Interleukin-32 Increases Human Gastric Cancer Cell Invasion Associated with Tumor Progression and Metastasis

Chung-Ying Tsai¹, Chia-Siu Wang⁶, Ming-Ming Tsai², Hsiang-Cheng Chi³, Wan-Li Cheng³, Yi-Hsin Tseng¹, Cheng-Yi Chen¹, Crystal D. Lin⁶, Jun-I. Wu⁵, Lu-Hai Wang⁵, and Kwang-Huei Lin¹

Abstract

Objective: The proinflammatory cytokine interleukin-32 (IL-32) is a novel tumor marker highly expressed in various human carcinomas, including gastric cancer. However, its effects on prognosis of patients with gastric cancer and cancer metastasis are virtually unknown at present. The main aim of this study was to explore the clinical significance of IL-32 in gastric cancer and further elucidate the molecular mechanisms underlying IL-32–mediated migration and invasion.

Experimental Design: Gastric cancer cells with ectopic expression or silencing of IL-32 were examined to identify downstream molecules and establish their effects on cell motility, invasion, and lung metastasis in vivo.

Results: IL-32 was significantly upregulated in gastric cancer and positively correlated with aggressiveness of cancer and poor prognosis. Ectopic expression of IL-32 induced elongated morphology and increased cell migration and invasion via induction of IL-8, VEGF, matrix metalloproteinase 2 (MMP2), and MMP9 expression via phosphor-AKT/phospho-glycogen synthase kinase 3β/active β-catenin as well as hypoxia-inducible factor 1α (HIF-1α) signaling pathways. Conversely, depletion of IL-32 in gastric cancer cells reversed these effects and decreased lung colonization in vivo. Examination of gene expression datasets in oncomine and staining of gastric cancer specimens demonstrated the clinical significance of IL-32 and its downstream molecules by providing information on their coexpression patterns.

Conclusions: IL-32 contributes to gastric cancer progression by increasing the metastatic potential resulting from AKT, β-catenin, and HIF-1α activation. Our results clearly suggest that IL-32 is an important mediator for gastric cancer metastasis and independent prognostic predictor of gastric cancer.

Introduction

Gastric cancer is the fourth most common cancer type worldwide and ranks second in terms of global cancer-related mortality (1). In Taiwan, gastric cancer was recorded as the sixth leading cause of cancer-related deaths in 2010 (2). The prognosis of gastric cancer is poor, and the key players in molecular pathogenesis are predominantly unknown at present. The tumor–node–metastasis (TNM) staging system is the only tool routinely used for predicting prognosis in the clinical setting. Currently, surgery is the major known cure for the disease. Therefore, elucidation of the molecular mechanisms involved in gastric cancer and identification of valuable prognostic markers as well as novel therapeutic strategies is of essential clinical value (3).

Increasing evidence has confirmed the hypothesis that several pro- and anti-inflammatory cytokines promote tumor progression and affect the host antitumor response (4). Inflammatory tumor microenvironments play a pivotal role, not only in tumor development but also metastasis. Cytokines released in response to infection affect tumor development in different ways. IL-8 produced by gastric cancer cells contributes to sustained angiogenesis and tumor invasion and metastasis via either autocrine or paracrine mechanisms (5). VEGF promotes breast cancer cell invasion through an autocrine pathway (6). Recent studies have revealed higher expression of IL-32, a novel pro-inflammatory cytokine, in human stomach (7, 8), lung (9), pancreas (10), liver (11), and esophagus (12) cancer tissues, compared with normal tissue or serum.
IL-32, originally designated natural killer cell transcript 4 (NK4) in humans, was found to be absent in rodents. The recently described cytokine is mainly produced by T cells, NK cells, epithelial cells, and monocytes after stimulation with IL-2, IL-18, or IFN-γ (13). The IL-32 gene is located on human chromosome 16p13.3, and has a full length of 705 bp. Moreover, the gene is organized into 8 exons and has 6 splice variants, IL-32α, IL-32β, IL-32γ, IL-32β, IL-32ε, and IL-32f, among which the γ isoform exhibits the highest cytokine producing activity (14). IL-32β is the major secreted isoform, whereas the α isoform is located intracellularly (15). However, the functional differences between these isoforms are yet to be established. The cytokine induction activity of IL-32 is activated via partial cleavage by proteinase-3 (16). Although a receptor for IL-32 has not been described, it is defined as a proinflammatory cytokine, in view of its stimulation of TNF-α, IL-1β, IL-6, and IL-8 production, and activation of the NF-kB and the p38 mitogen-activated protein kinase pathways (17). Overexpression of IL-32 in stomach cancer tissue has been reported (7). However, the specific role of this cytokine in gastric cancer has received little attention, and its clinicopathologic significance and underlying molecular mechanisms have rarely been studied to date. Therefore, we focused on the clinical and functional significance, as well as the signaling pathway of IL-32 in gastric cancer.

Materials and Methods

Subjects

After obtaining informed consent, 120 patients (67 males and 53 females; median age, 66 years; range 28–86 years) diagnosed pathologically with gastric cancer at the Chang Gung Memorial Hospital (CGMH) from 2000 to 2005 were enrolled in the study. None of the patients had received chemotherapy or radiotherapy before operation and all of them were subjected to gastric resection that included total gastrectomy in 35 patients and subtotal gastrectomy in 85. The pathologic examination and further biological studies were carried out under informed consents of patients. This study protocol was approved by the Medical Ethics and Human Clinical Trial Committee of the CGMH (IRB NO. 95-0472B).

