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GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Conjugated Bile Acids Promote Invasive Growth of Esophageal Adenocarcinoma Cells and Cancer Stem Cell Expansion via Sphingosine 1-Phosphate Receptor 2—Mediated Yes-Associated Protein Activation

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Esophageal adenocarcinoma (EAC) is the sixth leading cause of cancer deaths worldwide and has been dramatically increasing in incidence over the past decade. Gastroesophageal reflux and Barrett esophagus are well-established risk factors for disease progression. Conjugated bile acids (CBAs), including taurocholate (TCA), represent the major bile acids in the gastroesophageal refluxate of advanced Barrett esophagus and EAC patients. Our previous studies suggested that CBA-induced activation of sphingosine 1-phosphate receptor 2 (S1PR2) plays a critical role in promoting cholangiocarcinoma cell invasive growth. However, the role of CBAs in EAC development and underlying mechanisms remains elusive. In the current study, we identified that the expression level of S1PR2 is correlated to invasiveness of EAC cells. TCA significantly promoted cell proliferation, migration, invasion, transformation, and cancer stem cell expansion in highly invasive EAC cells (OE-33 cells), but had less effect on the lower invasive EAC cells (OE-19 cells). Pharmacologic inhibition of S1PR2 with specific antagonist JTE-013 or knockdown of *S1PR2* expression significantly reduced TCA-induced invasive growth of OE-33 cells, whereas overexpression of *S1PR2* sensitized OE-19 cells to TCA-induced invasive growth. Furthermore, TCA-induced activation of S1PR2 was closely associated with YAP and β -catenin signaling pathways. In conclusion, CBA-induced activation of the S1PR2 signaling pathway is critically involved in invasive growth of EAC cells and represents a novel therapeutic target for EAC. (*Am J Pathol* 2018; 188: 2042–2058; <https://doi.org/10.1016/j.ajpath.2018.05.015>)

Esophageal cancers, including esophageal squamous cell carcinoma and esophageal adenocarcinoma (EAC), are the seventh most frequently diagnosed cancers and the sixth leading causes of cancer death worldwide.^{1,2} EAC comprised more than half of all esophageal cancers, and the incidence of EAC has increased more than sixfold in developed regions, including the United States, over the past decade.^{1,3} Although the treatment based on esophagectomy, with addition of chemoradiotherapy, has been shown to improve the survival rate of patients with EAC, the estimated median of the overall survival time is <1 year because of late diagnosis, inherent resistance to chemotherapy, lack of targeted therapy, metastasis, and recurrence.^{4,5}

Several risk factors of EAC, including Barrett esophagus (BE), gastroesophageal reflux diseases (GERDs), male sex,

tobacco consumption, diet, and obesity, have been established and extensively investigated.^{6,7} BE, characterized by columnar type and intestinal-like epithelial tissue metaplasia, is a premalignant condition of EAC resulting from chronic GERD.^{7,8} Patients with high dysplasia grade BE and EAC reflux more bile acids in the esophagus compared with patients with nondysplasia BE and patients with GERD, but without BE. Several studies reported that the conjugated bile acids (CBAs), including taurocholate (TCA)

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Table 1 List of Antibodies Used in This Study

Antibody	Species	Source	Catalog number	Application (dilution)
S1PR1	Rabbit	Abcam (Cambridge, UK)	Ab23695	WB (1:500)
S1PR2	Rabbit	Santa Cruz Biotechnology (Dallas, TX)	sc-25491	WB (1:200)
S1PR3	Rabbit	Santa Cruz Biotechnology	sc-30024	WB (1:200)
E-cadherin	Rabbit	Santa Cruz Biotechnology	sc-7870	WB (1:200)/IF (1:50)
Vimentin	Rabbit	Cell Signaling Technology (Danvers, MA)	mAb 5741	WB (1:1000)
CTGF	Rabbit	Sigma-Aldrich (St. Louis, MO)	Sab1300909	WB (1:500)
β-Catenin	Mouse	Santa Cruz Biotechnology	sc-7963	WB (1:500)
YAP	Rabbit	Sigma-Aldrich	Y0771	WB (1:1000)
p-YAP	Rabbit	Sigma-Aldrich	Y4645	WB (1:1000)
Lamin B	Goat	Santa Cruz Biotechnology	sc-6216	WB (1:500)
β-Actin (JLA20)	Mouse	DSHB at University of Iowa (Iowa City, IA)	JLA20	WB (1:500)

CTGF, connective tissue growth factor; DSHB, Developmental Studies Hybridoma Bank; IF, immunofluorescence; mAb, monoclonal antibody; p-YAP, phosphorylated YAP; S1PR, sphingosine 1-phosphate receptor; WB, Western blotting.

and glycocholic acid (GCA), represent the major bile acids in the esophageal refluxate of esophagitis and BE patients. However, the role of CBAs in EAC development and the potential underlying mechanisms remain elusive.^{9–11}

Sphingosine 1-phosphate receptors (S1PRs), a family of five G-protein–coupled receptors, have been implicated in the progression of various cancers.¹² It has been extensively studied and shown that S1P-mediated activation of S1PR1 and S1PR3 plays an important role in promoting cancer cell proliferation and migration.¹³ Recent studies indicated that sphingosine 1-phosphate receptor 2 (S1PR2) plays differential roles in different types of cancers because of tissue/cell-type specific expression and coupling with different G proteins.^{14–21} S1PR2 is the most predominant S1PR in cholangiocarcinoma cells.²² CBAs and S1P activate S1PR2 and further promote cholangiocarcinoma cells' invasive growth through activating extracellular signal-regulated kinase and AKT signaling pathways and inducing cyclooxygenase-2.^{22,23} However, the expression of S1PR2 in EAC and its role in EAC progression have not been studied.

