RGS-GAIP–interacting protein controls breast cancer progression

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Running title: Role of GIPC in breast cancer progression

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Abstract

While the importance of RGS-GAIP–interacting protein (GIPC) in the biology of malignant cells is well known, the molecular mechanism of GIPC in the inhibition of tumor progression has not been identified. This study focused on elucidating the molecular role of GIPC in breast cancer progression. By using a human breast tumor specimen, an in vivo mouse model, and breast cancer cell lines, we showed for the first time that GIPC is involved in breast cancer progression through regulation of breast cancer cell proliferation, survival, and invasion. Furthermore, we found that the Akt/Mdm2/p53 axis, insulin-like growth factor-1 receptor (IGF-1R), matrix metalloproteinase-9 (MMP-9), and Cdc42 were downstream of GIPC signaling in breast cancer cells. Moreover, we showed that wild-type p53 reduced GIPC-induced breast cancer cell survival, whereas mutant p53 inhibited GIPC-induced cell invasion. Finally, we demonstrated that a myristylated GIPC peptide (CR1023, Myristoyl-PSQSSSEA) capable of blocking the PDZ domain of GIPC successfully inhibited MDA-MB-231 cell proliferation, survival, and further in vivo tumor growth. Taken together, these findings demonstrate the importance of GIPC in breast tumor progression, which has a potentially significant impact on the development of therapies against many common cancers expressing GIPC, including breast and renal cancer.
Introduction

RGS-GAIP–interacting protein (GIpc) was originally identified as a protein that interacts with GAIP (RGS 19), a regulator of G protein signaling thought to play a role in the trafficking of clathrin-coated vesicles (1). Since then, different studies have suggested an important role for GIpc in the biology of normal and malignant cells (2-4). It is well known that GIpc mRNA is relatively highly expressed in gastric, pancreatic, colorectal, and lung cancer (3). However, the expression of GIpc mRNA is downregulated in primary kidney, colorectal, gastric, and prostate tumors (3), which hints at the complexity of GIpc function. Thus, a number of experiments have been initiated to further elucidate the role of GIpc in the tumor environment.

Several published reports suggest that GIpc is a novel cancer-associated antigen and therapeutic target. One study reports a role for GIpc in cancer cell invasion and metastasis (5). Also, a recently published study revealed that the cytoplasmic domain of type III transforming growth factor-beta receptor (TGF-βRIII) plays a role in TGF-βRIII-mediated suppression of breast cancer progression in vivo. The interaction between the cytoplasmic domain of TGF-βRIII and GIpc is critical in mediating the suppressive effects of TGF-βRIII on signaling and invasion (6). The PDZ domain of GIpc can specifically interact with the COOH terminus of GAIP. Our laboratory has shown that targeting GIpc with short interfering RNA or an inhibitory peptide targeting the PDZ domain substantially reduces pancreatic adenocarcinoma growth in vivo (4). Taken together, these reports indicate that targeting GIpc and its PDZ domain, which
can interact with tyrosine kinase receptors such as IGF-1R, could be a promising new treatment option for breast cancer and other GIPC-expressing cancers.

In this study, we explored the molecular role of GIPC in breast cancer progression. Furthermore, we evaluated the role of a myristylated GIPC peptide (Myristoyl, PSQSSSEA), designated as CR1023, in GIPC-mediated breast tumor growth. Our results suggest that GIPC plays an important role in breast cancer progression through the involvement of breast cancer cell proliferation, survival, migration, and invasion, and that CR1023 could be used as a generalized and novel approach for the treatment of a variety of cancers characterized by the overexpression of GIPC.

**Materials and Methods**

**Antibodies:** Anti-GIPC, anti-p53, anti-p21, anti-Mdm2, anti-IGF-1Rβ, anti-phospho-IGF-1Rβ, and anti-epidermal growth factor receptor (EGFR) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-Akt1/PKB and anti–phospho-Akt1/PKB(Ser473) were purchased from Upstate Biotechnology (Waltham, MA), and anti–β-actin was purchased from Sigma Chemical (St. Louis, MO).

**Breast tissue specimens and patient data:** Human breast tumor specimens were purchased from US Biomax (Rockville, MD). Of the 90 patient tissues examined, 88 were from females. With grading performed according to pathology diagnosis, four tumors were grade 1 (well differentiated), 43 were grade 2 (moderately differentiated), 43 were grade 2 (moderately differentiated),
16 were grade 3 (poorly differentiated), and five were grade 4 (undifferentiated). GIPC expression was scored semi-quantitatively in respect to intensity and extent of staining.

