GPR48, a poor prognostic factor, promotes tumor metastasis and activates β-catenin/TCF signaling in colorectal cancer

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G-protein-coupled receptor 48 (GPR48) is an orphan receptor belonging to the G-protein-coupled receptors family, which plays an important role in the development of various organs and cancer development and progression such as gastric cancer and colorectal cancer (CRC). However, the prognostic value of GPR48 expression in patients with CRC has not been reported. In this study, we observed that GPR48 was overexpressed in primary CRC and metastatic lymph nodes and closely correlated with tumor invasion and metastasis. Multivariate analysis indicated that high GPR48 expression was a poor prognostic factor for overall survival in CRC patients. In vivo and in vivo assays demonstrated that enforced expression of GPR48 contributed to enhanced migration and invasion of cancer cells and tumor metastasis. In addition, we found that GPR48 increased nuclear β-catenin accumulation, T-cell factor 4 (TCF4) transcription activity, and expression of its target genes including Cyclin D1 and c-Myc in CRC cells. Correlation analysis showed that GPR48 expression in CRC tissues was positively associated with β-catenin expression. Upregulation of GPR48 resulted in increased phosphorylation of glycogen synthase kinase 3β, Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) in CRC cells, while inhibition of PI3K/Akt and mitogen-activated protein kinase /ERK1/2 pathways was sufficient to abolish the effect of GPR48 on β-catenin/TCF signaling. Taken together, GPR48 could serve as both a prognostic biomarker and a therapeutic target for resectable CRC patients.

Introduction

Colorectal cancer (CRC) is the third most common malignancy and fourth most common cause of cancer mortality worldwide (1). Despite improvements in surveillance and clinical treatment over the past decades, almost 50% of CRC patients eventually develop recurrent disease and distant metastasis after curative resection (2). Metastasis of tumor cells to distant organs is the main cause of treatment failure and the high mortality rate (3). The 5-year survival rate for patients with regional and distal metastases is only ~60 and 10%, respectively (4). Hence, the identification of proteins associated with CRC metastasis would contribute to understanding the mechanisms involved in CRC malignancy, leading to the development of novel biomarkers and therapeutic targets for CRC (5).

Leucine-rich repeat-containing G-protein-coupled receptors (LGRs) belong to the G-protein-coupled receptors superfamily and are characterized by the presence of a seven-transmembrane domain and a large extracellular domain with a long N-terminal segment containing leucine-rich repeats motifs that are involved in receptor–ligand interactions (6–8). LGRs can be classified into three subtypes, including three known glycoprotein hormone receptors (LH, FSH and TSH), LGR4-6 and LGR7-9 (9). G-protein-coupled receptor 48 (GPR48), also known as LGR4, is closely related to LGR5 and LGR6 with ~50% amino acid identity between them (10) and is widely expressed in diverse tissues at both the embryonic and adult stages (11). Substantial evidence demonstrates that GPR48 plays an important role in the development of multiple organs including the male reproductive tract, eyelid, hair follicle, ocular anterior segment and mouse intestinal crypt (9,11–15). During eyelid development, GPR48 can promote epithelial cell proliferation and migration through activation of the epidermal growth factor receptor (EGFR) pathway (16). Recent studies showed that GPR48 was overexpressed in several types of cancer including colorectal and gastric cancer (17,18). Upregulation of GPR48 mediated by p27⁰⁷⁰ contributed to enhance colorectal carcinoma cell invasiveness and metastasis (17). In addition, Steffen et al. (18) found that GPR48 was differentially expressed in gastric cancer and closely associated with node involvement. However, the prognostic value of GPR48 expression in patients with CRC has not been reported.

It has been shown that GPR48 and LGR5/GPR49 function as the cognate receptors of R-spondin to potentiate Wnt/β-catenin signaling (19,20). Conditional deletion of GPR48 and GPR49 genes in the mouse gut impairs Wnt target gene expression and results in the rapid demise of intestinal crypts, thus phenocopying Wnt pathway inhibition (15). β-Catenin serves as a central downstream effector of the Wnt signaling pathway and plays a key role in the regulation of growth and development (21). In addition, activation of β-catenin signaling could control the ability of melanomas to metastasize to lung, bowel and spleen (22). In the absence of Wnt ligand, levels of cytoplasmic β-catenin are tightly regulated by a multiprotein complex of adenomatous polyposis coli (APC), axin, casein kinase 1 and glycogen synthase kinase 3β (GSK-3β), which facilitates phosphorylation and proteasomal degradation of β-catenin (21). However, mutational loss of APC function, or less commonly, stabilizing mutations of β-catenin, mutations of axin 2 or dysregulation of Wnt ligands results in loss of β-catenin degradation in cells (23). Consequently, β-catenin that accumulates in the cytoplasm is translocated to the nucleus, where it binds to the T-cell factor (TCF)/lymphoid enhancer factor and thereby activates transcription of its target genes such as cyclin D1 and c-Myc (1,21). In addition to the Wnt-dependent regulation of β-catenin, it has been reported that certain growth stimuli or oncogenes can stabilize β-catenin through phosphorylation of GSK-3β at Ser⁰ mediated by extracellular signal-regulated kinase 1/2 (ERK1/2), AKT or PKC (24–26).

