapamycin and *H. pylori*. These results suggest that rapamycin plays an activating role in p53 tumor suppression and that its inhibition of tumorigenesis is strongest in the presence of a carcinogenic factor such as *H. pylori*. RKIP knockdown samples show a decrease in p53 expression, indicating a link between RKIP expression and other tumor suppressors. Perhaps in the absence of rapamycin, p53 tumor suppression increases to compensate for the absence of RKIP-mediated cell-cycle regulation. *H. pylori* infection alters the p53 protein size, perhaps by modification of the protein (Fig. 6). Rapamycin appears to have no effect despite its positive role in the Kinexus microarray.

Raf, a MAP3K that phosphorylates MEK kinases, acts as a proto-oncogene that regulates cell growth and differentiation.²⁸ RKIP, or Raf kinase inhibitor protein, is expected to inhibit downstream activity of Raf kinases in the MAPK/ERK signaling cascade. According to the Kinexus microarray data, Raf-1 (phospho site: S259) expression is variable across RKIP knockdown samples. Knockdown control samples show an increase in Raf-1 expression when compared to wild-type control samples. This may be attributed to RKIP's inhibitory effect on Raf-1; RKIP also decreases Raf-1 function by preventing its kinase activity. However, both rapamycin-treated and *H. pylori*-infected knockdown cells show a decrease in expression when compared to the wild type. It is possible that in the absence of RKIP, downstream feedback activators of Raf-1 are inactive. Western blot (Fig. 6) shows that in the presence of *H. pylori* infection, Raf-1 expression increases, and rapamycin has no effect

VEGF (vascular endothelial growth factor) promotes vasculogenesis and angiogenesis in tumors.²⁸ VEGF expression is activated by downstream signals of the mTOR pathway; treatment of murine skin flap with rapamycin suppresses lymphoangiogenesis.²⁹ In our AGS model, we found a similar decrease in the expression of VEGF-C (pan-specific) in rapamycin-treated samples, including ones infected with *H. pylori*. However, no effect on VEGF-C expression could be observed in knockdown samples treated with rapamycin. When similar treatment conditions were repeated for Western blot analysis, *H. pylori* infection induced VEGF expression in a dose-dependent manner. Rapamycin appeared to have no effect on VEGF expression despite the treatment of cells with the same dose and time period (10 nM, 2 h) as in the Kinexus samples. Nonetheless, the experiment provides evidence that *H. pylori* infection is activating, directly or indirectly, a downstream signal of mTOR. VEGF-mediated angiogenesis may be one of the mechanisms by which *H. pylori* induces tumorigenesis in gastric epithelial cells.

RKIP and pRKIP expressions follow the trends previously observed. In the presence of *H. pylori* infection and rapamycin treatment, RKIP expression remains uniform across samples (Fig. 6). However, infection increases the number of bands observed in the RKIP region, indicating that other forms of RKIP increase in expression. This is not inhibited by rapamycin treatment. Rapamycin treatment decreases pRKIP expression in AGS cells compared to control samples. *H. pylori* infection enhances pRKIP expression, which is diminished with rapamycin pretreatment.

VI. *H. PYLORI*-INFECTED, RKIP KNOCKOUT MICE SHOW SOME EARLY SIGNS OF INCREASED PREDISPOSITION TO GASTRIC CANCER

Our studies indicate that there is a robust induction of RKIP in mice infected for 2 months with *H. pylori* (Fig. 7) that is associated with an inhibition of the inflammatory response. Immunohistochemistry (IHC) of c57BL/6 mouse stomachs 2 months after *H. pylori* infection shows an increased level of RKIP and pRKIP in the glandular epithelial cells of the gastric mucosa. RKIP knockout mice do not show expression of RKIP or pRKIP after infection (Fig. 7). *H. pylori* infection was not able to induce the expression of RKIP in knockout mice.

