Deoxycholic acid activates epidermal growth factor receptor and promotes intestinal carcinogenesis by ADAM17-dependent ligand release

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Abstract
High fat diet is implicated in the elevated deoxycholic acid (DCA) in the intestine and correlated with increased colon cancer risk. However, the potential mechanisms of intestinal carcinogenesis by DCA remain unclarified. Here, we investigated the carcinogenic effects and mechanisms of DCA using the intestinal tumour cells and Apc

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min/+ mice model. We found that DCA could activate epidermal growth factor receptor (EGFR) and promote the release of EGFR ligand amphiregulin (AREG), but not HB-EGF or TGF-

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α in intestinal tumour cells. Moreover, ADAM-17 was required in DCA-induced promotion of shedding of AREG and activation of EGFR/Akt signalling pathway. DCA significantly increased the multiplicity of intestinal tumours and accelerated adenoma-carcinoma sequence in Apc

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min/+ mice. ADAM-17/EGFR signalling axis was also activated in intestinal tumours of DCA-treated Apc

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min/+ mice, whereas no significant change occurred in tumour adjacent tissues after DCA exposure. Conclusively, DCA activated EGFR and promoted intestinal carcinogenesis by ADAM17-dependent ligand release.

KEYWORDS
a disintegrin and metalloprotease-17, deoxycholic acid, epidermal growth factor receptor, intestinal carcinogenesis

1 INTRODUCTION

More than 95% of sporadic colorectal cancers (CRC) develop from adenomas over a number of years. Apart from hereditable components, environmental factors strongly determine the progression of intestinal neoplastic transformation, known as the adenoma-adenocarcinoma sequence. Several features include high fat diet (HFD), obesity and low levels of physical activity, are known risk factors for CRC, and dietary fat intake could increase the secondary bile acids such as deoxycholic acid (DCA) in the intestine. Previous studies including our data have shown that persistent and repeated exposure of intestinal epithelium to abnormally high concentrations of DCA appeared to induce DNA damage, genomic instability and alteration of the microbial community to promote CRC development. However, despite intensive researches over the years, the molecular mechanisms of intestinal cancer promotion by DCA remain to be further elucidated.

Epidermal growth factor receptor (EGFR), which plays an important role in tumorigenesis, is overexpressed in many types of cancers, especially in CRC. EGFR tyrosine kinase activation leads to...
the activation of numerous intracellular signals, which are critical to tumour progression, including cell growth, epithelial-mesenchymal transition (EMT), metastasis and angiogenesis. These changes are mediated by the downstream targets of EGFR, including extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt protein kinase. The proteolytic processing of EGFR soluble ligands, amphiregulin (AREG), heparin-binding (HB)-EGF or transforming growth factor (TGF)-α requires a disintegrin and metalloprotease (ADAM-17), which is also known as tumour necrosis factor-α converting enzyme (TACE). As a member of the ADAM family of metalloproteases, ADAM-17 involves in cell adhesion, migration, cellular signalling and proteolysis, recently emerging as a potential therapeutic target in several tumour types.

Our previous studies have already showed that DCA enhanced the multiplicity of intestinal tumours and accelerated intestinal adenoma-adenocarcinoma sequence in Apc<sup>min/+</sup> mice. And recent research provided evidence that DCA induced EGFR/STAT3 signalling to promote gastrointestinal cancer progression. In this study, we investigated whether DCA promoted intestinal carcinogenesis through activation of ADAM-17/EGFR signalling axis. Here, we provided the evidence that DCA up-regulated the release of AREG in accordance with EGFR/Akt activation, and ADAM-17 was required in the shedding AREG for activation of EGFR. These data defined a mechanism of DCA in promoting intestinal tumour development through ADAM-17-mediated AREG release, leading to activation of EGFR, and represented a potential target for the bile acid-related intestinal cancer prevention and therapy.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

