Gankyrin Activates IL-8 to Promote Hepatic Metastasis of Colorectal Cancer

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Abstract

Hepatic metastasis is responsible for the majority of colorectal cancer (CRC)-related mortalities. Although Gankyrin (PSMD10) has been implicated in cancer metastasis, its exact role and underlying mechanisms of CRC hepatic metastasis remain largely unknown. Herein, we showed that the expression of Gankyrin was higher in primary CRC with hepatic metastasis compared with CRC without metastasis. RNAi-mediated silencing of Gankyrin expression in highly metastatic human CRC cells impaired their migratory and metastatic capacity in vivo. Genome-wide transcriptome profiling revealed activation of the interleukin (IL)-8 signaling pathway by Gankyrin. Protein levels of IL-8 and cyclin D1 (CCND1), the two important molecules in the IL-8 pathway, were positively correlated with Gankyrin expression in human CRC specimens. Furthermore, genetic deletion of cyclin D1 showed its requirement in Gankyrin-mediated cell migration. Finally, administration of recombinant IL-8 rescued the migratory defect of CRC cells where Gankyrin expression was silenced. Together, our findings highlight the importance of Gankyrin in hepatic metastasis of CRC and point out its candidature as a potential prognostic marker and therapeutic target to improve the clinical management of metastatic CRC. Cancer Res; 73(14); 4548–58. ©2013 AACR.

Introduction

Gankyrin (also known as PSMD10 or p28gank or nas6) was initially identified as the p28 component, a regulatory subunit of the 26S proteasome complex (1). Gankyrin consists of 7 ankyrin repeats, which are required for protein–protein interactions (2–4). The expression of Gankyrin has been investigated in a variety of cancers with nearly universal findings that showed elevated protein and/or mRNA levels in cancers when compared with normal counterparts (4–5). Gankyrin has been shown to be involved in carcinogenesis and oncogene-induced tumor onset and progression (4, 6–10). Overexpression of Gankyrin promotes cellular proliferation by targeting retinoblastoma protein (Rb), leading to subsequent Rb degradation, activation of E2F transcription factor, and DNA synthesis of genes required for G1–S-phase transition (11–13). Overexpression of Gankyrin promotes oncogenic transformation in NIH3T3 cells (4).

Multiple mechanisms by which Gankyrin acts as an oncoprotein have been proposed: (i) Gankyrin enhances Rb phosphorylation and inactivation, and disengages E2Fs from Rb repression complex (4). E2F, as an oncoprotein, initiates the expression of genes, which encode proteins required for cell-cycle progression. (ii) Gankyrin targets p53 for degradation by ubiquitination-proteasome pathways (14). (iii) Gankyrin participates in C/EBP protein degradation. C/EBP is a tumor suppressor in liver, and reduced protein expression of C/EBP is required for carcinogen-induced tumor initiation and progression (15). Thus, Gankyrin acts as an oncogene by negatively regulating tumor suppressors through posttranslational modification and subsequent degradation of these proteins. (iv) Overexpression of Gankyrin prevents nuclear translocation of NF-κB subunit and inhibits the transcriptional activity of NF-κB (2). (v) Gankyrin mediates Ras-induced tumorigenesis by suppressing ROCK activity (16), and (vi) Gankyrin enhances PI3K/AKT/HIF1 signaling pathway in hepatocellular carcinoma (HCC; refs. 17–19).

