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Role of a novel bile acid receptor TGR5 in the development of oesophageal adenocarcinoma

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ABSTRACT

Background and aims Mechanisms of the progression from Barrett's oesophagus to oesophageal adenocarcinoma (OA) are not fully understood. Bile acids may have an important role in this progression. This study aimed at examining the role of NADPH oxidase NOX5-S and a novel bile acid receptor TGR5 in taurodeoxycholic acid (TDCA)-induced increase in cell proliferation.

Methods Human Barrett's cell line BAR-T and OA cell line FLO were transfected by the Lipofectamine 2000 or Amaxa-Nucleofector-System. mRNAs were measured by real-time PCR. H_2O_2 was measured by a fluorescent assay. Cell proliferation was determined by measurement of thymidine incorporation.

Results NOX5-S was present in FLO cells. TDCA significantly increased NOX5-S expression, H_2O_2 production and thymidine incorporation in FLO and BAR-T cells. This increase in thymidine incorporation was significantly reduced by knockdown of NOX5-S. TGR5 mRNA and protein levels were significantly higher in OA tissues than in normal oesophageal mucosa or Barrett's mucosa. Knockdown of TGR5 markedly inhibited TDCA-induced increase in NOX5-S expression, H_2O_2 production and thymidine incorporation in FLO and BAR-T cells. Overexpression of TGR5 significantly enhanced the effects of TDCA in FLO cells. TGR5 receptors were coupled with Gαq and Gαi3 proteins, but only Gαq mediated TDCA-induced increase in NOX5-S expression, H_2O_2 production and thymidine incorporation in FLO cells.

Conclusions TDCA-induced increase in cell proliferation depends on upregulation of NOX5-S expression in BAR-T and FLO cells. TDCA-induced NOX5-S expression may be mediated by activation of the TGR5 receptor and Gαq protein. These data may provide potential targets to prevent and/or treat Barrett's OA.

The incidence of oesophageal adenocarcinoma (OA) has increased more than sixfold in the past three decades.[1] Gastro-oesophageal reflux disease complicated by Barrett's oesophagus (BO) is a major risk factor for OA.[2] The refluxate in gastro-oesophageal reflux disease may contain oro-oesophageal (saliva, oesophageal secretions), gastric (acid, pepsin, mucus) and duodenal (bile salts, trypsin and lipase) components.[3] Acid and bile acids, present in refluxate, may be major risk factors for the progression from BO to OA.[4–6] However, the mechanisms of this progression are not fully understood.

Acid reflux may mediate the progression from metaplasia to dysplasia and to OA in patients with BO for the following reasons: (*a*) cultured biopsy specimens of intestinal metaplastic cells demonstrate a significant increase in tritiated thymidine uptake when explants are briefly exposed to acid[7]; (*b*) long-term inhibition of oesophageal acid exposure by administration of proton pump inhibitors to patients with BO has been shown to decrease proliferation of metaplastic cells[8]; (*c*) a prospective study showed that proton pump inhibitor treatment significantly reduced the incidence of dysplasia in patients with BO, when compared with no treatment or treatment with H_2 receptor antagonists.[9]

In addition to acid reflux, there is increasing evidence that bile acids may also contribute to the progression from BO to OA[10 11] since (*a*) in animal models, diversion of duodenal contents into the lower oesophagus leads to OA[12–14]; (*b*) reflux of bile acids into the oesophagus causes short-term damage to the mucosa and also induces long-term oxidative stress, and cellular DNA damage[15 16]; (*c*) bile salts may induce upregulation of cyclo-oxygenase-2 and c-myc expression,[17 18] and activate mitogen-activated protein kinase and NF-κB pathways,[19 20] thereby increasing cell proliferation and decreasing cell apoptosis. However, mechanisms whereby bile acids promote the development of OA are not known.

Recently, a novel cell membrane receptor of bile acid, TGR5 (a G-protein-coupled receptor) has been shown to be important in bile acid-regulated lipid metabolism, energy homoeostasis and glucose metabolism.[21–23] Whether TGR5 receptor mediates bile acid-induced increase in cell proliferation is not known. Bile acids also have been reported to induce production of reactive oxygen species (ROS) in OA cells.[24] In our previous studies, we have demonstrated that acid exposure increases H_2O_2 production and cell proliferation, an increase which is blocked by knockdown of NOX5-S, suggesting that NADPH oxidase NOX5-S mediates an acid-induced increase in H_2O_2 production and cell proliferation.[4 5] Whether NADPH oxidases mediate a bile acid-induced increase in cell proliferation and ROS production is not known. We now show that a taurodeoxycholic acid (TDCA, a bile salt)-induced increase in cell proliferation depends on upregulation of NOX5-S expression. To our knowledge, we are the first to report that TDCA-induced NOX5-S expression is mediated by activation of the TGR5 receptor and Gaq protein in OA cells.

MATERIAL AND METHODS

Cell culture and TDCA treatment

Human Barrett's adenocarcinoma cell line FLO was derived from human oesophageal Barrett's

adenocarcinomas[25] and generously provided by Dr David Beer (University of Michigan Medical School). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics at 37°C with 5% CO_2 humidified atmosphere.

Human Barrett's cell line BAR-T was derived from oesophageal mucosal biopsies of patients with BO (intestinal metaplasia) and immortalised with telomerase, as described previously.[26] Cells were cultured in wells precoated with collagen IV (1 μg/cm^2; BD Bioscience, Bedford, Maryland, USA) and in Keratinocyte Medium-2 (Ca^{2+}-free solution, Cambrex, Rockland, Maine, USA) supplemented with 1.8 mM $CaCl_2$, 5% fetal bovine serum, 400 ng/ml hydrocortisone, 20 ng/ml epidermal growth factor, 0.1 nM cholera toxin, 20 μg/ml adenine, 5 μg/ml insulin, 70 μg/ml bovine pituitary extract and antibiotics.