Our previous proteomics study showed that GPR48 expression was much higher in the metastatic colon cancer cell line SW620 than in the matched primary colon cancer cell line SW480 (27). In this study, we have investigated the predictive values of GPR48 for the prognosis of CRC patients. GPR48 overexpression in CRC is associated with adverse clinical outcome, supporting its potential roles as a prognostic biomarker and a therapeutic target.

Abbreviations: APC, adenomatous polyposis coli; CI, confidence interval; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GPR48, G-protein-coupled receptor 48; GSK-3β, glycogen synthase kinase 3β; HR, hazards ratio; LGRs, Leucine-rich repeat-containing G-protein-coupled receptors; MAPK, mitogen-activated protein kinase; OS, overall survival; SD, standard deviation; TCF, T-cell factor.

1These authors contributed equally to this work.

Carcinogenesis vol.34 no.12 pp.2861–2869, 2013
doi:10.1093/carcin/bgt229
Advance Access publication June 26, 2013
Materials and methods
Clinical specimens
All colorectal carcinomas, corresponding adjacent normal tissues and metastatic lymph nodes were obtained from the patients who underwent surgical resections during 2005 at the Sichuan Provincial People’s Hospital (Chengdu, China). A total of 113 patients were involved in the study. Fresh frozen tumor tissues and the corresponding normal tissue were randomly selected from five patients for western blot analysis. Formalin-fixed paraffin-embedded tissues from the other 108 patients including 65 paired normal mucosa were used for immunohistochemical analysis. Tumor stage was determined according to the TNM classification system of the International Union against Cancer (UICC) (23). Histological differentiation was determined according to the criteria of World Health Organization (29). During the follow-up period, 47 (43.5%) patients died of CRC, and 10 (9.26%) patients were lost to follow-up because of an incorrect address in the register or other reasons. This study was approved by the Institutional Review Board (IRB) of West China Hospital, Sichuan University and informed consent for tissue procurement was obtained from all patients, or their relatives, before study initiation.

Plasmid constructions and cell transfection
GPR48-plasmid encoding full-length human GPR48 and GPR48 control plasmid were purchased from Gene Copeoa (Guangzhou, China). For GPR48 overexpression, GPR48 vectors were separately transfected into HCT116 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and the stable transfectants were selected in the presence of 600 μg/ml G418 (Amresco, Solon, OH). Knockdown vectors for GPR48 (pGPU6/GFP/Neo-sh-GPR48) were constructed by introduction of 355-CATCAGCCAGTGAAGCCAT-373 (NM_018490.2) into the pGPU6/ GFP/Neo vectors by GenePharma Co. (Shanghai, China). pGPU6/GFP/Neo-sh-NC (control shRNA) that targets a sequence not found in the human, mouse or rat genome databases (5′-GTTTCGCAACGCTGTACGT-3′) was used as a negative control. The GPR48 shRNA and control shRNA were transfected into HCT116, LoVo and HEK293T cells using Lipofectamine 2000 according to the manufacturer’s instructions. Construct-carrying clones were selected in G418-containing medium beginning 48 h after transfection.

Immunohistochemistry and evaluation of immunohistochemical staining
Tissues were formalin fixed and paraffin embedded, and serial 4 μm thickness sections were taken for immunohistochemistry analysis using an EnVisionTM Detection System, Peroxidase/DA/B, Rabbit/Mouse (K5007; DAKO) according to the manufacturer’s instructions. The protocol for immunohistochemical staining has been described previously (30). Immunostaining was considered negative if <10% of the tumor cells were stained. The immunohistochemistry staining was evaluated independently by two board-certified clinical pathologists (L.S. and D.D.) blinded to the clinical parameters. CRC was considered positive for GPR48 staining when >10% of tumor cells. Staining for GPR48 was assessed using a scoring method as described previously (31,32). Briefly, the sections were scored using a four-tier scale according to percentage of positive cells and staining intensity; (+) or 0, tissue specimens without staining (0–10%); (+) or 1, tissue specimens with weak staining (10–25%); (++) or 2, tissue specimens with moderate staining (25–50%) and (+++) or 3, tissue specimens with strong staining (>50%) (31,32). (-) and (+) were defined as low expression, and (++) and (+++) were defined as high expression (or overexpression). Any discrepancy between the two evaluators was resolved by reevaluation and careful discussion until agreement was reached.

