Reduced hGC-1 Protein Expression Is Associated with Malignant Progression of Colon Carcinoma

Wenli Liu, Yueqin Liu, Jianqiong Zhu, Elizabeth Wright, Ivan Ding, and Griffin P. Rodgers

Abstract

Purpose: hGC-1 (human granulocyte colony–stimulating factor–stimulated clone 1) is a gastrointestinal protein that is a member of the olfactomedin glycoprotein family. Its biological function remains poorly understood. Aberrant expression of hGC-1 in some human carcinomas has been recently reported. The purpose of this study was to examine hGC-1 expression in colon carcinoma and explore the relationship between hGC-1 expression and the clinicopathologic features of patients with colon cancer.

Experimental Design: The expression of hGC-1 in colon adenocarcinoma tissues was examined by dot-blot analysis, in situ hybridization, and immunohistochemistry. The association of hGC-1 expression pattern with patient differentiation grade, tumor stage, metastasis, and survival were examined. To further investigate the involvement of hGC-1 in colon cancer progression, human colon carcinoma (HT-29) cells overexpressing hGC-1 were established and cell proliferation, adhesion, and migration were studied.

Results: Compared with normal colon mucosa, the up-regulation of hGC-1 was more frequently detected in more differentiated colon cancers, whereas down-regulation or no expression was associated with poorly differentiated colon cancers. Interestingly, hGC-1 down-regulation was also found in late tumor-node-metastasis stage, metastasis, and in patients with shorter survival. The morphology and cortical actin distribution of HT-29 cells were altered by hGC-1 overexpression. However, this did not change cell proliferation, but decreased cell adhesion and migration.

Conclusion: Our findings indicate that hGC-1 is involved in colon cancer adhesion and metastasis, and that hGC-1 may be a useful marker for tumor differentiation and progression of human colon carcinoma.

Colorectal cancer is one of the leading causes of cancer death and distant metastasis is the main cause of death in the later stages. Malignant progression of cancers involves the accumulation of multiple, irreversible genetic hits (1). The identification of new genes functionally involved in tumor development and progression may help to find alternative approaches for prognostic and diagnostic evaluation.

Olfactomedin was first cloned from the olfactory epithelium of the bullfrog in 1993 (2). Now, >100 olfactomedin domain–containing proteins have been identified in a variety of species (3). Five members of a human olfactomedin gene family, which includes hOlfA, hOlfB, hOlfC, hOlfD and the myocilin/trabecular meshwork–inducible glucocorticoid response (TIGR) gene, have been revealed by a GenBank search (4).

These olfactomedin-related proteins are secreted glycoproteins that have been shown to play important roles in normal tissue development and disease (5–9). hGC-1 (human granulocyte colony–stimulating factor–stimulated clone 1), also known as GW112, hOlfD, and OLFM4, was originally cloned from human hematopoietic myeloid cells (10). hGC-1 is mainly expressed in human bone marrow, gastrointestinal tract, and prostate. An association of hGC-1 expression with inflammatory disease and cancers has been recently documented. hGC-1 is up-regulated in the crypt epithelium of inflammatory bowel disease (11) and in gastric biopsies from patients infected with Helicobacter pylori (12). hGC-1 mRNA expression has been found to be up-regulated in gastric cancer (13), colorectal adenomas (14), and colon, lung, and breast cancers (15). hGC-1 has been reported to interact with GRIM-19, a gene associated with retinoid-IFN mortality, and to attenuate retinoic acid-IFN β–induced cellular apoptosis in prostate cancer cells (16). However, a clear correlation of hGC-1 expression with colon cancer and metastasis has not been shown. The potential biological implication of hGC-1 in gastrointestinal carcinoma remains to be elucidated.

In this study, we examined the expression of hGC-1 in normal colon, primary colon cancers, lymph node, and remote metastases. The potential relationship of hGC-1 expression with patient clinicopathologic features and survival data was also investigated. To further study the physiologic role of hGC-1 in colon cancer, we established a human colon carcinoma HT-29 cell line stably transfected with an hGC-1-expressing...
vector to examine malignant phenotypic changes. Our results show that down-regulation or no expression of hGC-1 may predict a malignant progression and poor prognosis in colon cancer, and suggests that this protein is involved in colon tumor differentiation and metastasis.