Epithelial-to-mesenchymal transition (EMT), a conserved process during tissue development characterized by loss of epithelial polarity, disruption of cell-cell adhesion, and gain of migratory mesenchymal phenotypes with invasive properties, was critical for cancer metastasis initiation, chemoresistance, and expansion of cancer stem cell (CSC) population.^{24–26} Emerging evidence indicates that multiple signaling pathways, including transforming growth factor β (TGF-β)–mediated signaling through Smads, recruitment of transcription repressors Snails and histone deacetylases, and activation of Rho-A/Rho-associated protein kinase signaling, are involved in EMT and metastasis.^{27–29} Previous studies suggested that EMT, at the edges of EAC, contributed to highly local invasiveness: lymphatic metastasis at early stages.³⁰ Enrichment of CSC also promoted reoccurrence and chemoresistance of EAC.^{31,32} However, underlying mechanisms remain to be identified.

In the present study, expression levels of S1PRs were examined in normal esophageal epithelial cells and EAC cell lines, highly proliferative OE-19 and highly migratory OE-33.

Effects of CBAs, especially TCA, on EAC cell transformation, migration, invasion, EMT process, and sphere-culture–induced expansion of CSCs, were determined. The results suggested a potential correlation between high S1PR2 expression and the invasive mesenchymal phenotype of EAC cells. Furthermore, CBAs significantly promoted invasive growth of EAC cells via activation of S1PR2.

Materials and Methods

Materials

JTE-013 and S1P were purchased from Cayman Chemicals (Boston, MA). W146 and TY52156 were obtained from SelleckChem (Houston, TX). TCA, GCA, and common chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Table 2 List of Real-Time Primer Sequences Used in This Study

Target	Genbank ID*	Sequences
HPRT1	M31642	F: 5′-TATGGCGACCCGACGCCCT-3′ R: 5′-CATCTCGAGCAAGACGTTTCAG-3′
S1PR1	NM_001400	F: 5′-TCCTCGCCATCGCCATTG-3′ R: 5′-GAGAGCAGAAGCAGAG-TGAAG-3′
S1PR2	NM_004230	F: 5′-CTGCCTTCATCAGCTCTC-3′ R: 5′-GCTCTTGTCGCTGCCATAC-3′
S1PR3	BC069579	F: 5′-CTACGCACGCATCTACTTC-3′ R: 5′-CACGCTCACCACAATCACC-3′
E-cadherin	NM_004360	F: 5′-AACAAAGACAAAGAAGGCAAGG-3′ R: 5′-CAGCGTGAGAGAAGAGAGTG-3′
CD44	KR709963	F: 5′-GGAAGAAGGATGGATATG-3′ R: 5′-CAGAGTAGAAGTTGTTGGA-3′
LGR5	AF061444	F: 5′-AACACTGACTCTGAATGG-3′ R: 5′-ACTTGGAGATTAGGTAAGTTC-3′
CD133	NM_006017	F: 5′-ATAATAAGCATTTGGCATCTTC-3′ R: 5′-TTGTCCTTGCTAGTGTG-3′

*GenBank, <https://www.ncbi.nlm.nih.gov/genbank>.

F, forward; HPRT1, hypoxanthine phosphoribosyltransferase 1; ID, identification; LGR5, leucine rich repeat containing G protein–coupled receptor 5; R, reverse; S1PR, sphingosine 1-phosphate receptor.

Fetal bovine serum was purchased from Atlanta Biotechnology (Atlanta, GA). All other supplemental materials for cell culture were obtained from Gibco (Thermo Fischer Scientific, Waltham, MA). Antibodies used in this study are listed in Table 1.

Cell Lines and Cell Culture

CP-A cells (KR-42421), obtained from ATCC (ATCC-CRL-4027; Manassas, VA), were cultured in MCDB-153

medium, supplemented with hydrocortisone (0.4 $\mu\text{g/mL}$), cholera toxin (8.4 $\mu\text{g/L}$), adenine (20 mg/L), human recombinant epidermal growth factor (20 ng/mL), $1\times$ Insulin-Transferrin-Selenium supplement (Sigma Aldrich), and 4 mmol/L L-glutamine. Het-1A cells, obtained from ATCC (ATCC-CRL-2692), were cultured in bronchial epithelial cell growth medium (Lonza, Allendale, NJ). OE-19 and OE-33 cells were obtained from Sigma Aldrich and were cultured in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum.

Figure 1 Differential expression of sphingosine 1-phosphate receptors (S1PRs) in esophageal adenocarcinoma (EAC) cells. **A:** The relative mRNA levels of *S1PR1*, *S1PR2*, and *S1PR3* in Het-1A, CP-A, OE-19, and OE-33 cells. **B:** Representative images of immunoblots and relative protein levels of *S1PR1*, *S1PR2*, and *S1PR3* in Het-1A, CP-A, OE-19, and OE-33 cells. Effects of conjugated bile acids and S1PRs on EAC cell proliferation. **C** and **D:** OE-19 and OE-33 cells were treated with different doses of S1PR2 antagonists W146, JTE-013, and TY52156 for 24 hours. **E** and **F:** OE-19 and OE-33 cells were treated with 100 $\mu\text{mol/L}$ taurocholate (TCA), 100 $\mu\text{mol/L}$ glycolic acid (GCA), and 200 nmol/L S1P, with or without 5 $\mu\text{mol/L}$ JTE-013. Relative cell numbers, compared with control (Con) group, are shown. Data are expressed as means $\pm$ SEM (**A–F**). $n = 4$ (**A** and **B**); $n = 8$ (**C–F**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus S1PR1; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$ versus groups without JTE-013 treatment.

RNA Isolation and Real-Time PCR

Total RNA was isolated using TRIzol reagent (Qiagen, Valencia, VA) and reverse transcribed into first-strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA), according to the manufacturer's instruction. The mRNA levels of *S1PRs*, E-cadherin, *CD44*, *LGR5*, and *CD133* were determined by real-time RT-PCR using iQTM SYBR Green Supermix reagents (Bio-Rad, Hercules, CA) and normalized using *HPRT1* as an internal control, as described previously.³³ Primer sequences of real-time PCR are listed in Table 2.

