GPR50 Promotes Hepatocellular Carcinoma Progression via the Notch Signaling Pathway through Direct Interaction with ADAM17

Subbroto Kumar Saha,1 Hye Yeon Choi,1 Gwang-Mo Yang,1 Polash Kumar Biswas,1 Kyeongseok Kim,1 Geun-Ho Kang,1 Minchan Gil,1 and Ssang-Goo Cho1

1Department of Stem Cell and Regenerative Biotechnology, Konkuk University, Seoul 05029, Republic of Korea.

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide, and it is thus critical to identify novel molecular biomarkers of HCC prognosis and elucidate the molecular mechanisms underlying HCC progression. Here, we show that G-protein-coupled receptor 50 (GPR50) in HCC is overexpressed and that GPR50 knockdown may downregulate cancer cell progression through attenuation of the Notch signaling pathway. GPR50 knockdown was found to reduce HCC progression by inactivating Notch signaling in a ligand-independent manner through a disintegrin and metalloproteinase metalloproteinase domain 17 (ADAM17), a proteolytic enzyme that cleaves the Notch receptor, which was corroborated by GPR50 overexpression in hepatocytes. GPR50 silencing also downregulated transcription and translation of ADAM17 through the AKT/specificity protein-1 (SP1) signaling axis. Notably, GPR50 was found to directly interact with ADAM17. Overall, we demonstrate a novel GPR50-mediated regulation of the ADAM17-Notch signaling pathway, which can provide insights into HCC progression and prognosis and development of Notch-based HCC treatment strategies.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth-leading cause of cancer-related death worldwide.1 Despite significant progress, more than 70% of HCC patients cannot be diagnosed at an early stage, which is easier to treat via local ablation, hepatic resection, or liver transplantation.2–5 Treatment is more difficult during later stages, and cancer recurrence and metastatic rates increase, leading to increased mortality.5–6 Therefore, the identification of novel molecular biomarkers of HCC and its prognosis and the elucidation of the detailed molecular mechanisms underlying HCC progression are of great importance.

G-protein-coupled receptors (GPCRs), or seven transmembrane (7TM)-spanning proteins, represent the largest class of cell surface receptor proteins, with approximately 800 members. GPCRs are important proteins in drug development and are reported to be key targets for more than 30% of drugs on the market.1 GPCRs were found to play dynamic roles in cancer development and progression, including survival and tumor growth.8,9 Moreover, around 100 GPCRs have been classified as orphan GPCRs, because their endogenous ligands remain unidentified; however, several of them were reported to function in a ligand-independent manner.10,11 They can facilitate signal transduction from the extracellular environment to intracellular effectors12 and mediate physiological and disease progression.13 A recent endeavor has strengthened the need to explore the vital role of GPCRs and their ligands, such as chemokines, lysophosphatidic acid (LPA), serine proteases (PAR1), and sphingosine 1-phosphate (S1P), in metastasis.14–16 Previously, pro-metastatic functions have been ascribed to numerous orphan GPCRs, such as GPR64, GPR116, and GPR161,17,18 highlighting the value of studying these receptors as novel therapeutic targets for preventing cancer metastasis.

GPR50, another member of orphan GPCRs, exhibits high sequence similarity with the melatonin receptors MT1 and MT2, however, melatonin is not a GPR50 ligand.19,20 GPR50 was reported to be associated with bipolar-affective disorder, lipid metabolism, thermogenesis, adiopogenesis, and neuronal development.21–23 In our previous study, several GPCRs, including GPR50, were claimed to be involved in the reprogramming of somatic cells to cancer stem cells and in the maintenance of stemness function.24 Recent studies also reported that GPR50 can act as a tumor suppressor in breast cancer (BRC);25,26 however, there is limited research on the role of GPR50 in cancer progression.

In this study, we aimed to uncover the role of GPR50 in HCC progression and prognosis. As GPR50 was described as a tumor suppressor in breast cancer, we examined whether GPR50 plays an oncogenic or a tumor-suppressor role in HCC. We found that GPR50 is overexpressed in HCC and that GPR50 knockdown can suppress HCC progression by downregulating the Notch signaling pathway. Our
Figure 1. GPR50 Is Differentially Expressed in Various Cancer Types

(A) Oncomine database Log2 median-centered expression intensities for GPR50 genes in various cancers, such as bladder (BLC; n = 9), brain and CNS cancer (BCC; n = 16), breast (BRC; n = 19), cervical (CEC; n = 7), colorectal (COC; n = 23), esophageal (ESC; n = 4), gastric (GAC; n = 5), head and neck (HNC; n = 6), kidney (KIC; n = 8), leukemia (legend continued on next page).
findings also indicate that GPR50 forms a novel molecular complex with a disintegrin and metalloproteinase (ADAM) metallopeptidase domain 17 (ADAM17) and regulates ADAM17 activity, activating the Notch signaling pathway in HCC in a ligand-independent manner. This pathway is also partially regulated by GPR50-mediated ADAM17 transcription via the noncanonical AKT/specificity protein 1 (SP1) axis. Thus, our results support the potential of targeting HCC via the GPR50/ADAM17/Notch signaling pathway.