Gastric tissue of wild-type and RKIP knockout (K/O) mice was scored for precancerous morphology, including inflammation (infiltrating leukocytes), metaplasia (chief and parietal cell disappearance), hyperplasia (elongation of gastric gland), and an overall score of histologic activity index (HAI), was generated for each sample by combining all scores of pre-cancerous morphology (Table 2). RKIP K/O gastric tissue shows more evidence for precancerous morphology after 2 months of *H. pylori* infection (bacterial strain PMSS1), with all mice expressing higher levels of inflammatory cells (Fig. 8). The infected K/O mice show an average HAI score that is eight times higher than the uninfected average HAI (8.4 versus 0.33). PMSS1 #12 and #15 mice show the greatest progression toward gastric cancer, expressing inflammation, epithelial defects, mucosal metaplasia, oxyntic atrophy, hyperplasia, pseudopyloric metaplasia, and some early evidence of dysplasia/neoplasia. RKIP wild-type mice show almost no precancerous morphology after 2 months of infection, indicating that RKIP may prevent the development of pre-cancerous lesions in gastric tissue in the presence of *H. pylori* infection. RKIP knockout mice show minimal pathological abnormalities unless infected by *H. pylori*.

Even though wild-type infected mice express higher levels of pRKIP (Fig. 7), it is known that the phosphorylated form of RKIP does not induce tumorigenesis, but rather seems to prevent it. This indicates that an increase in RKIP expression and its phosphorylation in the presence of *H. pylori* infection may result from the cell's activation of protective mechanisms. In addition, pRKIP is most likely activating transcriptional activity of RKIP. It is still unclear whether the phosphorylation of RKIP occurs by the mTOR pathway, or whether the cell responds to pro-survival signaling in mTOR by activating apoptotic proteins such as pRKIP. Treatment with rapamycin indicates that expression of pRKIP decreases, which raises the question of whether treatment of gastric adenocarcinoma cells with the drug would enhance tumorigenesis by removing this seemingly significant cell cycle regulator. It is possible that rapamycin suppresses pro-survival and pro-proliferation signaling by mTOR sufficiently enough to compensate for the loss of pRKIP expression.

Many new insights into the cellular and molecular pathogenesis of gastric cancer have been gained from *H. pylori* infected mouse models that have been corroborated in human patients, in whom disease outcome is largely determined by the expression of host pro-inflammatory cytokines.³⁰ *H. pylori*-initiated chronic gastritis is characterized by the enhanced expression of pro-inflammatory cytokines, whose expression is largely mediated by transcription factor NF- κ B and STAT.^{31,32} Maintenance of NF- κ B activity in tumors requires STAT3, which is

constitutively activated in many cancers.^{31,33} Both STAT3 and NF- κ B are activated by Cag A, which suggests that there is considerable cross talk between signaling pathways to maintain the pro-inflammatory state after *H. pylori* infection. We did not observe a reduction in RKIP levels in wild-type mice after 8.5 and 9.5 months of infection (Fig. 9). These mice did display inflammation, indicating an involvement of Cag A (Table 3),^{34,35} suggesting that loss of RKIP may promote the inflammatory response in infected mice. This is supported by the HAI of infected mice, which were 11.25 compared to 0 for non-infected mice (Table 3).

To investigate the involvement of Cag A in this process, AGS wild-type and RKIP KD cells were untreated or infected with *H. pylori*. We observed a significant enhancement of Cag A expression after *H. pylori* infection (Fig. 10), indicating that in addition to inhibiting STAT3 and NF- κ B activity (Fig. 1), RKIP may also affect the regulation of Cag A to inhibit inflammation associated with *H. pylori* infection.

VII. CONCLUSIONS

Our results indicate that *H. pylori* infection induces the phosphorylation of RKIP and STAT3 in AGS cells, which activates STAT3 transcriptional activity. RKIP overexpression inhibits STAT3 transcriptional activity, which is enhanced in the presence of infection (Figs. 1 and 11). It appears that *H. pylori* infection may induce gastric epithelial cells to activate apoptosis in order to prevent further damage. At the same time, however, STAT3 pro-survival signaling may be induced by *H. pylori*'s injected virulence factors to promote the survival of the cells. Induction of STAT3 expression may induce the inflammatory and pre-cancerous response observed in the gastric tissue of RKIP-KO mice. It is possible that RKIP mutations occur after long-term infection with *H. pylori*, which causes gastric epithelial cells' cancer progression. However, initial studies indicate that RKIP can simultaneously inhibit STAT3 and NF- κ B activities and suppress Cag A protein levels after *H. pylori* infection and abrogate the pro-inflammatory response.