Western Blot Analysis

Total cellular, nuclear, and cytosolic proteins were isolated, as previously described.³⁴ Western blot analysis was performed

using specific primary antibodies and Bio-Rad horseradish peroxidase-conjugated secondary antibodies, as described previously.^{22,23} Immunoreactive bands were detected using electrochemiluminescence reagents (Thermo Scientific, Wilmington, DE) and the Bio-Rad ChemiDoc XRS imaging system. The density of immunoblots was analyzed using Image Lab software version 6.0.0 build 25 (Bio-Rad).

Cell Counting Kit-8 Assay

Cells were cultured in 96-well plates in serum-free medium and treated with CBAs or S1P with or without specific antagonists of individual S1PRs. Cell viability was determined using Cell Counting Kit-8 from Dojindo Molecular Technologies, Inc. (Rockville, MD). Absorbance at 450 nm was obtained using Victor³ Multilabel Plate Counter (Perkin Elmer, Waltham, MA).

Figure 2 Sphingosine 1-phosphate (S1P) and taurocholate (TCA) promote anchorage-independent growth of esophageal adenocarcinoma cells. **A:** OE-19 and OE-33 cells were cultured in soft agar for 15 days. Representative images and magnified images (**insets**) for colonies at day 15 (D15) are shown. **B:** The numbers and size distribution of OE-19— and OE-33—transformed colonies were counted and analyzed. **C–F:** OE-19 (**C** and **E**) and OE-33 (**D** and **F**) cells were cultured in soft agar and treated with 200 nmol/L S1P and 100 μ mol/L TCA, with or without 5 μ mol/L JTE-013. Representative images, magnified views of a colony, and the number and size distribution of colonies are shown. Data are expressed as means $\pm$ SEM (**B**, **E**, and **F**). $n = 3$ (**B**, **E**, and **F**). $***P < 0.001$ versus OE-19 cells; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus control; $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$, and $^{\ddagger\ddagger\ddagger}P < 0.001$ versus counterpart groups without JTE-013 treatment. Original magnifications: $\times 4$ (**A**, **left** and **middle** columns); $\times 20$ (**A**, **right** column). D5, day 5.

Soft Agar Colony Formation Assay

Warm 0.6% agarose in complete culture medium was plated into 12-well cell culture plates and solidified on ice as the bottom layer. Cells (1×10^5) were resuspended in complete culture medium, mixed with the same volume of 0.6% agarose, and plated on the top of the bottom layer. After solidification of the top layer, complete medium was added into each well as the feeding layer. After 24 hours, cells were treated with 100 $\mu\text{mol/L}$ TCA or 100 nmol/L S1P, with or without 5 $\mu\text{mol/L}$ JTE-013, and the feeding layer was replaced every 2 days. The colony formation was monitored every day under a microscope. Images of the formed colonies were obtained on day 5 and day 15 after

treatment. The number and size distribution of the formed colonies were analyzed using IPLab 4.0 imaging software (Scanalytics, Inc., Rockville, MD).

Wound Healing Assay

Cells were cultured to form a confluent monolayer. After starvation, the cell monolayer was scratched to mimic a wound and then imaged (0 hour) using an Olympus 1X71 microscope (Olympus, Center Valley, PA). Cells were then treated with 100 $\mu\text{mol/L}$ TCA or 100 nmol/L S1P, with or without 5 $\mu\text{mol/L}$ JTE-013. After 8 or 24 hours, the wound area was imaged and quantified using IPLab 4.0 imaging software, as described previously.²²

Figure 3 Sphingosine 1-phosphate (S1P) and taurocholate (TCA) promote migration of esophageal adenocarcinoma cells. Wound healing assays were performed on OE-19 and OE-33 cells. **A:** Representative images of 0-, 10-, and 24-hour (h) time points after scratch are shown. Wound area healing over time was analyzed. **B and C:** Wound healing assays were performed in OE-19 (**B**) and OE-33 (**C**) cells. The cells were then treated with 200 nmol/L S1P and 100 $\mu\text{mol/L}$ TCA, with or without 5 $\mu\text{mol/L}$ S1P receptor 2 antagonist JTE-013, for 24 hours (OE-19 cells) or 10 hours (OE-33 cells). Representative images and analyzed wound closure areas are shown. **Black solid lines** and **black dotted lines** indicate wounded area at 0 hour; **white dotted lines** indicate wounded area at the end of treatment. Data are expressed as means $\pm$ SEM (**A** and **B**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus OE-19 or control; † $P < 0.05$, †† $P < 0.001$ versus groups without JTE-013 treatment.

Cell Invasion Assay

Cells were seeded in the upper chamber of a BD Biocoat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA) and cultured in serum-free medium. Complete medium containing 10% fetal bovine serum was used as a chemo-attractant. After 4 hours of incubation, OE-19 cells were then treated with 100 $\mu\text{mol/L}$ TCA or 100 nmol/L S1P, with or without 5 $\mu\text{mol/L}$ JTE-013 for 48 hours, whereas OE-33 cells were treated for 24 hours. At the end of treatment, noninvasive cells were removed from the top surface of the upper chamber. Invasive cells were fixed with 3.7% para-formaldehyde and stained with 0.1% crystal violet. Stained cells were counted and analyzed, as described previously.²²

Sphere-Culture of Cells

For primary sphere formation, OE-19 and OE-33 cells were resuspended in Dulbecco's modified Eagle's medium/F12 medium (1:1), with 1 $\times$ B27 supplement, 1% penicillin-streptomycin, 20 ng/mL epidermal growth factor, and 10 ng/mL fibroblast growth factor. Then, they were plated in nontreated, low adhesion, 24-well plates, with a density of 5×10^4 cells/well. Cells were treated 4 hours after plating. Images of formed spheres were taken on day 5. The number and size of spheres were analyzed using IPLab 4.0 imaging software. Total RNA from four identical wells was isolated for further study.