RESULTS

GPR50 Is Differentially Expressed in Various Cancers and Associated with Liver Cancer Prognosis

Using the Oncomine database (https://www.oncomine.org/resource/login.html) to examine the expression status of GPR50 in various cancers, we found dysregulated GPR50 expression (Wooster cell line dataset) that was especially enhanced in BRC, cervical (CEC), esophageal (ESC), liver (HCC), and lung (LUC) cancers (Figure 1A). Subsequently, we analyzed GPR50 mRNA expression in these cancers using several Gene Expression Omnibus (GEO) datasets. The GEO data showed that GPR50 expression was significantly upregulated in liver cancers (i.e., HCC) and downregulated in breast, cervical, esophageal, and lung cancers (Figure 1B; Table S1), which is in contrast with the expression patterns in the Oncomine database. Moreover, we analyzed the association between prognosis and GPR50 expression in various cancer patients using The Cancer Genome Atlas (TCGA) database via the SurvExpress web. Among the indicated cancers, high GPR50 expression exhibited a significant (p = 0.0118), poor prognostic role in HCC, whereas a nonsignificant prognostic role was found for other cancers, including breast, cervical, esophageal, and lung cancers (Figure 1C), suggesting a differential prognostic role of GPR50 in various cancers. Thus, these results indicate that GPR50 may have an oncogenic role in liver cancer.

We further examined GPR50 expression in liver cancer using TCGA dataset through the SurvExpress web and confirmed GPR50 overexpression (Figure 1D). We then examined mutation and copy number alterations (CNAs) in the liver cancer TCGA dataset through the SurvExpress web. Among the indicated cancers, we found dysregulated GPR50 expression (Figure 1E). Moreover, we checked GPR50 mRNA and protein expression levels in the normal hepatocyte cell line MIHA and several HCC cell lines, including HepG2, SNU449, and SNU475, via reverse-transcriptase PCR (RT-PCR) and western blot analyses and found that GPR50 expression was clearly overexpressed in the HCC cell lines (Figure 1F). Overall, these results indicate that GPR50 expression is dysregulated in various cancers and specifically upregulated in HCC.

GPR50 Knockdown Decreases Cell Proliferation, Migration, Sphere Formation, and Drug Resistance

To examine the role of GPR50 in HCC, we knocked down GPR50 using GPR50-targeted short hairpin RNA (shRNA) in two HCC cell lines: HepG2 and SNU475. GPR50 knockdown was confirmed (approximately 95%) via RT-PCR and western blot analysis (Figure 2A). We found that cell proliferation was decreased upon GPR50 silencing in both HepG2 and SNU475 cells (Figure 2B). In addition, cell migration, sphere formation, and drug resistance (Figures 2C, 2D, and 2F) were attenuated in GPR50-knockdown HepG2 and SNU475 cells compared with their normal counterparts. We also found that expression of stemness markers, such as NANOG, SOX2, OCT4, and KLF4, and drug-resistance markers, such as P-GP, ABCG2, ABCCI, ALDH1A1, and ABCB5, was decreased upon GPR50 knockdown (Figures 2E and 2G; Figure S1), suggesting that GPR50 has oncogenic ability and regulates HCC progression.

GPR50 Is a Novel Regulator of the Notch Signaling Pathway

As GPR50 knockdown attenuated cancer progression, we attempted to uncover the underlying signaling mechanism. For that, we checked several signaling pathways that potentially contribute to cancer progression. We found that expression of HES1, a Notch signaling target gene, was significantly downregulated upon GPR50 knockdown (Figure 3A). We did not detect any significant differences in the expression of target genes of other signaling pathways, including Wnt, Hedgehog, and Hippo, which are predominantly involved in cancer progression, stemness, and metastasis.29–33 Expression of other Notch pathway target genes, such as NOTCH1, MAML1, and RBPJK, was also suppressed upon GPR50 knockdown in HCC cells (Figure 3B). Western blotting confirmed the reduced protein expression levels of Notch intracellular domain (NICD) and HES1 upon GPR50 knockdown (Figure 3C). Moreover, transcription activity of HES1 and HES5 was reduced upon GPR50 silencing (Figures 3D and 3E).

Next, we induced the Notch signaling pathway in GPR50-knockdown cells using NICD to test whether NICD overexpression can relieve GPR50 suppression effects. FLAG-NICD overexpression in SNU475 cells was confirmed by western blot analysis using a FLAG antibody (Figure 3F). We then analyzed HES1 and HESS promoter activity and found that the shGPR50-induced reduction in HES1 and HES5 transcriptional activity was increased upon NICD overexpression (Figures 3G and 3H). Moreover, shGPR50-induced reduction in cell
Figure 2. GPR50 Knockdown Led to Suppressed Cancer Properties in HCC

(A) GPR50 expression in scramble and shGPR50-transduced cells was analyzed by RT-PCR and western blotting in the indicated cancer cell lines. GAPDH/actin was used as a loading control. (B) Cell proliferation was analyzed using trypan blue. Cells were counted over 5 days. (C) Wound-healing assay to test migration of the indicated cells. Cell migration was observed at the indicated time points and presented as percentage (%) wound enclosure (right panel). Photos were acquired using inverted light microscopy.