Postoperatively, the patients with stage 2 to 3 diseases received adjuvant chemotherapy with 5-fluorouracil–based regimens, and those with stage 4 diseases were treated with palliative chemotherapy of various regimens as indicated. All patients were followed up regularly in the outpatient service of our hospital, of every 3 months in the first 2 years, every 6 months between the 3rd and 5th years and then once a year thereafter.

Clinicopathologic studies

Resected specimens were examined pathologically using the criteria of the 6th edition of the American Joint Committee on Cancer (pTNM) classification system and the Japanese General Rules for gastric cancer Study (18). Clinicopathologic parameters included patient age and gender, tumor location, and size, gross (Borrmann) tumor type, wall invasion, resection margin, histologic type, lymph node metastasis, vascular invasion, lymphatic invasion, and perineural invasion. After discharge, all patients had periodic follow-up visits at the outpatient department of CGMH until death or the beginning of preparation of this article.

Tumor samples

Fresh specimens of tumor tissue and adjacent noncancerous mucosa were harvested immediately following gastric resection. Samples were dissected from resected specimens by a pathologist at CGMH, and immediately snap-frozen in liquid nitrogen. Frozen specimens were stored at −70°C in a tumor bank until use.

Real-time quantitative reverse transcription PCR

Quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR green as described previously (19). Fluorescence emitted by SYBR green was detected using the ABI PRISM 7500 sequence detection system (Applied Biosystems). The following primers were used: human IL-32 qPCR (forward primer, 5'-TGAAGATAAAAAATG ACATCCC-TAAAGA-3', and reverse primer, 5'-CTGCCGGTGGGCTTCGTTT-3'); human IL-8 qPCR (forward primer, 5'-CGACCTCGTATACCTACCA-3', and reverse primer, 5'-CGACCTCGTATACCTACCA-3'). Human Clinical Trial Committee of the CGMH (IRB NO. 95-0472B).

Immunoblot analysis

Total cell lysates from tumors and adjacent noncancerous mucosa or cell lines were extracted by lysis buffer (150 mM NaCl/50 mM Tris, pH 7.8/1% Triton X-100/1 mM EDTA, 1 mM phenylmethylsulfonylfluoride) and quantified by Bradford method. Equal amounts of protein were electrophoresed through a 10% Tris-SDS gel and transferred onto a...
polyvinylidene difluoride membrane. The membranes were blocked overnight with PBS containing 0.1% Tween 20 in 5% skimmed milk at 4°C, and subsequently immunoblotted with primary antibody listed in Supplementary Table S1. After further washing, the membrane was incubated for 1 hour with a horseradish peroxidase-conjugated, affinity-purified anti-rabbit antibody (1:5,000 dilution in TBS; Santa Cruz Biotechnology Inc.). Immune complexes were visualized via chemiluminescence using an ECL Detection Kit (Amersham Inc.).

RNA interference

The lentiviral pLKO, control shLuc, and shRNA were obtained from the National RNAi Core Facility at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica. Target information is listed in Supplementary Table S2. Recombinant lentivirus carrying shRNA was produced by cotransfecting 293FT cells with a mixture of plasmid DNA consisting of pMD-G (VSV-G envelope), pCMV-ψR8.91 (Gag/Pol/Rev), and pLKO/1-shRNA vectors using TurboFect reagent (Fermentas) according to the manufacturer’s recommendations. Virus-containing culture supernatants were collected 2 days after transfection and used to infect AGS and TSGH9201 cells in combination with 10 μg/mL polybrene (Sigma-Aldrich). Stable cell lines were selected by culturing cells in 2 μg/mL puromycin (Calbiochem) for 1 week. Western blot analysis or qPCR was used to determine the effects of gene expression knockdown.

Cell lines and culture

TSGH 9201 human gastric cancer cell line (BCRC 60146) was obtained from the Bioresource Collection and Research Center of Taiwan. Human gastric carcinoma cell line AGS was obtained from American Type Culture Collection (CRL-1739). The cell line was authenticated by assessments of short tandem repeat loci following database comparison. All cell lines were cultured in RPMI 1640 medium (Invitrogen), supplemented with 10% FBS (Invitrogen), 100 IU/mL penicillin G, and 100 mg/mL streptomycin sulfate (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO2. All kinase inhibitors used in cell culture are listed in Supplementary Table S4.

In vivo tumor metastasis model

TSGH9201 cells with or without silencing IL-32 were harvested and washed twice with PBS. Cells (1 × 106/150 μL per mouse) were injected intravenously via a 30-gauge needle into the tail vein of 4 to 6 weeks old male severe combined immunodeficiency (SCID; C.B17/Irscid) mice. Five weeks later, mice were sacrificed by CO2 and the lung was removed, fixed with 3.7% formaldehyde and sectioned. Paraffin sections were stained with hematoxylin and eosin (H&E; Sigma). Lung metastasis was quantified by counting the total tissue area per lung section (A1) and metastasis present in the same area (A2). The metastatic index was calculated by the ratio A2/A1.

Statistical analysis

The Mann–Whitney U test or Fisher exact test was used for between-group comparisons, where appropriate, and the correlation between the results obtained with the 2 different analyses analyzed with Spearman test. Follow-up studies of patients were performed until the time of writing or patient death. The cancer-specific survival outcome was evaluated by applying the Kaplan–Meier method for all patients, except those who died from surgical complications. The log-rank test was used to compare the prognostic significance of individual variables on survival. Cox’s proportional hazards model was applied in a multivariate analysis to identify independent predictors of survival. A P-value of <0.05 was considered statistically significant.