TGF- β –Induced EMT

Cells were cultured in low serum medium (3% fetal bovine serum) and treated with vehicle or 3 ng/mL TGF- β alone or in combination with 100 $\mu\text{mol/L}$ TCA and 5 $\mu\text{mol/L}$ JTE-013 for 72 hours. At the end of the treatment, cells were imaged for morphologic assessment, and then harvested for total protein isolation.

Immunofluorescence Staining

Cells were plated on coverslips and cultured overnight. After treatment with TCA, TGF- β , or both, with or without JTE-013 for 24 hours, cells were fixed, permeabilized, and blocked with 2.5% normal goat serum, as previously described.³⁵ Cells were then incubated with mouse anti-E-cadherin primary antibody (1:100 dilution) overnight at 4°C, followed by incubation of Alexa Fluor 488 conjugated goat anti-mouse secondary antibody (Life Technologies) for 45 minutes. Nuclei were counterstained with DAPI. Images were recorded using a Zeiss Axio Scope A1 microscope (Carl Zeiss, Thornwood, NY).

Knockdown and Overexpression of S1PR2

Cells were cultured overnight to 70% confluence. OE-33 cells were transiently transfected with scrambled control shRNA or shRNA specifically targeting human *S1PR2*. OE-19 cells were transiently transfected with pcDNA3.1

Figure 4 Sphingosine 1-phosphate (S1P) and taurocholate (TCA) promote invasion of esophageal adenocarcinoma cells. OE-19 and OE-33 cells were plated in upper chambers of Matrigel-coated transwells. **A:** Representative images of invading cells, 12, 24, and 48 hours (h) after seeding, are shown. Invasion index was calculated as described in *Materials and Methods*. **B** and **C:** Twelve hours after plating, OE-19 (**B**) and OE-33 (**C**) cells were then treated with 200 nmol/L S1P and 100 $\mu\text{mol/L}$ TCA, with or without 5 $\mu\text{mol/L}$ S1P receptor 2 antagonist JTE-013, for 48 and 24 hours, respectively. Representative images of invading cells and the invasion index are shown. Data are expressed as means $\pm$ SEM (**A–C**). $n = 3$ (**A–C**). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus OE-19 or control; $^\dagger P < 0.01$, $^\dagger\dagger P < 0.001$ versus counterpart groups without JTE-013 treatment.

plasmid (control plasmid) or pcDNA3.1-3xHA-tagged S1PR2 plasmid. PolyJet DNA In Vitro Transfection Reagent (SignaGen Laboratories, Rockville, MD) was used. Cells were harvested or subcultured after 24 hours for further experiments. Western blot was used to detect knockdown or overexpression efficiency.

Statistical Analysis

All results represent at least three independent experiments and are expressed as means $\pm$ SD. One-way analysis of variance was used to analyze the differences among different sets of data using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). $P \leq 0.05$ was considered statistically significant.

Results

Differential Expression of S1PRs in Normal Esophageal Epithelial Cells, BE, and EAC Cells

Emerging evidence indicates that S1PR-mediated signaling pathways play critical roles in regulating cancer cell growth and metastasis. To determine whether S1PRs are involved in EAC cell growth, the expression levels of individual S1PRs were first examined in normal esophageal epithelial cells

(Het-1A), Barrett esophageal cell line (CP-A), and EAC cell lines (OE-19 and OE-33). S1PR1, S1PR2, and S1PR3 were expressed in all tested cells, but S1PR4 and S1PR5 were too low to be detected. The relative expression levels of S1PR1-3 were different in different cells. S1PR3 was the predominant S1PR in Het-1A cells (Figure 1, A and B). S1PR1 was predominantly expressed in CP-A cells. Interestingly, S1PR2 was the predominant S1PR only in OE-33 cells. Compared with other cells, OE-33 expressed the highest level of S1PR2 (Figure 1B).

CBAs and S1P Promote EAC Cell Proliferation through S1PR2

To determine the role of individual S1PRs in EAC cell growth, specific chemical antagonists of S1PR1-3, W146, JTE-013, and TY52156 were used to treat OE-19 and OE-33 cells for 24 hours, and the relative variable cell number was measured using Cell Counting Kit-8 assay. TY52256, an S1PR3 antagonist, inhibited proliferation of OE-19 cells at a relatively high dose (10 to 20 $\mu\text{mol/L}$) (Figure 1C). However, in OE-33 cells, JTE-013, an S1PR2 antagonist, significantly and dose dependently inhibited cell proliferation (Figure 1D). W146, an S1PR1 antagonist, had no effect on cell growth in both OE-19 and OE-33 cells. These results suggest that S1PR2 is critically involved in

Figure 5 Effects of sphingosine 1-phosphate receptor 2 (S1PR2) overexpression and knockdown on taurocholate (TCA)-induced invasion in OE-19 and OE-33 cells. **A:** Protein levels of S1PR2 in normal OE-19 cells (NT) and OE-19 cells transfected with control pcDNA (Ct pcDNA) or pcDNA-S1PR2 were determined by Western blot. **B:** Protein levels of S1PR2 in normal OE-33 cells (NT) and OE-33 cells transfected with control shRNA (Ct shRNA) and S1PR2 shRNA were determined by Western blot. Representative immunoblot images are shown. Relative S1PR2 levels are normalized using β -actin as the loading control. **C:** OE-19 and OE-33 cells were plated in upper chambers of Matrigel-coated transwells. The cells were then treated with vehicle control or 100 $\mu\text{mol/L}$ TCA for 48 hours (OE-19 cells) or 24 hours (OE-33 cells). Data are expressed as means $\pm$ SEM (A–C). $n = 3$ (A–C). * $P < 0.05$, *** $P < 0.001$ versus the Ct pcDNA or Ct shRNA group; †† $P < 0.01$ versus NT; ‡‡ $P < 0.01$ versus S1PR2 pcDNA control; §§§ $P < 0.001$ versus Ct pcDNA TCA or Ct shRNA TCA.