(D) Cell-sphere formation assay was performed using noncoated culture dishes. Spheres were counted after 5 days of culture with crystal violet staining and presented as the percent (%) of colonies. Photos were acquired by inverted light microscopy. (E) mRNA expression of stemness markers was analyzed by RT-PCR. GAPDH was used as an internal standard. (F) Effect of GPR50 knockdown on drug resistance was measured by cell counting after 48 h of doxorubicin (DOX) treatment (0.5 µM). (G) mRNA expression levels of drug-resistance marker genes were analyzed by RT-PCR using GPR50-knockdown cells. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Molecular Therapy: Oncolytics

**A**

|          | HepG2 | SNU475 |
|----------|-------|--------|
| **Scramble** | **shGPR50-(2)** | **shGPR50-(2)** |
| DKK1     | 1.2   | 0.8    |
| HES1     | 1.1   | 0.5    |
| GLI1     | 1.0   | 0.5    |
| YAP1     | 0.9   | 0.5    |

**B**

|          | HepG2 | SNU475 |
|----------|-------|--------|
| **shGPR50-(2)** | **+** | **+** |
| NOTCH1    | 0.7   | 0.5    |
| MAML1     | 0.8   | 0.5    |
| RBP/j     | 0.9   | 0.5    |
| HES1      | 0.8   | 0.5    |
| GAPDH     | 1.0   | 1.0    |

**C**

|          | HepG2 | SNU475 |
|----------|-------|--------|
| **shGPR50-(2)** | **-** | **+** |
| NICD      | 1.0   | 0.19   |
| HES1      | 1.0   | 0.51   |
| Actin     | 1.0   | 0.55   |

**D**

|          | SNU475 |
|----------|--------|
| **50-(2)** | **+** |
| HES1-luc activity (RLUs) | 50    |
| shGPR50-(2) | **-** |
| HES1-luc activity (RLUs) | 10    |

**E**

|          | SNU475 |
|----------|--------|
| **50-(2)** | **+** |
| HES1-luc activity (RLUs) | 50    |
| shGPR50-(2) | **-** |
| HES1-luc activity (RLUs) | 10    |

**F**

|          | SNU475 |
|----------|--------|
| **Flag-NICD o/e** | **+** |
| HES1-luc activity (RLUs) | 50    |
| shGPR50-(2) | **-** |
| HES1-luc activity (RLUs) | 10    |

**G**

|          | SNU475 |
|----------|--------|
| **50-(2)** | **+** |
| HES1-luc activity (Fold) | 100   |
| shGPR50-(2) | **-** |
| HES1-luc activity (Fold) | 10    |

**H**

|          | SNU475 |
|----------|--------|
| **50-(2)** | **+** |
| HES1-luc activity (Fold) | 25   |
| shGPR50-(2) | **-** |
| HES1-luc activity (Fold) | 5    |

**I**

|          | SNU475 |
|----------|--------|
| **50-(2)** | **+** |
| Cell viability (Fold) | 1.6   |
| shGPR50-(2) | **-** |
| Cell viability (Fold) | 1.2   |

**J**

|          | SNU475 |
|----------|--------|
| **50-(2)** | **+** |
| Sphere formation efficiency (Fold) | 4.0   |
| shGPR50-(2) | **-** |
| Sphere formation efficiency (Fold) | 1.0   |

(legend on next page)
viability and sphere formation was also reversed upon NICD overexpression (Figures 3I and 3J). These results indicate that attenuation of HCC progression via shGPR50 can be regulated through the Notch signaling pathway.

The GPR50-Regulated Notch Signaling Pathway Is Notch Ligand Independent

We further assessed the Notch ligand dependency of the GPR50-regulated Notch signaling pathway, because this pathway can be regulated in a ligand-dependent34–36 or -independent37 manner. First, we focused on ligand-dependent Notch signaling activation upon GPR50 knockdown. mRNA expression of the Notch signaling ligands Jagged 1 (JAG1) and 2, as well as delta-like ligand 1 (DLL1), 3, and 4, was not significantly altered in shGPR50-HepG2 and SNU475 cells compared with their normal counterparts (Figure 4A). To confirm, we overexpressed JAG1 and DLL1 in scramble and shGPR50-SNU475 cells, validated by RT-PCR (Figures 4B and 4C), and found that Notch ligand overexpression did not significantly affect the shGPR50-induced reduction in HES1 mRNA expression, cell viability, and sphere formation (Figures 4D–4F). Taken together, these results indicate that suppression of Notch signaling via GPR50 knockdown occurs in a ligand-independent manner.