H. pylori infection induces the expression of cellular pro-survival signaling proteins, including pERK and Raf-1, which is somewhat unexpected given that infection induces pRKIP expression, which should be able to block pro-survival signals in the Raf/MAPK pathway. Again, this raises the possibility that the phosphorylated form of RKIP is actually inactive and increases expression of MAPK after longer-term infection. Since rapamycin reduces pRKIP expression, and thereby reduces RKIP expression, according to our hypothesis, one would expect an increase in MEK/MAPK/ERK signaling due to decreased feedback inhibition. We observed an increase in pERK expression after 4 h of rapamycin treatment in the presence and absence of *H. pylori* infection.

H. pylori infection also alters the expression of several key proteins involved in tumorigenesis including EGFR and VEGF. The bacterium may be inducing cell growth through EGFR and angiogenesis through VEGF as a mechanism by which to acquire more nutrients from its host. However, after long-term infection, hyperactive expression of these factors leads to the formation of pre-cancerous lesions in the stomach. Other protein candidates identified by microarray analysis, including p53, may be explored further to uncover novel causes of tumorigenesis by *H. pylori* in gastric adenocarcinoma cells.

IHC of 2 month-infected wild-type mice shows that despite induction of RKIP and pRKIP, the inflammatory response is not activated. This indicates that RKIP serves as a protective protein that inhibits gastritis in infected stomachs. Pretreatment of AGS cells with rapamycin *in vitro* decreases pRKIP expression, indicating that phosphorylation of RKIP may proceed through the mTOR pathway. It is still puzzling why the activation of an apoptotic protein, RKIP, proceeds through a cell-survival pathway such as mTOR. It is possible that mTOR's hyperactivity in the cell induces a feedback response in the cell to activate apoptotic signaling, which suggests that mTOR's downstream signaling includes important feedback regulation (Fig. 11). Therefore, overtreatment of a cell with rapamycin may lead to the loss of this protective system and further induce tumorigenesis. Further work is needed to confirm the long-term consequences of rapamycin treatment in gastric adenocarcinoma cells in the presence and absence of *H. pylori* infection. Identification of proteins/microRNAs that are regulated by the presence or absence of RKIP during *H. pylori* infection as well as those responsible for the phosphorylation of RKIP after infection may reveal putative targets involved in gastric cancer progression.

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References

1. National Cancer Institute. [30 April 2012] *Helicobacter pylori* and cancer. Available from <http://www.cancer.gov/cancertopics/factsheet/Risk/h-pylori-cancer>
2. Wen S, Moss S. *Helicobacter pylori* virulence factors in gastric carcinogenesis. *Cancer Lett.* 2009; 282:1–8. [PubMed: 19111390]
3. Dicken B, Bigam D, Cass C, Mackey JR, Joy AA, Hamilton SM. Gastric adenocarcinoma: Review and considerations for future directions. *Ann Surg.* 2005; 241:27–39. [PubMed: 15621988]
4. Parsonnet J, Friedman G, Vandersteen DP, Chang Y, Vogelstein JH, Orentreich N, Sibley RK. *Helicobacter pylori* infection and the risk of gastric carcinoma. *New Engl J Med.* 1991; 325:1127–30. [PubMed: 1891020]
5. Wei J, Nagy T, Vilgelm A, Zaika E, Ogden SR, Romero-Gallo J, Piazzuelo MB, Correa P, Washington MK, El-Rifai W, Peek RM, Zaika A. Regulation of p53 tumor suppressor by *Helicobacter pylori* in gastric epithelial cells. *Gastroenterology.* 2010; 139:1333–43. [PubMed: 20547161]
6. Nakachi N, Klein T, Friedman H, Yamamoto Y. *Helicobacter pylori* infection of human gastric epithelial cells induces IL-8 and TNF α , but not TGF β 1 mRNA. *FEMS Immunol Med Microbiol.* 2000; 29:23–6. [PubMed: 10967256]
7. Li SP, Chen XJ, Sun AH, Zhao JF, Yan J. CagA + *H. pylori* induces Akt1 phosphorylation and inhibits transcription of p21 WAF1/CIP1 and p27KIP1 via PI3K/Akt1 pathway. *Biomed Environ Sci.* 2010; 23:273–8. [PubMed: 20934114]
8. Chatterjee D, Sabo E, Tavares R, Resnick MB. Inverse association between Raf kinase inhibitory protein and signal transducers and activators of transcription 3 expression in gastric adenocarcinoma patients: implications for clinical outcome. *Clin Cancer Res.* 2008; 14:2994–3001. [PubMed: 18483365]
9. Yeung K, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, Fee F, Katsanakis KD, Rose DW, Mischak H, Sedivy JM, Kolch W. Suppression of Raf-1 kinase activity and MAP kinase signaling by RKIP. *Nature.* 1999; 401:173–7. [PubMed: 10490027]