Results

High expression of IL-32 in gastric cancer is correlated with tumor progression and poor prognosis

In our previous study (21), differential gene expression patterns from genome-wide microarray analyses revealed upregulation of several inflammation-associated genes in gastric cancer tissues, compared with adjacent nontumorous mucosa tissues. CXCL1 (22), SLIP (23), and SPARC (21) have been verified and studied for clinical significances in our setting. Furthermore, we focused on a novel pro-inflammatory gene, IL-32, as a potential tumor marker for gastric cancer development. To verify the enhanced expression of IL-32 in gastric cancer, IHC (n = 120) and real-time qPCR (n = 66) were performed on clinical specimens. Among the 120 patients analyzed using IHC, the mean score in tumor tissues was 107.2 ± 4.8, which was significantly greater than that (32.33 ± 3.98) in the matching adjacent mucosa. IL-32 was consistently detected in the majority of tumor regions, whereas levels were reduced in nontumor areas (Supplementary Fig. S1A). Four pairs of representative cases from IHC analysis are shown in Supplementary Fig. S1B. Strong staining of IL-32 was mostly detected in the cytosol of gastric tumor cells. In contrast, absent or weak staining for IL-32 was observed in nontumor gastric epithelial cells. Staining was more intense in the advanced stages (Supplementary Fig. S1B, right), relative to early stages of the disease (Supplementary Fig. S1B, left). Data from qPCR experiments revealed high IL-32 expression (≥1.5-fold) in 48 of 66 (74%) gastric cancer tissues, compared with paired nontumorous tissues (P < 0.001; Supplementary Fig. S1C).

Because the IL-32 isoforms cannot be distinguished using qPCR and IHC, we further analyzed expression of IL-32 protein in paired specimens via Western blotting.
(Supplementary Fig. S1D). As expected, IL-32 expression in tumor areas was higher than that in matched noncancerous adjacent mucosa in 3 representative patients. Notably, all IL-32 isoforms, including α, β, and γ, were expressed in the tumor, with the β isoform as the dominant variant, as reported previously (15). Similar results were obtained from a RT-PCR assay (data not shown).

To determine the association between IL-32 expression and tumor characteristics, the IHC score for IL-32 was analyzed using clinicopathologic parameters. Table 1 lists the characteristics of study patients (n = 120). Tumor size (maximum diameter) was on average 5.6 cm (median, 5.2 cm; range, 0.3–20 cm). Tumors were located in the proximal third of the stomach in 24 cases (20%), middle third in 29 (24.1%), distal third in 61 (50.8%), and the whole stomach in 6 (5%). Histologic tumor types were intestinal 29 (24.1%), distal third in 61 (50.8%), and the whole stomach in 6 (5%).

Pathologic staging was demonstrated in 22 (18.3%) cases, and liver metastasis was diagnosed in 24 (20%) cases (mucosal in 10 and submucosal in 14), whereas advanced cancers (T2; muscle proper and subserosa) was diagnosed in 18 (15%) cases, and serosa (T3) in 59 (49.1%), and invasion to adjacent organs (T4) in 19 (15.8%) cases. Lymph node metastasis was diagnosed in 87 cases (72.5%). During surgery, peritoneal seeding was diagnosed in 22 (18.3%) cases, and liver metastasis was diagnosed in 2 (1.6%) patients. Pathologic staging was distributed as follows: I, 30 cases, 25%; II, 10 cases, 8.3%; III, 47 cases, 39.1%; and IV, 33 cases, 27.5%.

Interestingly, higher levels of IL-32 were detected in T3 and T4 groups where the serosal surface of the gastric wall was invaded by cancer, compared with T1 and T2 groups where no invasion was evident (P = 0.016; Table 1 and Fig. 1A). In addition, IL-32 was significantly increased in patients with metastasis to the lymph nodes (P = 0.005; Table 1 and Fig. 1B) and those at the more advanced pathologic stages (III and IV) of gastric cancer, compared with those in the earlier pathologic stages (I and II; P = 0.011; Table 1 and Fig. 1C).

Among the 120 patients, 65 died: 56 died because of gastric cancer progression; 2 died from surgical complications; and 7 died because of noncancerous causes. Median follow-up duration for survivors (n = 55) was 83.1 months (range, 64–137 months). Overall cumulative 5-year survival rate for the 120 patients was 53.4%. After dichotomization based on the median IL-32 expression level, the IL-32-low group (n = 73) presented significantly shorter cumulative survival, compared with the IL-32-high group (n = 47) using univariate analysis (Table 1 and Fig. 1D; 66.4% vs. 40.4%, log-rank P = 0.0045). Other factors that predicted outcome significantly for cumulative survival in univariate analysis included tumor location, gross type, tumor size, histologic type, depth of invasion, lymph node metastasis, distant metastasis, pathologic stage, liver metastasis, peritoneal seeding, vascular invasion, lymphatic invasion, and perineural invasion (Table 1). Cox regression analysis (multivariate) was performed to determine the prognostic potential of IL-32 independently for gastric cancer in relation to the significant clinicopathologic parameters in univariate analysis, including histologic type, serosal invasion, distant metastasis, lymph node metastasis, peritoneal seeding, vascular invasion, lymphatic invasion, perineural invasion, gross type, and tumor size. Distant metastasis [P < 0.001; HR = 3.325; 95% confidence interval (CI), 1.838–6.014], higher IL-32 expression (P = 0.006; HR = 2.340; 95% CI, 1.277–4.290), serosal invasion (P = 0.014; HR = 2.893; 95% CI, 1.239–6.752), and histologic type (P = 0.005; HR = 2.918; 95% CI, 1.387–6.141) significantly emerged as independent prognostic markers for gastric cancer in a stepwise forward-conditional multivariate regression model (Table 1). These data indicate IL-32 as well as 3 pathologic parameters (distant metastasis, serosal invasion, and histologic type) displayed an independent prognostic value for 5-year cumulative survival. Our results support a strong association between IL-32 expression and gastric cancer progression, and support the utility of IL-32 as a useful prognosis marker for gastric cancer.