GPR50 Regulates ADAM17 Transcription through the AKT/SP1 Axis

Next, we investigated the ligand-independent activation of Notch signaling by analyzing mRNA expression levels of ADAM metallopeptidases, including ADAM9,-10,-12, and -17, which have been reported to regulate the Notch signaling pathway.37–41 Among these ADAMs, ADAM17 mRNA expression was significantly downregulated upon GPR50 knockdown in both HCC cell lines, HepG2 and SNU475 (Figure 5A). Moreover, we analyzed the correlation between ADAMs and GPR50 mRNA expression in HCC using TCGA database through the cBioPortal web and found that mRNA expression levels of the studied...
Figure 5. GPR50 Regulates ADAM17 Transcription via the AKT/SP1 Axis

(A) Expression levels of Notch signaling regulating genes ADAM9, ADAM10, ADAM12, and ADAM17 were analyzed using qRT-PCR; GAPDH was used as an internal standard. (B) Protein expression levels of AKT, phosphorylated (p)-AKT, GSK3β, p-GSK3β, ERK, p-ERK, p38, and p-p38 were analyzed using western blotting; actin was used as an internal standard.
ADAMs were positively correlated with GPR50 expression, with ADAM17 showing the most significant correlation (Figure S2).

Therefore, we aimed to uncover how GPR50 regulates ADAM17 transcription by examining whether GPR50 knockdown affects the AKT, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), and p38/MAPK signaling pathways, as previous studies reported that transcription of ADAM17 can be regulated through these pathways.21-23 We found that AKT phosphorylation was downregulated in shGPR50-HepG2 cells compared with that in scramble-HepG2 cells, whereas the ERK/MAPK and p38/MAPK signaling pathways were not altered upon GPR50 knockdown (Figure S3B). Glycogen synthase kinase 3β (GSK3β) phosphorylation at Ser9 was also altered upon GPR50 suppression (Figure S5B). To confirm these findings, we treated GPR50-knockdown cells with the AKT inhibitor LY294002 (20 μM).45 LY294002 treatment drastically reduced AKT phosphorylation in GPR50-knockdown cells compared with control cells (Figure S5C). LY294002 treatment also induced GSK3β phosphorylation in shGPR50 cells compared with that in control cells (Figure S5C), which may subsequently regulate other signaling pathways, including GSK3β/mammalian target of rapamycin (mTOR) or Wnt/β-catenin, which are yet to be investigated.

As the ADAM17 promoter region contains a high guanine-cytosine (GC)-rich sequence that was depicted to bind various transcription factors, including SP1,46,47 we tested whether GPR50 silencing regulates SP1 expression. We examined the mRNA expression levels of all SP family genes (SP1, -2, -3, and -4) and found that SP1 expression was significantly downregulated upon GPR50 suppression (Figure 3D). Moreover, SP1 expression was significantly attenuated upon LY294002 treatment in GPR50-knockdown cells compared with that in control cells (Figure 5D). Cell proliferation, wound healing/migration, drug resistance, and sphere formation (Figures 5E–5H) were also widely attenuated upon LY294002 treatment. We also analyzed ADAM expression and found that ADAM17 was significantly downregulated upon LY294002 treatment in GPR50-silenced cells compared with that in control cells (Figure S5I). Thus, these results support the notion that GPR50 knockdown mediates ADAM17 downregulation through the AKT/SP1 axis.

**GPR50 Directly Interacts with ADAM17 and Regulates ADAM17 Activity**

As GPR50 knockdown suppressed ADAM17 transcription, we analyzed ADAM17 protein levels upon GPR50 silencing and found that ADAM17 protein levels were attenuated in shGPR50 cells (Figure 6A). ADAM17 is a proteolytic enzyme that can cleave Notch receptors and subsequently activate the Notch signaling pathway.48 Thus, we examined ADAM17 activity in GPR50-knockdown cells and found reduced ADAM17 activity (Figure 6B). We theorized that GPR50 and ADAM17 can directly interact with each other (as both are cell membrane proteins), which was confirmed via communoprecipitation (coIP; Figure 6C), indicating that ligand-independent Notch signaling activation is mediated through the GPR50-ADAM17 interaction.