Statistical analysis
Values are presented as mean ± standard deviation (SD). Unpaired t-test or Pearson’s correlation test was used to compare quantitative variables; Pearson chi square test or Fisher’s exact test was applied to compare qualitative variables. Patients’ survival curve was plotted using the Kaplan–Meier method, and the log-rank test was used to determine the significant difference among groups; the chi square test or Fisher’s exact test was applied to compare qualitative variables studied such as patient age (P < 0.05). In addition, we found that the expression of GPR48 protein in CRC tissues was ~5 times higher than that in the normal tissues (Figure 1C; P < 0.001). Upregulation of GPR48 expression was further confirmed in 108 paraffin-embedded, archival primary CRC tissues and 65 normal colorectal mucosa tissues by immunohistochemical analysis, respectively (the clinicopathological parameters are summarized in Supplementary Table 1, available at Carcinogenesis Online). As shown in Figure 1D and Supplementary Figure 1A, available at Carcinogenesis Online, most of the normal colorectal mucosa tissues exhibited low membrane and cytoplasmic GPR48 expression in crypt epithelial cells. In CRC tissues, GPR48 expression was observed in the membrane and cytoplasm of tumor cells (Figure 1D). GPR48 was expressed at higher levels in 75% (81/108) of CRC tissues compared with those in 29.2% (19/65) of normal mucosa tissues, and the difference was statistically significant (Figure 1E; P < 0.001). In addition, we compared the intensity of staining for GPR48 in normal mucosa and tumor tissues, and the results showed that GPR48 expression was significantly higher in tumor cells than in normal mucosal epithelial cells (Supplementary Table 2, available at Carcinogenesis Online). These data suggest that GPR48 is overexpressed in human CRC.

GPR48 is correlated with several clinicopathologic features of CRC patients
We next investigated the relationship between GPR48 expression and the clinicopathologic features of 108 CRC patients (Table I). GPR48 expression was significantly correlated with tumor stage (P = 0.010) and lymph node status (P = 0.010) (Table I). However, no significant correlation was found between GPR48 expression and other clinicopathologic variables studied such as patient age (P = 0.149), gender (P = 1.000), tumor location (P = 0.315), tumor differentiation (P = 1.000), and depth of invasion (P = 0.098) (Table I; P > 0.05). In addition, we found that GPR48 expression in moderately and poorly differentiated CRC was higher than that observed in the primary CRC cell line (SW480) from the same patient (27). Herein, we confirmed that the expression of GPR48 was markedly increased in highly metastatic cell lines (SW620 and KM12L4A) compared with poorly metastatic cell lines (SW480 and KM12C) at both the mRNA and protein levels (Figure 1A and B). In addition, we observed that GPR48 expression in a series of CRC cell lines (SW480, SW620, KM12L4A, KM12C, LoVo and HCT116) was significantly higher than that in a human normal colorectal epithelial cell line (NCM460) (33) at both mRNA and protein levels (P < 0.001) (Figure 1A and B), indicating a differential expression pattern of GPR48 in CRC cells.

To investigate the expression pattern of GPR48 in human CRC, paired non-tumor and tumor tissues from frozen tissue samples were analyzed by immunoblot analysis. The results showed that the expression of GPR48 protein in CRC tissues was ~5 times higher than that in the normal tissues (Figure 1C; P < 0.001). Upregulation of GPR48 expression was further confirmed in 108 paraffin-embedded, archival primary CRC tissues and 65 normal colorectal mucosa tissues by immunohistochemical analysis, respectively (the clinicopathological parameters are summarized in Supplementary Table 1, available at Carcinogenesis Online). As shown in Figure 1D and Supplementary Figure 1A, available at Carcinogenesis Online, most of the normal colorectal mucosa tissues exhibited low membrane and cytoplasmic GPR48 expression in crypt epithelial cells. In CRC tissues, GPR48 expression was observed in the membrane and cytoplasm of tumor cells (Figure 1D). GPR48 was expressed at higher levels in 75% (81/108) of CRC tissues compared with those in 29.2% (19/65) of normal mucosa tissues, and the difference was statistically significant (Figure 1E; P < 0.001). In addition, we compared the intensity of staining for GPR48 in normal mucosa and tumor tissues, and the results showed that GPR48 expression was significantly higher in tumor cells than in normal mucosal epithelial cells (Supplementary Table 2, available at Carcinogenesis Online). These data suggest that GPR48 is overexpressed in human CRC.