Materials and Methods

Patient samples, tissue arrays, and immunohistochemistry. A normal colon tissue array and a colon adenocarcinoma array with combinations of margin and normal tissues were purchased from Cybrdi. The colon adenocarcinoma arrays consisted of a total of 176 cases of primary colon adenocarcinoma, 55 cases of lymph node metastasis, 8 cases of liver metastasis, 2 cases of ovary, and 2 cases of lung metastasis patient samples with clinicopathologic data were obtained from the customer services of Cybrdi. Colon cancer tissues from 28 cases of primary tumor with corresponding adjacent normal tissues and metastatic tissues from 11 cases of liver metastasis, 6 cases of ovary, and 6 cases of lung metastasis were collected from the Cooperative Human Tissue Network (CHTN Mid-Atlantic, Charlottesville, VA). In addition to the abovementioned samples, 42 cases of stage II colon cancer patients with a 96-month survival follow-up obtained from Cybrdi and Cureline were included in this study.

The slides were stained with hGC-1 polyclonal antibody (17) using the Envision System (DAKO) as previously described (17). hGC-1 expression in colon cancer cells was compared with normal colon tissue, which shows a moderate intensity of expression and served as an internal reference and was graded as +/- according to the following classification: +, very strong or strongly positive; +/-, moderate/heterogeneously positive; -, weak or negative.

Cell culture and stable transfection. Human colon cancer cells, HT-29, were purchased and maintained as previously described (18). HT-29 cells were transfected with pcDNA3.1/lacZ-V5 or pcDNA3.1/hGC-1-V5 vector (18) using LipofectAMINE 2000 (Invitrogen). Cells were then cultured in the presence of 750 μg/mL of G418 to establish stable cell lines. The expression of hGC-1 protein in these stable cell lines was confirmed by Western blot as previously described (18). One lacZ-HT-29 and two hGC-1-HT-29 stable cell lines were included in this study.

Fig. 1. hGC-1 expression in colon and rectal adenocarcinoma. A, dot-blot was done to detect hGC-1 mRNA expression in cancer tissues from 10 patients with colon cancer and 10 patients with rectal cancer (7) compared with corresponding normal tissues (5) from the same individuals (left). The same blot was stripped and hybridized with a β-actin probe as a control (right). Enhanced (+), unchanged (+/-), and reduced (-) expression of hGC-1 in colon cancer tissues compared with corresponding normal tissues. B, in situ hybridization assay to detect hGC-1 mRNA expression in normal colon tissue and colon cancer tissues. In the normal colon tissue, a moderate staining was observed in the crypts, whereas the surface epithelium stained negative (a). Enhanced (b), moderate (c), and reduced/loss (d) of hGC-1 expression were observed in colon cancer tissues. C, immunohistochemistry was done to detect hGC-1 protein expression in normal and cancerous colon tissues. A moderate cytoplasmic staining in the crypts was detected in normal tissue (a). Representative staining of a well-differentiated colon cancer (b), moderately differentiated colon cancer (c), and poorly differentiated colon cancer (d).
**Table 1. hGC-1 protein expression in colon adenocarcinoma and its relationship with clinicopathologic data**

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<tr>
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</tr>
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<td>III + IV = 68</td>
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<td><strong>Remote metastasis</strong></td>
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<td>7</td>
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**NOTE:** hGC-1 expression in primary colon cancer tissues and metastatic tissues was detected by immunohistochemistry on tissue microarrays. +, strong staining; +/-, moderate staining; +/-, weak or absent staining. 

*P* values are based on a comparison of the value reported under (-) column with the value under the (+) column.

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**Dot-blot hybridization.** The BD Clontech Cancer Profiling Array was purchased from BD Biosciences Clontech. The array was hybridized with a [32P]dCTP-labeled hGC-1 probe amplified by PCR using DNA labeling beads (Amersham Biosciences). The primers used for PCR were a sense primer of 5′-GATTACTCTCCCCACATC-3′ and an antisense primer of 5′-CTCTTTACCCCTAACCTC-3′. Prehybridization, hybridization, washes, and exposures were done according to the instructions of the manufacturer.