For TDCA treatment, FLO and BAR-T cells were incubated with different concentrations of TDCA for 24 h. Then, the culture medium and cells were collected for measurements.

Human oesophageal tissues

Normal oesophageal mucosa, Barrett's mucosa and OA tissues were obtained from patients with OA undergoing oesophagogastrectomy. The experimental protocols were approved by the Human Research Institutional Review Committee at Rhode Island Hospital.

Construction of pCDNA3.1-TGR5 plasmid

The DNA fragment encoding the TGR5 gene (G-protein-coupled bile acid receptor 1) (GenBank accession number BC033625) was PCR-amplified from human cDNA with the following primers: TGR5-sense: 5′-GCAAGCTTATGACGCCCAACAGCACTGGC GAG-3′ (the introduced *Hind III* is underlined) and TGR5-antisense: 5′-GCTCTAGAGTTCA AGTCCAGGTCGACACTGCT-3′ (the introduced *Xba I* is underlined). The cDNA fragments obtained above were first cloned into pGEM-T Easy Vector (Promega, Madison,Wisconsin,USA), verified by sequencing, and then subcloned into pCDNA3.1 between *Hind III* and *Xba I* to obtain TGR5 expression plasmid pCDNA3.1-TGR5.

Detecting of NOX5 in FLO OA cells

The primers used for detecting NOX5 in FLO OA cells were as follows: 5-ATGGGCTACGTGGTAGTGGGGC-3 (2F), 5-ATGG AGAACCTGACCATCAGC-3 (3F), 5-TTGGGCCCATGAAAGAT GAGCA-3 (2R), 5-GTGTGAGCCACAGTGTGCACG-3 (3R), 5-A GCCCCACTACCACGTAGCCC-3 (4R), 5-AGTGGGCAGCGCT GATGGTC-3 (5R) and 5-CTAGAAATTCTCTTGGAAAAATCT G-3 (6R). Three primers (3R for RT, 4R and 5R for nested PCR) were used to amplify the 5′-end of NOX5 using a 5′-RACE kit (Invitrogen, Grand Island, New York, USA). PCR products were gel-extracted, and sequenced by GENEWIZ (South Plainfield, New Jersey, USA).

Small interfering RNA (siRNA) and plasmid transfection

Twenty-four hours before transfection at 70—80% confluence, cells were trypsinised (1—3×10^5 cells/ml) and transferred to 12-well plates. Transfection of siRNAs was carried out with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. To each well, 75 pmol of siRNA duplex of NOX5, TGR5, Gαq, Gαi3 or control siRNA formulated into liposomes was applied; the final volume was 1.2 ml/well. Forty-eight hours after transfection, cells were treated without or with TDCA (10^{-11} M) in culture medium (pH 7.2, without phenol red) for 24 h, and then the culture medium and cells were collected for measurements. Transfection efficiencies were determined by fluorescence microscopy after transfection of Block-it fluorescent oligonucleotide (Invitrogen, Grand Island, New York, USA) and were about 70% at 48 h.

For transfection of pCDNA3.1-TGR5 plasmid, FLO cells (70% confluence, approximately 5×10^6 cells) were transfected with 2 μg of pCDNA3.1-TGR5 or control plasmids using Amaxa-Nucleofector-System (Lonza, Allendale, NJ, USA), according to the manufacturer's instructions. Twenty-four hours after transfection, cells were treated with TDCA (10^{-11} M) for an additional 24 h and then the culture medium and cells were collected for measurements. Transfection efficiencies were determined by fluorescence microscopy after transfection of pmax-GFP (Lonza, Allendale, NJ, USA) and were about 90% at 48 h.

Reverse transcription-PCR

Total RNA was extracted by TRIzol reagent (Invitrogen, Grand Island, New York, USA) and purified by the total RNA purification system (Invitrogen). According to the protocols of the manufacturers, 1.5 μg of total RNAs from cultured cells was reversely transcribed by a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Grand Island, New York, USA).

Quantitative real-time PCR

Quantitative real-time PCR was carried out on a Stratagene Mx4000 multiplex quantitative PCR system (Stratagene, La Jolla, CA, USA). The primers used were: NOX5-S sense (5′-AAGACT CCATCACGGGGCTGCA-3′), NOX5-S antisense (5′-CCTTCA GCACCTTGGCCAGA-3′), TGR5 sense (5′-CTGGCCCTGGCA AGCCTCAT-3′), TGR5 antisense (5′-CTGCCATGTAGCGCTC CCCGT-3′), 18S sense (5′-CGGACAGGATTGACAGATTGATA GC-3′) and 18S antisense (5′-TGCCAGAGTCTCGTTCGTT ATCG-3′). All reactions were performed in triplicate in a 25 μl total volume containing a 1×concentration of Brilliant SYBR Green QPCR Master Mix (Stratagene); the concentrations of each sense and antisense primer were 100 nM, 1 μl cDNA, and 30 nM reference dyes. Reactions were carried out in a Stratagene Mx4000 multiplex quantitative PCR system for one cycle at 94°C for 5 min; 40 cycles at 94°C for 30 s, 59°C for 30 s and 72°C for 30 s; one cycle at 94°C for 1 min; and one cycle at 55°C for 30 s. The transcript level of each specific gene was normalised to GAPDH or 18S amplification.

Coupling to immunoprecipitation matrix and immunoprecipitation experiments

TGR5 antibody (5 μg) was coupled to 40—50 μl of suspended IP matrix (Santa Cruz Biotechnology, Santa Cruz, California, USA) in 500 μl phosphate-buffered saline, as recommended by the manufacturer, incubated at 4°C on a rotator overnight. Forty-eight hours after pCDNA3.1-TGR5 transfection, cells were lysed in Triton X lysis buffer containing 50 mM Tris.HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerol phosphate, 40 mM p-nitrophenylphosphate, 200 μM sodium orthovanadate, 100 μM phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 1 μg/ml aprotinin. The supernatants were mixed with TGR5 antibody—IP matrix complex and incubated at 4°C on a rotator for 5 h. The immunoadsorbents were recovered by centrifugation for 5 min at 700×*g*, washed three times with cell lysis buffer, mixed with sodium dodecyl sulphate loading buffer (Sigma, St Louis, Missouri, USA), and heated at 100°C for 5 min.