**Tissue microarray analysis:** Paraffin-embedded specimens were de-paraffinized and rehydrated. Endogenous peroxidases were quenched with 3% H$_2$O$_2$ for 10 minutes. Antigen retrieval was done with 0.01mol/L citric buffer for 10 minutes under heat. Specimens were incubated with anti-GIPC (Proteus BioSciences, Ramona, CA) for 60 minutes at room temperature and developed using the IHC Select DAB kit (Millipore).

**Cell culture:** The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from American Type Culture Collection (Manassas, VA). MDA-MB-231 (wild-type and stable transfectants) and MCF-7 cells were maintained in RPMI medium and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, respectively.

**Plasmids:** The expression plasmid for GIPC short hairpin RNA (shRNA) was purchased from Open Biosystems. The targeting sequence for GIPC was 5'-GCAAATGCAATAATGCCCTCA-3' (shGIPC).

**Virus production and infection:** Retroviruses for shGIPC and the control GFP shRNA were prepared, and MDA-MB-231 cells were infected as described (7). After infection, 2 µg/mL puromycin was added to the medium for antibiotic selection. Stable clones were isolated and expanded for subsequent experiments.

**RNA interference:** Breast cancer cells plated at 50% confluence were transfected in Opti-MEM medium with siRNA-targeting GIPC, p53, or a scrambled control using DharmaFECT 4 (Dharmacon, Lafayette, CO). After 24 hours, Opti-MEM was removed and replaced with complete medium for another 72 hours.
Proliferation and viability assay: Viability of the cells was measured using an MTS assay (Promega). For proliferation, thymidine incorporation was used.

Migration and invasion assay: Cell migration was performed as described previously (7). FBS (10%) was added as the chemoattractant in the lower well of the chambers. The invasion assay was performed similarly except that the transwell chambers were coated with 4 mg/mL Matrigel (BD Biosciences, San Jose, CA), and the incubation time was 16 hours.

Flow cytometry analysis: siRNA-transfected cells were collected and apoptosis was assessed using the Annexin V-FITC/PI Apoptosis Detection Kit (BioVision, Inc., Mountain View, CA) according to the manufacturer's instructions. Apoptotic cells and necrotic/late-apoptotic cells were measured using the FACSCalibur analysis system (Becton Dickinson, CA).

Rho family GTPase activities assay: Cells were seeded at roughly 1 x 10^6 cells per 60 mm in a tissue culture dish. After a 24-hour incubation in serum-free medium, RhoA, Cdc42, and Rac1 activities were assessed using the G-LISA RhoA, Cdc-42, and Rac1 Activation Assay Biochem Kits (Cytoskeleton, Inc., Denver, CO) according to the manufacturer's protocol.

Myristlated peptide design: We have designed an octapeptide (Myristoyl-PSQSSSEA, designated as CR1023) possessing the SEA-binding motif, a control peptide (Myristoyl-SESPSASQ, designated as CR2055), and a fluorescein isothiocyanate (FITC)-conjugated GIPC peptide (Myristoyl-K(FITC)PSQSSSEA, designated as CR1171), as described previously (8).
In vitro cell treatment with GIPC peptide: MDA-MB-231 cells were treated with GIPC peptide (CR1023), control peptide (CR2055), and FITC-conjugated GIPC peptide (CR1171) at different doses (50 to 200 µM) for 2 days.

Subcutaneous tumor model: Six-week-old female nude mice were obtained from the National Cancer Institute-Frederick Animal Production Program. Mice were housed in the institutional animal facilities, and all animal work was performed under protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee. To establish tumor growth in mice, 2 x 10^6 cells of each clonal shRNA transfectant were resuspended in phosphate-buffered saline (PBS) and injected into the mammary fat pad with growth-factor–reduced Matrigel (BD Biosciences). To examine the effect of GIPC peptide on tumor growth, 2 x 10^6 MDA-MB-231 wild-type cells were injected into the mammary fat pad of 4- to 6-week-old female SCID mice. When the tumor size reached about 5 to 10 mm, 500 μg CR1023 suspended in 50 μl dimethyl sulfoxide (DMSO)-PBS (80% DMSO in PBS) or DMSO-PBS were injected directly into the tumor every other day for 1 month. Tumors were measured every 5 days. Primary tumor volumes were calculated with the formula \( V = \frac{1}{2} a \times b^2 \), where \( a \) is the longest tumor axis and \( b \) is the shortest tumor axis. After 30 days, mice were sacrificed and tumors were collected for the evaluation for epidermal growth factor receptor (EGFR) and IGF-1R expression by Western blot analysis.