GPR48 is overexpressed in human CRC
In our previous proteomic studies, the expression of GPR48 in a cell line (SW620) generated from a lymph node metastasis was found to be much higher than that observed in the primary CRC cell line (SW480) from the same patient (27). Herein, we confirmed that the expression of GPR48 was markedly increased in highly metastatic cell lines (SW620 and KM12L4A) compared with poorly metastatic cell lines (SW480 and KM12C) at both the mRNA and protein levels (Figure 1A and B). In addition, we observed that GPR48 expression in a series of CRC cell lines (SW480, SW620, KM12L4A, KM12C, LoVo and HCT116) was significantly higher than that in a human normal colorectal epithelial cell line (NCM460) (33) at both mRNA and protein levels (P < 0.001) (Figure 1A and B), indicating a differential expression pattern of GPR48 in CRC cells.

GPR48 is a poor prognostic factor for patients with CRC
The median follow-up time of 108 patients was 42 months (Figure 2B). Kaplan–Meier survival analysis revealed a close correlation between GPR48 expression and overall survival (OS) (Figure 2C; log-rank P = 0.002). The 5-year OS rates of patients with high GPR48 expression (40.7%) were significantly lower than those with low GPR48 expression (73.1%). The univariate analysis of prognostic markers of CRC is summarized in Table II. GPR48 expression (P = 0.003, hazards ratio (HR): 3.365, 95% confidence interval (CI): 1.511–7.496) and lymph node status (P < 0.001, HR: 4.533, 95% CI: 2.887–7.263) were prognostic factors for OS by univariate analysis. Factors showing significant by univariate analysis were adopted in multivariate Cox proportional hazards analysis. Multivariate analysis revealed that GPR48 expression (P = 0.015, HR: 2.740, 95% CI: 1.219–6.158) and lymph node status (P < 0.001, HR: 4.080, 95% CI: 2.225–7.483) were independent prognostic factors that could affect the OS of CRC patients (Table II). Furthermore, we evaluated the correlation between GPR48 and Ki67, a marker of cell proliferation (34). The result showed that expression of Ki67 was unrelated to GPR48 expression (R² = 0.022, P = 0.174; Figure 2D).
Prognostic role of GPR48 in human colorectal cancer

GPR48 is upregulated in the invasive front and metastatic lymph nodes of CRC

When analyzing the levels of GPR48 expression in CRC tissues by immunohistochemistry, we found an interesting phenomenon where GPR48 expression was markedly upregulated at the tumor invasive front compared with the tumor center (88.9 versus 45.4%; \( P < 0.001 \)) (Figure 2E and Supplementary Table 3, available at Carcinogenesis Online). Expression levels of GPR48 were normalized to those of \( \beta \)-actin. Error bars represented triplicate experiments \( \pm \) SD (\( P \) value calculated by Student’s \( t \)-test).

Next, we performed immunohistochemical analysis of GPR48 expression on 25 matched primary CRC and metastatic lymph nodes from individual patients. The results showed that GPR48 expression was much higher in metastatic lymph nodes than in matched primary tumors (92 versus 76%; \( P < 0.001 \); Figure 2F). These findings suggest that GPR48 expression might be involved in invasion and metastasis of human CRC.

Overexpression of GPR48 promotes migration and invasion of CRC cells and tumor metastasis

To investigate the role of GPR48 in the aggressive phenotype of CRC cells, HCT116 cells stably expressing full-length GPR48
Table 1. Relationship between the clinicopathological features and the expression of GPR48 in colorectal cancer

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>GPR48 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>No. of patients (%)</td>
<td>No. of patients (%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>17 (30.9)</td>
<td>38 (60.1)</td>
</tr>
<tr>
<td>≥65</td>
<td>10 (18.9)</td>
<td>43 (81.1)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16 (23.4)</td>
<td>48 (76.6)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (25.0)</td>
<td>33 (75.0)</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>10 (20.4)</td>
<td>39 (79.6)</td>
</tr>
<tr>
<td>Rectum</td>
<td>17 (28.8)</td>
<td>42 (71.2)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>21 (34.4)</td>
<td>40 (65.6)</td>
</tr>
<tr>
<td>III</td>
<td>6 (12.8)</td>
<td>41 (87.2)</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well/moderate</td>
<td>26 (25.5)</td>
<td>76 (74.5)</td>
</tr>
<tr>
<td>Poor</td>
<td>1 (16.7)</td>
<td>5 (83.3)</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>3 (60.0)</td>
<td>2 (40.0)</td>
</tr>
<tr>
<td>T3–T4</td>
<td>24 (23.3)</td>
<td>79 (76.7)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
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</tr>
<tr>
<td>N0</td>
<td>21 (34.4)</td>
<td>40 (65.6)</td>
</tr>
<tr>
<td>N1/N2</td>
<td>6 (12.8)</td>
<td>41 (87.2)</td>
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</table>

*P < 0.05 was considered statistically significant. The P values of chi-square test.