Young adult mouse colonic epithelium cell line (YAMC) cell line was generated using a mouse harbouring thermobaluble mutation (tsAS58) under the control of an interferon (IFN-γ)-inducible H-2Kb promoter and a temperature-sensitive simian virus 40 large T antigen (Immortalouse). Immorto-Min colonic epithelial cell line (IMCE) cell line was derived from the colonic epithelium of F1 Immorto-Apc<sup>min/+</sup> mouse hybrid and carried both the mutant Min gene and a temperature-sensitive mutant of the SV40 large T antigen. ADAM17-deficient MCE (ADAM17<sup>−/−</sup>MCE) cell line was derived from the colonic epithelium of Adam17<sup>−/−</sup>-Apc<sup>min/+</sup> mouse and was crossed to the Immortalouse. All these cell lines were cultured in 1640 medium (Invitrogen, Carlsbad, CA, USA), mixed with Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and then blotted to PVDF membrane (Invitrogen, Carlsbad, CA, USA). Membranes of total protein were blocked with 5% BSA. Then, membranes were incubated overnight with primary antibodies: EGFR, Akt, phospho-EGFR (Tyr1068), phospho-Akt (Cell Signaling Technology). The membranes were flowed by horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology). The band density was detected using an Image processor program (ImageJ) and was determined by comparing the O.D. of the samples to the standard curve. The concentrations of measured protein in the samples were then determined by comparing the O.D. of the samples to the standard curve. The concentrations of the indicated ligands in serum were calculated as pg or ng of ligand/mL serum.

2.2 | ELISA assay

Cell culture media was collected after the arranged treatment to determine the levels of EGFR ligands, including HB-EGF, TGF-α and AREG, using the corresponding ELISA kits (R&D Systems, Inc.) according to the manufacturer’s instructions. Briefly, standard and sample proteins were binded with the corresponding antibody coated with the bottom of each well. Further, by adding secondary antibody, the amount of metabolized colour was measured spectrophotometrically at a wavelength of 450 nm. The concentrations of measured protein in the samples were then determined by comparing the O.D. of the samples to the standard curve. The concentrations of the indicated ligands in serum were calculated as pg or ng of ligand/mL serum.

2.3 | Western blot analysis

The cells lysates were solubilized using RIPA buffer with protease inhibitors (Solarbio, Beijing, China) and homogenized. The protein concentrations were determined using Bicinchoninic acid protein assay (Solarbio, Beijing, China). Western blotting was performed on SDS-PAGE Electrophoresis System. The total cellular lysates were mixed with Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and then blotted to PVDF membrane (Invitrogen, Carlsbad, CA, USA). Membranes of total protein were blocked with 5% non-fat milk and phospho-protein with 5% BSA. Then, membranes were incubated overnight with primary antibodies: EGFR, Akt, phospho-EGFR (Tyr1068), phospho-Akt (Cell Signaling Technology, Beverly, MA), cleavage caspase 3, intact PARP (Cell Signaling Technology), cleavage PARP, ADAM-17 (Cell Signaling Technology), or with anti-β-actin antibody (Cell Signaling Technology). The membranes were flowed by horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology). The band density was detected using an Image processor program (ImageJ) and was determined by comparing the density of the indicated band to the internal control band.

2.4 | Real-time PCR analysis

Total RNA was extracted using the RNasy mini kit (Qiagen, Carlsbad, CA, USA), and cDNA reverse transcription was carried out using
the TIANScript RT Kit (TIANGEN, Inc. Beijing, China) according to the manufacturer’s instructions. The Oligonucleotide primers for target genes (GAPDH and AREG) were shown in Table S1. The ΔΔCt method was used to calculate relative mRNA expression.

2.5 Animal treatment and tissue processing

Apcmin/+ mice on a C57BL/6J background were purchased from Animal Model Institution of Nanjing University, China. The mice were provided with either sterile water (n = 10) or 0.2% DCA in drinking water (n = 10) under specific pathogen free (SPF) conditions for 12 weeks as previously described. Signs of illness were monitored daily and body weight was recorded weekly. Mice were killed for intestinal tumour burden assessment and tissue collection as previously described. Tissue sections were prepared for haematoxylin and eosin (H&E) and immunohistochemical staining. Adenomas of distal small intestinal section were excised, immediately frozen in liquid nitrogen and then stored at −80°C until analysis for protein expression. Animal protocols were approved by the Institutional Animal Care and Use Committee at Tianjin Medical University, Tianjin, China.