Statistical analysis: Statistical analyses were performed with the statistical SPSS 11.0 software (SPSS, Inc.). The independent-sample t test was used to test the probability of significant differences between groups.
Results

The role of GIPC in tumor progression. To assess the role of GIPC in breast cancer progression, we purchased human breast tumor specimens from US Biomax and screened tissue microarrays of normal and tumor tissues using immunohistochemistry. As shown by arrows in Figure 1A, GIPC expression was observed mainly on the luminal portion of the infiltrating ductal carcinoma (IDC) and was confined to epithelial cells. Importantly, there was significantly higher cytoplasmic GIPC expression in tumor samples compared to normal breast tissue and in undifferentiated IDC compared to moderately differentiated IDC (Figure 1A and Supplemental Table). These results suggest that GIPC expression correlates with disease progression.

Furthermore, we investigated whether GIPC was required for breast cancer growth in an in vivo tumor xenograft model. Stable MDA-MB-231 transfectants were prepared with shRNA expressing the GFP vector alone or shRNA silencing GIPC expression. Protein lysates from these cells were prepared and analyzed by Western blotting to verify the knockdown of GIPC (Figure 1B). These cell lines were then injected subcutaneously into female nude mice to evaluate the role of GIPC in breast tumor growth. Tumor volumes were recorded weekly. Mice injected with the GIPC-depleted transfectants developed smaller tumors, as determined by a decrease in size compared with mice injected with control GFP-labeled MDA-MB-231 cells (Figure 1B). These results provide evidence that GIPC is involved in breast cancer progression.

Expression of GIPC in breast cancer cell lines. MDA-MB-231 and MCF-7 were
selected to examine the role of GIPC in breast cancer cell growth and invasion. Using equal amounts of cell lysate, we examined GIPC expression by Western blotting. As shown in Figure 2A, GIPC is strongly expressed in both cell lines.

**GIPC contributes to breast cancer cell proliferation, survival, and invasion.** We then examined the influence of GIPC on the proliferation of these breast cancer cell lines. Cell proliferation was examined by MTS assay after the cells were transfected with GIPC-targeting siRNA (GIPC siRNA) oligonucleotides. In both cell lines, cell viability and proliferation were decreased in cells treated with GIPC siRNA compared with those treated with control siRNA (Figure 2B).

The decrease in proliferation in the absence of GIPC suggests that GIPC may also mediate breast cancer cell survival. To examine this possibility, apoptotic levels of MDA-MB-231 cells and MCF-7 cells treated with GIPC siRNA were assessed with flow cytometry. As shown in Figures 2B and 2C, while GIPC siRNA treatment in MCF-7 resulted in a twofold reduction in apoptosis compared with its control, no significant cell death was observed in MDA-MB-231. These results suggest that GIPC mediates the survival of MCF-7 cells but not MDA-MB-231 cells.

The role of GIPC in the migration and invasion of MDA-MB-231 was investigated. As shown in Figures 2B and 2D, knockdown of GIPC was able to inhibit MDA-MB-231 cell migration after 8 hours by about 55%. Furthermore, knockdown of GIPC with GIPC siRNA significantly reduced Matrigel invasion of MDA-MB-231 compared with the cells transfected with control siRNA (44.5% vs 100.00%). These data show that GIPC contributes to MDA-MB-231 cell migration and invasion. We also tried to examine the
effect of GIPC on MCF-7 cell invasion, but did not observe any significant change with or without GIPC (data not shown). GIPC is a PDZ domain-containing protein. The protein-protein interactions mediated by PDZ-containing proteins leads to diverse biology outcomes (43), which depends on its upstream binding partners and downstream signaling in the host cell. MCF-7 is a non-metastatic cell line, suggesting that the important related invasion signals may be absent despite expressing GIPC.

These in vitro assays including cancer cell proliferation, survival, migration, and invasion recapitulate many of the steps crucial to tumorigenesis in vivo, suggesting that GIPC mediates breast cancer progression.