Western blot analysis

Cells were lysed in Triton X lysis buffer. The suspension was centrifuged at 15 000×*g* for 5 min, and the protein concentration in the supernatant was determined. Western blotting was done as described previously.[27]

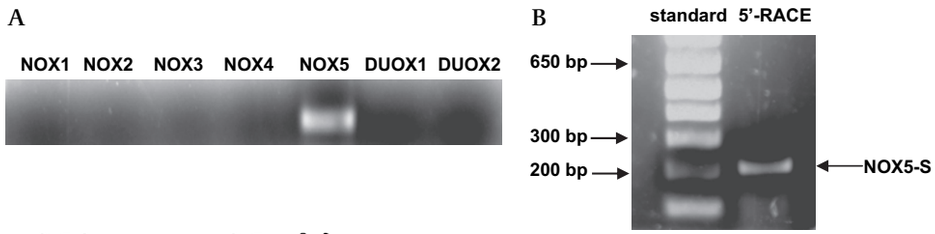

Figure 1 Expression of NADPH oxidases in FLO oesophageal adenocarcinoma (OA) cells. (A) Reverse transcriptase-PCR showed that only NOX5 was detectable in human FLO OA cells. (B) Only one band was detected in FLO OA cells by 5′-RACE using the primers abridged universal amplification primer and 5R (11—31 from ATG). The size of this PCR product was consistent with NOX5-S but not with NOX5 α, β, δ or γ.

Primary antibodies used were as follows: TGR5 antibody (1:1000), Gαq antibody (1:2000), Gαs antibody (1:1000), Gα13 antibody (1:1000), Gαi3 antibody (1:1000), Gαi1-2 antibody (1:1000) and GAPDH antibody (1:2000). NOX5 antibody was prepared against a mixture of unique NOX5 peptides (NH2-YESFKASDPLGRGSKRC-COOH and NH2-YRHQKRKHTCPS-COOH)[28] and used at a dilution of 1:1000.

[^{3}H]Thymidine incorporation

Twenty-four hours after transfection with control siRNA, NOX5 siRNA or TGR5 siRNA, cells were treated without or with TDCA for 24 h and then incubated with methyl [^{3}H] thymidine (0.05 μCi/ml) for 4 h. After washing three times with phosphate-buffered saline, cells were collected and homogenised with a lysis buffer containing (pH 7.4) 50 mM HEPES, 50 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 0.1 mM phenylmethylsulphonyl fluoride and 1 mM dithiothreitol. Methyl [^{3}H] thymidine uptake was measured in a scintillation counter. The level of protein in the homogenates was also determined and the level of methyl [^{3}H]thymidine incorporation was normalised to protein content.

Amplex red hydrogen peroxide fluorescent assay

Levels of H_2O_2 in culture medium were determined by the Amplex Red H_2O_2 assay kit (Molecular Probes, Eugene, Oregon, USA), according to the manufacturer's instruction. Fluorescence was measured with a fluorescence microplate reader using excitation at 550 nm and emission detection at 590 nm.