cell proliferation in OE-33 cells. Activation of S1PR2 by CBAs promotes cholangiocarcinoma invasive growth by induction of cyclooxygenase-2 and activation of epidermal growth factor receptor—extracellular signal-regulated kinase—AKT pathways.^{22,23} To determine the effect of CBA- and S1P-induced activation of S1PR2 on EAC cell growth, both OE-19 and OE-33 cells were treated with TCA, GCA, or S1P in the absence or presence of JTE-013 for 24 hours (Figure 1E). TCA and GCA, but not S1P, slightly induced proliferation of OE-19. However, TCA, GCA, and S1P had a more profound effect on promoting OE-33 cell proliferation (Figure 1F). Cell proliferation was significantly induced by TCA, GCA, and S1P by approximately 1.5-fold (Figure 1F). As expected, JTE-013 significantly inhibited TCA-, GCA-, and S1P-induced proliferation of both OE-19 and OE-33 cells.

CBA- and S1P-Induced Activation of S1PR2 Enhances Malignant Transformation of EAC Cells

Anchorage-independent growth (or colony-forming capacity in semisolid media) is the most important marker of malignant transformation of tumor cells and is connected with *in vivo* tumorigenic and metastatic potentials.³⁶ OE-33 cells formed more and larger colonies than OE-19 after 5 days of soft agar culture (Figure 2, A and B). On day 15, both OE-19 and OE-33 cells formed similar numbers of colonies, but the colony size in OE-33 cells was significantly larger than that in OE-19 cells. The number and size of OE-19—formed colonies were only induced by S1P for 40% and 20%, respectively (Figure 2, C and E). TCA had no effect on anchorage-independent growth of OE-19 cells. However, both S1P and TCA significantly enhanced malignant transformation of OE-33 cells, indicated by a 70% to 90% increase of the number of colonies and an approximately 60% increase of the colony size, which was completely inhibited by S1PR2 antagonist JTE-013 (Figure 2, D and F).

CBA- and S1P-Induced Activation of S1PR2 Enhances EAC Cell Migration and Invasion

To determine the effect of TCA and S1P on cell motilities and whether S1PR2 is involved in this process, wound healing and Matrigel invasion assays were performed. The wounded area was healed approximately 7% and 20% in an OE-19 monolayer over 10 and 24 hours, respectively, whereas the wounded area was healed 20% and 80% in OE-33 monolayer over the same time periods, respectively (Figure 3A). Both TCA and S1P significantly increased wound healing in OE-19 and OE-33 monolayers by approximately twofold (Figure 3, B and C), which was completely abrogated by JTE-013. In addition, JTE-013 alone significantly inhibited OE-33 cell migration by 50%. Furthermore, OE-33 cells were more invasive compared with OE-19 cells, indicated by a significantly higher

invasion index at all time points (Figure 4A). In addition, both S1P and TCA had minimal effects on OE-19 invasion, but OE-33 cell invasion was significantly promoted after 24 hours of treatment with S1P or TCA by 1.8-fold and >2-fold, respectively, which was completely inhibited by JTE-013 (Figure 4, B and C).

To further investigate the potential role of S1PR2 in EAC invasion, S1PR2 was overexpressed in OE-19 cells using pcDNA3-hS1PR2 plasmid and down-regulated in OE-33 cells using specific shRNA that targeted human *S1PR2* (Figure 5, A and B). Overexpression of S1PR2 not only promoted the basal invasiveness of OE-19 cells by almost twofold, but also sensitized cells to TCA-induced invasion. In contrast, down-regulation of S1PR2 in OE-33 cells significantly inhibited invasive growth of OE-33 cells and completely abrogated TCA-enhanced invasion (Figure 5C).

CBA-Induced Activation of S1PR2 Potentiates TGF- β —Induced EMT in EAC Cells

It is well documented that EMT is a key mechanism underlying cancer metastasis, which allows cancer cells to dedifferentiate and acquire migratory and invasive properties. Tumor-infiltrating immune cell—derived inflammatory cytokines, such as TGF- β , are potent inducers of EMT.³⁷ To determine the role of CBA- and S1P-induced activation of S1PR2 in TGF- β —induced EMT in EAC cells, the basal expression levels of E-cadherin, a marker of epithelial phenotype, were first examined in OE-19 and OE-33 cells. Both mRNA and protein levels of E-cadherin were significantly higher in OE-19 cells than those in OE-33 cells, which was further confirmed by immunofluorescence staining of E-cadherin (Figure 6, A—C). OE-19 cells had strong and integrated cell-cell adhesion, but not OE-33 cells. In addition, OE-33 cells, but not OE-19 cells, were morphologically presenting mesenchymal phenotype (Figure 6C). Furthermore, TGF- β significantly triggered EMT in both OE-19 and OE-33 cells, indicated by morphologic changes and loss of E-cadherin expression and cell-cell adhesion (Figure 6D). Western blot analysis further showed that the protein level of E-cadherin was decreased, but protein levels of vimentin and connective tissue growth factor were increased, in TGF- β —challenged OE-19 and OE-33 cells. In addition, TGF- β induced nuclear translocation of β -catenin (Figure 6E). However, TCA had no effect on TGF- β —induced EMT in OE-19 cells. In contrast, in OE-33 cells, TCA alone slightly suppressed E-cadherin expression and induced connective tissue growth factor, but significantly potentiated TGF- β —induced EMT, indicated by a remarkable decrease of E-cadherin and increase of vimentin and connective tissue growth factor as well as nuclear translocation of β -catenin. The effect of TCA on TGF- β —induced EMT activation was inhibited by JTE-013 in OE-33 cells (Figure 6, D and E).