10. Yeung KC, Rose DW, Dhillon AS, Yaros D, Gustafsson M, chatterjee D, McFerran B, Wyche J, Kolch W, Sedivy JM. Raf kinase inhibitor protein interacts with NF- κ B-inducing kinase and TAK1 and inhibits NF- κ B activation. *Mol Cell Biol.* 2001; 21:207–17.
11. Slupsky JR, Quitterer U, Weber CK, Gierschik P, Lohse MJ, Rapp UR. Binding of G $\beta\gamma$ subunits to cRaf1 downregulates G-protein-coupled receptor signaling. *Curr Biol.* 1999; 9:971–4. [PubMed: 10508586]
12. Schuierer MM, Bataille F, Hagan S, Kolch W, Bosserhoff AK. Reduction in Raf kinase inhibitor protein expression is associated with increased Ras-extracellular signal-regulated kinase signaling in melanoma cell lines. *Cancer Res.* 2004; 64:5186–92. [PubMed: 15289323]
13. Keller ET, Fu Z, Yeung K, Brennan M. Raf kinase inhibitor protein: a prostate cancer metastasis suppressor gene. *Cancer Lett.* 2004; 207:131–7. [PubMed: 15151133]
14. Hagan S, Al-Mulla F, Mallon E, Oien K, Ferrier R, Gusterson B, Garcia JJ, Kolch W. Reduction of Raf-1 kinase inhibitor protein expression correlates with breast cancer metastasis. *Clin Cancer Res.* 2005; 11:7392–7. [PubMed: 16243812]
15. Minoo P, Zlobec I, Baker K, Tornillo L, Terracciano L, Jass JR, Lugli AL. Loss of Raf-1 kinase inhibitor protein expression is associated with tumor progression and metastasis in colorectal cancer. *Am J Clin Pathol.* 2007; 127:820–7. [PubMed: 17439843]
16. Chatterjee D, Bai Y, Wang Z, Beach S, Mott S, Roy R, Braastad C, Sun Y, Mukhopadhyay A, Aggarwal BB, Darnowski J, Pantazis P, Wyche J, Fu Z, Kitagawa Y, Keller ET, Sedivy JM, Yeung KC. RKIP sensitizes proteins and breast cancer cells to drug-induced apoptosis. *J Biol Chem.* 2004; 279:17515–23. [PubMed: 14766752]
17. Eves EM, Shapiro P, Naik K, Klein UR, Trakul N, Rosner MR. Raf kinase inhibitory protein regulates aurora B kinase and the spindle checkpoint. *Mol Cell.* 2006; 23:561–74. [PubMed: 16916643]
18. Corbit KC, Trakul N, Eves EM, Diaz B, Marshall M, Rosner MR. Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. *J Biol Chem.* 2003; 278:13061–8. [PubMed: 12551925]
19. Moen E, Anwar T, Cross-Knorr S, Brilliant K, Birnbaum F, Rahaman S, Sedivy JM, Moss SF, Chatterjee D. Regulation of RKIP function by *Helicobacter pylori* in gastric cancer. *PLoS One.* 2012; 7:37819–37819.
20. Murayama T, Inokuchi M, Takagi Y, Yamada H, Kojima K, Kumagai J, Kawano T, Sugihara K. Relation between outcomes and localisation of p-mTOR expression in gastric cancer. *Br J Cancer.* 2009; 100:782–8. [PubMed: 19223902]
21. Sarbassov D, Ali S, Sabatini D. Growing roles for the mTOR pathway. *Curr Opin Cell Biol.* 2005; 17:596–603. [PubMed: 16226444]
22. Guertin D, Sabatini D. An expanding role for mTOR in cancer. *Trends Mol Med.* 2005; 11:353–61. [PubMed: 16002336]
23. Van Den Ent F, Amos L, Lowe J. Prokaryotic origin of the actin cytoskeleton. *Nature.* 2001; 413:39–44. [PubMed: 11544518]
24. Feng W, Brown R, Trung CD, Li W, Wang L, Khoury T, Alrawi S, Yao J, Xia K, Tan D. Morphoproteomic profile of mTOR, Ras/Raf kinase/ ERK, and NF- κ B pathways in human gastric adenocarcinoma. *Ann Clin Lab Sci.* 2008; 38:195–209. [PubMed: 18715846]
25. Kincade CW, Castillo-Martin M, Puzio-Kuter A, Yan J, foster TH, Gao H, Sun Y, Ouyang X, Gerald WL, Cordon-Cardo C, Abate-Shen C. Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model. *J Clin Invest.* 2008; 118:3051–64. [PubMed: 18725989]
26. [30 April 2012] Kinex™ Antibody Microarray Services. Available at http://www.kinexus.ca/ourServices/microarrays/antibody_microarrays/details/index.html
27. Zhang H, Berezov A, Wang Q, Zhang G, Drebin J, Murali R, Greene MI. ErbB receptors: from oncogenes to targeted cancer therapies. *J Clin Invest.* 2007; 117:2051–8. [PubMed: 17671639]
28. Li P, Wood K, Mamon H, Haser W, Roberts T. Raf-1: a kinase currently without a cause but not lacking in effects. *Cell.* 1991; 64:479–82. [PubMed: 1846778]
29. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol.* 2001; 280:C1358–66. [PubMed: 11350730]