Gankyrin-mediated regulation of cancer metastasis has been proposed; however, the mechanisms through which Gankyrin regulates cell migration/invasion and cancer metastasis remain largely unknown. Colorectal cancer (CRC) remains to be one of the most lethal forms of cancers worldwide. In United States, the predicted new cases and CRC-related deaths in 2012 were 143,460 and 51,690, respectively. Synchronous hepatic metastases are detected in 23% of all patients with CRC and additional 25% patients with CRC without distant metastasis at initial diagnosis subsequently...
develop hepatic metastasis (20). Attempted chemotherapy for hepatic metastatic CRC has been showing a disappoint-
ing patient responsiveness rate at only 10% to 40% (21). The
efforts for screening for cellular molecules that are respon-
sible for CRC progression in recent years have added several
proteins that could be targeted pharmacologically. Chemo-
kines and the corresponding receptors have been implicated
in cancer metastasis. The involvement of chemokines/recep-
tors has been summarized in a comprehensive review article
(22). Wnt signaling pathway has a well-established role in
CRC progression and metastasis (23). The interleukin (IL)-8
(22). Wnt signaling pathway has a well-established role in
levels in the CRC tissue showed a signi-
ficant correlation with the microvesSEL density of primary tumor and hepatic
metastasis (24). Cyclin D1, a component of both IL-8 and
Wnt signaling pathways (25–26), is increased in CRC and its
overexpression predicts poor clinical outcome for metastat-
ic CRC (27). The identi

cation of these signaling molecules has
changed the clinical management of patients with CRC
with hepatic metastasis; however, only modest effectiveness
was achieved with these advances. Therefore, there is still an
urgent need to develop biomarkers that predict CRC metas-
tasis and to discover novel therapeutic targets for treating
metastatic CRC.

Herein, we showed that Gankyrin could serve as a biomarker
for differentiating CRC with hepatic metastasis potential from
nonmetastatic CRC. Gankyrin was found to be highly expressed
in colorectal cancers, and nuclear translocation of Gankyrin in
the primary tumor correlates with hepatic metastasis. We
further showed that Gankyrin is required for cell proliferation,
migration, and invasion in both in vitro and in vivo models. Targeting Gankyrin expression impaired tumor growth
and metastasis. Finally, transcriptome regulated by Gankyrin pop-
ulated signaling pathways responsible for cellular proliferation
and migration. Together, these results established Gankyrin as
a biomarker that can be used to evaluate hepatic metastasis
potential in primary CRC. This study also provided mechanistic
insights how Gankyrin functions as an oncoprotein to promote
tumor metastasis through targeting IL-8 and cyclin D1.

Materials and Methods

Clinical specimens, immunohistochemical staining, scoring, and statistical analysis

Formalin-fixed and paraffin-embedded samples recruited in this study were from the tissue bank maintained in the
Department of Pathology at the 90th Hospital of Jinan (Jinan,
China). All tumors were primary and untreated before surgery. All of the specimens were anonymous and the tissues were
collected in compliance with the Institutional review board
regulations from the hospital. The characteristics of patients
with CRC recruited to this study are summarized in Supple-
mentary Table S1.

Immunohistochemical (IHC) staining and scoring for Gan-
kyrin, cyclin D1, and IL-8 was conducted as previously
described (28). A minimum of 100 cells were evaluated in
calculating the H-score (29). Means of continuous variables
for Gankyrin staining intensity between CRCs, matched nor-
mal tissue, and colorectal adenoma (CRA) samples were
compared by one-way ANOVA (multiple comparisons). The
comparison of Gankyrin staining intensity between CRC with
and without hepatic metastasis was evaluated with the Mann-
Whitney U test. The correlation between Gankyrin expression
and that of IL-8 and cyclin D1 was determined using Spearman
Bivariate correlation test. All statistical tests were 2-sided, and
P values <0.05 were considered as statistically significant. The
statistical analyses were conducted using SPSS 13.0 software
(SPSS Inc).

Plasmids, antibodies, and reagents

pLKO-TRC short hairpin RNA (shRNA) clones targeting
Gankyrin were purchased from Open Biosystems. shRNA
targeting human Gankyrin sequence are A, 5′-GCTCAAGT-
GAATGCTGTCAAT-3′, sequence; B, 5′-CAAGGGTACCTT-
GAAGATGAT-3′, or the scrambled derivative 5′-AAGCCAGA-
GCGTTACGTA-3′. The shRNA targeting mouse Gankyrin
sequence is 5′-GCCATACAAAAATGGTGAAT-3′.