**Akt-Mdm2-p53 signaling is involved in the GIPC signaling pathway.** The phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway is a common signaling pathway that mediates cell proliferation, survival, and migration. It is also well-known that serine 166 and serine 186 of Mdm2 serve as targets for direct Akt phosphorylation (9, 10). Also, phosphorylation of Mdm2 by Akt enables Mdm2 translocation from the cytoplasm into the nucleus and the subsequent inactivation of nuclear p53 (9). Because Mdm2 is a major physiological regulator of p53 function, we tested whether GIPC acts through Akt-mediated Mdm2 phosphorylation to inhibit the apoptotic effects of p53 activity and subsequently increase cell survival. As shown in Figure 3A, knockdown of GIPC significantly reduced Akt phosphorylation, downregulated phospho-Mdm2, and increased p53 expression in both MDA-MB-231 and MCF-7 cells, suggesting that GIPC-mediated breast cancer cell proliferation, survival, and migration/invasion may be due to the involvement of the Akt/Mdm2/p53 pathway.
IGF-1R is downstream of GIPC signaling in breast cancer cells. IGF-1R plays important roles in cell signaling, proliferation, differentiation, and apoptosis (11). It was previously reported that GIPC is associated with IGF-1R and is important to its stability (4, 8). We investigated whether GIPC regulates IGF-1R protein expression in both MDA-MB-231 and MCF-7. As shown by Western blot, IGF-IRβ levels significantly decreased in both cell lines after treatment with GIPC siRNA (Figure 3B). Furthermore, we analyzed the effect of GIPC on IGF-IRβ phosphorylation on MDA-MB-231 cell and found that knockdown of GIPC also reduced the phosphorylation of IGF-IRβ (Figure 3B).

Involvement of MMP-9 and Cdc42 in GIPC-mediated breast cancer cell invasion. Matrix metalloproteinase-9 (MMP-9) preferentially degrades denatured collagens and native collagen type IV, a major component of extracellular matrix and basement membranes whose disruption is central to tumor invasion and metastasis (12, 13). Our analysis of conditioned medium with gelatin zymography revealed that the release of MMP-9 was significantly reduced in MDA-MB-231 cells deficient in GIPC expression (Figure 3C).

Small Rho family GTPases (RhoA, Rac1, and Cdc42) are associated with the morphological transformation of tumor cells and have been linked to tumor cell migration and invasion (14). We thus investigated the effects of GIPC on the activities of RhoA, Rac1, and Cdc42 in MDA-MB-231 by ELISA. We found that knockdown of GIPC reduced Cdc42 activity (p<0.01) (Figure 3D), whereas the activities of Rac-1 and RhoA
were not reduced (data not shown). These results suggest that activation of MMP-9 and Cdc42 in GIPC signaling is important for breast cancer cell invasion.

**p53 status is critical in determining the functional role of GIPC in breast cancer cells.** It is well known that tumor behavior is based upon oncogenes, tumor suppressors, and signal transduction pathways (15, 16). Loss of function from chromosome 17 is the most common genetic alteration observed in breast cancer cells. MDA-MB-231 cells carry a single TP53 (17p13.1) allele (17), while MCF-7 cells have two p53 alleles that are most likely wild-type (17, 18). Our results here show that GIPC mediates MDA-MB-231 migration and invasion but not survival, whereas it mediates MCF-7 cell survival but not migration and invasion. This suggests that the functional role of GIPC is determined by p53 status. We thus sought to compare the effects of wild-type p53 and mutant p53 on the cellular response to GIPC. After co-transduction of the cells with GIPC siRNA and p53 siRNA, we found that the apoptosis of MCF-7 cells was reduced in the cells co-transfected with either GIPC or p53 siRNA (Figure 4A) compared with cells transfected with GIPC siRNA alone, which suggests that wild-type p53 in MCF-7 is involved in cell survival through GIPC signaling. We also checked cell survival in MDA-MB-231 cells solely transfected with p53 siRNA and found that the knockdown of mutant p53 has no effect on their cell survival (data not shown), suggesting that this mutant p53 has lost its tumor suppressive ability. Similar to apoptosis, MDA-MB-231 invasion was increased in cells that were deficient in both GIPC and p53 (Figure 4B). This suggests that mutant p53 in MDA-MB-231 (TP53) is involved in the GIPC-mediated cell invasion.
Mutant p53 regulates MMP-9 activation in GIPC signaling. Wang et al. reported that mutant p53 inactivated Slug degradation, led to Slug accumulation, and increased cancer cell invasiveness in highly invasive cancer cell lines (19). We thus investigated the effect of mutant p53 on the expression level of Slug in shRNA-transfected MDA-MB-231 cells. Dual knockdown of GIPC and p53 did not reduce Slug expression (data not shown). Moreover, we failed to demonstrate an association between p53 and Slug by co-immunoprecipitation (data not shown). The inconsistency of our result with Wang’s study suggests that the effect of the mutant p53-mediated GIPC signaling on breast cancer invasion may function through different signaling pathways. We thus checked whether MMP-9 was downstream of the mutant p53 in GIPC signaling. Knockdown of p53 in stable MDA-MB-231 cells deficient in GIPC led to an induction of MMP-9 expression compared with the GIPC knockdown alone. These data suggest that mutant p53 is involved in the regulation of MMP-9 in MDA-MB-231.