2.6 Histopathology and immunohistochemistry

Formalin-fixed tissues were dehydrated and embedded in paraffin according to standard H&E protocols. Low-grade dysplasia (LGD) is confined to the lower half of the epithelium, and in high-grade dysplasia (HGD), the abnormal cells occur in the upper half and exhibit a greater degree of atypia. And intramusosal carcinoma is diagnosed when the tumour invades into the lamina propria, but not through the muscularis mucosae. The histopathologic analysis was performed in a blinded manner by the same pathologist (Yujie Zhang). The tissue sections were incubated with primary antibodies, mouse monoclonal ki-67 (Abcam, Cambridge, MA, USA), ADAM-17 (Abcam, Cambridge, MA, USA), AREG (R&D Systems, USA), EGFR, phospho-EGFR (Tyr1068), Akt and phosphor-Akt (Cell Signaling Technology, Inc.). The biotinylated anti-rabbit or anti-goat secondary antibody was applied followed by horseradish peroxidase (HRP)-streptavidin solution. Finally the sections were counterstained with haematoxylin. Five random areas from a single section were checked for the percentage of positive cells. Data were quantified by calculating the average percentages of positive cells in each mouse as the positive rate of cells.

2.7 TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (Roche Applied Science, Mannheim, Germany) was used to detect apoptotic cells. Apoptotic cells were determined by counting percentage of positive-stained cells in five randomly selected fields in each tumour. At least three tumours in each mouse were randomly selected.

2.8 Statistical analysis

Statistical analysis was performed on SPSS 22.0 (SPSS, Chicago, IL, USA). Data were presented as mean ± SD. Differences among groups were tested by one-way ANOVA for multiple comparison and t test for paired samples. P < .05 was considered significant.

3 RESULTS

3.1 DCA activated EGFR in intestinal tumour cells

The effects of DCA on EGFR signalling pathway were examined in IMCE and HCT-116 cell lines. DCA exposure dramatically increased phosphorylation levels of EGFR and Akt along with time variation, while total EGFR and Akt expressions were not significantly changed. In IMCE cells with DCA treatment, phospho-EGFR level peaked at 0.5 hours. Just as the downstream signalling, maximal level of phospho-Akt was also observed 0.5 hours after DCA treatment (Figure 1A-B). While, the peak levels of phospho-EGFR and phosphor-Akt occurred 2 hours after DCA treatment in HCT-116 cells (Figure 1C-D). After AREG blocking antibody used (R&D Systems, af262 for HCT-116 cells and af989 for IMCE cells), significant inhibition was found of DCA-induced EGFR phosphorylation and the downstream signalling (Figure S1).

To elucidate the ligand responsible for activation of EGFR, we examined the effects of the tumour-promoting DCA on AREG, TGF-α and HB-EGF by ELISA assay in IMCE and HCT-116 cell lines. DCA exposure increased the release of AREG, but not TGF-α or HB-EGF. The peak level of AREG was found at 2 and 4 hours after incubation with DCA and then declined in both cell lines (Figure 2A-C,E). Real-time PCR analysis was performed to measure AREG mRNA levels in intestinal tumour cells treated with DCA. It showed that AREG mRNA levels peaked at 2 hours of incubation with DCA in HCT-116 cells, whereas no significant change occurred after DCA exposure in IMCE cells (Figure 2D,F). These interesting data suggested that DCA affected the mRNA expression of AREG only in intestinal cancer cells, but not in pre-cancerous cells. DCA up-regulated soluble and mature AREG through shedding of ligand in the culture medium, independent of the mRNA levels in IMCE cells, while the mRNA expression and shedding of AREG were significantly promoted by DCA in HCT116 cells. Moreover, the trend of DCA-stimulated AREG shedding activity was consistent with the finding of DCA-stimulated EGFR signalling activation. It indicated a requirement of AREG for the DCA-induced effects on EGFR-Akt signalling pathway activation in intestinal tumour cells. Besides, there were no significant changes of ELISA for HB-EGF and TGF-α of HCT-116 cells and the mRNA expression of HB-EGF and TGF-α by DCA in both HCT-116 cells and IMCE cells as well after DCA treatment (Figure S2).
3.2 | EGFR kinase activity and ADAM-17 were required for DCA-induced intestinal epithelial cell apoptosis resistance

To further elucidate if EGFR kinase activity might mediate DCA-induced intestinal epithelial cell apoptosis resistance, AG1478 (150 nmol/L), an EGFR receptor kinase inhibitor was used. Cleavage of PARP and caspase-3 are correlated with cell apoptosis. Western blot analysis showed that DCA did not affect apoptosis in YMCE cells, while after treatment of AG1478, the expression of cleavage PARP and caspase-3 were significantly increased (Figure 3A). These results indicated that EGFR was required for the apoptosis resistance of colon epithelial cells induced by DCA. Next, we determined the role of ADAM-17 in cells apoptosis resistance. It showed ADAM-17 activity in ADAM-17+/−MCE cells expressing wt ADAM-17 but not control vector. Importantly, Western blot analysis of cleavage PARP and caspase-3 showed that apoptosis was prevented in ADAM-17+/−MCE cells expressing wt ADAM-17 but not with control vector after DCA treatment (Figure 3B). In addition, TUNEL analysis in HCT-116 cells was used to detect apoptosis which was shown in the Figure S3. These data suggested that EGFR kinase activity and ADAM-17 were essential to DCA induction of intestinal epithelial cell apoptosis resistance.