Ectopic expression of IL-32 in TSGH9201 cells promotes migration and invasion

Clinicopathologic data indicate that IL-32 is involved in cell invasiveness. To confirm whether IL-32 is associated with invasiveness of gastric cancer cell lines, we established stable IL-32–expressing sublines. Because all IL-32 isoforms are expressed in clinical specimens, the largest γ variant that can be spliced into IL-32β and IL-32α (24) was introduced in TSGH9201 cells (Supplementary Fig. S2A). All cells overexpressing IL-32 displayed protrusions at the leading edge and elongated spindle-like morphology, compared with control cells (Supplementary Fig. S2B).

On the basis of these morphologic characteristics, we propose that IL-32 enhances cell motility (25). Indeed, IL-32–overexpressing cells exhibited significantly higher migration (Supplementary Fig. S2C) and invasive (Fig. 2A) abilities toward a serum gradient, as well as wound healing ability (Supplementary Fig. S2D), relative to control cells. Similar findings were consistently obtained in doxycycline-inducible IL-32–overexpressing cells (Supplementary Fig. S3A), whereby cell migration ability was increased following induction of IL-32 with doxycycline (Supplementary Fig. S3B). Our results clearly indicate that IL-32 is involved in gastric cancer cell migration and invasion.

IL-32 accelerates cell invasion through IL-8, VEGF, MMP9, and MMP2 in an AKT-dependent manner

In general, cancer cells utilize an autocrine mechanism by releasing several important factors to enhance proliferation and metastasis (26). Accordingly, we investigated whether IL-32 induces secretory factors to facilitate gastric cancer cell invasion. Following the addition of concentrated conditioned medium to the lower Boyden chamber as a chemoattractant, IL-32 and the induced secretory proteins significantly enhanced the invasion ability of parental TSGH9201 cells (Supplementary Fig. S2E). To further define the chemoattractant in the IL-32–induced secretome, several well-known invasion-associated factors, such as IL-8, VEGF, MMP2, and MMP9, were determined in conditioned medium. As expected, ectopic expression of
Table 1. Clinicopathologic correlations of IL-32 expression and 5-year survival rate in patients with gastric cancer

<table>
<thead>
<tr>
<th>Parameters</th>
<th>n</th>
<th>Mean ± SE*</th>
<th>P</th>
<th>5-y S.R. c</th>
<th>Log-rank Pd</th>
<th>Univariate analysis</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>58</td>
<td>108.7 ± 6.9</td>
<td>0.876</td>
<td>45.1</td>
<td>0.2329</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥65</td>
<td>62</td>
<td>107.0 ± 6.6</td>
<td></td>
<td>56.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>67</td>
<td>109.0 ± 6.7</td>
<td>0.880</td>
<td>52.0</td>
<td>0.7656</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>53</td>
<td>106.4 ± 6.7</td>
<td></td>
<td>48.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper third</td>
<td>24</td>
<td>110.0 ± 8.9</td>
<td>0.985</td>
<td>52.2</td>
<td>0.0005</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle third</td>
<td>29</td>
<td>106.6 ± 10.5</td>
<td>54.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower third</td>
<td>61</td>
<td>106.7 ± 8.8</td>
<td></td>
<td>53.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>6</td>
<td>112.7 ± 24.6</td>
<td></td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gross type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>46</td>
<td>99.7 ± 8.4</td>
<td>0.095</td>
<td>77.4</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltrative</td>
<td>74</td>
<td>112.9 ± 5.6</td>
<td></td>
<td>33.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5 cm</td>
<td>65</td>
<td>102.4 ± 6.8</td>
<td>0.187</td>
<td>72.3</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥5 cm</td>
<td>55</td>
<td>114.3 ± 6.5</td>
<td></td>
<td>24.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Histologic type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>42</td>
<td>101.3 ± 8.8</td>
<td>0.657</td>
<td>77.5</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>78</td>
<td>108.7 ± 5.6</td>
<td></td>
<td>35.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Depth of invasion (pT)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>24</td>
<td>83.8 ± 12.5</td>
<td><strong>0.033</strong></td>
<td>91.5</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>18</td>
<td>108.6 ± 12.5</td>
<td>68.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>59</td>
<td>115.3 ± 6.3</td>
<td></td>
<td>39.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>19</td>
<td>114.2 ± 9.6</td>
<td></td>
<td>11.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serosal invasion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (T1, T2)</td>
<td>42</td>
<td>93.5 ± 9.0</td>
<td><strong>0.016</strong></td>
<td>82.4</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (T3, T4)</td>
<td>78</td>
<td>115.6 ± 5.3</td>
<td></td>
<td>32.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node status (pN)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>33</td>
<td>88.5 ± 10.5</td>
<td><strong>0.024</strong></td>
<td>87.4</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>43</td>
<td>115.4 ± 7.4</td>
<td></td>
<td>48.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>23</td>
<td>106.5 ± 10.9</td>
<td>43.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td>21</td>
<td>124.3 ± 7.3</td>
<td></td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (N0)</td>
<td>33</td>
<td>88.5 ± 10.5</td>
<td><strong>0.005</strong></td>
<td>87.4</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (N1, N2, N3)</td>
<td>87</td>
<td>115.2 ± 5.0</td>
<td></td>
<td>36.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Distant metastasis (pM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>91</td>
<td>106.9 ± 5.8</td>
<td>0.631</td>
<td>64.1</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>29</td>
<td>110.7 ± 7.4</td>
<td></td>
<td>5.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pathologic stage (pStage)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>30</td>
<td>84.3 ± 11.2</td>
<td><strong>0.007</strong></td>
<td>92.7</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>116.5 ± 11.0</td>
<td>80.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>47</td>
<td>121.6 ± 7.5</td>
<td></td>
<td>47.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>33</td>
<td>107.0 ± 6.9</td>
<td></td>
<td>4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stages I, II</td>
<td>40</td>
<td>92.4 ± 9.1</td>
<td><strong>0.011</strong></td>
<td>89.4</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stages III, IV</td>
<td>80</td>
<td>115.6 ± 5.3</td>
<td></td>
<td>30.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Liver metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>118</td>
<td>108.0 ± 4.8</td>
<td>0.853</td>
<td>51.5</td>
<td>0.0135</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>100.0 ± 50.0</td>
<td></td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continued on the following page)
AKT in IL-32–induced cell invasion