Western blotting and IHC assays were conducted using
following antibodies as indicated: anti-FLAG (M2 clone, Sig-
ma), anti-C/EBPα, anti-Gankyrin and anti-cyclin D1 (Santa
Cruz Biotechnology), anti-IL-8 (Abgent), and anti-α-tubulin or
anti-GAPDH (BD Bioscience).

Cell culture

The human embryonic kidney 293T cells (HEK293T) and 3T3
cells were maintained in Dulbecco’s Modified Eagle Medium
(DMEM) culture solution with 10% FBS (FBS). 3T3 cells derived
from C3H10T1/2 cells were previously described (30). Human
colon cancer cell line SW480 was purchased from the American
Type Culture Collection. Mouse colon cancer cell line MC38
was kindly provided by Dr. J. Schlom, National Cancer Institute,
Bethesda, MD (31). CT26 cell line was previously described (32).
The basal culture mediums are: L15 (SW480) and DMEM
(MC38 and CT26). For cell maintenance, the basal medium
was supplemented with 10% FBS.

Cell transfection and transduction

For transient transfection, Superfect transfection reagent
was used following manufacturer’s protocol (Qiagen). For cell
transduction, retroviruses were prepared by transient cotrans-
fecition with helper viral vector into HEK 293T cells using
calcium phosphate precipitation. HEK 293T cells were trans-
fected with plasmid DNA and cultured at 37°C for 6 hours, the
medium was replaced, and after 36 hours, the supernatant was
collected and filtered through a 0.45 μm filter. Cells were infected at approximately 70% confluence in DMEM supple-
mented with 8 μg/mL of polybrene. The following day, the
medium was changed to basal medium supplemented with 10% 
FBS and cultured for further assay.

The siRNAs of Gankyrin and control Photinus pyralis
luciferase gene were from Invitrogen, and the target
sequences were described previously (10). The Lipofecta-
mine reagent (Invitrogen) was used for transfections, and the
final concentration of siRNA was 50 nmol/L. MC38-luc/
shGankyrin and CT26-luc/shGankyrin stable cell lines used
for in vivo experiments were generated by the sequential
induction of luciferase 2 (Luc2) and shGankyrin expression
in MC38 and CT26 cells.
RNA isolation and quantitative real-time PCR
Total RNA was prepared using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. One microgram of total RNA was subjected to reverse transcription to synthesize cDNA using the SuperScript II Reverse Transcriptase Kit (Invitrogen). A 25 μL volume reaction consisted of 1 μL reverse transcription product and 100 nmol/L of each primer. The primers used for quantitative real-time PCR (qRT-PCR) are listed in Supplementary Table S3.

Cell migration and 3-dimensional invasion assays
Cell migration assays were carried out as previously described (10). Each determination represents the average of 3 individual experiments. For 3-dimensional invasion assay, 100 μL of 1.67 mg/mL rat tail collagen Type I (BD biosciences) was pipetted in the top chamber of a 24-well 8 μm pore Transwell (Corning). The Transwell was incubated at 37° C overnight to allow the collagen to solidify. A total of 3 x 10^4 cells were then seeded on the bottom of the Transwell membrane and allowed to attach for 4 hours. Serum-free growth medium was placed in the bottom chamber, whereas 5% serum was used as a chemoattractant in the growth medium of the top chamber. The cells were then chemoattracted across the filter through the collagen above for 3 days. Cells were fixed in 4% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS then stained with 40 μg/mL propidium iodide (PI) for 2 hours. Fluorescence was analyzed using confocal z-sections (4 μm interval) at × 10 magnification from the bottom of the filter. 3-Dimensional reconstructions of the PI-stained cells were done using Carl Zeiss Zen software (2007 Light Edition).

Mouse models for in vivo tumor growth and metastasis
MC38 is a murine colon adenocarcinoma derived from immunocompetent C57BL/6 mice. MC38 cells (2 x 10^6) were injected subcutaneously behind the anterior forelimb of C57BL/6 mice. Each animal group consisted of 8 animals. Tumor volumes were calculated and the growth curves for tumors were plotted as mean ± SD of tumors from 8 mice.