S1PR2 Activation Promotes the Enrichment of CSCs in Sphere-Cultured EAC Cells

CSCs are a small subpopulation of cancer cells capable of self-renewal, differentiation, and tumorigenesis, which drive cancer cell survival and metastasis. CSCs are the major cause of tumor formation and recurrence. Nonadherent tumor spheres from different cancer cell lines cultured in stem cell conditioned medium have been established as valuable *in vitro* CSC models, which possess CSC properties.³⁸ In the current study, sphere cultures of OE-19 and OE-33 cells were used to examine the effect of bile acid–induced activation of S1PR2 on CSCs. The OE-33 cells formed more spheres compared with OE-19 cells after a 5-day culture in stem cell conditioned media (Figure 7, A and B). The size of OE-33–formed spheres was much bigger than that of OE-19–formed spheres. Both S1P and TCA induced sphere formation in OE-19 and OE-33 cells, but had a more profound effect on OE-33 cells, indicated by a larger number and the larger size of the spheres (Figure 7, C and D). In addition, inhibition of S1PR2 activation using JTE-013 completely abolished S1P- and TCA-induced sphere formation in both OE-19 and OE-33 cells. In addition, as expected, overexpression of S1PR2 in OE-19 cells increased TCA-induced sphere formation and down-regulation of S1PR2 and not only significantly inhibited sphere formation, but also abolished the effect of TCA on promoting sphere formation and growth in OE-33 cells (Figure 7E).

To confirm that the spheres formed in OE-19 and OE-33 cells possess stem cell properties, total RNA was isolated from both monolayer cultured EAC cells and EAC-formed spheres. The mRNA expression levels of commonly used CSC markers, including *CD44*, *CD133*, and *LGR5*, were detected by real-time RT-PCR. The CSC marker genes were differentially expressed in monolayer cultured OE-19 and OE-33 cells. *CD44*, a potential marker for CSC with mesenchymal phenotype, was highly expressed in OE-33 cells, which is consistent with the higher motility of OE-33 cells (Figure 8A). *LGR5* was moderately expressed in OE-19 cells, but not in OE-33 cells, whereas *CD133* was nondetectable in both OE-19 and OE-33 cells. Although mRNA expression levels of *CD44* and *LGR5* were not affected by S1P and TCA treatment, JTE-013 significantly inhibited the basal expression levels of *CD44* and *LGR5* in both monolayer cultured OE-19 and OE-33 cells (Figure 8B). Sphere culture significantly enriched the CSC

subpopulation in EAC cells, with a more profound impact on OE-33 cells compared with OE-19 cells (Figure 8, C and D). Interestingly, both TCA and S1P markedly induced *CD44* and *LGR5* expression via activation of S1PR2 in sphere-cultured OE-33 cells (Figure 8C). However, S1P and TCA had no significant effect on *CD44* and *LGR5* expression in sphere-cultured OE-19 cells (Figure 8D). Inhibitory effects of JTE-013 on S1P- and TCA-induced up-regulation of *CD44* mRNA and protein levels in sphere-cultured OE-33 cells were confirmed by real-time PCR (Figure 8C) and Western blot analysis (Figure 8E).

TCA and S1P Activate YAP Signaling Pathways via S1PR2

The Hippo-Yap signaling pathway plays a critical role in tumorigenesis.³⁹ Previous study identified a potential role of S1PR2 in YAP activation.⁴⁰ Activation of YAP is also linked to activation of β -catenin. Because S1PR2 is highly expressed in OE-33 cells, it was further examined if TCA- and S1P-induced activation of S1PR2 had any effect on YAP activation. OE-33 cells were treated with S1P or TCA for different periods; nuclear, cytosolic, and total proteins were isolated. The protein levels of YAP, phosphorylated YAP, and β -catenin were determined by Western blot analysis. S1P and TCA decreased YAP phosphorylation and induced YAP nuclear translocation, which were accompanied by nuclear translocation of β -catenin (Figure 9A). In addition, TCA-induced activation of YAP and β -catenin was dose dependent (Figure 9B). Inhibition of S1PR2 activation by JTE-013 abrogated S1P- and TCA-induced YAP activation and nuclear translocation (Figure 9C). To further delineate the role of S1PR2 in YAP and β -catenin activation, the effect of overexpression of S1PR2 was examined on YAP and β -catenin activation in OE-19 cells. TCA significantly induced YAP stabilization and YAP and β -catenin nuclear translocation in OE-19 cells overexpressing S1PR2 (Figure 10A). In contrast, knockdown of S1PR2 using shRNA in OE-33 cells completely abolished TCA-mediated activation of YAP and β -catenin (Figure 10B).

Discussion

Accumulating evidence indicates that GERD is an important contributor in BE and EAC pathogenesis and

Figure 6 Sphingosine 1-phosphate receptor 2 (S1PR2) activation promotes transforming growth factor (TGF)- β –induced epithelial-to-mesenchymal transition in esophageal adenocarcinoma cells. **A:** Relative mRNA levels of E-cadherin in OE-19 and OE-33 cells. **B:** Representative images and relative protein levels of E-cadherin in OE-19 and OE-33 cells. **C:** Representative bright-field images and immunofluorescence images against E-cadherin of OE-19 and OE-33 cells. **D:** OE-19 and OE-33 cells were treated with 2.5 μ g/mL TGF- β , 100 μ mol/L taurocholate (TCA), or both for 72 hours. Representative bright-field images and immunofluorescence images of E-cadherin are shown. **E:** Protein levels of E-cadherin and vimentin in total lysate and β -catenin in nuclear and cytosolic fractions in OE-19 and OE-33 cells were determined by Western blots. Relative protein levels were analyzed using Lamin B as loading control for nuclear fraction and β -actin as loading control for cytosolic fraction and total lysate. Data are expressed as means $\pm$ SEM (**A**, **B**, and **E**). ** P < 0.01 versus OE-19; * P < 0.05, † P < 0.01 versus control; ‡ P < 0.05 versus no JTE-013 (S1PR2 antagonist); § P < 0.05 versus TGF- β . CTGF, connective tissue growth factor; E-Cad, E-cadherin.