Effect of GIPC blocking peptide on breast cancer progression. GIPC has a central PDZ domain that specifically interacts with the C-terminus of RGS-GAIP (4,8). The C-terminus of GAIP is unique and possesses a distinct SEA motif and it is this motif that specifically interacts with the PDZ domain. To further examine and confirm the role of GIPC in breast cancer progression, a myristylated GIPC octapeptide peptide containing the SEA motif (CR1023, Myristoyl-PSQSSSEA) was designed to inhibit GIPC by specifically binding to its PDZ domain. Our lab has previously shown that this peptide can block the PDZ domain of GIPC and is functional active and capable of suppressing
tumor growth in an *in vitro* and *in vivo* model of pancreatic cancer (4,8).

After treating MDA-MB-231 cells with increasing concentrations of CR1023, we examined its effect on IGF-1R protein expression, cell proliferation, cell survival, and breast cancer growth in vivo. From Western blot analysis, a 48-hour treatment with increasing concentrations of CR1023 (50 and 100 µM) led to a decrease in IGF-1R expression (Figure 5A). To then determine if CR1023 affected cell viability, we performed the MTS assay on MDA-MB-231 cells after peptide treatment. With increasing levels of CR1023 (50 to 200 µM), we detected an inhibition of cell survival compared with samples treated with the control CR2055 peptide or DMSO alone (data not shown). Subsequently, we examined cell proliferation after treatment with CR1023 (50 to 100 µM) and control CR2055. After peptide treatment for 48 hours, the cells were pulsed with ³H-thymidine. Results from the dose-dependent cell proliferation are shown in Figure 5B. CR1023 significantly inhibited MDA-MB-231 proliferation, an effect that was not exhibited in control peptide-treated samples or samples with DMSO alone.

After observing the inhibition of cell proliferation and the downregulation of IGF-1R expression in the presence of CR1023, we investigated whether the blocking peptide induced apoptosis in MDA-MB-231. With annexin V-FITC and propidium iodide staining, a significant induction of apoptosis (roughly 40%) was observed after breast cancer cells were incubated with 200 µM of CR1023 for 48 hours (Figure 5C). No apoptotic increase was detected even with a 200-µM dose of control CR2055.

To determine the localization of the peptides in wild-type MDA-MB-231 cells, the GIPC CR1023 peptide was conjugated to 5-carboxyfluorescein. Fluorescence images were collected; Figure 5D shows the internalizaton of CR1023 (green) in relation to 4’,6-
diamidino-2-phenylindole (DAPI)-stained nuclei (blue). No fluorescence was detected in the untreated control cells.

Taken together, our in vitro results from peptide treatment suggest that GIPC expression correlates with apoptosis, proliferation, and cell viability. We then extended our peptide investigation to an in vivo tumor model. MDA-MB-231 cells were injected into both sides of the mammary fat pads of female SCID mice. When tumors reached 5 to 10 mm, CR1023 peptide was intratumorally injected on alternate days over 1 month. Figure 6A shows that administration of CR1023 peptides suppressed the tumor growth over time. However, tumor growth increased when DMSO-PBS was administered. Tumors were then harvested; their average mass and volume are summarized in Figures 6B and C. It is evident from these figures that peptide-treated mice clearly demonstrate significant tumor regression compared with DMSO-PBS–treated mice. A portion of each tumor was evaluated for IGF-1R expression, and a significant reduction of IGF-1R expression was observed in mice treated with increasing levels of CR1023 (Figure 6D).

Discussion

Key early events in tumor progression involve the proliferation, survival, and invasion of tumor cells. GIPC is a novel modulator of tumor progression, and inhibition of GIPC signaling is considered a promising strategy for inhibiting tumor development. To date, GIPC has not been studied as a target for inhibiting breast cancer progression. To the best of our knowledge, our report is the first to show that GIPC acts through multi-signaling pathways to regulate breast cancer cell proliferation, survival, and invasion,
leading to tumor growth in vivo. This further strengthens GIPC as a promising therapeutic target in cancer. Our study also reveals that the p53 status critically affects the tumorigenic properties of breast cancer development.