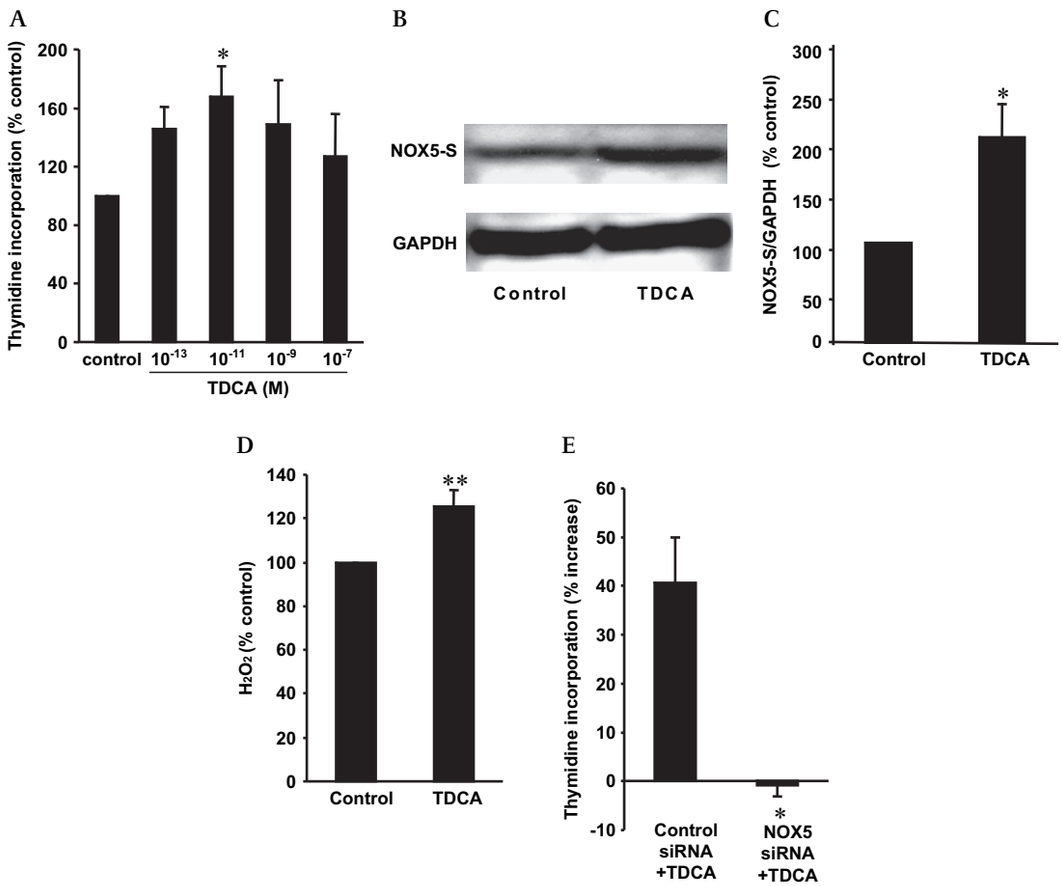

Figure 2 NOX5-S expression, H_2O_2 production and cell proliferation after taurodeoxycholic acid (TDCA) treatment in FLO oesophageal adenocarcinoma (OA) cells. (A) 10^{-11} M TDCA (24 h) significantly increased [^{3}H]thymidine incorporation. (B) Western blot analysis, (C) summarised data and (D) H_2O_2 measurement showed that 10^{-11} M TDCA significantly increased NOX5-S expression and H_2O_2 production. (E) Knockdown of NOX5-S by NOX5 small interfering RNA (siRNA) blocked TDCA-induced increase in thymidine incorporation. The data suggest that TDCA may increase NOX5-S expression, H_2O_2 production and cell proliferation in FLO cells and that TDCA-induced increase in cell proliferation may depend on activation of NOX5-S. n=3, ANOVA. *$p<0.05$, **$p<0.01$, compared with control.

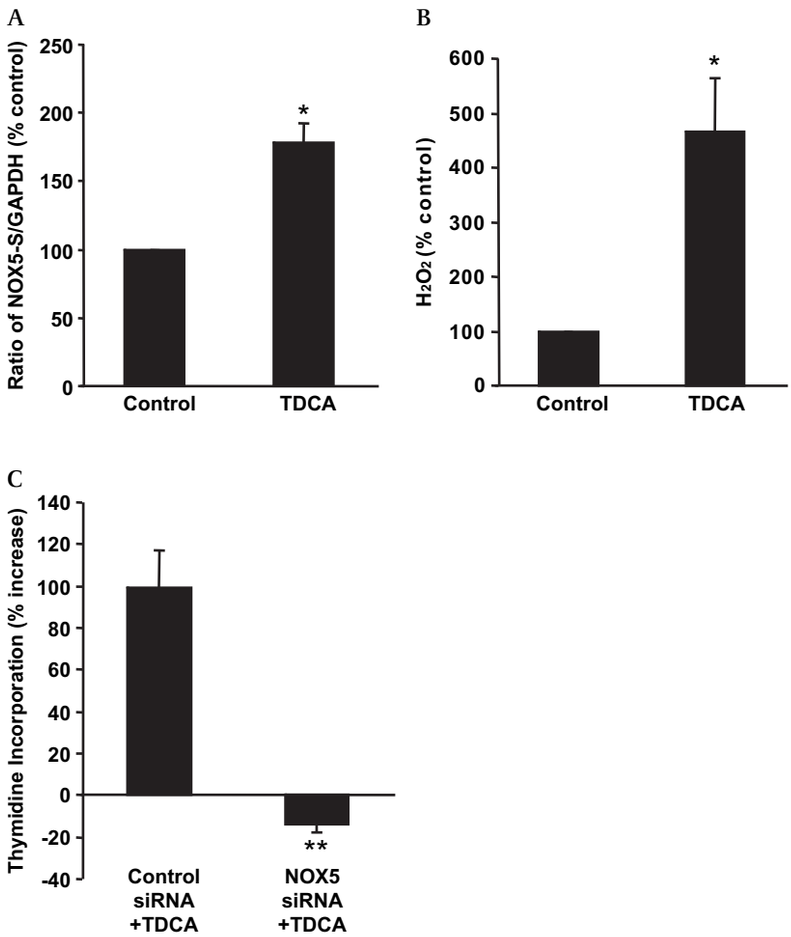

Figure 3 NOX5-S expression, H_2O_2 production and cell proliferation after taurodeoxycholic acid (TDCA) treatment in BAR-T cells. (A) Real time PCR data and (B) H_2O_2 measurement showed that TDCA (10^{-11} M) significantly increased NOX5-S mRNA level and H_2O_2 production. (C) Knockdown of NOX5-S by NOX5 small interfering RNA (siRNA) blocked TDCA-induced increase in thymidine incorporation in BAR-T cells. The data suggest that TDCA (low dose) may increase NOX5-S expression, H_2O_2 production and cell proliferation in BAR-T cells and that TDCA-induced increase in cell proliferation may depend on activation of NOX5-S. n=3, ANOVA. *p<0.01, compared with control; **p<0.05, compared with control siRNA+TDCA.

Materials

Methyl [^{3}H]thymidine was purchased from PerkinElmer (Waltham, Massachusetts, USA) and human NOX5 siRNA from Ambion (Austin, Texas, USA); TGR5 siRNA, TGR5 antibody, Gα13 antibody and GAPDH antibody were bought from Santa Cruz Biotechnology (Santa Cruz, California, USA). Gαq antibody, Gαs antibody, Gαi3 antibody, and Gαi1-2 antibody were purchased from EMD Chemicals (Gibbstown, New Jersey, USA). TDCA, hydrocortisone, epidermal growth factor, cholera toxin, adenine, insulin, bovine pituitary extract, Triton X-100, phenylmethylsulphonyl-fluoride and other reagents were purchased from Sigma (St Louis, Missouri, USA).

Statistical analysis

Data are expressed as mean±SE. Statistical differences between two groups were determined by the Student t test. Differences between multiple groups were tested using analysis of variance (ANOVA) and checked for significance using Fisher's protected least significant difference test.

RESULTS

Effect of TDCA on NOX5-S expression and ROS production

We have previously shown that NADPH oxidase NOX5-S is overexpressed in BO with high-grade dysplasia, mediates acid-induced hydrogen peroxide production and increases cell proliferation.[4] To test which NADPH oxidase isoforms exist in FLO OA cells, we performed RT-PCR and rapid amplification of 5' complementary DNA ends (5′-RACE). RT-PCR demonstrated that NOX5 was the only NADPH oxidase expressed in FLO OA cells (figure 1A).