(HCT116/GPR48 cells) or the empty vector (HCT116/vector cells) were established and subjected to in vitro migration and invasion assays. In the wound-healing assay, the number of HCT116/GPR48 cells migrating into the wound area was much higher than control cells (Figure 3A). Consistent with this observation, the number of HCT116/GPR48 cells migrating into the lower chamber was 10-fold higher than that of control cells in a transwell chamber migration assay (Figure 3B). A similar result was observed in HCT116/GPR48 shRNA and HCT116/control shRNA cells (Supplementary Figure 2A, available at Carcinogenesis Online). A Matrigel invasion assay was used to evaluate the effects of GPR48 on invasion of CRC cells. The results revealed a 19-fold increase in invasive capacity of HCT116/GPR48 cells compared with the control cells (Figure 3C). A tail vein metastatic assay was performed in nude mice to examine the in vivo metastatic potential of HCT116/GPR48 and HCT116/vector cells. As shown in Figure 3D, the number of lung metastatic lesions derived from HCT116/GPR48 cells was markedly increased compared with control cells. The average number of both the micrometastases and macrometastases in mice lungs following injection of HCT116/GPR48 cells was >2-fold greater than when injected with control cells as determined by H&E staining (P < 0.01; Figure 3D). Additionally, we also assessed the effect of GPR48 on proliferation and found that tumor cell growth in an in vitro colony formation assay and in vivo xenograft tumor model was not influenced by overexpression of GPR48 (Supplementary Figure 2B and C, available at Carcinogenesis Online). Collectively, these results show that GPR48 could promote migration and invasion of CRC cells and tumor metastasis but did not enhance cell proliferation and tumor growth.

GPR48 upregulates the expression of β-catenin in vitro and in vivo

It has recently been reported that GPR48 is involved in regulation of Wnt/β-catenin signaling in HEK293T cells (20). We sought to determine whether this also applied to CRC cells. Firstly, we measured total and nuclear protein levels of β-catenin in HCT116/GPR48 and HCT116/vector cells. Immunoblot analysis showed that total and nuclear β-catenin was increased 2- to 3-fold in HCT116/GPR48 cells compared with control cells (Figure 4A and B). Deletion of GPR48 in LoVo, HCT116 and HEK293T cells decreased total β-catenin protein levels (Figure 4A and Supplementary Figure 3A, available at Carcinogenesis Online). Subsequently, the subcellular distribution of β-catenin in HCT116/GPR48 cells and HCT116/vector cells was examined by immunofluorescence staining. We observed that β-catenin was mainly distributed in the cytoplasm and the nucleus of HCT116/GPR48 cells compared with the cytoplasm and membrane of HCT116/vector cells (Supplementary Figure 3B, available at Carcinogenesis Online), indicating that GPR48 could induce nuclear β-catenin accumulation in CRC cells.

To determine whether β-catenin expression is upregulated in vivo by GPR48, primary CRC tissues from 34 patients were stained for β-catenin. As shown in Figure 4C, β-catenin protein in CRC tissues was mainly accumulated in the cytoplasm and nuclear compartment, whereas moderate membranous and weak cytoplasmic β-catenin staining was observed in noncancerous mucosa. The expression of β-catenin in the cell membrane as appeared normal, whereas the expression in cytoplasm and nuclei was ectopic. High β-catenin expression in the cell membrane, cytoplasm and nuclei was observed in 18 (52.9%), 24 (70.6%) and 9 (26.5%) cases of CRC, respectively (Supplementary Table 5, available at Carcinogenesis Online). Correlation analysis showed that expression of GPR48 was positively related to β-catenin expression (R² = 0.166; P = 0.017; Supplementary Figure 3C, available at Carcinogenesis Online). These data suggest that GPR48 expression was associated with activation of β-catenin in CRC.

GPR48 activates β-catenin/TCF signaling pathway and increases expression of its downstream target genes in CRC cells

Next, we determined whether overexpression of GPR48 affected β-catenin signaling in CRC cells using the luciferase-based TOPflash/FOPflash assay. TCF4 transcription activity was increased 1.5- to 2.7-fold in HCT116/GPR48 and HEK293T/GPR48 cells compared with control cells (Figure 4D). In contrast, knockdown of GPR48 resulted in reduced TCF4 transcriptional activity in LoVo/GPR48 shRNA and HEK293T/GPR48 shRNA cells relative to that in control cells (Figure 4E). In addition, we examined the expression of β-catenin target genes by reverse transcription–polymerase chain reaction. Expression levels of the β-catenin target genes including Cyclin D1 and c-Myc were much higher in HCT116/GPR48 cells than in the corresponding control cells (Supplementary Figure 3D, available at Carcinogenesis Online). These results demonstrate that GPR48 could activate β-catenin/TCF signaling and increase expression of its target genes (Cyclin D1 and c-Myc) in CRC cells.