**In situ hybridization.** Digoxigenin-labeled RNA probe was prepared by using pcDNA-hGC-1 (18) as a template along with a MAXScript kit (Ambion). Digoxigenin-11-UTP was purchased from Roche. Colon cancer and adjacent normal tissues were hybridized with antisense and sense Dig-labeled hGC-1 probe (2 ng/μL) at 62°C for 15 h with rotation using an in situ hybridization kit (Biochain). The result was detected with anti-dig antibody (1:1,000) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

**hGC-1 gene mutation analysis.** Colon cancer tissues and corresponding normal tissues from 28 patients with colon cancer were obtained from the Cooperative Human Tissue Network (CHTN Mid-Atlantic). Genomic DNA was extracted with the Wizard Genomic DNA purification kit (Promega). The promoter and five exons of hGC-1 (exons 1-5) were amplified by PCR and sequenced.

**Methylation analysis of hGC-1 gene.** The methylation status of eight CpG sites in the hGC-1 promoter and exon 1 was analyzed by Pyrosequencing (EpigenDx). Briefly, 1 μg of genomic DNA was bisulfite-treated with Zymo Research’s EZ DNA Methylation kit. The DNA elute was diluted 1:10. One microliter of the diluted DNA was used for PCR with HotStar Taq Polymerase (Qiagen). Three pairs of PCR primers were designed to amplify the fragments that cover the eight CpG dinucleotide sites. The reverse PCR primer was biotin-labeled on the 5′-end and was high-performance liquid chromatography-purified. Pyrosequencing reactions were run on the PSQ 96HS system. The Pyro Q-CpG software was used for the analysis.

**Confocal fluorescence microscopy.** hGC-1-V5 and lacZ stably transfected HT-29 cells were grown, fixed, and blocked as previously described (18). TRITC-conjugated phalloidin (Chemicon International) was added and the cells were incubated overnight at 4°C. After three washes, the cells were counterstained with 10 ng/mL of 4′,6-diamidino-2-phenylindole. The slides were washed thrice and observed under a confocal fluorescence microscope (LSM510 META, Zeiss).

**Cell proliferation.** Exponentially growing transfected HT-29 cells were plated at a density of 1 × 10⁵ cells/well in six-well tissue culture plates and the culture medium was replenished every other day. Cell numbers for each well were counted with a trypan blue exclusion assay every day for a total of 5 days (from days 1 to 5). Three sets of experiments were done, each one in triplicate.

**Cell adhesion assay.** For cell surface adhesion assays, adherent hGC-1 and lacZ-transfected HT-29 cells were harvested by adding 10 mmol/L of EDTA in HBSS at room temperature for 10 min. A 100-μl aliquot of a 10⁶ cells/mL suspension of each sample in triplicate was seeded into a well of 96-well flat-bottomed noncoated bacteria plate. Plates were incubated at 37°C in a CO₂ incubator for 1 or 2 h. Adherent cells were quantified by 0.2% crystal violet staining and 570 nm absorbance determination as previously described (18).

**Cell migration assays.** Trans-well cell migration assays were done in a two-chamber migration assay plate (Chemicon International) according to the instructions of the manufacturer. In brief, 300 μL of a prepared cell suspension was added to each migration insert and 500 μL of DMEM with 10% fetal bovine serum or serum-free medium was added to the lower chamber. The plate was incubated for 6 h at 37°C in a CO₂ incubator. The migration insert was then incubated in 400 μL of a cell-staining solution for 20 min at room temperature. A 100-μL aliquot of the dye mixture was then transferred to a 96-well plate and the absorbance was measured at 560 nm.

The wound closure assay was carried out as previously described (19). Briefly, the cells were seeded into a six-well plate and grown to confluence. The cell monolayers were scraped with a sterile pipette tip. The width of the wound was observed and recorded with an inverted microscope (×200) at time 0 and after 16 h of incubation.