NOX5 has isoforms α, β, δ and γ or NOX5-S.[29] Figure 1B showed that only one band (around 200 bp) was detected by 5′-RACE (figure 1B) using the primers abridged universal amplification primer and 5R (+11 to +31 from ATG). The size of this PCR product was consistent with NOX5-S but not with the other NOX5 isoforms. NOX5-S in FLO cells was sequenced, and this sequence was consistent with previously reported (GenBank accession number AF317889) (data not shown). These data confirmed that NOX5-S may be the major isoform of NOX5 present in FLO OA cells.

To determine whether bile salts increase NOX5-S expression and induce production of ROS, FLO cells were exposed to TDCA as described in "Material and methods". We found that a low dose of TDCA (10^{-11} M), but not higher doses, significantly increased thymidine incorporation (figure 2A), suggesting that a low dose of TDCA may increase cell proliferation. Similarly, 10^{-11} M TDCA significantly increased thymidine incorporation in BAR-T cells (figure 3C). In addition, a low dose of TDCA significantly increased NOX5-S expression (figures 2B,C and 3A) and H_2O_2 production (figures 2D and 3B) in FLO OA cells and BAR-T cells. Figures 2E and 3C showed that knockdown of NOX5-S with NOX5 siRNA significantly decreased TDCA-induced increase in thymidine incorporation in FLO OA cells and BAR-T cells. NOX5 siRNA has been shown by us to effectively knockdown NOX5-S protein in OA cells.[4] The data suggest that a low dose of TDCA

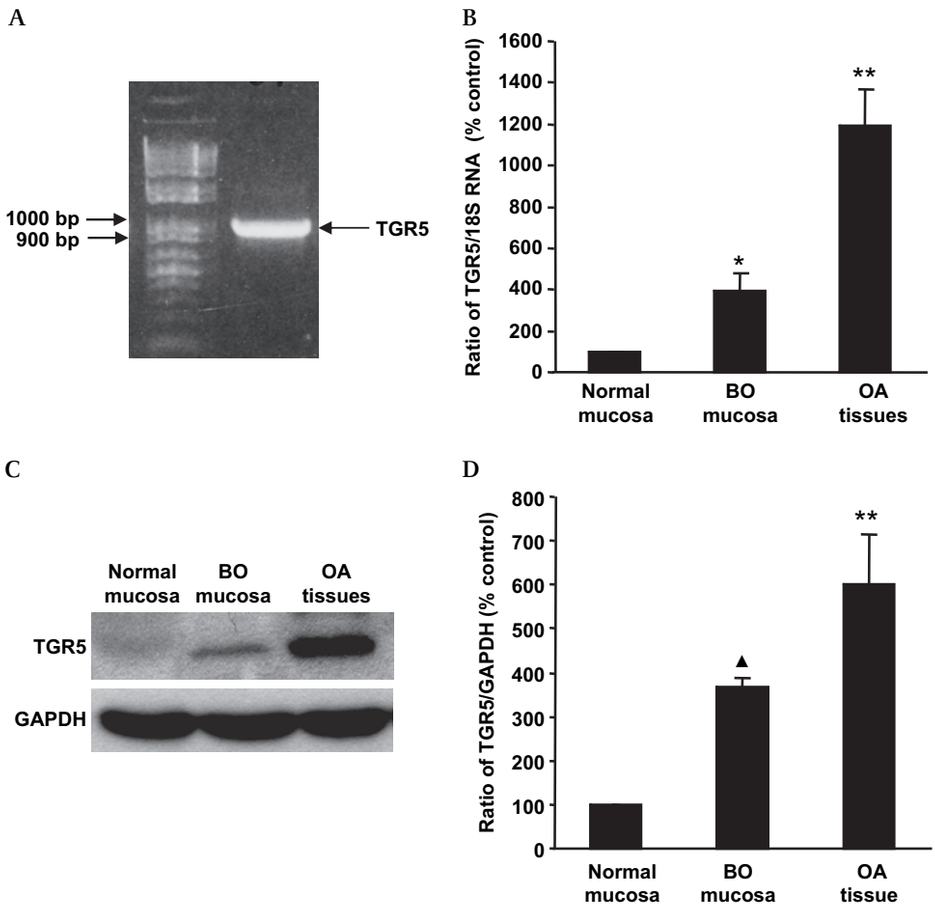

Figure 4 Expression of TGR5. (A) RT-PCR showed that TGR5 was detectable in human FLO oesophageal adenocarcinoma (OA) cells. (B) Real time RT-PCR (n=12), (C) Western blot analysis and (D) summarised data (n=3) showed that the levels of TGR5 mRNA and protein expression were significantly increased in Barrett's oesophagus (BO) mucosa, when compared with normal oesophageal mucosa. TGR5 mRNA and protein levels are significantly higher in OA tissue than in normal oesophageal mucosa or in BO mucosa, supporting the importance of TGR5 in the development of OA. ANOVA. *p<0.01, ▲p<0.05, compared with normal oesophageal mucosa, **p<0.01, compared with BO mucosa.

may increase NOX5-S expression, H_2O_2 production and cell proliferation in FLO cells and BAR-T cells.

Role of TGR5 receptors in TDCA-induced NOX5-S expression and ROS production

The G-protein-coupled bile acid receptor 1 (TGR5) has been reported to be a cell surface receptor and mediates different biological functions of bile acids.[21 30] To test whether this receptor is present in FLO cells, we carried out RT-PCR. RT-PCR showed that TGR5 mRNA was detectable in FLO cells (figure 4A). The PCR product was sequenced and confirmed to be TGR5. Western blot analysis also showed that TGR5 protein was present in FLO cells (figure 5A). Next we measured TGR5 mRNA levels and protein levels in different tissues by real-time PCR and western blot analysis. We found that TGR5 mRNA and protein expression were significantly increased in Barrett's mucosa (figure 4B, n=12; figure 4C,D, n=3), in comparison with normal mucosa. Furthermore, TGR5 expression was much higher in human OA tissue than in normal mucosa or Barrett's mucosa. The data suggest that TGR5 receptors may be important in the development of OA.