**Overexpression of ADAM17 Relieves GPR50-Knockdown Effects**

We next overexpressed ADAM17 in GPR50-knockdown SNU475 cells to test whether ADAM17 overexpression can relieve the effects of shGPR50. Overexpression of FLAG-ADAM17 was confirmed by western blot analysis using a FLAG antibody (Figure 6D). Afterward, Notch signaling activation upon ADAM17 overexpression in GPR50-knockdown cells was analyzed by examining HES1 and HES5 transcription activity via luciferase assays (Figures 6E and 6F). shGPR50-induced suppression of HES1 and HES5 transcription activity was reversed upon ADAM17 overexpression (Figures 6E and 6F). Moreover, treatment with marimastat (4 μM),49 an ADAM17 inhibitor, suppressed the effects of ADAM17 overexpression specially in shGPR50-SNU475 cells (Figures 6E and 6F). Similarly, shGPR50-induced reduction in ADAM17 activity was relieved upon ADAM17 overexpression in SNU475 cells; conversely, marimastat (4 μM) treatment of ADAM17-overexpressed SNU475 cells reduced ADAM17 activity, which was marked in shGPR50-SNU475 cells (Figure 6G). Moreover, the shGPR50-induced reduction in cell viability (Figure 6H) and sphere formation (Figure 6I) was rescued upon ADAM17 overexpression in SNU475 cells, whereas marimastat alone or marimastat + shGPR50 suppressed the effects of ADAM17 overexpression in SNU475 cells. These results indicate that the shGPR50-induced suppression of HCC progression is mediated through ADAM17-dependent Notch signaling suppression.

**Overexpression of GPR50 Induces Cancer Progression through ADAM17-Dependent Notch Signaling Pathway Activation**

Finally, we assessed the role of the ADAM17-activated Notch signaling pathway during GPR50 overexpression in HCC. We first confirmed GPR50 overexpression in the MIHA and HepG2 cell lines using RT-PCR and western blot analysis (Figure 7A) and then analyzed the effect of GPR50 overexpression in MIHA and HepG2
To confirm the GPR50-induced regulation of the Notch signaling pathway through ADAM17, we treated GPR50-overexpressed HepG2 cells with marimastat (4 μM) and/or the Notch signaling (γ-secretase) inhibitor DAPT (20 μM). GPR50 protein expression was not significantly altered upon marimastat and/or DAPT treatment in GPR50-overexpressed HepG2 cells, whereas the GPR50 overexpression-induced upregulation of ADAM17, NICD, and HES1 was dramatically relieved upon marimastat and/or DAPT treatment (Figure 7H). Moreover, the GPR50 overexpression-induced ADAM17 activity was significantly attenuated upon marimastat treatment (Figure 7I), whereas enhanced HES1 and HES5 transcriptional activity was significantly relieved upon treatment with marimastat and/or DAPT in HepG2 cells (Figures 7J and 7K). These results strongly suggest that GPR50 regulates HCC progression through the ADAM17-mediated Notch signaling pathway.

**DISCUSSION**

An orphan GPCR (GPR50) was shown to regulate bipolar-affective disorder, lipid metabolism, thermogenesis, adipogenesis, and neuronal development, although it has been claimed that GPR50 has high sequence similarity with melatonin receptors (MT1 and MT2), but melatonin does not bind to GPR50. GPR50 was also reported to interact directly with various proteins, such as TIP60, NOGO-A, MT1, and MT2, via heterodimerization through the large carboxyl-terminal tail (C-tail) of GPR50. Moreover, previous studies displayed that GPR50 can directly interact with transforming growth factor-β1 (TGF-β1) and constitutively activate the canonical SMAD2/3 signaling pathway, which contributes to regulation of breast cancer progression, indicating that GPR50 can act as a tumor suppressor in breast cancer. In addition, our previous study was given a clue that several GPCRs, including GPR50, are involved in the reprogramming of somatic cells to cancer stem cells and in the maintenance of stemness function. However, little is known regarding the biological significance of GPR50 in liver cancer.

Here, we investigated the expression patterns of GPR50 in various cancer cells and found dysregulated GPR50 expression. Moreover, GPR50 expression was lower in normal hepatocytes than in HCC cells. We also found that GPR50 knockdown attenuates cell proliferation, sphere formation, migration, and drug resistance via the Notch signaling pathway. We identified, for the first time, a novel GPR50-mediated Notch signaling activation pathway that is activated in a ligand-independent manner.

Notch signaling is involved in most multicellular processes in cancer cells, including cell fate, proliferation, metastasis, invasion, and stemness. Recent studies have depicted that the Notch signaling pathway can play either pro-oncogenic or tumor-suppressive roles in cancer cells. Notch signaling is normally triggered by a Notch-activating ligand that is subsequently processed through two proteolytic cleavage events via ADAM17 and the γ-secretase complex. This cleavage can then result in release of the NICD, which is imported to the nucleus and binds to cotranscription factors, including MAML1, RBP-J, p300, and CSL, to trigger expression of Notch signaling target genes. Notably, the Notch signaling pathway is significantly associated with liver cirrhosis and HCC. Although various members of the Notch signaling pathway may act as inhibitors or enhancers of HCC, the Notch signaling pathway generally plays a carcinogenic role in HCC, and activation of Notch signaling has been associated with a more malignant phenotype.