For intrasplenic transplantation, 4 x 10^6 SW480-Luc/shControl and SW480-Luc/shGankyrin cells were transplanted in the spleen of 8-week-old NMRI nu/nu mice at day 0 (4 mice/group). Furthermore, 2 x 10^6 MC38-luc/shControl and MC38-luc/shGankyrin cells were transplanted in the spleen of 6-week-old male C57BL/6 mice at day 0 (6 mice/group). A total of 2 x 10^6 CT26-luc/shControl and CT26-luc/shGankyrin cells were transplanted in the spleen of 6-week-old male BALB/c mice at day 0 (6 mice/group). The animals were staged for 70 days (SW480) or 28 days (MC38 and CT26 cells) to allow for the development of distant metastases. After animals were sacrificed, tumors and metastasis target organs were removed. All experiments were carried out in accordance with the relevant guidelines and approved by our Institute (National Center of Biomedical Analysis).

Genome-wide gene expression analysis
Gene expression profiling was conducted in cells with knockdown of Gankyrin using Affymetrix Human Genome U133 Plus 2.0 Array. Microarray data have been deposited in GEO (Accession No. GSE44029; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44029). The core set of probe set clusters was used with annotation version na30, dated December 2009. The dataset was imported into Matlab version R2010b (The Mathworks), and one-way ANOVA was used to evaluate the significance of differential expression between biologic conditions. Genes with a differential expression P value of 0.05 or less and an absolute fold change of 1.5 or more were clustered and visualized using a clustergram heatmap. The differentially expressed gene list was loaded into Ingenuity Pathway Analysis (IPA) 8.0 software (http://www.ingenuity.com) to conduct biologic network and functional analyses.

Bioluminescence imaging
Animals were injected with 10 mg/kg D-luciferin (Xenogen) in PBS intraperitoneally and anesthetized by isofluorane using the XGI-8 gas anesthesia system (Xenogen). Bioluminescence images were acquired by using the IVIS Imaging System (Xenogen) 10 to 15 minutes after injection.

ELISA
Cells were plated at 60% confluence at day 1 and changed to serum-free medium at day 2. After 24 hours, cell culture medium was harvested and IL-8 concentration was determined by ELISA using a kit purchased from R&D Systems.

Results
Elevated Gankyrin expression in primary colorectal cancers with hepatic metastasis
We previously reported that Gankyrin is overexpressed in human lung and breast cancers and plays an essential role in breast cancer metastasis (10, 16). To determine the role of Gankyrin in tumor metastasis, we extended our observation to CRC given that Gankyrin promoted cell proliferation and tumor growth in a colon cancer cell line HCT116 as recently reported (33). IHC staining was used to determine the expression and subcellular distribution of Gankyrin in a large cohort of CRC specimens. The expression levels of nuclear and cytoplasmic Gankyrin were scored separately, showing that Gankyrin is expressed in both normal and cancer tissues. The expression levels of Gankyrin were significantly increased in cancer cells compared with adjacent normal epithelium (P = 0.0000, Supplementary Fig. S1) or CRA (P = 8.659E-14, Supplementary Fig. S1). In normal epithelial cells, the majority of Gankyrin was found in the cytoplasm, whereas in cancerous tissue, Gankyrin was detected in both cytoplasm and nucleus. These observations showed that Gankyrin is highly expressed in CRCs and the nuclear distribution marks the difference between cancer and normal cells.