Using tissue microarrays and immunohistochemistry, our results show a high level of GIPC in human tissue samples of breast cancer compared with normal breast tissue. Furthermore, we showed that knocking down GIPC inhibits breast cancer growth in an orthotopic mouse model, which supports the results of human breast tumor tissue samples and our assumption that GIPC mediates breast cancer progression. On the basis of these results, we used breast cancer cell lines as an _in vitro_ model system and found that the knockdown of GIPC leads to a proliferative decrease in two different breast cancer cell lines, a result that could be attributed to IGF-1R downregulation. The downregulation of IGF-1R can also lead to an increase in apoptosis. Interestingly, our experiments with siRNA to knock down GIPC did not induce apoptosis in MDA-MB-231 cells but did in MCF-7. The main difference between these two cell lines is their p53 status. The MDA-MB-231 cell has a mutant p53 (TP53), whereas MCF-7 has a wild-type p53. Therefore, this result suggests that wild-type p53 mediates breast cancer cell apoptosis through GIPC, while mutant p53 loses its tumor-suppressive role. A previous study (20) found that wild-type p53 is crucial in regulating cell growth and apoptosis. Blockage of the PI3K pathway caused G1-S-phase arrest, decreased cell growth, and increased chemo- and radio-therapeutic sensitivity in MCF-7 cells that express wild-type p53. However, it did not increase the sensitivity to adriamycin in MDA-MB-453 breast cancer cells that express mutant p53. This result supports our findings.
Some research has implicated GIPC in metastasis and, thus, in the cancer process. Consistent with this finding, our results directly demonstrated that GIPC mediates MDA-MB-231 migration and invasion. Furthermore, previous studies have shown that tumors emerging in mutant-p53 knock-in mouse models display aggressive, metastatic traits that are never detected in tumors developing in a p53-null background (21, 22). These results are supported by molecular epidemiologic data in humans showing that mutant p53-expressing tumors are aggressive and associated with a poor prognosis (23). These cumulative data are evidence that mutant p53 may mediate a gain-of-function metastatic phenotype. However, we found that knockdown of GIPC increased p53 expression levels in MDA-MB-231 cells, and the lack of GIPC and p53 in MDA-MB-231 cells exhibited strong invasive capabilities. This finding indicated that p53 suppresses the GIPC invasion signal. We presume that the inconsistency is due to the different mechanisms involved in mutant p53-mediated GIPC invasion signaling in MDA-MB-231 cells. The regulation of MMP activities by p53 has received little consideration. Mutant p53 activates synovial cell MMP-1 and MMP-13 (24). Wild-type p53 inhibits MMP-2 in melanoma cells (25) though, in contrast, wild-type p53 has also been shown to activate the MMP-2 promoter (26). A recent study reported that wild-type p53 inhibits nuclear factor-kappa β-induced MMP-9 promoter activation (27). Our studies show that mutant p53 partly inhibits GIPC-induced MMP-9 expression in MDA-MB-231 cells. Although the mechanisms of mutant p53 involvement in GIPC at least partially account for the role of GIPC in breast cancer cell growth and metastasis, it would be intriguing to determine whether additional molecules function with mutant p53 to inhibit its migration and invasive capabilities.
It is well established that GIPC/synectin mammalian protein is overexpressed in pancreatic and breast cancer cells and that it also regulates the expression of IGF-1R, which is very important for pancreatic and breast cancer cell survival (1, 4, 8). It is well known that IGF-1R is overexpressed in pancreatic and breast carcinoma and that its expression is regulated by GIPC. Our group has shown that knockdown of GIPC by siRNA or shRNA downregulates the expression of IGF-1R in pancreatic cancer (8). Accordingly, we have examined the IGF-1R expression of MBA-MD231-WT cells by Western blot after treatment with GIPC peptide (CR1023) and observed the downregulation of IGF-1R expression in MDA-MB-231 cells. This GIPC peptide can inhibit the function of GIPC/synectin and, consequently, we have observed the induction of apoptosis, inhibition of cell proliferation, and cell survival of MDA-MB-231 cells and, finally, significant reduction of tumor growth in an in vivo breast cancer model after treatment with GIPC peptide (CR1023). These results confirm that GIPC is a central protein in breast cancer cells. Our observations suggest that GIPC may be a novel target for breast cancer therapeutics and a prime target for the development of small-molecule inhibitors, as it can block the IGF-1R axes, inhibiting breast cancer growth, angiogenesis, metastasis, and chemotherapy resistance.