GPR48-mediated β-catenin/TCF signaling is associated with activation of PI3K/Akt and mitogen-activated protein kinase/ERK1/2 pathway in CRC cells

GPR48 could activate EGFR and EGFR downstream signaling pathways, including mitogen-activated protein kinase (MAPK/ERK1/2 and STAT3 pathways (16,35). It is known that ERK and Akt can phosphorylate GSK-3β at Ser3, resulting in phosphorylation and degradation of β-catenin (24–26). To better understand the mechanism underlying by which GPR48 activates β-catenin/TCF signaling, we next examined whether EGFR downstream pathways including PI3K/Akt and MAPK/ERK1/2 pathways are activated by GPR48 in CRC cells. Immunoblot analysis showed that upregulation of GPR48 in HCT116/GPR48 cells was associated with increased phosphorylation of GSK-3β (at Ser3), ERK1/2 and Akt, whereas total GSK-3β, ERK1/2 and Akt levels were unchanged (Figure 4F). In contrast, depletion of GPR48 in LoVo and HEK293T cells led to decreased phosphorylation levels of GSK-3β, ERK1/2 and Akt (Figure 4F and Supplementary Figure 3E, available at Carcinogenesis Online),
suggesting that GPR48 could activate MAPK/ERK1/2 and PI3K/AKT pathways in CRC cells.

To investigate whether two pathways participate in activation of β-catenin/TCF signaling in CRC cells, pharmacological inhibitors of PI3K (LY294002) or MEK (U0126) were used in TCF4 transactivation assays. As shown in Figure 4G (left), treatment of HCT116/GPR48 cells with PI3K inhibitor LY294002 (10 μmol/l) and MEK inhibitor U0126 (10 μmol/l) alone, or in combination, markedly decreased GPR48-induced TCF4 transactivation activity, suggesting that PI3K/Akt and MAPK/ERK1/2 pathways were required for GPR48-mediated β-catenin signaling. To further confirm that GSK-3β, a downstream target of PI3K/AKT and MAPK/ERK1/2 pathways, is involved in GPR48-mediated β-catenin signaling, we cotransfected HCT116 cells with GPR48 cDNA and SuperTop TCF4 luciferase reporter plasmid and treated these with LiCl, a GSK-3β inhibitor. The results showed that GPR48 and LiCl alone or in combination increased TCF4 transactivation activity compared with HCT116/vector cells (Supplementary Figure 3F, available at...
These findings suggest that GPR48 activates β-catenin/TCF signaling via regulation of GSK-3β phosphorylation through MAPK/ERK1/2 and PI3K/Akt pathways in CRC cells.

Table II. Univariate and multivariate analysis for OS

<table>
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<th>Factor</th>
<th>OS</th>
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<td></td>
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<td>P value</td>
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<td>Gender (male versus female)</td>
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<td>Tumor differentiation (well/moderate versus poor)</td>
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<td>Depth of invasion (T2 versus T3T4)</td>
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<td>LN metastasis (N0 versus N1N2)</td>
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<tr>
<td>GPR48 (low versus high)</td>
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<td>2.740</td>
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NA, not adopted. Multivariate analysis and Cox proportional hazards regression model were used. Variables were adopted for their prognostic significance by univariate analysis with forward stepwise selection (forward, likelihood ratio). Variables were adopted for their prognostic significance by univariate analysis (P<0.05).

Discussion

G-protein-coupled receptors, the largest family of cell-surface molecules with key roles in signal transmission, are emerging as crucial players in tumor growth and metastasis (36). Recent studies have highlighted the role of GPR48, a member of the G-protein-coupled receptor family, in carcinogenesis and development of CRC and gastric carcinoma (17,18), but the correlation between GPR48 expression and clinical outcomes in human CRC has not been fully investigated. Here, we show that GPR48 is highly expressed in primary CRC and correlates with the poor prognosis of patients with CRC.