Therefore, identification of reliable Notch pathway regulatory mechanisms is critical for the application of Notch-based HCC therapeutic strategies, as regulation of the Notch pathway can potentially suppress HCC progression and aggressiveness.
We further found that the GPR50-regulated Notch signaling pathway is mediated by ADAM17, independent from the Notch ligands JAG and DLL. ADAM, a single-pass transmembrane protein, is involved in multiple cellular functions, such as migration, proteolysis of extracellular matrix (ECM) components, and shedding of membrane proteins (e.g., cytokines and growth factors); fertilization; development; inflammation; asthma; and neurodegenerative diseases, such as Alzheimer’s disease.63–65 Several ADAM family proteins have been identified and are characterized by their domain organization, including a pro-domain; a metalloprotease, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like domain; and transmembrane domains, and a C-terminal cytoplasmic tail. Several reports have demonstrated that ADAMs play an important role in HCC pathogenesis.66 ADAM17, also known as TACE (tumor necrosis factor α [TNF-α]-converting-enzyme), has been reported to be a Notch receptor molecular scissor, which leads to tumorigenesis and tumor progression.67,68 Moreover, ADAM17 activates the Notch signaling pathway in a ligand-independent manner69 and is reported to regulate cell proliferation, angiogenesis, invasion, and apoptosis of cancer cells by regulating Notch signaling.70–72 However, ligand-independent Notch signaling activation via ADAM17 is not fully elucidated.

Consistent with our results, a number of studies depicted that ligand-independent Notch activation is mediated by ADAM17.73–75 Another study demonstrated Notch signaling activation without cell-cell contact in the presence of soluble JAG1.74 Moreover, a study demonstrated that several GPCRs, including orphan GPCRs, resulted in ADAM17 activation and subsequently induced TGF-β shedding in HEK293 cells.71 Furthermore, GPCRs, including GPR50, were reported to interact with other receptors from the same family or different receptor and transporter proteins through heterodimerization, eventually forming molecular complexes.24,52,72 Similarly, our findings demonstrated that GPR50 can form a molecular complex with ADAM17 through direct interaction in HCC (see Figure 6C), which subsequently activates Notch signaling via ADAM17 activity, strongly supporting a novel signaling pathway between GPR50 and the ligand-independent, ADAM17-mediated Notch signaling.

We further demonstrated that GPR50 can regulate ADAM17 transcription and translation through the AKT/SP1 signaling axis, which is in agreement with previous studies that ADAM17 can be transcriptionally regulated via the AKT, ERK/MAPK, and p38/MAPK signaling pathways.42–44 Moreover, the ADAM17 promoter has a high GC-rich sequence where a number of transcription factors, including SP1, can bind.66,67 SP1 has been shown to have a potential role in cancer by regulating the transcription of several genes that have high GC-rich sequences in their DNA-binding promoter regions.78–80 SP1 is regulated by its upstream effectors, including the phosphatidylinositol 3-kinase (PI3K)/AKT, ERK, and p38/MAPK signaling pathways.77–79 Moreover, a study demonstrated that GPCRs can regulate SP1 via their downstream proteins and effectors (i.e., β-arrestin 1), which represses leukemic cell senescence.81 Therefore, by taking into consideration these previous studies and our findings, there is ample evidence supporting GPR50-mediated ADAM17 transcription and translation via the AKT/SP1 axis.

In conclusion, we demonstrated how the orphan receptor GPR50 regulates the ligand-independent activation of Notch signaling through GPR50-mediated modulation of ADAM17 activity in HCC (Figure 8). A signaling cascade initiating from GPR50 was uncovered, wherein GPR50 was also found to regulate ADAM17 transcription and translation via the AKT/SP1 axis in HCC (Figure 8). Thus, our findings revealed the molecular basis underlying the GPR50 and ADAM17 complex-mediated, ligand-independent modulation of the Notch signaling pathway, which can be exploited in Notch-based HCC therapeutic strategies.

MATERIALS AND METHODS

Bioinformatic Analysis

The expected expression levels of the GPR50 gene in various cancers were retrieved from the Oncomine database (https://www.oncomine.org/resource/login.html).82,83 Fold changes in mRNA expression in cancer tissues compared with their normal counterparts were acquired using a default threshold. Gene expression was also analyzed using GEO, a web database that gathers submitted high-throughput gene-expression data of chips, microarray, or next-generation sequencing (https://www.ncbi.nlm.nih.gov/geo/).84 Microarray datasets with accession numbers GEO: GSE1477, GSE7803, GSE20347, GSE45436, and GSE2514 containing gene-expression information of clinical human BRC, CEC, ESC, HCC, and LUC patients, respectively, were used in this study. The other GEO datasets are listed in Table S1. The raw data were retrieved and replotted using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA).

With the use of a web-based tool for survival analysis, SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp),85
we conducted an integrative analysis of GPR50 mRNA expression levels and clinical outcomes. This database contains more than 39,000 samples and 225 datasets covering tumors from more than 26 different tissues. With the use of this platform, survival plots were generated for GPR50 in specific cancer types using TCGA data. We further performed an integrative analysis of GPR50 and clinical characteristics using cBioPortal, an open-access resource found at http://www.cbioportal.org/, which currently provides access to data from more than 48,668 tumor samples and 172 cancer studies in TCGA pipeline. The query interface, combined with customized data storage, enabled us to explore genetic alterations interactively across samples curated from national and international cancer studies for specific genes. The primary search parameters included alterations (amplification, deep deletion, and missense mutations), CNAs, gene-gene correlation from Genomic Identification of Significant Targets in Cancer (GISTIC), and RNA-seq data using the default settings.