**Statistical analyses.** Pearson correlation coefficients were used to compare results obtained by in situ hybridization to those obtained for the same cases using immunohistochemistry. Correlations between the expression of hGC-1 and patient clinicopathologic data were analyzed for statistical significance by the Mantel-Haenszel χ² test using the SAS System. Experimental results of cell adhesion and migration assays were analyzed for significance usingANOVA. Cancer-related survival was measured from the date of primary treatment (surgery) to the end of follow-up or death. Survival curves were calculated using the Kaplan-Meier method and the log-rank test was used to test the difference between groups. The calculations were done using SAS version 9.1. P < 0.05 was considered significant in all the statistical analyses.

**Results**

**hGC-1 mRNA expression in colorectal carcinoma.** A previous study had shown hGC-1 up-regulation in colon cancer through analysis of the Cancer Genome Anatomy Project database (16). To experimentally examine the hGC-1 expression in colorectal cancers, a dot-blot analysis using a [32P]-labeled hGC-1 cDNA and commercially available multiple tissue blots with RNA from various human cancer tissues and corresponding normal tissues from the same individual was done (Fig. 1A, left). Among a total of 20 colon and rectal cancer cases, 9 cases (45%) showed increased hGC-1 expression (+), 3 cases (15%) remained unchanged (+/-), whereas 8 cases (40%) showed decreased expression (-) in cancer tissues compared with corresponding normal tissues. Interestingly, hGC-1 expression was totally absent in six cancer cases (30%). As a control, the
same blot was stripped and hybridized with a β-actin probe (Fig. 1A, right). These results showed that hGC-1 expression was changed (up-regulated or down-regulated) in a majority of colon and rectal cancers. However, various levels of hGC-1 expression also exist in normal tissues from different individuals. One explanation might be due to tissue heterogeneity or biopsy discrepancy, as it has been shown in a previous study (17), that hGC-1 expression is abundant in the crypt and becomes less abundant toward the surface epithelium.

Therefore, we did an in situ hybridization to detect hGC-1 mRNA expression at the cellular level in 16 cases of colon cancer and adjacent normal tissues. For 3 of 16 normal colon tissues, no specific signal was detected. In the other 13 cases, hGC-1 was modestly expressed in the crypt and was negative in the surface epithelium with antisense hGC-1 probe (Fig. 1B, a). The sense hGC-1 probe staining was negative (data not shown). The hGC-1 expression pattern in the 13 normal colon tissues was consistent. Compared with adjacent normal tissues, hGC-1 mRNA showed enhanced (Fig. 1B, b), moderate (Fig. 1B, c), and reduced expression (Fig. 1B, d) in colon cancer tissues.

Localization of hGC-1 protein expression in normal and cancerous colon tissues. Next, we examined the hGC-1 protein expression in normal colon and colon cancer tissues by immunohistochemistry. In normal colon tissues, hGC-1 was modestly expressed throughout the cytoplasm of the crypt epithelium. The expression decreased and became heterogeneous toward the surface epithelium (Fig. 1C, a). To further elucidate the relationship of hGC-1 expression in colon cancers, we studied hGC-1 expression in colon tissues from 176 cases of primary colon cancer, 55 cases of lymph node metastasis, and 35 cases of remote metastasis (liver, ovary, and lung) by immunohistochemistry. Consistent with the mRNA in situ hybridization results (Fig. 1B), hGC-1 protein expression in colon cancer tissues showed three distinct patterns compared with normal colon tissue: enhanced expression (Fig. 1C, b), moderate or unchanged expression (Fig. 1C, c), and reduced or undetectable expression (Fig. 1C, d).