Figure 7 Sphingosine 1-phosphate (S1P) and taurocholate (TCA) promote growth of sphere-cultured esophageal adenocarcinoma cells. **A:** Representative images and magnified views of sphere-cultured OE-19 and OE-33 cells. **B:** The number and size distribution of OE-19 and OE-33 cell-derived spheres are shown. **C and D:** Sphere-cultured OE-19 (**C**) and OE-33 (**D**) cells were treated with 200 nmol/L S1P and 100 μ mol/L TCA, with or without 5 μ mol/L S1P receptor 2 (S1PR2) antagonist JTE-013, for 5 days. Representative images and the number of OE-19 and OE-33 cell-derived spheres are shown. **E:** OE-19 cells transfected with control pcDNA (Ct pcDNA) or S1PR2 pcDNA and OE-33 cells transfected with Ct shRNA or S1PR2 shRNA were sphere cultured for 5 days, with or without 100 μ mol/L TCA treatment. Representative images and the number of spheres are shown. Data are expressed as means $\pm$ SEM (**B–E**). $n = 3$ (**B–E**). * $P < 0.05$, ** $P < 0.01$ versus OE-19 or control; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus counterpart groups without JTE-013; ‡ $P < 0.05$ (versus Ct pcDNA control or Ct shRNA control); § $P < 0.05$ versus S1PR2 pcDNA control; ¶ $P < 0.05$, ¶¶ $P < 0.001$ versus Ct pcDNA TCA or Ct shRNA TCA.

development.^{41–44} Conjugated primary bile acids, including TCA and GCA, but not unconjugated bile acids and secondary bile acids, were found to be predominant bile acids in GERD refluxate in BE patients, as well as in the rat duodenal reflux EAC model.^{45,46} The effect of secondary

bile acids, such as deoxycholic and lithocholic acids, on carcinogenesis of EAC has been extensively studied.⁴⁷ However, the effect of CBAs on EAC progression, which has more pathological relevance, has not been well studied. CBAs promote cholangiocarcinoma cell invasive growth

Figure 8 Sphingosine 1-phosphate (S1P) and taurocholate (TCA) promote enrichment of cancer stem cell-like cells in sphere-cultured esophageal adenocarcinoma (EAC) cells. **A:** Relative mRNA levels of *CD44*, *CD133*, and *LGR5* in monolayer (Mono)-cultured OE-19 and OE-33 cells. **B:** The monolayer-cultured OE-19 and OE-33 cells were treated with 200 nmol/L S1P and 100 μ mol/L TCA, with or without 5 μ mol/L S1P receptor 2 antagonist JTE-013, for 2 days, and relative mRNA levels of *CD44* in OE-33 cells and *LGR5* in OE-19 cells were determined using RT-PCR. OE-33 and OE-19 cells were sphere cultured and treated with 200 nmol/L S1P and 100 μ mol/L TCA, with or without 5 μ mol/L JTE-013, for 5 days. **C** and **D:** Relative mRNA levels of *CD44* and *LGR5* in OE-33 (**C**) and OE-19 (**D**) cells were determined using RT-PCR. **E:** Relative protein levels of CD44 in OE-33 cell total lysate were detected by Western blot and analyzed using β -actin as loading control. Representative immunoblot images are shown. Data are expressed as means $\pm$ SEM (**A–E**). $^{***}P < 0.01$, $^{***}P < 0.001$ versus control or monolayer-cultured EAC cells; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus sphere-cultured EAC cell control; $^{\ddagger}P < 0.05$, $^{\ddagger\dagger}P < 0.01$, and $^{\ddagger\dagger\dagger}P < 0.001$ versus sphere-cultured EAC cell counterpart without JTE-013. HPRT1, hypoxanthine phosphoribosyltransferase 1; LGR5, leucine rich repeat containing G protein-coupled receptor 5; ND, nondetectable.

through activation of S1PR2.²² In the current study, S1PR2 was found to be highly expressed in a poorly differentiated mesenchymal-like EAC cell line, OE-33, but was only moderately expressed in OE-19 cells, a moderately differentiated epithelial-like cell line. TCA significantly enhanced invasive growth of both cell lines, facilitated TGF- β -induced EMT, and induced expansion of CD44-expressing CSCs in spherical culture in S1PR2-expressing OE-33 cells. OE-33 cells express much higher S1PR2, lower E-cadherin, and higher CD44 levels and are more migratory and invasive as well as more sensitive to TCA-

induced stimulation when compared with OE-19 cells. Overexpression of S1PR2 enhanced the response of OE-19 cells to TCA, whereas shRNA-mediated *S1PR2* knockdown inhibited TCA-induced promotion of cell proliferation and invasion in OE-33 cells. These results suggested that CBAs play a vital role in EAC invasive growth, metastasis, and CSC expansion via activation of S1PR2.

S1P/S1PR-mediated signaling has been linked to various diseases. S1PRs are differentially expressed in different types of cells. Individual S1PRs exert their diverse cellular functions via coupling to different G proteins that activate

Figure 9 Effects of sphingosine 1-phosphate (S1P) and taurocholate (TCA) on YAP and β-catenin activation. Representative immunoblot images of YAP and β-catenin in the nuclear fraction, cytosolic fraction, and total lysate are shown. **A:** OE-33 cells were treated with S1P or TCA for 1, 2, 4, or 8 hours (h). **B:** OE-33 cells were treated with different doses of TCA for 2 hours. **C:** OE-33 cells were treated with 100 μmol/L TCA, with or without 5 μmol/L S1P receptor 2 antagonist JTE-013, for 2 hours. Relative protein levels were analyzed using Lamin B as loading control for nuclear fraction and β-actin as loading control for cytosolic fraction and total lysate. Data are expressed as means ± SEM (A–C). $n = 3$ (A–C). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus OE-19 or Ct or 0-hour; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ versus counterpart groups without JTE-013. Ct, control; p-YAP, phosphorylated YAP.