Patients with hepatic metastases from primary CRC were considered to be one of the clinical challenges. To explore the correlation between Gankyrin expression and subcellular localization with metastasis in CRC, we investigated an independent cohort of patient samples. This cohort consisted of 48 cases with hepatic metastasis and 33 cases randomly selected from patients with CRC without developing hepatic metastasis in a minimal 5-year follow-up...
The hepatic metastasis lesions were surgically resected at the time of diagnosis. Immunohistochemistry was conducted to determine the Gankyrin expression in these specimens. There was a significant increase of Gankyrin levels in the primary tumors from 48 cases with hepatic metastasis compared with cancer tissues from 33 cases without hepatic metastasis ($P = 0.009$, Fig. 1). The expression levels of Gankyrin were significantly upregulated in the nucleus of CRCs with hepatic metastasis compared with those without metastasis ($P = 0.002$, Fig. 1B). No observable difference in Gankyrin expression was found between the metastatic cancer tissues when compared with tissues from the primary tumors. These results suggest that the highly expressed Gankyrin and its nuclear translocation could define a subtype of CRCs that are potentially metastatic.

**Gankyrin knockdown impairs CRC cell migration, invasion, and tumor metastasis in vivo**

Our observation that Gankyrin expression correlates with hepatic metastasis prompted us to further investigate the role of Gankyrin in cellular migration and invasion. To do so, we conducted Transwell migration assay using SW480 cells stably expressing Gankyrin shRNA (shGankyrin) or control shRNA (shControl). We found that Gankyrin knockdown reduced the number of migrating cells by 2-fold (Supplementary Fig. S2). We examined the effects of Gankyrin on CRC cell movement using wound-healing assays. Representative examples of cell moving toward the wound were shown (Fig. 2A). Gankyrin knockdown significantly reduced the velocity of cell movements ($P = 1.337E-08$, $n = 24$), suggesting that Gankyrin is required for cell movement in cancer cells, a key aspect of cancer metastasis. As shown in Fig. 2B, the shRNA of Gankyrin achieved nearly 100% knockdown efficiency at protein levels.

To further test whether the endogenous Gankyrin contributes to the invasiveness of SW480 cells, we carried out a modified Transwell assay by coating the device with extracellular matrix (ECM). The cells were seeded in the top chamber in serum-free medium. The medium supplemented with 10% FBS was provided in the bottom chamber, serving as chemoattractant. Compared with control group, the transmigration distance through the ECM of SW480 Gankyrin-knockdown cells was significantly reduced (Fig. 2C).

To evaluate the effect of Gankyrin in tumor metastasis in vivo, we adopted a well-established spleen-hepatic metastasis model. We transduced Gankyrin knockdown and control SW480 cells with a retroviral plasmid DNA–encoding luciferase to visualize the metastasis. In each group of the experiment, $4 \times 10^6$ cells were inoculated into the distal tip of the spleen in immunodeficient nu/nu mice. At day 70, mice were sacrificed and the spleen and liver organs were removed. Luciferin substrates were injected into mice 10 minutes after organ removal. Luciferase activity was examined for the presence of “primary” splenic tumor and hepatic metastasis lesions. We found that the “primary” tumors were detected in spleen of both Gankyrin knockdown and control groups and hepatic metastasis was only present in mice implanted with SW480 control group. The Gankyrin knockdown completely abolished the hepatic metastasis (Fig. 2D). Together, these results suggest that Gankyrin is required for cell migration and invasion and hepatic metastasis of CRC in vivo.

**Figure 1.** Gankyrin expression is increased in metastatic CRC.

A, IHC staining of primary CRC specimens for Gankyrin protein expression and subcellular localization. Comparison was made between cancer samples from patients with or without hepatic metastasis at the time of diagnosis. B, boxplot of IHC staining score for Gankyrin showing increased expression in primary cancer in patient who has hepatic metastasis.
Gankyrin is required for tumor metastasis in immunocompetent mice

The immunodeficient mouse models are ideal for evaluating tumor growth using human cancer cells; however, these models do not reflect the nature of colon cancer in hosts developing antitumor immune response. Two murine colon carcinoma cells were tested in immunocompetent allograft models. The murine carcinoma-38 (MC38) colon cancer cell line was derived from a high-grade murine colon tumor, induced by carcinogen and able to grow in C57BL/6 mice. MC38 cells were transfected with shRNA vector targeting mouse Gankyrin, and stable clone was established by antibiotic selection. qRT-PCR showed a successful knockdown of Gankyrin expression (Supplementary Fig. S3A). MC38/shGankyrin and MC38/shControl cell were implanted subcutaneously. As shown in Fig. 3A, knockdown of Gankyrin significantly reduced tumor growth rate reflected by tumor size at each measuring time point (left) and tumor weight at day 28 (middle and right).