30. Huber S, Bruns CJ, Schmid G, Hermann PC, Conrad C, Niess H, Huss R, Graeb C, Jauch KW, Heeschen C, Guba M. Inhibition of the mammalian target of rapamycin impedes lymphangiogenesis. *Kidney Int.* 2007; 71:771–7. [PubMed: 17299523]
31. Houghton J, Wang TC. *Helicobacter pylori* and gastric cancer: a new paradigm for inflammation-associated epithelial cancers. *Gastroenterology.* 2005; 128:1567–78. [PubMed: 15887152]
32. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer.* 2009; 9:798–809. [PubMed: 19851315]
33. Lee H, Herrmann A, Deng JH, Kujawski M, Niu G, Li Z, Forman S, Jove R, Pardoll DM, Yu H. Persistently activated Stat3 maintains constitutive NF-kappaB activity in tumors. *Cancer Cell.* 2009; 15:283–93. [PubMed: 19345327]
34. Rogers AB, Cormier KS, Fox JG. Thiol-reactive compounds prevent nonspecific antibody binding in immunohistochemistry. *Lab Investigation.* 2006; 86:526–33.
35. Fox JG, Rogers AB, Whary MT, Ge Z, Ohtani M, Jones EK, Wang TC. Accelerated progression of gastritis to dysplasia in the pyloric antrum of TFF2-/- C57BL6 x Sv129 *Helicobacter pylori*-infected mice. *Am J Pathol.* 2007; 171:1520–8. [PubMed: 17982128]

ABBREVIATIONS

Cag A	cytotoxin associated antigen A
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinases
HAI	histology activity index
IHC	immunohistochemistry
KD	knockdown
KO	knockout
MAPK	mitogen activated protein
MOI	multiplicity of infection
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
RKIP	Raf kinase inhibitor protein
STAT3	signal transducer and activator of transcription 3
Vac A	vacuolating exotoxin
VEGF	vascular endothelial growth factor

FIG. 1.

(A, C) AGS gastric cancer cells were transfected with STAT3 or NF- κ B luciferase reporter constructs and in some samples a RKIP expression vector. After 48 h, cells were infected with *H. pylori* (MOI: 200:1) for 16 h. Cells were harvested and luciferase activity measured with Dual reporter luciferase activity kit (Promega). (B) Western blot analysis of AGS gastric cancer cells transfected with an expression plasmid for RKIP for 48 h and then infected with *H. pylori* (MOI 200:1) for 3 h. Whole cell lysates were prepared and Western blot analysis performed to examine the levels of the indicated proteins. The upper band in the gel of RKIP transfected cells is ectopic and the lower band is endogenous RKIP.