different downstream signaling pathways, such as extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, AKT, phospholipase C, and c-Jun N-terminal kinase.⁴⁸ The contribution of S1PR1 and S1PR3 to cancer cell proliferation, migration, and invasion has been extensively studied in different types of cancers.⁴⁹ However, the role of S1PR2 in cancer remains unclear. Identification of conjugated bile acids as natural activators of S1PR2 opened up a new direction for both the sphingosine lipid and bile acid research fields.^{22,23}

EAC is well known for its local invasive growth to submucosa and adjacent tissues, and lymphatic metastasis at early stages.⁶ Loss of E-cadherin and further activation of EMT at the invasion margin are closely associated with lymph node/distant metastasis in esophageal cancers.^{30,31} In line with these findings, OE-33 cells, which have low E-cadherin expression and mesenchymal-like morphology, are highly invasive when compared with OE-19 cells. TCA also potentiated TGF- β -induced EMT through activation of S1PR2 in OE-33 cells. Previous studies demonstrated that TGF- β triggers Snail and zinc finger E-box-binding homeobox signaling to transcriptionally regulate E-cadherin repression and EMT marker expression. Activation of Snail and ZEB signaling also cooperated with Ras-mitogen-activated protein kinase, phosphatidylinositol 3-kinase/AKT, and Wnt- β -catenin pathways in epidermal growth factor receptor, hepatocyte growth factor receptor, fibroblast growth factor receptor, and other signaling-induced EMTs.^{27,28} Our study suggests that activation of S1PR2 by TCA may facilitate TGF- β -induced EMT through interaction with Snail/ZEB signaling pathways.

In addition, the current study also demonstrated that TCA-induced activation of S1PR2 is also linked to the YAP signaling pathway in EAC cells. Consistent with our findings, previous studies reported that S1PR2 induced YAP nuclear translocation through G12/13-mediated Rho A-RhoA/Rho-associated protein kinase activation and is independent of YAP upstream kinases.⁴⁰ YAP activation rescued cytoplasmic β -catenin from a repressive complex and results in nuclear translocation of β -catenin. Nuclear β -catenin plays a critical role in regulating the transcription of target genes involved in cell proliferation, migration, and EMT process and eventually leads to the generation of CSCs.⁵⁰⁻⁵²

Figure 10 Effects of sphingosine 1-phosphate (S1P) and taurocholate (TCA) on YAP and β -catenin activation. OE-19 cells transfected with control pcDNA (Ct pcDNA) or pcDNA-S1P receptor 2 (S1PR2; **A**) and OE-33 cells transfected with Ct shRNA or S1PR2 shRNA (**B**) were treated with vehicle control or 100 μ mol/L TCA for 2 hours. Representative immunoblot images of YAP and β -catenin in nuclear fraction, cytosolic fraction, and total lysate are shown. Relative protein levels were analyzed using Lamin B as a loading control for nuclear fraction and β -actin as a loading control for cytosolic fraction and total lysate. Data are expressed as means $\pm$ SEM (**A** and **B**). $n = 3$ (**A** and **B**). * $P < 0.05$, ** $P < 0.01$ versus Ct pcDNA or Ct shRNA; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ versus S1PR2 pcDNA control or S1PR2 shRNA control; $^{\S}P < 0.05$, $^{\#}P < 0.01$ versus Ct pcDNA TCA or Ct shRNA TCA. p-YAP, phosphorylated YAP.

Recent studies suggested that high tumor initiating capacity of CSCs contributed to recurrence, metastasis, and chemoresistance of esophageal cancers. CD44 and leucine rich repeat containing G protein-coupled receptor 5 (LGR5) were recently identified as markers of CSCs in esophageal cancers.^{5,53,54} Our results indicate that *CD44* is expressed in OE-19 and OE-33 cells, but *LGR5* is only detected in OE-19 cells (Figure 8A). However, both *CD44* and *LGR5* were significantly up-regulated in sphere-cultured OE-19 and OE-33 cells (Figure 8C). CD44 is not only a marker of CSCs, but also plays a critical role in controlling CSC stemness.⁵⁵ Several studies demonstrated that up-regulation of CD44 leads to mesenchymal phenotype of CSCs, enhances tumorigenicity, and elevates metastasis incidence in many cancers, including esophageal cancer.^{56,57} Although tremendous effort has been put in to identify the potential mechanisms involved in regulating CD44 expression and function, the current understanding of transcriptional regulation of *CD44* expression is still limited.^{55,58,59} In the current study, it was identified that TCA- and S1P-mediated activation of S1PR2 plays an important role in regulation of CD44 expression and YAP activation in sphere-cultured OE-19 and OE-33 cells, which is further supported by recent findings that the Hippo-YAP pathway abrogation reduces CD44 expression in intestinal stem cells and neural crest cells.^{60,61} A recent study also reported that aberrant expression and nuclear localization of YAP were observed in EAC cells, but not BE cells.⁵⁰ However, further studies are required to investigate the underlying mechanisms by which TCA-/S1P-induced activation of S1PR2/YAP signaling regulates CD44 expression.

Chronic inflammation is another important player in tumor progression. Numerous studies have reported that, in addition to cytokines and chemokines, lipid mediators, especially sphingolipids, also greatly contribute to inflammation.⁶² Several studies reported that activation of S1PR2 is associated with vascular inflammation.^{63–65} Also, S1PR2 is involved in regulating hepatic inflammation under cholestatic conditions.^{34,66}

In summary, the combination of CBAs in gastroesophageal refluxate and an inflammatory microenvironment contributes to the mesenchymal-like phenotype of high S1PR2-expressing EAC cells. CBAs and S1P promote invasive growth and metastasis of EAC via activation of S1PR2, which further activates YAP and β -catenin and induces CSC formation. The current study not only provides new insights into the pathogenesis of EAC and the role of CBAs in disease progression, but also strongly suggests that targeting CBA-S1PR2 signaling pathways represents a novel therapeutic strategy for the treatment of EAC.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.05.015>.

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