Activated AKT is capable of phosphorylating and inactivating GSK3β, resulting in nuclear translocation and activation of β-catenin (27). Several studies have reported that β-catenin regulates IL-8, VEGF, MMP2, and MMP9 expression (28, 29), and acts as a major pathway frequently altered in human gastric cancer (30). Accordingly, we hypothesized that β-catenin operates in IL-32-phospho-AKT signaling. Interestingly, expression of active β-catenin was dramatically increased in the nuclei of IL-32–overexpressing cells in which p-AKT was activated as well as the intermediate regulator, phospho-GSK-3β (Fig. 3A). This finding was further confirmed using doxycycline-inducible IL-32–overexpressing cells in which active nuclear β-catenin was increased via inducing IL-32 production over a short period of time, compared with empty vector control cells (Supplementary Fig. S4A). To provide evidence of the mechanistic role of β-catenin on the regulation of IL-32/p-AKT–induced IL-8, VEGF, MMP2, and MMP9 expression, our results collectively indicate that AKT activation by IL-32 is required to induce IL-8, VEGF, MMP2, and MMP9 expression for cell invasion.

Active β-catenin is a downstream effector of phospho-AKT in IL-32–induced cell invasion

Next, we investigated the mechanism underlying AKT-dependent invasiveness in IL-32–overexpressing cells. Activated AKT is capable of phosphorylating and inactivating β-catenin (27). Several studies have reported that β-catenin regulates IL-8, VEGF, MMP2, and MMP9 expression (28, 29), and acts as a major pathway frequently altered in human gastric cancer (30). Accordingly, we hypothesized that β-catenin operates in IL-32-phospho-AKT signaling. Interestingly, expression of active β-catenin was dramatically increased in the nuclei of IL-32–overexpressing cells in which p-AKT was activated as well as the intermediate regulator, phospho-GSK-3β (Fig. 3A). This finding was further confirmed using doxycycline-inducible IL-32–overexpressing cells in which active nuclear β-catenin was increased via inducing IL-32 production over a short period of time, compared with empty vector control cells (Supplementary Fig. S4A). To provide evidence of the mechanistic role of β-catenin on the regulation of IL-32/p-AKT–induced IL-8, VEGF, MMP2, and MMP9 expression, our results collectively indicate that AKT activation by IL-32 is required to induce IL-8, VEGF, MMP2, and MMP9 expression for cell invasion.

Active β-catenin is a downstream effector of phospho-AKT in IL-32–induced cell invasion

Next, we investigated the mechanism underlying AKT-dependent invasiveness in IL-32–overexpressing cells. Activated AKT is capable of phosphorylating and inactivating β-catenin (27). Several studies have reported that β-catenin regulates IL-8, VEGF, MMP2, and MMP9 expression (28, 29), and acts as a major pathway frequently altered in human gastric cancer (30). Accordingly, we hypothesized that β-catenin operates in IL-32-phospho-AKT signaling. Interestingly, expression of active β-catenin was dramatically increased in the nuclei of IL-32–overexpressing cells in which p-AKT was activated as well as the intermediate regulator, phospho-GSK-3β (Fig. 3A). This finding was further confirmed using doxycycline-inducible IL-32–overexpressing cells in which active nuclear β-catenin was increased via inducing IL-32 production over a short period of time, compared with empty vector control cells (Supplementary Fig. S4A). To provide evidence of the mechanistic role of β-catenin on the regulation of IL-32/p-AKT–induced IL-8, VEGF, MMP2, and MMP9 expression, our results collectively indicate that AKT activation by IL-32 is required to induce IL-8, VEGF, MMP2, and MMP9 expression for cell invasion.