To test whether TGR5 is involved in TDCA-induced NOX5-S expression and H_2O_2 production, we used TGR5 siRNA to knockdown TGR5. TGR5 siRNA significantly decreased TGR5 protein expression 48 h after transfection (figure 5A,B), indicating that TGR5 siRNA knocked down TGR5 effectively. Knockdown of TGR5 with TGR5 siRNA significantly reduced NOX5-S expression (figures 5C,D and 6A), H_2O_2 production (figures 5E and 6B) and thymidine incorporation (figures 5F and 6C) in response to TDCA treatment both in FLO and BAR-T cells. These data suggest that TGR5 receptors may contribute to TDCA-induced increase in NOX5-S expression, H_2O_2 production and cell proliferation in FLO and BAR-T cells.

To further confirm the role of TGR5 in TDCA-induced NOX5-S expression and H_2O_2 production, we constructed pCDNA3.1-TGR5 plasmid and transfected this recombinant plasmid into FLO cells. Transfection of pCDNA3.1-TGR5 plasmid significantly increased TGR5 protein expression in FLO cells in comparison with pCDNA3.1 control (figure 7A,B), indicating that TGR5 receptors are successfully overexpressed in FLO cells. Overexpression of TGR5 significantly enhanced NOX5-S expression (figure 7C,D), H_2O_2 production (figure 7E) and thymidine incorporation (figure 7F) in response to TDCA treatment, compared with the pCDNA3.1 group treated with TDCA. These data indicate that TGR5 receptor may mediate TDCA-induced increase in NOX5-S expression, H_2O_2 production and cell proliferation.

Gαq proteins coupled With TGR5 receptor in FLO cells

To determine which G-proteins are coupled with the TGR5 receptor, we used the TGR5 antibody to immunoprecipitate TGR5 and then to detect different G-protein in the immunoprecipitates by western blot analysis. We found that Gαq and Gαi3 proteins were co-immunoprecipitated with TGR5 from FLO cell lysate (figure 8A–E), indicating that TGR5 receptors are coupled with Gαq and Gαi3 protein in FLO OA cells.

To test whether Gαq and Gαi3 participate in TDCA-induced NOX5-S expression and H_2O_2 production, we used Gαq and Gαi3