3.3 | DCA accelerated intestinal carcinogenesis in Apc<sup>min/+</sup> mice

To determine the effect of DCA-induced carcinogenesis, we evaluated the intestinal tumour development in Apc<sup>min/+</sup> mice with or without DCA treatment. DCA significantly enhanced the multiplicity of intestinal tumour (36.3 ± 3.16 vs 18.5 ± 1.35, P < .001) and the numbers of all sizes of tumours were also increased. In addition, tumour numbers in proximal, middle and distal portions of the small intestine in DCA groups were increased by 91% (8.7 ± 1.32 vs 4.4 ± 0.73, P < .001), 104% (10.8 ± 1.18 vs 5.3 ± 0.83, P < .01) and 88% (15.6 ± 0.91 vs 8.3 ± 0.64, P < .001), respectively. DCA mainly increased middle (1-2 mm, 30.2 ± 3.26 vs 14.6 ± 2.14, P < .05) and large (>2 mm, 2.1 ± 0.15 vs 1.1 ± 0.15, P < .001) tumours (Figure 4A). Tumours were histologically identified as benign adenomas with or without LGD in untreated Apc<sup>min/+</sup> mice (control). However, HGD, including intramucosal carcinoma, was confirmed in 70% (7/10) mice in DCA-treated Apc<sup>min/+</sup> mice compared with 0% (0/10) in untreated Apc<sup>min/+</sup> mice.
Deoxycholic acid stimulated the release of amphiregulin (AREG), but not transforming growth factor (TGF-α) or heparin-binding (HB)-EGF in intestinal tumour cells. A–C,E. The concentration of epidermal growth factor receptor ligand TGF-α, HB-EGF and AREG by ELISA assay in Immorto-Min colonic epithelial cell line (IMCE) and human colorectal cancer cell line (HCT-116) cell lines treated with deoxycholic acid (DCA) along with time variation. D,F. Real-time PCR results showed that AREG mRNA levels peaked at 2 h of incubation with DCA in HCT-116 cells, whereas no change significantly occurred after DCA exposure in IMCE cells. DCA, deoxycholic acid. *, $P < .05$, **, $P < .01$, ***, $P < .001$. Data were quantified from at least 3 separate experiments.
mice (Figure 4B-C), which suggested that DCA enhanced intestinal adenoma to adenocarcinoma progression.

We further assessed tumour cell proliferation and apoptosis for the DCA-induced tumour development in Apc\superscript{min/+} mice. Immunohistochemistry showed that DCA significantly increased the percentage of Ki-67 positive cells (80.67/C6 4.03 vs 49.83/C6 2.76, \( P < .001 \)) (Figure 4D), suggesting that DCA significantly promoted tumour cell proliferation in Apc\superscript{min/+} mice. The percentage of apoptotic cells in tumours detected by TUNEL staining were significantly reduced by 2.4-fold in DCA group compared with that in control group (1.80/C6 0.52 vs 6.20/C6 0.92, \( P < .001 \), Figure 4E). Thus, these results suggest that promotion of proliferation and inhibition of apoptosis by DCA might play a role in the promotion of tumour development in Apc\superscript{min/+} mice.