Next, in assays using spleen-hepatic metastasis model, 2 × 10⁶ MC38/shGankyrin and MC38/shControl cells were injected into the spleen of C57BL/6 mice, respectively. Liver was removed at day 28 and the metastasis was visualized by bioluminescence imaging, showing dramatic decrease of hepatic metastasis in mice with injection of MC38/shGankyrin cells (P = 0.017, Fig. 3B). The observation was further confirmed by the second immunocompetent mouse model. CT26 cell line was derived from a carcinogen-induced colon cancer in BALB/c mice. Stable transfection of shRNA reduced the gene expression, as evidenced by qRT-PCR (Supplementary Fig. S3B). Both CT26/shGankyrin and CT26/shControl cells were inoculated into the spleen of BALB/c mice. Similar to the results above, knockdown of Gankyrin in CT26 cells also significantly reduced hepatic metastasis (Fig. 3C). To clearly display the hepatic metastasis lesions, both liver and spleen organs were removed and the number of liver surface metastatic foci was counted, showing a significant difference between the 2 groups (P = 0.003, Fig. 3D). These results suggest that Gankyrin expression is required for hepatic metastasis of CRC in vivo.

IL-8 signaling pathway is regulated by Gankyrin in CRC cells

The nuclear translocation of Gankyrin observed in clinical specimens raises the question whether Gankyrin functions as a regulator of gene transcription. It has been previously reported that Gankyrin regulates transcription indirectly through NF-κB and Wnt signaling pathways (2, 17). To identify the transcriptional targets of Gankyrin, we conducted genome-wide screening using SW480 cells with Gankyrin knockdown on an
Affymetrix gene expression array, which identified 937 genes that were differentially regulated by Gankyrin knockdown (1.5-fold, \( P < 0.05 \)). Six hundred and sixty three were decreased and 274 increased in SW480/shGankyrin cells when compared with vector control cells (Fig. 4A and Supplementary Table S2). IPA revealed that the molecular and cellular functions were affected by Gankyrin knockdown. These pathways include cell death, cellular movement, cell cycle, and cell-to-cell signaling and interaction (Fig. 4B). The genes regulated by Gankyrin also populated canonical pathways including IL-8 signaling, AMPK signaling, molecular mechanisms of cancer, xenobiotic metabolism signaling, and PI3K/AKT signaling (Fig. 4C and D). It is worth noting that IL-8 signaling, including IL-8 and cyclin D1, has been linked to CRC metastasis. Some of key genes involved IL-8 pathways were validated by qRT-PCR (Supplementary Fig. S4).

Gankyrin promotes colorectal cell migration through targeting IL-8 and cyclin D1

To investigate the functional interaction between Gankyrin and IL-8 signaling, IHC staining was conducted on CRC samples to determine the correlation between Gankyrin and IL-8 expression, showing that Gankyrin has a significantly positive correlation with IL-8 expression (\( r = 0.312, \ P = 0.021 \), Fig. 5A and B). Consistently, Gankyrin overexpression in SW480 cells increased the IL-8 production (Fig. 5C, left), and Gankyrin knockdown reduced IL-8 levels (Fig. 5C, right). To further determine whether IL-8 induced by Gankyrin contributes to cell migration, recombinant IL-8 protein was added to the medium in Transwell migration assays. As shown in Fig. 5D, IL-8 partly rescued the migratory defect of SW480/shGankyrin cells. Together, these results show that IL-8 plays an important role in Gankyrin-mediated CRC cell migration.
Cyclin D1, encoded by *CCND1* gene, has been reported to be either amplified or overexpressed in CRC, which correlates to enhanced cellular proliferation, migration, and metastasis (34–37). Overexpression of Gankyrin induces cyclin D1 protein expression in hepatic carcinoma (17). To test whether Gankyrin requires Cyclin D1 in promoting cell migration, we overexpressed Gankyrin in 3T3 fibroblast derived from *Ccnd<sup>+/+</sup>* and *Ccnd<sup>−/−</sup>* mice (30). Consistent with the role of Gankyrin in CRC cells, overexpression of Gankyrin enhanced cell migration in *Ccnd<sup>+/+</sup>* 3T3 fibroblast, and the enhanced cell migration by Gankyrin was decreased in *Ccnd<sup>−/−</sup>* cells (Fig. 6A). These results suggest that cyclin D1 is also involved in Gankyrin-mediated CRC cell migration.