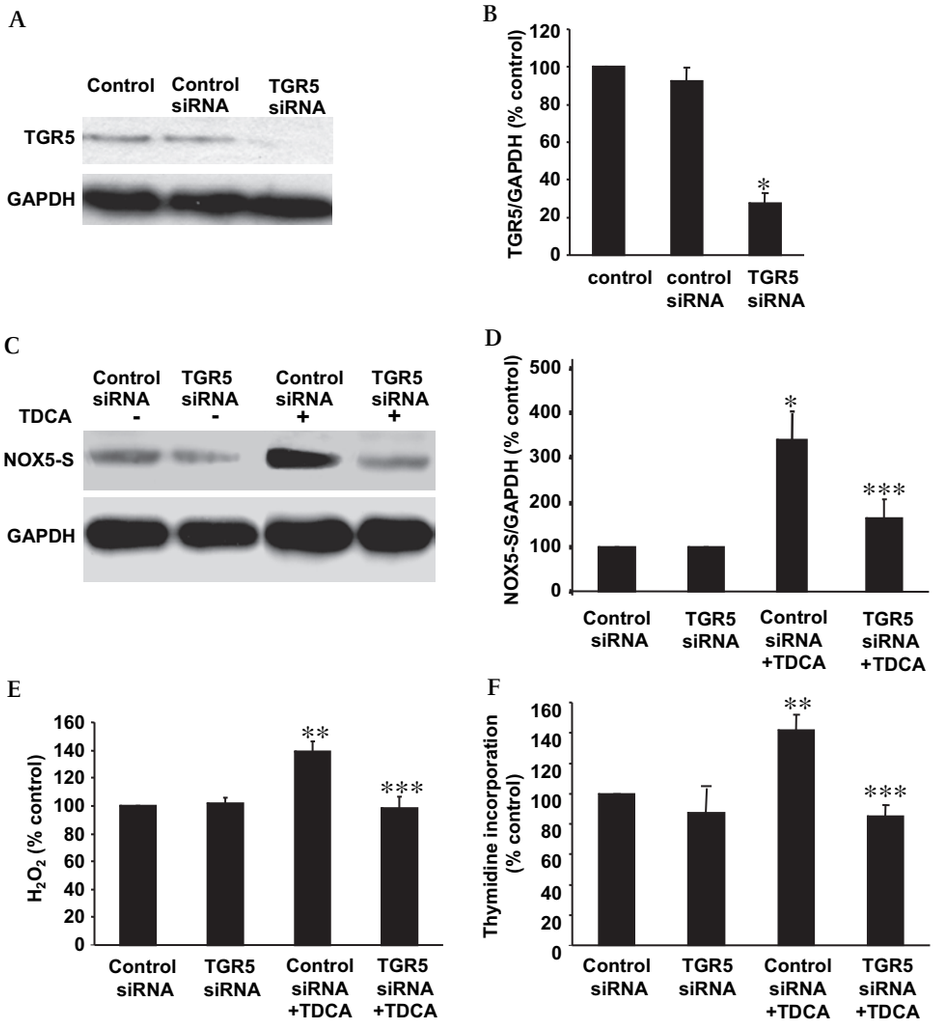

Figure 5 Role of TGR5 in taurodeoxycholic acid (TDCA)-induced NOX5-S expression in FLO oesophageal adenocarcinoma (OA) cells. (A) Western blot analysis and (B) summarised data showed that transfection with TGR5 small interfering RNA (siRNA) significantly decreased TGR5 protein expression in FLO cells, indicating that TGR5 siRNA effectively knocked down TGR5 protein expression (n=3). (C) Western blot analysis and (D) summarised data showed that knockdown of TGR5 significantly decreased TDCA-induced NOX5-S expression. (E) Knockdown of TGR5 significantly decreased TDCA-induced H_2O_2 production. (F) Knockdown of TGR5 significantly decreased TDCA-induced thymidine incorporation. The data suggest that TGR5 receptor may mediate TDCA-induced NOX5-S expression, H_2O_2 production and cell proliferation in FLO OA cells. n=3, ANOVA, *p<0.001, **p<0.01, compared with control siRNA group; ***p<0.001, compared with control siRNA+TDCA group.

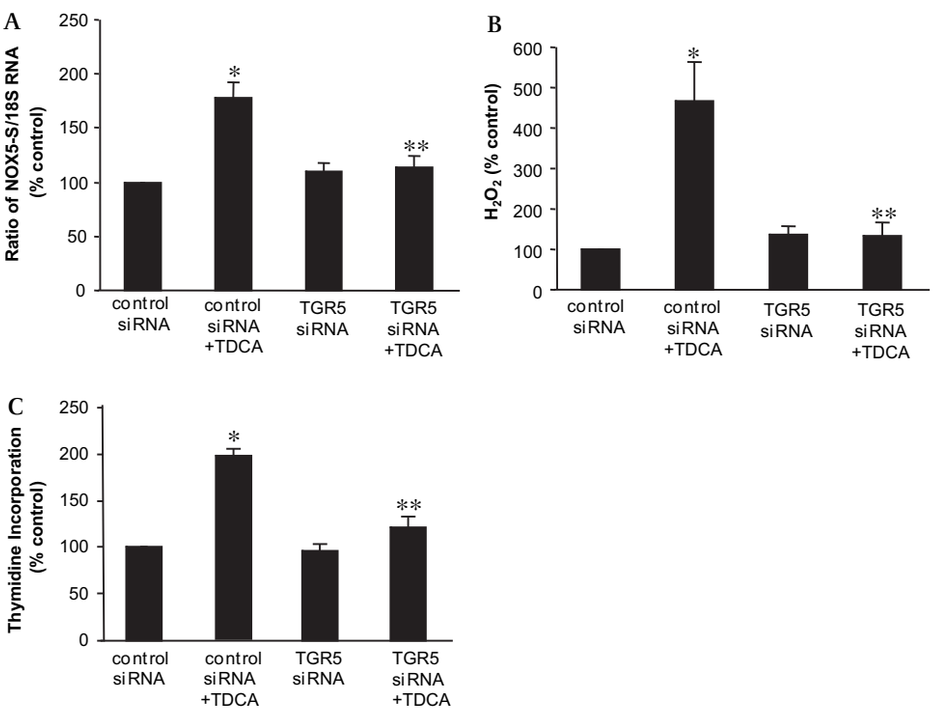

Figure 6 Role of TGR5 in taurodeoxycholic acid (TDCA)-induced NOX5-S expression in BAR-T cells. (A) Real time PCR data showed that knockdown of TGR5 significantly decreased TDCA-induced NOX5-S expression. (B) Knockdown of TGR5 significantly decreased TDCA-induced H_2O_2 production. (C) Knockdown of TGR5 significantly decreased TDCA-induced thymidine incorporation. The data suggest that TGR5 receptor may mediate TDCA-induced NOX5-S expression, H_2O_2 production and cell proliferation in BAR-T cells. n=3, ANOVA. *p<0.01, compared with control siRNA; **p<0.01, compared with control siRNA+TDCA.