3.4 ADAM-17/EGFR signalling axis was activated in intestinal tumours of DCA-treated Apc\superscript{min/+} mice

We then investigated the effects of DCA on ADAM-17/EGFR signalling axis in intestinal tumour development. Immunohistochemistry showed that DCA treatment increased the percentage of positive cells of ADAM-17 in intestinal tumour in Apc\superscript{min/+} mice (75.00 ± 2.35 vs 36.33 ± 1.94, \( P < .001 \), Figure 5A). Similarly, the AREG expression increased with DCA treatment (56.83 ± 2.37 vs 27.00 ± 1.75, \( P < .001 \), Figure 5B). These results further supported the importance of ADAM-17 in DCA-induced shedding activity of AREG. Furthermore, phosphorylation of EGFR and Akt in intestinal tumours was also up-regulated by DCA treatment. The average percentages of p-EGFR-positive cells in DCA and untreated groups were 57.83/C6 1.97 vs 27.83/C6 1.68 (\( P < .001 \), Figure 5C), and p-Akt stained cells were 70.17/C6 1.17 vs 33.17/C6 1.62 (\( P < .001 \), Figure 5D), respectively. Besides, Western blot analysis showed that DCA increased the phosphorylation of EGFR and Akt in Apc\superscript{min/+} mice as well (Figure 5E). These results further showed that release of AREG mediated by ADAM-17 could activate EGFR-Akt pathway to further promote intestinal tumour development after DCA treatment. Notably, the up-regulation of ADAM-17 (the average percentages of positive cells: 15.4/C6 0.98 vs 18.1/C6 1.12, \( P = .09 \)) and AREG (14.6/C6 1.88 vs 16.8/C6 1.67, \( P = .39 \)) were not found in tumour adjacent tissues of DCA-treated mice, which suggested the specific effects of DCA on intestinal tumour cells.
A

ADAM17

Control

DCA

(% ADAM17 positive cells)

Control

DCA

***

B

AREG

Control

DCA

(% AREG positive cells)

Control

DCA

***

C

p-EGFR (Tyr1068)

Control

DCA

(% p-EGFR (Tyr1068) positive cells)

Control

DCA

***

D

p-Akt

Control

DCA

(% p-Akt positive cells)

Control

DCA

***

E

EGFR

Tyr1068

Total EGFR

p-Akt

Total Akt

β-actin

Fold change (Control = 1)

Control

DCA

Tyr1068 to total EGFR

Phosphorylated to total Akt

**

Control

DCA

**
Immunohistochemistry showed that DCA treatment increased the percentage of positive cells of ADAM-17 in intestinal tumour in Apc\textsuperscript{min/+} mice. C, D, Phosphorylation of epidermal growth factor receptor (EGFR) and Akt in intestinal tumours was up-regulated after DCA treatment. E, Western blot analysis showed that DCA increased the phosphorylation of EGFR and Akt in Apc\textsuperscript{min/+} mice. DCA, deoxycholic acid. Scale bar: 20 µm. ***P < .001, **P < .01, *P < .05, n = 10/group.

**FIGURE 6**  Model of ADAM-17/EGFR signalling axis activation induced by deoxycholic acid in intestinal carcinogenesis. DCA stimulates ADAM-17 activation and AREG release, which is required for EGF receptor activation, EGFR/Akt signalling pathway activation and apoptosis resistance in intestinal tumour cells. ADAM-17, a disintegrin and metalloprotease domain-containing protein 17; AREG, amphiregulin; DCA, deoxycholic acid; EGFR, epidermal growth factor receptor [Colour figure can be viewed at wileyonlinelibrary.com]
positive correlation between ADAM-17 and AREG expression in mice inferred the importance of ADAM-17 on cleaving AREG.

Researches had shown that the EGFR pathway was the most important target for CRC therapy.\(^\text{41}\) Two FDA-approved monoclonal antibodies against EGFR have become clinically routine, but only a small part of patients had an effective result.\(^\text{42,43}\) Thus, the development of combinatorial therapeutic target should be introduced into CRC therapy.\(^\text{44}\) In this study, we found that DCA accelerated intestinal carcinogenesis through activation of ADAM-17/EGFR signalling axis. Further studies should be arranged to study the relationship between DCA and upstream components of ADAM-17/EGFR signalling axis, as specific nuclear receptors (FXR, PXR and vitamin D receptor) and G-protein-coupled receptors (TGR5, sphingosine-1 phosphate receptor 2 and muscarinic receptors).\(^\text{45}\)

In conclusion, we investigated the importance of ADAM-17/EGFR signalling activation in the process of intestinal carcinogenesis by DCA treatment. This report also shows that DCA can stimulate release of AREG mediated by ADAM-17. Consequently, it may provide new insights that DCA promotes adenoma to adenocarcinoma progression. And ADAM-17/EGFR signalling axis will represent a potential target for the bile acid-related CRC therapy.

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**CONFLICT OF INTEREST**

All the authors declare that they had no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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