Active β-catenin is a downstream effector of phospho-AKT in IL-32–induced cell invasion

Next, we investigated the mechanism underlying AKT-dependent invasiveness in IL-32–overexpressing cells. Activated AKT is capable of phosphorylating and inactivating β-catenin (27). Several studies have reported that β-catenin regulates IL-8, VEGF, MMP2, and MMP9 expression (28, 29), and acts as a major pathway frequently altered in human gastric cancer (30). Accordingly, we hypothesized that β-catenin operates in IL-32-phospho-AKT signaling. Interestingly, expression of active β-catenin was dramatically increased in the nuclei of IL-32–overexpressing cells in which p-AKT was activated as well as the intermediate regulator, phospho-GSK-3β (Fig. 3A). This finding was further confirmed using doxycycline-inducible IL-32–overexpressing cells in which active nuclear β-catenin was increased via inducing IL-32 production over a short period of time, compared with empty vector control cells (Supplementary Fig. S4A). To provide evidence of the mechanistic role of β-catenin on the regulation of IL-32/p-AKT–induced IL-8, VEGF, MMP2, and MMP9 expression, our results collectively indicate that AKT activation by IL-32 is required to induce IL-8, VEGF, MMP2, and MMP9 expression for cell invasion.

Active β-catenin is a downstream effector of phospho-AKT in IL-32–induced cell invasion

Next, we investigated the mechanism underlying AKT-dependent invasiveness in IL-32–overexpressing cells. Activated AKT is capable of phosphorylating and inactivating β-catenin (27). Several studies have reported that β-catenin regulates IL-8, VEGF, MMP2, and MMP9 expression (28, 29), and acts as a major pathway frequently altered in human gastric cancer (30). Accordingly, we hypothesized that β-catenin operates in IL-32-phospho-AKT signaling. Interestingly, expression of active β-catenin was dramatically increased in the nuclei of IL-32–overexpressing cells in which p-AKT was activated as well as the intermediate regulator, phospho-GSK-3β (Fig. 3A). This finding was further confirmed using doxycycline-inducible IL-32–overexpressing cells in which active nuclear β-catenin was increased via inducing IL-32 production over a short period of time, compared with empty vector control cells (Supplementary Fig. S4A). To provide evidence of the mechanistic role of β-catenin on the regulation of IL-32/p-AKT–induced IL-8, VEGF, MMP2, and MMP9 expression, our results collectively indicate that AKT activation by IL-32 is required to induce IL-8, VEGF, MMP2, and MMP9 expression for cell invasion.
introducing either β-catenin or IL-8 shRNA (Fig. 3B). The data indicate that β-catenin and IL-8 act as downstream targets of p-AKT in the IL-32 signaling pathway and induce VEGF, MMP2, and MMP9 production. To determine whether active β-catenin and IL-8 are required for IL-32–induced cell invasiveness, we performed an invasion assay with β-catenin or IL-8 knockdown cells. Consistent with their well-established role in cancer cell invasion, depletion of β-catenin and IL-8 significantly suppressed IL-32–induced matrix invasion ability, but had little effect on invasiveness of control cells (Fig. 3C). Interestingly, elongated IL-32–overexpressing cells was partly restored to a round shape upon β-catenin knockdown, but not IL-8 depletion (Fig. 3D). Taken together, these results indicate that IL-32 enhances the activity of AKT, and subsequent β-catenin activation results in the production of IL-8, VEGF, MMP2, and MMP9 to promote cell invasion.

Silencing of IL-32 reduces the invasive ability of human gastric cancer cells mediated via inactivation of the p-AKT/β-catenin pathway

To investigate the significance of IL-32 in gastric cancer metastasis, we transduced AGS or TSGH cells with 2 independent shRNAs targeting human IL-32. Introduction of IL-32 siRNA resulted in reduced IL-32 expression (Supplementary Fig. S6A and S6B), and more importantly, was accompanied by decreased cell invasion, compared with shLuc control, in both gastric cancer cell lines (Fig. 4A and B). Consistent with the molecular changes in IL-32–overexpressing cells, IL-32 silencing resulted in decreased expression of p-AKT, active β-catenin, IL-8, and VEGF (Fig. 4C). We additionally re-expressed a siRNA-resistant form of IL-32 by introducing a silent mutation in the siRNA target region into IL-32 knockdown cells. Immunoblot analyses confirmed that expression of IL-32 is restored to the control cell level (Supplementary Fig. S6A). As expected, re-expression of IL-32 was sufficient to restore the invasive properties of TSGH IL-32 knockdown cells (Fig. 4A). Next, we compared in vivo metastasis formation of TSGH shLuc control and IL-32 knockdown cells to further establish the relationship between IL-32 expression levels and metastasis formation. Following injection of cells into the tail vein of immunodeficient mice for 5 weeks, the number of experimental lung metastatic nodules was examined macroscopically and microscopically for the occurrence of metastasis. The number decreased significantly in animals injected with IL-32 knockdown cells, compared with those injected with shLuc control cells (Fig. 4D). Our results clearly show that silencing of IL-32 decreases invasive activity in vitro and pulmonary metastatic potential to a substantial extent in vivo in human gastric cancer cells.