LGRs, including GPR48, GPR49 and LGR6, have previously been reported to be involved in many different physiological and pathophysiological processes (18). GPR49, a marker of cancer stem cells, was found to be overexpressed in several types of cancer including hepatocellular carcinoma, ovarian cancer and CRC, and its overexpression was associated with lymph node metastasis, liver metastasis and poor prognosis for CRC patients (37,38). Although GPR48 shares ~50% sequence homology with GPR49, few studies have been reported on the potential of GPR48 as a marker for the prognosis of patients with cancer. Recent studies have shown that GPR48 expression is upregulated in gastric cancer at the transcription and translation levels (10,18). In addition, Gao et al. found that GPR48 mRNA expression was increased in CRC tissues compared with normal tissues and was associated with tumor differentiation and lymphatic involvement (17). In this study, GPR48 protein was frequently upregulated in primary CRC tissues, and its high expression closely correlated with advanced tumor stage and lymph node metastases, suggesting that GPR48 is involved in CRC development. In log-rank test, we found that CRC patients with low GPR48 expression lived longer than those with high GPR48 expression. Multivariate analysis indicated that GPR48 served as an independent prognostic marker for OS in patients with CRC. In contrast, Steffen et al. did not find that...
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Fig. 4. GPR48 induces nuclear β-catenin accumulation and activates β-catenin/TCF signaling in CRC cells via regulation of PI3K/Akt and MAPK/ERK1/2 signaling pathways. (A) Immunoblot analysis of β-catenin expression in HCT116/vector and HCT116/GPR48 or LoVo/control shRNA and LoVo/GPR48 shRNA cells. β-Actin was used as a loading control. (B) Cytoplasmic and nuclear fractions of HCT116/vector and HCT116/GPR48 cells were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then immunoblotted with anti-β-catenin, β-actin and histone-3 (right). β-Actin was used as a cytoplasmic protein loading control, and histone-3 was used for nuclear protein loading control. (C) Immunohistochemical staining of GPR48 and β-catenin in the sample of primary CRC (lower) and adjacent normal tissues (upper). Original magnification, ×200. (D) Luciferase assay for HCT116 and HEK293T cells expressing empty vector or GPR48 vector, which were transfected with the Super TOP Flash TCF4 reporter construct along with a mutant control FOP Flash reporter construct. Luciferase reporter activity was measured after 48 h. Results were presented relative to FOP Flash luciferase activity. Mean values ± SD from three separate experiments were shown. *P < 0.05; **P < 0.01; ***P < 0.001, Student’s t-test. (E) Luciferase assay for LoVo and HEK293T cells expressing control shRNA or GPR48 shRNA. (F) Expression levels of GSK-3β, p-GSK-3β, Akt, p-Akt, ERK1/2 and p-ERK1/2 protein in HCT116 cells and LoVo cells determined by immunoblotting. β-Actin was used as a loading control. (G) Luciferase assay for HCT116/vector and HCT116/GPR48 cells (left). Cells were then treated with Ly294002 (10 μmol/l) and U0126 (10 μmol/l) alone, or in combination, for an additional 24 h. Luciferase reporter activity was measured after 48 h. **P < 0.01; ***P < 0.001, Student’s t-test. (H) A model to illustrate the regulation of β-catenin signaling by GPR48 in CRC cells. In the absence of Wnt protein (left panel), β-catenin associates with the APC/Axin/GSK-3β-complex and is phosphorylated by GSK-3β, which leads to its ubiquitylation and degradation by the proteasome. Overexpression of GPR48 leads to the transactivation of EGFR (data not shown) and the subsequent activation of PI3K/Akt and MAPK/ERK1/2 pathways (right panel), which could inhibit GSK-3β activity, thereby blocking the proteosomal degradation of β-catenin. Accumulated β-catenin then translocates into the nucleus and activates Wnt target genes in concert with TCF/LEF cofactors, including Cyclin D1 and c-Myc.
GPR48 expression had positive predictive values for OS of gastric cancer patients (18). This discrepancy might be due to the different cancer types and the different cutoff values used (they used negative or positive GPR48 expression, whereas we used high or low GPR48 expression) for distinguishing differential subgroups.

Cancer cells undergo a series of events during metastasis, including invasion, entry into the circulatory system, arrest at a distant site, proliferation and induction of angiogenesis (39). Lymph node metastasis is one of the most important factors that influences the survival of surgical patients with CRC (40). Positive correlation between GPR48 expression and lymph node metastasis has been reported for human gastric cancer and CRC (17,18). In our study, we found that GPR48 expression in lymph node metastasis was much higher than that in matched primary CRC, suggesting that GPR48 might be a potential marker of lymph node metastasis. The invasive front of a tumor is a region containing useful prognostic information, presumably because the most invasive cells are located there (41). In this study, we found that GPR48 was overexpressed at the invasive front of tumors, indicating that GPR48 might play a role in acquisition of migrating and invading cell phenotype that is a prerequisite for malignancy. Previous studies have reported that tumor budding often occurs ahead of the defined invasive edge in CRC tissues and the presence of budding was significantly correlated with a higher rate of lymph node metastasis and poor prognosis of patients (42,43). We found that GPR48 was overexpressed in tumor budding and its expression at the invasive front correlated with grade of tumor budding, which further confirmed that GPR48 expression was associated with more aggressive tumor phenotype. In vitro and in vivo study further validated the role of GPR48 in CRC invasion and metastasis. In line with these results, depletion of endogenous GPR48 in HeLa and Lewis lung carcinoma cells markedly diminished their invasive and metastatic activities (17). These data, in concert with the present findings, further support a critical role of GPR48 in regulating invasion and metastasis of CRC. However, so far, the relevance of GPR48 expression in CRC metastasis to distant organs such as liver and lung has not been investigated and the precise mechanism by which GPR48 promotes tumor invasion and metastasis remains unclear; further studies are needed to reveal this.