Immunohistochemistry was also done on the same 16 cases in which in situ hybridization was performed. The immunohistochemistry results were consistent with the detection of mRNA in approximately two-thirds (11 of 16) of the samples by in situ hybridization. The results of two methods exhibited a close correlation that reached statistical significance by Pearson coefficients analysis (n = 16; coefficient = 0.76). Three cases showed enhanced mRNA expression by in situ hybridization, but moderate or reduced expression in immunohistochemistry. These minor differences observed for the two methods might be attributed to differences in sensitivity and specificity.

hGC-1 protein expression is correlated with colon tumor differentiation, stage, and metastasis. The correlation of hGC-1 expression in colon cancer tissue with patient clinicopathologic data including differentiation, stage, and metastasis (lymph node and remote) was investigated (Table 1). Enhanced expression of hGC-1 was more often observed in well-differentiated cancer, whereas moderate or heterogeneous expression was associated with moderately differentiated cancer.
and down-regulation was most often observed in poorly differentiated cancer ($P < 0.001$). We also compared hGC-1 expression in normal colon tissue, cancer tissue, and marginal tissue of the same individual. In a representative well-differentiated cancer, an increased expression of hGC-1 was detected in cancer tissue (Fig. 2A, b), and a moderate heterogeneous staining was observed in inflammatory margin tissue (Fig. 2A, c) as well as in normal tissue (Fig. 2A, a) from the same individual. In contrast, hGC-1 expression was absent in a poorly differentiated cancer tissue (Fig. 2A, e), whereas the margin tissue (Fig. 2A, f) and normal tissue (Fig. 2A, d) from the same individual had moderate staining. The loss/reduction of hGC-1 expression at the front of the invasion was observed in some cases. A significant reduction of hGC-1 expression (Fig. 2B, a, d) was observed at the front of the invasion, close to the colon serosa compared with uninvolved mucosa (Fig. 2B, b) and cancer tissues (Fig. 2B, a, c).

In addition to the close association of hGC-1 expression with the state of differentiation, down-regulation of hGC-1 in colon cancer tissue was seen in cases with late tumor-node-metastasis stage (III + IV) and lymph node metastasis in a significantly higher number of cases ($P < 0.001$; Table 1). Moreover, hGC-1 expression was also down-regulated in 22 of 35 (62.9%) remote metastasis tissues including 19 cases of liver metastasis, 8 cases of ovary, and 8 cases of lung metastasis (Table 1). Thirteen out of 19 cases of liver metastasis (68.4%) showed reduced hGC-1 expression, which was significantly higher than primary colon cancer (27.6%) and lymph node metastases.

Table 2. Methylation pattern in the promoter region of hGC-1 in the colon cancer tissues and the adjacent normal tissues

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**NOTE:** Methylation status of eight CpG sites in the promoter and exon 1 of hGC-1 in colon cancer tissues (T) and adjacent normal tissues (N) were detected and quantitated by Pyrosequence. Down and Up in hGC-1 expression indicate down-regulation and up-regulation of hGC-1 in cancer tissues compared with adjacent normal tissues. N/A, no data available due to weak or no signal.
metastasis (52.7%). These results indicated that hGC-1 expression is correlated with colon cancer differentiation, stage, and metastasis.

**hGC-1 expression is correlated with patient survival.** The above results indicated that down-regulated or no expression of hGC-1 was associated with poorly differentiated and highly metastatic colon cancer, and may be of prognostic value for tumor malignancy. Therefore, we further studied the association of hGC-1 expression in colon cancer tissue with patient survival in 42 cases of stage II colon cancer patients over a follow-up period of as long as 96 months. Patients with weak or negative hGC-1 expression [L (-)], 12 cases] had a significantly reduced survival rate compared with that found for patients with a moderate [M (+/-), 16 cases], and/or an enhanced expression [H (+), 14 cases; Fig. 3]. Patients with strong or moderate hGC-1 staining survived longer (62.5% survived for 5 years) than patients with down-regulation or no expression (33.3% survived for 5 years).

**Mutation and methylation analysis of hGC-1 gene in patients with colon cancer.** To determine the genetic and epigenetic mechanism of abnormal hGC-1 expression in colon cancer, the promoter (1 kb) and five exons of the hGC-1 gene were amplified and sequenced. No mutation or alteration was found in cancer tissues compared with corresponding normal tissues from 28 colon cancer patients with various hGC-1 expression levels and clinicopathologic features. These results suggest that the abnormal expression of hGC-1 might be regulated at the transcriptional or posttranscriptional level rather than at the genomic level.