FIG. 2.

AGS cells were treated with rapamycin alone (R), *H. pylori* (HP), and both *H. pylori* and rapamycin (R+HP). Control samples are labeled "C." Cells were pretreated with 10 nM rapamycin (R) for 1 h and then infected with 200 MOI of *H. pylori* for 0.5 or 3 h. Actin was used as a loading control.

FIG. 3.

AGS cells were treated with rapamycin alone (R), *H. pylori* (H), and both *H. pylori* and rapamycin (RH). Cells were pretreated with 10 nM rapamycin (R) for 1 h and then infected with 200 MOI of *H. pylori* for 1 or 2 h. Actin was used as a loading control. Rapamycin reduces expression of pRKIP.

FIG. 4.

The results in Figure 4 were adjusted for differences in actin expression. pRKIP pixel density was divided by actin pixel density to find relative pRKIP expression for each sample condition.

FIG. 5.

AGS cells were treated with rapamycin alone (R), *H. pylori* (HP), and both *H. pylori* and rapamycin (R+HP). Control samples are labeled "C." Cells were pretreated with 10 nM rapamycin (R) for 1 h and then infected with 200 MOI of *H. pylori* for 0.5 or 3 h. Actin was used as a loading control.

FIG. 6.

Samples were treated with 10 nM rapamycin (R), or infected with *H. pylori* at 200 or 400 MOI (H200, H400), or pretreated with 10 nM rapamycin 1 h prior to being infected with *H. pylori* at 200 or 400 MOI (RH 200, RH400).

FIG. 7.

Immunohistochemistry of paraffin embedded sections of four representative stomachs of wild-type and RKIP knockout mice [from a total of 17 (seven wild type and 10 knockout)] infected with *H. pylori* for 2 months and examined for RKIP and pRKIP. Bar is 10 μ m. Significantly, in RKIP knockout mice there is more inflammation after *H. pylori* infection and the development of pre-neoplastic gastric pathological changes (Table 2).

FIG. 8. Hematoxylin and eosin stain of infected and uninfected wild-type mice and RKIP K/O mice 2 months postinfection. This figure shows a greater degree of inflammatory cells in the knockout mice when compared to wild type after *H. pylori* infection.

FIG. 9.

Immunohistochemistry of paraffin embedded sections of representative stomachs of wild type and mice infected with *H. pylori* for 8.5 and 9.5 months and examined for RKIP and pRKIP. Bar is 50 μ m. There is development of pre-neoplastic gastric pathological changes (Table 3).

FIG. 10. Western blot analysis of AGS wild-type and RKIP KD gastric cancer cells infected with *H. pylori* (MOI 200:1) for 3 h. Whole cell lysates were prepared and Western blot analysis performed to examine the levels of the indicated proteins.

FIG. 11.

Model depicting the complex signaling after *H. pylori* infection. The phosphorylation of STAT3 (1) and RKIP (2) occurs after infection. RKIP overexpression (3) abrogates *H. pylori*-mediated STAT3 phosphorylation. Rapamycin-mediated inhibition (4) of the mTOR pathway (Fig. 2) results in the simultaneous inhibition of RKIP phosphorylation by *H. pylori*.

TABLE 1

Samples analyzed by Kinex protein microarray

WT AGS cells		RKIP KD AGS cells	
1	WT Ctrl	5	KD Ctrl
2	WT HP	6	KD HP
3	WT Rapa	7	KD Rapa
4	WT HP + Rapa	8	KD HP + Rapa

Wild-type (WT) and RKIP Knockdown (KD) AGS cells were pretreated for 1 h with 10 nM Repamycin (Rapa) and/or infected with *H. pylori* (HP) at 200 MOI for 2 h.