Figure 1. IL-32 overexpression in human gastric cancer is correlated with prognosis of patients. A, scatter plot of IL-32 IHC scores according to depth of wall invasion (\(P = 0.016, \text{T1–T2 vs. T3–T4}\)). B, scatter plot of IL-32 IHC scores according to lymph node metastasis (\(P = 0.005, \text{N0 vs. N1–3}\)). C, scatter plot of IL-32 IHC scores according to pathologic stage (\(P = 0.011, \text{stage I–II vs. stage III–IV}\)). D, Kaplan-Meier survival curves of 2 groups of patients with gastric cancer defined by an IL-32 expression level cutoff value of 100 (the median), established on the basis of IHC scores. The 5-year survival rate of the lower expression groups (\(n = 47\)) was significantly better than that of the higher expression groups (\(n = 73\); 68.4% vs. 40.4%; log-rank \(P = 0.0045\)).
IL-32, CTTNB1 (β-catenin), IL-8, VEGF-A, MMP2, and MMP9 are coexpressed in human gastric malignancies

To determine the clinical significance of the IL-32/β-catenin/IL-8/VEGF-A/MMP2/MMP9 axis, we examined whether the observed correlation between IL-32 and these downstream effectors could be extended to human gastric cancer. Using oncomine, expression values for IL-32, CTTNB1, IL-8, VEGF-A, MMP2, and MMP9 were regained from the published Cho gastric microarray dataset (GEO accession: GSE13861; ref. 31). Spearman analysis performed on the plotted mRNA expression values revealed a significantly moderate correlation between expression of IL-32 and its downstream effectors in 65 gastric adenocarcinoma specimens (Fig. 5A and Supplementary Fig. S7). However, this finding remains to be experimentally confirmed. Further immunohistochemical analysis for IL-32 and IL-8 in 85 cases of gastric cancer disclosed a significantly moderate correlation between IL-32 and IL-8 (Fig. 5B and C; Spearman r = 0.562, P < 0.001). These results strongly suggest that the regulation of CTTNB1 (β-catenin), IL-8, VEGF-A, MMP2, and MMP9 by IL-32 occurs not only in gastric cancer cell lines in vitro, but also human gastric tumors in vivo.

Discussion

Gastric cancer is the second most common cause of cancer-induced death worldwide. The prognosis in the late
stage of gastric cancer remains extremely poor, and median survival rarely approaches 1 year, mainly because of metastasis (32). No effective therapeutic targets have been identified to date. The most important objective of gastric cancer research is to elucidate the molecular mechanism(s) underlying metastasis.

IL-32 was overexpressed in gastric cancer, whereas expression was significantly low in nontumor mucosa, as described previously (7). IL-32 expression in tumors from the Cho gastric microarray dataset was higher than that in nontumor specimens, in keeping with our data. The mechanism underlying IL-32 overexpression in gastric cancer is unclear at present. It has been reported that chromosome 16p13.3 region, which contains the IL-32 gene is frequently amplified in breast and small intestine cancer (33, 34). The above findings provide implications of IL-32 dysregulation in the mechanism of gastric cancer development, but more experimental evidence is needed to confirm this hypothesis. In our analyses, Kaplan–Meier survival analysis showed that higher IL-32 expression is significantly associated with poor prognosis of patients with gastric cancer. IL-32 was additionally identified as an independent prognostic marker in multivariate analysis in parallel with the known clinical and pathologic factors for gastric cancer. Further analysis of the predictive potential of IL-32 expression supported its utility as a predictor to identify aggressive gastric cancer, consistent with both Kaplan–Meier and Cox regression analyses. This study provides evidence that IL-32 is a useful independent biomarker for prognosis. Shortly before the publication of this article, a study also found higher IL-32 expression is correlated with poor postoperative outcomes of patients with gastric cancer (8). Although that study only focused on clinical significance, the findings lend support to our claim of the importance of the regulation and function of IL-32 in gastric cancer.

Ectopic expression of the largest γ variant of IL-32, which can be spliced into IL-32β and IL-32α in gastric cancer cells, leads to spindle-like morphology and increased cell migration ability. Analogous results were obtained upon ectopic expression of the β variant of IL-32, which generated both β and α isoforms without the γ isoform (data not shown). These results imply that all IL-32 isoforms may have similar functions in cell migration. A more recent study showed that IL-32 contains an RGD motif that potentially binds to and activates integrins and focal adhesion kinase (FAK) essential for cell anchorage and migration, consistent with our findings (35). Previous studies have identified Rac1 as an important downstream component of integrin/FAK signaling (30). Accordingly, we hypothesize that IL-32 induces integrin and Rac1 activation to enhance cell migration ability. Our experiments showed that the Rac1-specific guanine nucleotide exchange factor, Tiam1, is upregulated in IL-32–overexpressing cells. Furthermore, IL-32–induced cell migration was abolished by a selective Rac1/Tiam1 inhibitor, NSC23766 (Supplementary Fig. S8). However, activation of the Wnt/β-catenin pathway has also been shown to trigger changes in epithelial cell morphology (e.g., epithelial-mesenchymal transition) and activate migration and invasion (36). Accordingly, downregulation of β-catenin in IL-32–overexpressing cells partly reversed IL-32–induced cell elongation. Together, these results give possible molecular mechanisms by which IL-32 induce cell migration.

Data from this study indicate that IL-32 triggers cell invasion partially by inducing IL-8, VEGF, MMP2, and MMP9 expression, resulting in degradation of the extracellular matrix. These results are in line with previous findings showing that IL-32 is associated with acquisition of an invasive and metastasis phenotype in lung cancer mediated via coexpression of IL-8 and VEGF (9). Furthermore, IL-8 and VEGF are well defined pro-angiogenic and pro-metastatic factors in numerous human cancers. Ectopic expression of IL-32 is accompanied by hypoxia-inducible factors 1α (HIF-1α) activation, one of the most powerful regulators of angiogenesis (Supplementary Fig. S9). Furthermore, depletion of HIF-1α decreased IL-32–induced cell invasion and IL-8, VEGF, MMP9, MMP2 expression (Supplementary Fig. S9B and S9C). However, the expression of IL-32–induced phospho-AKT and active β-catenin did not change by HIF-1α depletion (Supplementary Fig. S9B). In addition, the expression of IL-32–induced HIF-1α also did not change by β-catenin or IL-8 depletion (Supplementary Fig. S9D). Notably, both active β-catenin and HIF-1α were inhibited whereas AKT was inactivated by MK-2206 (Supplementary Fig. S5A). Therefore, both β-catenin and HIF-1α involve in IL-32–induced cell invasion under phospho-AKT activation. Other investigators have demonstrated an involvement of IL-32 in the regulation of IL-8 in endothelial cells, indicative of a role in modulation of endothelial function (37). The results collectively support an important role of IL-32 in angiogenesis in gastric cancer.