The methylation of 5′ CpG dinucleotide sites in eukaryotic genomes is known to play a crucial role in gene regulation and oncogenesis (20). Therefore, we studied the methylation status of six CpG sites in the hGC-1 promoter (-681, -666, -562, -486, -446, and -91) and two CpG sites in exon 1 (+4 and +34) in 19 cases of colon cancer tissues, together with their corresponding adjacent normal tissues (Table 2). It was interesting to note that there were high levels of methylation in most of the CpG sites in normal colon tissues. In the 10 cases with reduced expression of hGC-1, there were 2 cases with hypermethylation, 1 case with hypomethylation, and 7 cases with no significant change. No significant correlation was observed in the reduced expression of hGC-1 with hypermethylation. However, in the nine cases with enhanced hGC-1 expression, six cases showed significant hypomethylation in most CpG sites, especially at -681, -666, +4, and +34 sites. This result suggested that the hypomethylation of CpG sites in the promoter and exon 1 of hGC-1 might contribute to the enhanced hGC-1 expression in colon cancer tissues.

**Morphologic and actin cytoskeleton changes in hGC-1–expressing HT-29 cells.** Having established that hGC-1 expression is correlated with colon cancer differentiation and metastasis, we then determined its involvement in the physiology of colon cancer cells. Overexpression of hGC-1 in HT-29 cells changes cell morphology and disturbs the cortical actin distribution. A, the expression of hGC-1 (lane 1, hGC-1/C3; lane 2, hGC-1/C5) and lacZ (lane 3) in HT-29 cells was shown by Western blot using an anti-V5 antibody (1:1,000). The hGC-1 expression in HT-29 cells without or with DTT treatment at different concentrations was shown by Western blot as above. The cell shape change (B) and cell response to serum starvation (C) in lacZ and hGC-1/C3 HT-29 cells as observed under a phase-contrast microscope. D, the cortical actin distribution in lacZ and hGC-1/C3 HT-29 cells stained by TRITC-conjugated phalloidin as observed by confocal fluorescence microscope, along with the corresponding 4,6-diamidino-2-phenylindole and overlap staining patterns.
cancer. HT-29 colon carcinoma cells that were deficient in hGC-1 expression (confirmed by reverse transcription-PCR) were transfected with hGC-1–V5 and lacZ-V5 cDNA to induce their overexpression. The expression of hGC-1 (in two cell lines; C3 and C5) and lacZ was confirmed by Western blot using V5 antibody (Fig. 4A, left). The hGC-1 existed in the cells as a monomer, dimer, and multimer form as shown in Western blots with treatment of different concentrations of DTT (Fig. 4A, right). The hGC-1 HT-29 cells rounded up with no or few surface protrusions and did not contact each other with podial protrusions as compared with lacZ HT-29 cells (Fig. 4B).

Next, we investigated the cell response to nutrient deprivation stress by starving the cells in serum-free medium (DMEM + 0.25% bovine serum albumin) for 6 h. The lacZ HT-29 cells responded by obvious podial extension, whereas the hGC-1 cells tended to cluster together without a notable response (Fig. 4C). Based on the cell morphology changes we observed with the expression of hGC-1, we next examined actin-based cytoskeletal changes in hGC-1 HT-29 cells by TRITC-phalloidin staining. A normal cortical filamentous actin distribution in the surface protrusions of lacZ HT-29 cells was observed, whereas hGC-1 HT-29 cells showed an even cortical filamentous actin distribution (Fig. 4D). These results suggested that overexpression of hGC-1 resulted in an alteration of the actin cytoskeleton and cell morphology.

hGC-1 expression inhibits cell adhesion and migration. We also determined whether hGC-1 overexpression might alter cell proliferation. The growth rate of hGC-1/C3 and C5 HT-29 cells did not differ from that of lacZ HT-29 cells (Fig. 5A). Because cell adhesion and cell migration are determining steps in cancer aggressivity and metastasis, we compared these features in hGC-1/C3, hGC-1/C5, and lacZ HT-29 cells. In a cell surface adhesion assay, the hGC-1/C3 and hGC-1/C5 HT-29 cells showed decreased adhesion compared with lacZ HT-29 cells with 10% fetal bovine serum in the lower chamber (*, P < 0.01). hGC-1/C3 cells (d) migrated more slowly than lacZ HT-29 cells (b) after 16 h as shown by a wider gap for the hGC-1/C3 cells in a cell wound closure assay. lacZ (a) and hGC-1/C3 (c), the 0 time points at which a layer of confluent cells were initially wounded by a tip scratch.