The Wnt/β-catenin signaling pathway plays major roles in stem cell biology, organogenesis, tissue homeostasis and cancer (44,45). In the canonical Wnt signaling pathway, Wnt ligands bind to Frizzled and low-density lipoprotein-related receptors 5 and 6, which in turn inhibits phosphorylation of β-catenin by disrupting the destruction complex consisting of the APC, axin and GSK-3β proteins, resulting in β-catenin accumulation in the nucleus and activation of the transcription of Wnt target genes such as cyclin D1 and c-Myc (1,21). Mutations in APC, β-catenin or axin result in increased β-catenin levels and activation of Wnt signaling and have been found in numerous human cancers including colorectal, gastric and ovarian cancer (46). However, mutations of Wnt pathway components are not the only factors that regulate activation of β-catenin signaling. Recent data suggest that GPR48 and GPR49 function as the cognate receptor of R-spondin to potentiate Wnt/β-catenin signaling by enhancing Wnt-induced LRP6 phosphorylation (19,20). Conditional deletion of GPR48 and GPR49 genes in the mouse gut impairs Wnt target gene expression and results in Wnt pathway inhibition (15). In this study, our data show that GPR48 induces nuclear β-catenin accumulation and augments β-catenin/TCF signaling accompanied by an increased expression of its target genes (including cyclin D1 and c-Myc) in CRC cells with mutations in either APC or β-catenin. It is noteworthy that in human CRC tissues, GPR48 was found to be associated with activation of β-catenin, which further confirmed that GPR48 was a positive regulator of β-catenin signaling in CRC.

Although recent data suggest that GPR48 could mediate Wnt/β-catenin signaling in HEK293T cells, the precise mechanism by which GPR48 increases β-catenin protein levels and modulates β-catenin/TCF pathway is still unclear. Mustata et al. found that downregulation of Wnt target genes caused by GPR48 knockout could be partially rescued by addition of LiCl but not by Wnt agonists Wnt3a and R-spondin, indicating that GPR48 may be involved in interaction between the Wnt and PI3K/Akt pathways (14). Our present data indicate that overexpression of GPR48 could increase phosphorylation levels of GSK-3β (at Ser9), Akt and ERK1/2, which can regulate phosphorylation and degradation of β-catenin (47). It has been reported that in response to certain growth stimuli and oncogenes, activated Akt and ERK1/2 can phosphorylate GSK-3β at Ser9, leading to inactivation of GSK-3β, upregulation of β-catenin and activation of Wnt/β-catenin signaling (24–26). Our data suggest that GPR48 activates β-catenin signaling in a similar manner. Either PI3K inhibitor or MEK inhibitor treatment could block activation of β-catenin signaling in CRC cells, whereas LiCl treatment further stimulated β-catenin signaling in the presence of GPR48, suggesting that GPR48 activates β-catenin signaling at least partially through the inhibition of GSK-3β in a PI3K- and ERK1/2-dependent manner. However, it remains to be elucidated whether other signaling pathways are also involved in GPR48-induced β-catenin signaling activation.

In summary, we observed that GPR48 was overexpressed in human CRC and this overexpression was associated with a more aggressive biological behavior. We report for the first time that GPR48 expression is associated with unfavorable prognosis in CRC patients postoperatively. Furthermore, our data demonstrate that GPR48 activates β-catenin signaling in CRC by regulating the phosphorylation of GSK-3β via PI3K/AKT and MAPK/ERK1/2 pathways. These results highlight the importance of GPR48 for a better understanding of CRC pathogenesis and its potential implication in the management of CRC, as a prognostic factor or a future therapeutic target.

Supplementary material
Supplementary Tables 1–5 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

Funding
National 973 Basic Research Program of China (2013CB911300, 2012CB518900, 2011CB910703); the National Science and Technology Major Project (2011ZX09302-001-01, 2012ZX09501001-003); Doctoral Fund of Ministry of Education of China (2012018110024); the National Natural Science Foundation of China (81072022, 81172173, 81225015).

Conflict of Interest Statement: None declared.

References
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Received January 27, 2013; revised May 6, 2013; accepted June 8, 2013