In summary, this study demonstrated the molecular mechanisms of GIPC in breast cancer progression and provided experimental evidence for the role of GIPC in breast cancer growth. These results should shed new light on the clinical treatment of breast cancer. It is a logical extension to target GIPC in tumor therapy as it is expressed in most common cancers.
Figure Legends

Figure 1. Role of GIPC in breast cancer progression. A. The expression of GIPC in human breast cancer specimens via immunohistochemical staining. Staining of GIPC in normal breast tissue (NBT) (a); Staining of GIPC in a moderately differentiated infiltrating ductal carcinoma (IDC) (b); Staining of GIPC in an undifferentiated IDC (c). The insets show a magnification of GIPC “luminal” staining pattern in human breast tumor specimens. B. The absence of GIPC decreased tumor volume in vivo. GIPC knockdown was confirmed in the cells prior to injection (upper panel).

Figure 2. Tumorigenic properties of GIPC in breast cancer development. A. Western blots showing native expression levels of GIPC in the breast cancer cell lines MDA-MB-231 and MCF-7. Human umbilical vein endothelial cells (HUVECs) were used as a positive control. B. GIPC mediates breast cancer cell viability and proliferation as assessed through an MTS assay. *p<0.01, in a student's t test compared with control (CTL). Western blot analysis confirmed GIPC expression after siRNA knockdown (upper panel). C. MCF-7 cells with downregulated GIPC expression showed an increase in apoptosis. No significant difference was observed in MDA-MB-231 cells. *p<0.05, **p<0.01 in a student's t test. D. NRP-1 and GIPC knockdown reduced migration and invasion in MDA-MB-231 cells. *p<0.01, **p<0.001 in a student's t test compared with control (CTL).
Figure 3. The effect of GIPC knockdown in MDA-MB-231 and MCF-7 cells. A number of molecules were analyzed after GIPC knockdown: **A.** Phosphorylated Akt, phosphorylated Mdm2, and p53; **B.** IGF-1Rβ and phospho-IGF-1Rβ in Western blot. β-actin was used as the loading control. The fold difference of p-IGF-1Rβ is normalized to β-actin expression; **C.** MMP-9 activity was assessed by gelatin zymography; **D.** Cdc-42 was analyzed.

Figure 4. p53 status determines the role of GIPC in MDA-MB-231. **A.** Wild-type p53 (wt-p53) mediates MCF-7 cell survival through GIPC. *p<0.05 and **p<0.01 compared with CTL siRNA group; **p<0.05 and ***p<0.01 compared with GIPC siRNA group as determined by the student’s t test; **B.** Mutant p53 (mut-p53) is involved in GIPC invasion signaling. In cells lacking both GIPC and mutant p53, invasion levels recovered. *p<0.05 compared with the control siRNA group; #p<0.01 compared with the GIPC siRNA group as determined by the student's t test; **C.** MMP-9 activity was assessed in supernatants from GIPC depleted MDA-MB-231 cells treated with p53 siRNA. Activity was quantified as a ratio to the CTL sample. Expression levels of p53 and GIPC are shown in the upper panel.

Figure 5. Myristylated GIPC peptide CR1023 treatment regulates IGF-1R expression, inhibits proliferation and cell viability. **A.** As determined by Western blotting, the expression level of IGF-1R decreased with CR1023 treatment; **B.** With a [³H]-thymidine incorporation assay, a dose-dependent inhibition of cell proliferation was observed in samples treated with CR1023. No inhibition was observed with control
CR2055 peptide treatment; C. Apoptosis assay of MDA-MB-231-WT cells treated with DMSO, CR1023 and CR2055 in a dose-dependent manner for 48 hours. A dose-dependent increase in apoptosis was observed after treatment with CR1023. No inhibition was observed with either the control peptide CR2055 or DMSO.

**Figure 6. In vivo effect of intratumoral injection of peptide on tumor weight, volume, and IGF-1R expression.** A. Effect of administration of CR1023 peptide on tumor growth over time; B and C. Tumor weight and tumor volumes decreased after CR1023 treatment compared with DMSO treatment alone; D. IGF-1R expression is decreased in harvested tumors after CR1023 treatment (T) compared with tumors treated with DMSO alone (U).
References


Figure 1

A

B

GFP    GIPC shRNA

Anti-GIPC

Anti-ß-actin

Tumor Volume (mm³)

0  100  200  300  400

4  6  8  10  12
(Weeks)