To investigate whether Gankyrin is correlated with cyclin D1 in CRC, we determined the expression levels of Gankyrin and cyclin D1 on consecutive slides of CRC samples. As shown in Fig. 6B, cyclin D1 and Gankyrin were both elevated and significantly correlated, suggesting these 2 proteins may functionally associate to regulate cell migration. Overexpression of Gankyrin increased the cyclin D1 abundance (Fig. 6C). Conversely, Gankyrin knockdown by shRNA in SW480 cells reduced the expression of cyclin D1 protein. It has been reported that oncoprotein Gankyrin contributes to the ubiquitin-proteasome–dependent elimination of C/EBPα during liver cancer development (15). C/EBPα inhibits *CCND1* gene expression through recruiting histone deacetylase 1 (HDAC1; ref. 38). To gain mechanistic insight how Gankyrin regulates cyclin D1 expression, we further examined the expression level of C/EBPα. Western blot analysis showed that C/EBPα expression was significantly reduced by Gankyrin overexpression. Furthermore, Gankyrin knockdown increased the protein abundance of C/EBPα (Fig. 6C and Supplementary Fig. S5). Together, these results suggest that Gankyrin induces cyclin D1 expression by downregulating the expression of C/EBPα, the known negative regulator of cyclin D1.
Discussion

The role of Gankyrin in tumorigenesis has been well established in a variety of human malignancies including HCC, esophageal squamous cell carcinoma, breast, pancreatic, oral, lung, and CRCs (4, 6–8, 10, 16, 19, 39, 40). Our results in the current study reveal the profound impact of Gankyrin in governing the capability of CRCs that metastasize to the liver. This study extended our understanding of Gankyrin functioning as an oncoprotein by showing that overexpression of Gankyrin promotes hepatic metastasis of CRC. Using genome-wide gene expression screening, we identified a transcriptome regulated by Gankyrin, populating IL-8 signaling pathway, which mediates the effect of Gankyrin in cell proliferation, migration, and metastasis in vivo.

Mechanistic studies have been intensively focusing on the function of Gankyrin as a component of proteasome complex in explaining its oncogenic activity. Gankyrin promotes Rb phosphorylation/inactivation through sequestering p16 from CDK4 complex and activates E2F family proteins (3, 41). Through binding ubiquitin ligase MDM2, Gankyrin targets p53 degradation (14, 42). In a carcinogen-induced liver cancer model, Gankyrin eliminates the tumor suppressor C/EBPα and promotes tumor development (15, 43). Despite these significant advances in understanding the role of Gankyrin in cell proliferation, tumor onset, and progression, the molecular mechanisms that underpin the function of Gankyrin in cell migration, invasion, and metastasis remain largely unknown. Our results show that Gankyrin overexpression defines a patient group whose tumors have increased potential to metastasize to the liver.