Cell Culture
The human HCC cell lines (HepG2, SNU449, and SNU475; American Type Culture Collection [ATCC], Manassas, VA, USA) and the normal hepatocyte cell line MIHA (ATCC) were cultured in DMEM (for MIHA and HepG2 cell lines; Sigma-Aldrich, St. Louis, MO, USA) or RPMI 1640 (for SNU449 and SNU475 cell lines; Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS; GE Healthcare HyClone, Pittsburgh, PA, USA), 100 U/mL penicillin (GE Healthcare HyClone), and 100 mg/mL streptomycin (GE Healthcare HyClone). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2. All cell lines were tested for possible mycoplasma contamination using the BioMycX Mycoplasma PCR Detection Kit (CS-D-25; Cellsafe, Suwon, Republic of Korea) and were authenticated using short tandem repeat (STR) profiling.

GPR50 Knockdown Using shRNA Constructs
Sense and antisense oligonucleotides were synthesized for control (scramble) or GPR50 knockdown (shGPR50-1 and -2); the sequences are listed in Table S2. The oligonucleotides were then annealed and cloned into a pGreenPuro lentiviral vector (System Biosciences, Mountain View, CA, USA) containing BamHI and EcoRI restriction enzyme sites, according to the manufacturer’s instructions. Briefly, two oligonucleotides were annealed at 95°C for 2 min in a heat block with annealing buffer, and then the samples were left to cool down to room temperature. The annealed, double-stranded DNA was then ligated into a lentiviral vector using the T4 DNA ligase enzyme (Promega, Madison, WI, USA). Sequences of the newly constructed plasmids were confirmed by sequencing analysis.

Overexpression of GPR50, JAG1, DLL1, NICD, and ADAM17
For overexpression of GPR50, the pGEM-T Easy vector (Promega) was used to clone the complete GPR50 coding sequence using the primers listed in Table S2. Afterward, the complete coding sequence (without the termination codon) was further subcloned into the pCDH-EF1-MCS-T2A-copGFP lentiviral vector (System Biosciences) using XbaI and EcoRI restriction enzymes and the primers listed in Table S2.

For overexpression of JAG1, DLL1, NICD, and ADAM17, cells were incubated overnight to a cell density of 2 × 10^5 cells per well in a 24-well culture plate. The cells were then transfected with the expression vectors for hemagglutinin (HA)-JAG1, GFP-DLL1, FLAG-NICD (kind gifts from Professor Hee-Sae Park, Chonnam National University, Republic of Korea), and FLAG-ADAM17 (plasmid number 31713; Addgene, Watertown, MA, USA) using the HyliMax transfection reagent in a 1:3 ratio (Dojindo, Kumamoto, Japan), according to manufacturer’s instructions. After 48 h of transfection, the transfected cells were ready for use in further experiments.

Lentivirus Production and Transduction
To generate the lentivirus, the Rev response element (RRE)/REV lentivirus expressing system was used. Briefly, 60%–70% confluent
HEK293T cells were cultured in 100 mm dishes on the day of transfection using the calcium phosphate transfection method. The medium was replaced with fresh medium and plasmids (RRE, REV, and target), after which, the calcium phosphate mixture was added dropwise into the dishes. After 12–16 h, the medium containing the plasmids was removed, and the cells were washed once with PBS. Then, an equal amount of medium was added. After 48 h, the cell supernatant (virus soup) was collected and filtered through a 0.45-μm pore capsule and used for infection, as previously described.90 Virus titers were also quantified as previously described.91 For virus infection, we used ~8.0 × 10^6 IU/mL viral particles for stable knockdown in the indicated cells. All experiments were started 72 h postinfection.92

RNA Extraction and Quantitative RT-PCR (qRT-PCR)
Total RNA was extracted using an Easy-Blue RNA Extraction Kit (iNtRON Biotechnology, Seongnam, Republic of Korea), and the purified total RNA (2 μg) was reverse transcribed into cDNA using a cDNA synthesis kit (Promega), according to the manufacturer’s instructions. For PCR analysis, 1 μL of synthesized cDNA, specific forward and reverse primers, and Taq Plus Master Mix (Elpis Biotech, Daejeon, Republic of Korea) were mixed and analyzed by thermocycler PCR, after which, the PCR products were analyzed by agarose gel electrophoresis. DNA was stained via ethidium bromide (EtBr), observed under UV light, and imaged. The images were then analyzed in Photoshop CS6 (Adobe, Mountain View, CA, USA), and the relative expression fold changes were measured using ImageJ; the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control, as described previously.90 qRT-PCR was performed using a thermal cycler (PTC-200) with a Chromo4 optical detector (MJ Research; Bio-Rad Laboratories, Hercules, CA, USA) using Fast 2X SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA); GAPDH was used as an internal control, as described previously.90,93 The primers used are listed in Table S3.