• : MDA-MB-231/GFP
■ : MDA-MB-231/GIPC shRNA
Figure 2

A

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<tbody>
<tr>
<td></td>
<td>Anti-GIPC</td>
<td>Anti-β-actin</td>
</tr>
<tr>
<td>MDA-MB-231</td>
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</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Control</th>
<th>GIPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Anti-β-actin</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Relative Viability**

**MDA-MB-231**
- CTLsiRNA: 1.6
- GIPCsiRNA: 0.8

**MCF-7**
- CTLsiRNA: 1.2
- GIPCsiRNA: 0.4

* indicates statistical significance.
Figure 2

C

Cell Number (%)

MDA-MB-231

- Live cells
- Early apoptotic cells
- Late apoptotic cells
- Dead cells

Cell Number

MCF-7

- Live cells
- Early apoptotic cells
- Late apoptotic cells
- Dead cells

Note: The figure shows the percentage of live cells, early apoptotic cells, late apoptotic cells, and dead cells in MDA-MB-231 and MCF-7 cell lines treated with CTLsiRNA and GIPC siRNA.
Figure 2

D

**Figure 2**

**Figure 2D**

% Migracion (%)

CTLsiRNA 100

GIPCsiRNA 60

% Invasion (%)

CTLsiRNA 100

GIPCsiRNA 60

MDA-MB-231
Figure 3

A

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Control</th>
<th>GIPC</th>
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<tbody>
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<tr>
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<td><img src="image3.png" alt="Image" /></td>
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<tr>
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<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Anti-β-actin</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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<tr>
<td>Anti-p53</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
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<tr>
<td>Anti-β-actin</td>
<td><img src="image11.png" alt="Image" /></td>
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</table>

MDA-MB-231

<table>
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<tbody>
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MCF-7

<table>
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Figure 3

B

**MDA-MB-231**

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<tr>
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<td><img src="image2.png" alt="Image" /> Anti-β-actin</td>
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**MCF-7**

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<td><img src="image4.png" alt="Image" /> Anti-β-actin</td>
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**MDA-MB-231**

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<tr>
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<td><img src="image6.png" alt="Image" /> Anti-β-actin</td>
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</table>

Bar graph showing the level of Phospho-IGF-IRβ protein expression:

- **Control siRNA**: Around 1.20
- **GI IPC siRNA**: Around 0.80
Figure 3

C

shRNA Control  GIPC
Anti-GIPC
Anti-β-actin

shRNA  CTL  GIPC
MMP-9

Ratio to CTL (%)
100  13.7

D

Cdc-42

Folds of Change

shRNA  CTL  GIPC

**

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Figure 4

A

![Graph showing cell number by siRNA treatment](image)

- Live cells
- Early apoptotic cells
- Late apoptotic cells
- Dead cells

B

![Graph showing percentage of invasion by siRNA treatment](image)

- CTL
- p53
- GIPC
- GIPC/wt-p53
- GIPC/mut-p53

* p-value < 0.05
** p-value < 0.01
*** p-value < 0.001
# p-value < 0.05 vs. CTL
## p-value < 0.01 vs. CTL
### p-value < 0.001 vs. CTL
Figure 4

C

<table>
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<tr>
<th>shRNA/siRNA</th>
<th>CTL</th>
<th>GIPC</th>
<th>CTL</th>
<th>GIPC</th>
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<tbody>
<tr>
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<td>Anti-β-actin</td>
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<td>MMP-9</td>
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</table>

Ratio to CTL (%) 100 13.7 236.7 82.5
Figure 5

C

% of Live Cells

<table>
<thead>
<tr>
<th></th>
<th>50 μM</th>
<th>100 μM</th>
<th>200 μM</th>
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</table>

D

FITC  DAPI  Merged

Control

CR1171
**Figure 6**

(A) Graph showing the growth of tumor weight over time for CR1023-T and CR1023-U treatments. The x-axis represents the number of days, and the y-axis represents the weight of the tumor (g).

(B) Bar graph comparing the weight of tumors treated with DMSO and CR1023. The weight of the tumor is shown in g, with DMSO on the left and CR1023 on the right. Asterisk indicates a significant difference.

(C) Bar graph comparing the size of tumors treated with DMSO and CR1023. The size of the tumor is shown in mm³, with DMSO on the left and CR1023 on the right. Asterisk indicates a significant difference.
Figure 6

D

<table>
<thead>
<tr>
<th></th>
<th>M-1</th>
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Molecular Cancer Research

RGS-GAIP-interacting protein controls breast cancer progression

Ling Wang, Julie S Lau, Chittaranjan Patra, et al.

Mol Cancer Res  Published OnlineFirst October 27, 2010.

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