Gankyrin regulation of protein stability through ubiquitin-proteasome system-mediated degradation has been recognized (4, 14, 42). Several lines of evidence suggest that Gankyrin regulates gene expression indirectly through targeting transcription factors and regulators such as E2F, NFκB, C/EBP, β-catenin, and Oct-4 (2, 4, 14–15, 17, 44–45). In this study, we conducted systematic analysis of gene expression regulated by Gankyrin using a genome-wide microarray approach. Nine hundred and thirty-seven genes were significantly regulated by Gankyrin. Pathway analysis of these genes confirmed the previous observations that Gankyrin regulates p53, C/EBPα, and HIFα-dependent gene expression. Recent study using transgenic zebrafish revealed that Gankyrin overexpression induced liver steatosis and
modulated gene and microRNA expression (46). Together, these studies suggest an important role of Gankyrin in regulating gene transcription. Future studies focusing the molecular details by which Gankyrin regulates gene transcription are warranted.

It has been previously reported that Gankyrin was mainly distributed to the cytoplasm in HCC (47–48). The IHC staining of Gankyrin showed similar subcellular distribution patterns in normal colon epithelium. A significant increase of nuclear Gankyrin distribution was observed in CRC cells when compared with the adjacent normal-like epithelium (Supplementary Fig. S1). In addition, increased nuclear Gankyrin differentiated the CRC patient population with increased risk of developing liver metastasis (Fig. 1). These observations suggest that the nuclear translocation of Gankyrin could play an important role in cancer progression. It is important to investigate the mechanisms that promote Gankyrin nuclear translocation in future studies. Targeting nuclear translocation of Gankyrin could potentially serve as a novel strategy to prevent hepatic metastasis of CRC.

It has been known for years that IL-8 production is increased in patients with CRC and that IL-8 promotes tumor growth and cancer metastasis (24). Microarray analysis revealed that genes in IL-8 signaling pathway are downregulated in Gankyrin knockdown cells, suggesting that Gankyrin governs this pathway likely through upregulation of IL-8 secretion. Cyclin D1 is required for Gankyrin promotion of cell migration. A, 3T3 cells derived from Ccnd1 wild-type (Ccnd1+/+) and knockout (Ccnd1−/−) were transduced with retroviral vectors expressing Gankyrin or control. Transwell migration assays were conducted. The number of cells migrating across the membrane was counted. Crystal violet dye staining of cells that migrated is shown. The data are shown as mean ± SEM of the number of cells migrated in 3 independent experiments. B, immunohistochemistry was conducted on CRC specimens. Representative images showed that Gankyrin and cyclin D1 were both overexpressed in CRC. There is a significant correlation between Gankyrin and cyclin D1 (Spearman ρ = 0.372, P = 0.005). C, Western blot analysis was conducted to examine the expression levels of cyclin D1 and C/EBPα in Gankyrin overexpressing and knockdown cells. D, schematic representation of Gankyrin functions. Gankyrin upon nuclear transformation promotes cellular proliferation and tumor growth likely through upregulation of cyclin D1. Gankyrin induction of IL-8 secretion promotes cell migration/invasion and cancer metastasis.
that the protein levels of cyclin D1 and Gankyrin are significantly correlated in human CRC specimens. We further showed the requirement of cyclin D1 and IL-8 in Gankyrin-mediated cell migration by showing that cyclin D1 deficiency blocked Gankyrin-dependent cell migration phenotype and IL-8 was able to rescue the migratory defect–associated Gankyrin knockdown.

In summary, our results show that Gankyrin distinguishes CRCs with hepatic metastasis from those with less metastatic potential. Knockdown of Gankyrin in CRC cells impairs the ability of the cells to migrate, invade, and metastasize in vivo. Our data provide novel insights into understanding the mechanisms by which Gankyrin regulates metastasis. In addition to the known targets, Gankyrin enhanced IL-8 pathways and thus increased cell proliferation and/or nuclear translocation in CRC hence represents a potential attractive biomarker for early detection of CRC with potential to develop hepatic metastasis, as well as the therapeutic approach, which would target the tumor growth and prevents cancer progression toward hepatic metastasis. By synthesizing our findings, we propose a model whereby Gankyrin overexpression enhances cellular proliferation, tumorigenesis, and metastasis by regulating cyclin D1 and IL-8–dependent pathways (Fig. 6D).

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

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