Fig. 5. Overexpression of hGC-1 in HT-29 cells decreased cell adhesion and migration. A, cells were plated in six-well plates and their growth was measured daily for 5 days. Points, mean from three independent experiments; bars, SD. B, cells were seeded into wells of 96-well flat-bottomed bacteria plates and cell adhesion to the plate was quantitated after 1 or 2 h. Columns, mean from three independent experiments; bars, SD. The cell surface adhesion was decreased in hGC-1/C3 (black columns) and hGC-1/C5 (gray columns) as compared with lacZ (white columns) HT-29 cells after 1 and 2 h (*, P < 0.01). C, trans-well cell migration assays were done in a two-chamber migration assay plate. The migration of hGC-1/C3 (black columns) and hGC-1/C5 (gray columns) cells were significantly inhibited as compared with lacZ (white columns) HT-29 cells with 10% fetal bovine serum in the lower chamber (*, P < 0.01). D, hGC-1/C3 cells (d) migrated more slowly than lacZ HT-29 cells (b) after 16 h as shown by a wider gap for the hGC-1/C3 cells in a cell wound closure assay. lacZ (a) and hGC-1/C3 (c), the 0 time points at which a layer of confluent cells were initially wounded by a tip scratch.
HT-29 cells (P < 0.01; Fig. 5B). Two separate experiments were also done to compare the cell migration of hGC-1/C3, hGC-1/C5 HT-29 cells, and lacZ HT-29 cells. In a two-chamber transwell assay, hGC-1/C3 and hGC-1/C5 HT-29 cell migration to a lower chamber containing 10% fetal bovine serum was significantly reduced as compared with lacZ HT-29 cells (P < 0.01; Fig. 5C). In a wound closure assay, hGC-1 HT-29 cell migration was inhibited, as shown by the wider wound gap observed in hGC-1/C3 HT-29 cells than in lacZ HT-29 cells 16 h after the wound was induced (Fig. 5D). Taken together, these results indicated that hGC-1 was involved in cell adhesion and migration.

**Discussion**

In this study, we investigated the potential involvement of hGC-1, a gastrointestinal glycoprotein, in the carcinogenesis and metastasis of colon cancer. Our results showed a correlation between hGC-1 expression and colon cancer differentiation, stage, metastasis, and survival. hGC-1 expression was up-regulated in the well-differentiated and early stage colon cancer and down-regulated in the poorly differentiated and advanced stage colon cancer. Moreover, hGC-1 expression was clearly down-regulated in lymph node and remote metastases. These results suggest that hGC-1 may play a different role in the early phases of tumor initiation and dissemination of tumor cells to distant organs.

The molecular events that suppress or induce tumor metastatic processes remain the least understood. A number of candidate antimetastasis genes have been studied in colorectal carcinoma, including KAI1 (21), nm23 (22), DCC (23), E-cadherin (24), and CD44 (25). Unlike these molecules, which are expressed ubiquitously in tissues, hGC-1 is expressed specifically in the gastrointestinal tract, prostate, and neutrophils (10). In this study, down-regulation of hGC-1 expression was found at a higher frequency in remote and lymph node metastasis compared with primary tumors, with down-regulation occurring more frequently in the liver and other remote metastasis compared with lymph node metastasis. This suggests that a reduction or loss of hGC-1 might aid the tendency of colon cancer tumor cells to metastasize. Inhibition of cell migration by overexpression of the hGC-1 gene in HT-29 cells further supports this possibility. More importantly, down-regulation of hGC-1 had clinical significance; the subgroup of patients with lower hGC-1 expression showed significantly reduced overall survival. These results have suggested an association of hGC-1 expression with tumor progression in colon cancer and might have a prognostic value.