Western Blotting
Total cell lysates were extracted from the indicated cells using lysis buffer. The cell lysates were then incubated at 4°C for 15 min, vortexed every 2–3 min, and centrifuged at 16,600 g for 15 min. Afterward, concentrations of the proteins were measured using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA), as previously described.90,93,94

Cell Proliferation and Viability Assays
For cell proliferation analysis, control (scramble-transduced), GPR50-knockdown, or GPR50-overexpressing cells (2 × 10^4 cells/well) were seeded onto 12-well plates. Cells were counted, starting from 24 h up until day 5 using a trypan blue kit. For cell viability assays, cells were seeded onto 96-well plates, and at the indicated time points, EZ-cytox WST-1 reagent (DoGen, Seoul, Republic of Korea) was added at a ratio of 1:10 and incubated for 1 h. Next, after washing with PBS, the cell viability was measured at 450 nm using a fluorescence microplate reader, as previously described.90,93–95

Wound Healing/Cell Migration Assay
For the wound-healing assay, ~90% confluent cells in 60 mm culture dishes were treated with mitomycin C (MMC; 10 μg/mL) for 3 h, after which, the cells were scratched with a 200-μL pipette tip. The indicated wound areas in the dishes were marked, and photos were taken every 12 h. The pictures were analyzed in ImageJ, and the wound closure percentage (%) was determined, as previously described.90

Sphere Formation Assay
The indicated cells (1 × 10^5 cells) were seeded onto noncoated Petri dishes with sphere-forming medium containing serum-free DMEM/F12 media with B27 supplement, 20 ng/mL EGF (Sigma-Aldrich), 10 μg/mL insulin (Sigma-Aldrich), and 1% bovine serum albumin (Sigma-Aldrich). After 6 days, colonies were gently collected into conical tubes (SPL Lifesciences, Pocheon, South Korea) and stained with crystal violet (Sigma-Aldrich). Finally, the colonies were dissociated using 0.25% trypsin-EDTA (1×; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), after which, the dissociated cells were counted and presented as the percent (%) of sphere-forming cells, as previously described.90,94

Drug-Resistance Assay
For drug-resistance analysis, 1 × 10^5 cells were seeded onto 12-well dishes and incubated overnight at 5% CO₂ and 37°C. The cells were then treated with 0.5 μM doxorubicin and incubated for another
48 h at 5% CO₂ and 37°C. After 48 h of incubation, the cells were counted and presented as the percent (%) of surviving cells, as previously described.90

**Luciferase Reporter Assay**

For the luciferase assay, the indicated cells (1 × 10⁵ cells/well) were seeded onto 12-well plates and transiently transfected with 1 µg of either HESI or HESS luciferase plasmid using HyliMax transfection reagent (1:3 ratio; Dojindo).96 The cells were harvested after 48 h post-transfection, and luciferase activity was measured using a luminometer (Veritas microplate luminometer; Turnor Biosystems, Sunnyvale, CA, USA). Luciferase activity was normalized to β-galactosidase expression levels.

**ADAM17 Activity Assay**

ADAM17 activity assay was performed using an ADAM17 Activity Assay Kit (CSB-E09315h; Cusabio Technology, Houston, TX, USA), according to the manufacturer’s instructions.97 Briefly, 1 × 10⁵ cells were harvested in 1 mL of ice-cold PBS (pH 7.2–7.4) with protease inhibitor and stored at −20°C overnight. After two freeze-thaw cycles, the cell lysates were centrifuged for 5 min at 5,000 × g at 2°C–8°C, and then the supernatant was collected and stored at −20°C until future use, after measurement of the protein concentrations in the supernatant. Approximately 15 mg of protein was mixed with substrate for 15–30 min, and the optical density (OD) of each well was determined within 5 min using a microplate reader (x-Mark spectrophotometer; Bio-Rad Laboratories).

**CoIP Assay**

To analyze protein interactions, a CoIP assay was performed with the indicated samples. Briefly, 400 µg of cell lysate was pretreated with 30 µL Protein A/G Agarose beads (Santa Cruz Biotechnology) to remove nonspecific IgG. The supernatant was then collected in new tubes and incubated overnight with 3–4 µg primary antibodies (anti-GPR50 or anti-ADAM17) and rabbit IgG on an agitator at 4°C. Subsequently, Protein A/G Agarose was added, and the mixture was incubated for an additional 3 h and spun down at 4,000 × g for 1 min. The pellets were then washed thrice with ice-cold cell lysis buffer, after which, the immunoprecipitated proteins were analyzed by western blotting, as described above.

**Statistical Analysis**

All experiments were performed three times, and the data are presented as the mean ± standard deviation (SD). For statistical analysis, an unpaired t test was performed between two groups (control versus treated), and p values <0.05 were considered statistically significant.

**Data Availability**

All data referenced in the manuscript can be downloaded from websites indicated in the Materials and Methods section.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.04.002.
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