TABLE 2
Histological scoring of wild-type and RKIP knockout mice infected with *H. pylori* for 2 months

ID	InfIM	EpDfct	MucMeta	OxAtroph	Hyper	PyMeta	Dyspl	HAI
RKIP wild type								
WT1	0	0	0	0	0	0	0	0
WT2	0.5	0	0	0	0	0	0	0.5
WT3	1	0.5	0	0.5	0.5	0	0	2.5
WT4	0	0.5	0	0	0	0	0	0.5
Average	0.375	0.25	0	0.125	0.125	0	0	0.875
PMSSI ST18	0.5	0.5	0	0	0	0	0	1
PMSSI ST19	0	0	0	0	0	0	0	0
PMSSI ST20	0	0	0	0	0	0	0	0
PMSSI ST21	0	0	0	0	0	0	0	0
PMSSI ST22	0	0	0	0	0	0	0	0
PMSSI ST23	0	0	0	0	0	0	0	0
PMSSI ST24	0.5	0	0	0	0	0	0	0.5
PMSSI ST25	0	0	0	0	0	0	0	0
Average	0.125	0.0625	0	0	0	0	0	0.2875
RKIP knockout								
Uninfected	0	0	0	0	0	0	0	0
Uninfected	0.5	0.5	0	0	0	0	0	1
Uninfected	0	0	0	0	0	0	0	0
Average	0.167	0.167	0	0	0	0	0	0.333
PMSSI #8	2	1.5	2	1	1	0.5	0.5	8.5
PMSSI ST10	1.5	1	0	1	1.5	1	0.5	6.5
PMSSI ST11	1.5	1	0	1	1.5	0.5	0.6	6
PMSSI ST12	2.5	1.5	2.5	2.5	2	1	1	13
PMSSI ST13	1	1	0	0.5	0.5	1	0.5	4.5
PMSSI ST15	3	2	3	3	2.5	1	1	15.5
PMSSI ST16	1	1.5	0	1	0.5	0.5	0.5	5
Average	1.7857	1.3571	1.0714	1.4286	1.3571	0.7857	0.6429	8.4286

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Histologic pathology was defined as follows: inflammation of infiltrating leukocytes in mucosa and submucosa; epithelial defects—surface erosions and not full-thickness ulceration, although there may also be subsurface gland atrophy and collapse; mucous metaplasia—progressive gastritis and cancer due to the loss of chief and parietal cells; oxyntic atrophy—chief cell disappearance always preceding that of parietal cells; hyperplasia—elongation of gastric gland units due to increased numbers of surface (foveolar) and/or antral-type epithelial cells; pseudopyloric metaplasia replacement of the oxyntic mucosa by glands with an antral phenotype; dysplasia/neoplasia—features include differences in overall cell and nuclear size (anisocytosis and anisokaryosis), hyperpleomorphism (highly variable cell shape and size), poorly defined cell junctions, loss of nuclear polarity, and hyperchromasia characterized by a tall columnar shape with increased nuclear:cytoplasmic ratio.^{33,34} A gastric HAI (histologic activity index) was generated for each mouse by combining all scores for gastric histopathology.

TABLE 3

Histological scoring of wild-type mice infected with *H. pylori* for 8.5 months

	Inflm	EpDfct	Hyalin	MucMeta	OxAtirph	Hyper	PyMeta	Dyspl	HAI
WT + Hp C	1	0.5	1	1	0.5	1	0.5	0	5.5
WT + Hp D	3	2	0	3	3	2.5	2.5	1.5	17.5
WT + Hp E	2	0.5	0	1.5	1	1.5	1.5	1.5	8.5
WT + Hp F	1	0.5	0	0.5	1	0.5	1.5	1.5	8
WT + Hp G	2	2	0	1	1	1	1	0.5	16.5
WT + Hp K	2	2	0.5	1.5	1.5	2	1	1	11.5
Average	1.66	1.16	0.25	1.41	1.33	1.41	1.5	1	11.25
WT L	0	0	0	0	0	0	0	0	0
WT O	0	0	0	0	0	0	0	0	0
WT P	0	0	0	0	0	0	0	0	0
WT Q	0	0	0	0	0	0	0	0	0
Average	0	0	0	0	0	0	0	0	0

Note: the increase in inflammation and enhanced pathology associated with gastric cancer progression. L-Q represents uninfected controls. HAI index: Uninfected: 0; Infected.