To our knowledge, this study has shown for the first time that IL-32 stimulates activation of AKT and β-catenin as well as HIF-1α to induce IL-8, VEGF, MMP2, and MMP9 secretion for gastric cancer metastasis (Fig. 5D). Clearly, p-AKT, activated β-catenin as well as HIF-1α are upstream regulators of IL-8 expression. However, we cannot exclude the possibility of cross-talk signaling between IL-8, VEGF, MMP2, and MMP9. Previous studies have reported suppression of IL-1β, TNF-α, and INF-γ–induced IL-32α expression via inhibition of the PI3K/AKT pathway in myofibroblasts (38). Gastric cancer cells may use the positive feedback loop to activate the PI3K/AKT pathway for cancer progression.

Figure 3. IL-32 induces IL-8, VEGF–A, MMP2, and MMP9 expression via β-catenin activation. A, expression of indicated molecules in IL-32–overexpressing and control cells was detected via Western blotting. B, expression of indicated proteins in v1 and IL-32–2 cells transfected with the gene silencers, shLuciferase (shLuc), β-catenin shRNA (shβ-catenin), or IL-8 shRNA (shIL8), was detected using Western blotting. C, invasion assay of indicated cells was performed. Magnification, ×100. Scale bar represents 200 μm. Quantified results are presented as a histogram in the right panel. One-way ANOVA with Tukey multiple comparison tests (mean ± SEM; 3 independent experiments). **, P < 0.01. D, quantification of cells displaying elongated morphology. Elongation is presented as a percentage (mean ± SEM; 3 independent experiments, n > 400) of cells with a length of more than 3 times the width. **, P < 0.05. Left: representative phase-contrast images. The arrows indicate the presence of a spindle shape. Magnification, ×400. Scale bar represents 50 μm.

www.aacajournals.org Clin Cancer Res; 20(9) May 1, 2014 2285
Figure 4. Depletion of IL-32 in gastric cancer cell lines reduces invasion ability in vitro and in vivo. A, invasion properties of the indicated cells (6 × 10^5) were analyzed. Magnification, ×100. Scale bar represents 200 μm. Quantified results are presented as a histogram in the right panel. One-way ANOVA with Tukey multiple comparison tests (mean ± SEM; 3 independent experiments). *, P < 0.05; ***, P < 0.001. B, the invasive properties of indicated cells were examined. Magnification, ×100. Scale bar represents 200 μm. Quantified results are presented as a histogram in the right panel. One-way ANOVA with Tukey multiple comparison tests (mean ± SEM; 3 independent experiments). ***, P < 0.001. C, expression of indicated molecules in control or IL-32 knockdown TSGH cells was detected via Western blotting. D, top: H&E staining of metastatic lesions upon intravenous administration of shLuc-1, KD-IL-32-1, and KD-IL-32-2 cells (1 × 10^6) into SCID mice. Microscopic images of the lung tumor nodules indicated with arrowheads. Magnification, ×100. Scale bar represents 200 μm. Bottom: lung sections of tumor-bearing mice were microscopically analyzed, and metastases per histologic section counted. N = 4; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
In summary, significant overexpression of IL-32 was observed in gastric cancer, suggesting a critical role in development and progression to malignancy. IL-32 may be a useful independent prognostic tumor marker to predict survival and metastasis of gastric cancer patients. Our findings collectively provide evidence that IL-32 functions as a potential therapeutic target and support its utility as a useful independent prognostic marker of patient survival.

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

Authors’ Contributions
Conception and design: C.-Y. Tsai, C.-S. Wang, Y.-H. Tseng, K.-H. Lin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-S. Wang, C.-Y. Chen, C.D. Lin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.-S. Wang, M.-M. Tsai, W.-L. Cheng, C.-Y. Chen, K.-H. Lin

Writing, review, and/or revision of the manuscript: C.-Y. Tsai, C.-S. Wang, K.-H. Lin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-C. Chi, Y.-H. Tseng, K.-H. Lin
Study supervision: C.-Y. Tsai, K.-H. Lin

Acknowledgments
The authors thank Dr. Y. Liang for immunohistochemistry and pathologic comments.

Grant Support
This work was supported by grants from Chang-Gung University, Taoyuan, Taiwan (CMRP/PG6B0011, NMRP 170441), and the National Science Council of the Republic of China (NSC 92-2314-B-182-077; 97-2314-B-182-009-MY2; 100-2314-B-182A-074).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 9, 2013; revised December 19, 2013; accepted January 25, 2014; published OnlineFirst March 6, 2014.
References

Interleukin-32 Increases Human Gastric Cancer Cell Invasion Associated with Tumor Progression and Metastasis

Chung-Ying Tsai, Chia-Siu Wang, Ming-Ming Tsai, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-1221

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/03/07/1078-0432.CCR-13-1221.DC1

Cited articles
This article cites 37 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/9/2276.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/20/9/2276.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.