A role for olfactomedin domain–containing proteins in normal development and cell differentiation has been documented (5, 26, 27). Noelin-1 (also called pancortin and OLFM1) is important in regulating the production of neural crest cells in chicken (27) and promotes neurogenesis in *Xenopus* (5). Olimedin (also known as OLFM3) has been suggested to play a role in the differentiation of neural cells (26). Here, we show that hGC-1 protein is mainly expressed in the crypts of normal colon mucosa and becomes decreased in expression with the maturation of epithelial cells toward the lumen surface. Moreover, the expression of hGC-1 in colon cancer tissues was significantly correlated with cell differentiation. Taken together, our results support the hypotheses that hGC-1 is a good marker for colon cancer cell differentiation and may play a role in the regulation of tumor cell differentiation.

Alterations in adhesive properties allow cancer cells to disobey the rules of tissue architecture and to advance in their malignant progression (21). Compared with normal epithelia, carcinoma cells often show diminished intercellular adhesiveness (28). In many instances, epithelial tumors lose E-cadherin–mediated adhesion as they progress toward malignancy (29). Recent studies have revealed that olfactomedin proteins interact with cell surface proteins and mediate cell adhesion. Gliomedin interacts with neurofascin and NrCAM, two axonal immunoglobulin cell adhesion molecules that mediate various cell adhesion and recognition events during the development and maintenance of the nervous system (30). Amassin mediates the massive Ca²⁺-dependent intercellular adhesion of sea urchin coelomocytes through binding to a cell surface protein (31). Optimedin induces the expression of N-cadherin and stimulates the aggregation of nerve growth factor–stimulated PC12 cells by binding to cell membrane proteins (26). We previously reported that hGC-1 binds to cadherin and lectins and affects cell adhesion in HEK293 cells (18). Cell adhesion mediated by cadherins occurs only when their intracellular domains are properly anchored with the cytoplasmic proteins, such as α- and β-catenins, and link to the actin cytoskeleton (32). In this study, overexpression of hGC-1 in HT-29 cells changed the filamentous actin distribution, cell shape, and cell adhesion. Therefore, these results suggested that hGC-1 is involved in cell cytoskeleton organization and cell adhesion. Although the detailed mechanisms of cell migration are not yet understood, it is now clear that dynamic and reciprocal interactions between cell adhesion molecules, extracellular matrix, and other factors are essential (33, 34). The reduction or loss of hGC-1 expression in colon cancer may contribute to cell adhesion and migration alteration and assist in colon cancer cell metastasis.

For the first time, we have reported that hGC-1 is expressed in colon cancer at both the mRNA and protein levels. During the preparation of our article, Koshida et al. reported that hGC-1 mRNA was overexpressed in 19 out of 28 (67.9%) colon cancer cases (15). Two reasons might explain why a previous report showed only up-regulation of mRNA levels in colon cancer as compared with our results showing increased/decreased protein expression (depending on the differentiation of the cancer). First, lower protein levels might reflect an additional level of regulation at the posttranscriptional or translational stage in poorly differentiated colon cancers. Second, our study includes more cases, and hence, might be more representative of the molecular changes that occur in colon cancers.

The hGC-1 gene is located at 13q14.3, adjacent to the tumor suppressor Rb gene. Chromosome 13q14 allele losses and mutations have been reported in different cancers such as prostate, breast, and B-cell chronic lymphoid leukemia (35, 36). Therefore, we did gene mutation analyses on hGC-1 by sequencing the 1 kb promoter and all five exons in 28 cases of primary colon cancer samples with corresponding normal tissues as a control. We did not find any mutations in the hGC-1 gene in any of the 28 samples with various hGC-1 expression levels. To determine if the regulation of hGC-1 expression is associated with promoter methylation, we
detected the methylation status of CpG sites in hGC-1 promoter and exon 1. Interestingly, a significant correlation was observed between the enhanced hGC-1 expression and CpG hypomethylation. The mechanism of hGC-1 down-regulation in colon cancer might involve other epigenetic pathways and needs to be addressed in the future.

The present study shows that hGC-1 expression correlates with colon cancer differentiation, metastasis, and prognosis.

hGC-1 may prove to be an important molecule in understanding the biology of the carcinogenesis and metastasis of colorectal cancer.

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References

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