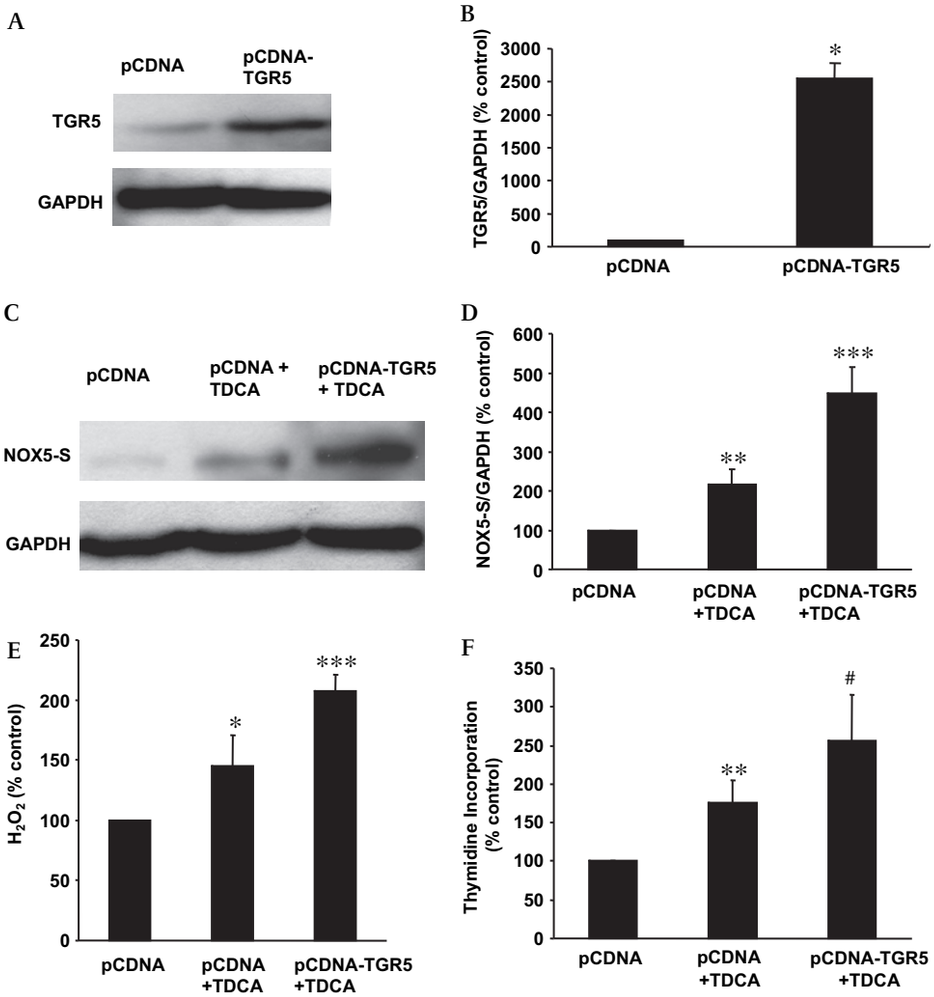

Figure 7 NOX5-S expression and thymidine incorporation in FLO oesophageal adenocarcinoma (OA) cells with TGR5 overexpression. (A) Western blot analysis and (B) summarised data showed that TGR5 protein was successfully overexpressed in FLO cells after transfection with pCDNA3.1-TGR5 (pCDNA-TGR5). (C) Western blot analysis and (D) summarised data showed that overexpression of TGR5 receptor significantly increased TDCA-induced NOX5-S expression, (E) H_2O_2 production and (F) thymidine incorporation. The data suggest that TDCA-induced increase in NOX5-S expression, H_2O_2 production and cell proliferation may depend on activation of TGR5 receptor. n=3, ANOVA. *p<0.01, **p<0.05, compared with control plasmid pCDNA3.1 (pCDNA); ***p<0.001, #p<0.05, compared with pCDNA3.1 (pCDNA)+TDCA group.

siRNA to knock down Gαq and Gαi3 expression, respectively. Gαq and Gαi3 siRNA significantly decreased its corresponding protein expression (figure 9A,B) and (figure 10A,B) 48 h after transfection. Knockdown of Gαq protein expression with Gαq siRNA significantly decreased NOX5-S expression (figure 9C,D), H_2O_2 production (figure 9E) and thymidine incorporation (figure 9F) in response to TDCA stimulation in FLO cells. However, knockdown of Gαi3 protein did not affect NOX5-S expression (figure 10C,D),

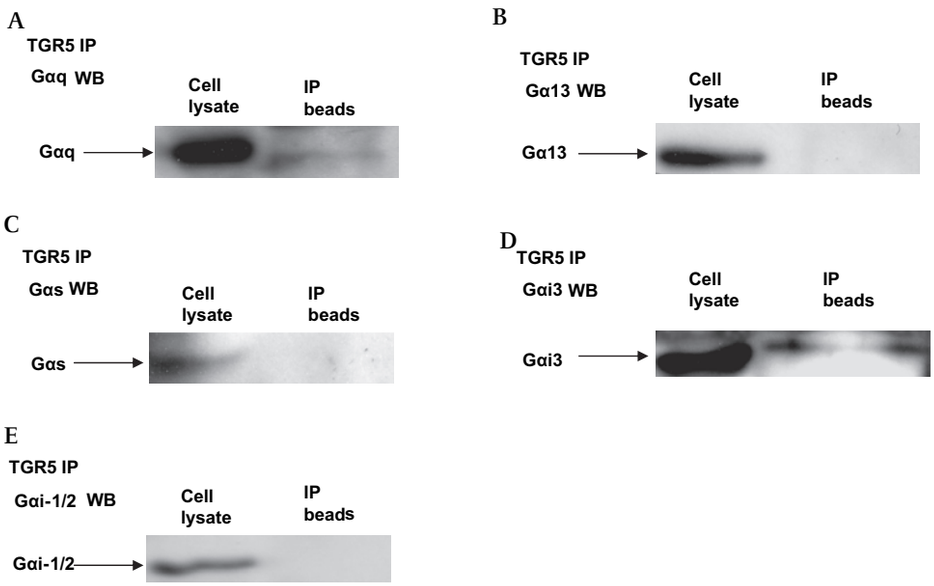

Figure 8 Immunoprecipitation of G proteins with TGR5 antibody from FLO cell lysate. Cell extracts were immunoprecipitated with an antibody against TGR5, followed by western blot analysis using the indicated antibodies. A typical example of western blot analysis of three experiments (A–E) showed that Gαq and Gαi3 protein were immunoprecipitated with TGR5 antibody from the FLO cell lysate, indicating that TGR5 receptor may be coupled with Gαq and Gαi3 protein in FLO oesophageal adenocarcinoma cells.

H_2O_2 production (figure 10E) and thymidine incorporation (figure 10F) induced by TDCA treatment. The data suggest that TDCA-induced NOX5-S expression and H_2O_2 production may be mediated by Gαq proteins, but not Gαi3 proteins.

DISCUSSION

ROS may play an important role in the development of OA since levels of ROS are increased in BO[31 32] and OA.[33 34] ROS may cause damage to DNA, RNA, lipids and proteins, which may result in increased mutation and altered functions of enzyme and proteins (eg, activation of oncogene products and/or inhibition of tumour suppressor proteins).[33 35] However, the sources of ROS in these conditions have not been well defined.

Low levels of ROS, seen in non-phagocytic cells, were thought to be byproducts of aerobic metabolism. More recently, however, superoxide-generating homologues of phagocytic NADPH oxidase catalytic subunit gp91^{phox} (NOX1, NOX3–NOX5, DUOX1, DUOX2) and homologues of other subunits (p41^{phox} or NOXO1, p51^{phox} or NOXA1) have been found in several cell types,[29 36 37] suggesting that ROS generated in these cells may have distinctive cellular functions related to immunity, signal transduction and modification of the extracellular matrix. NOX5

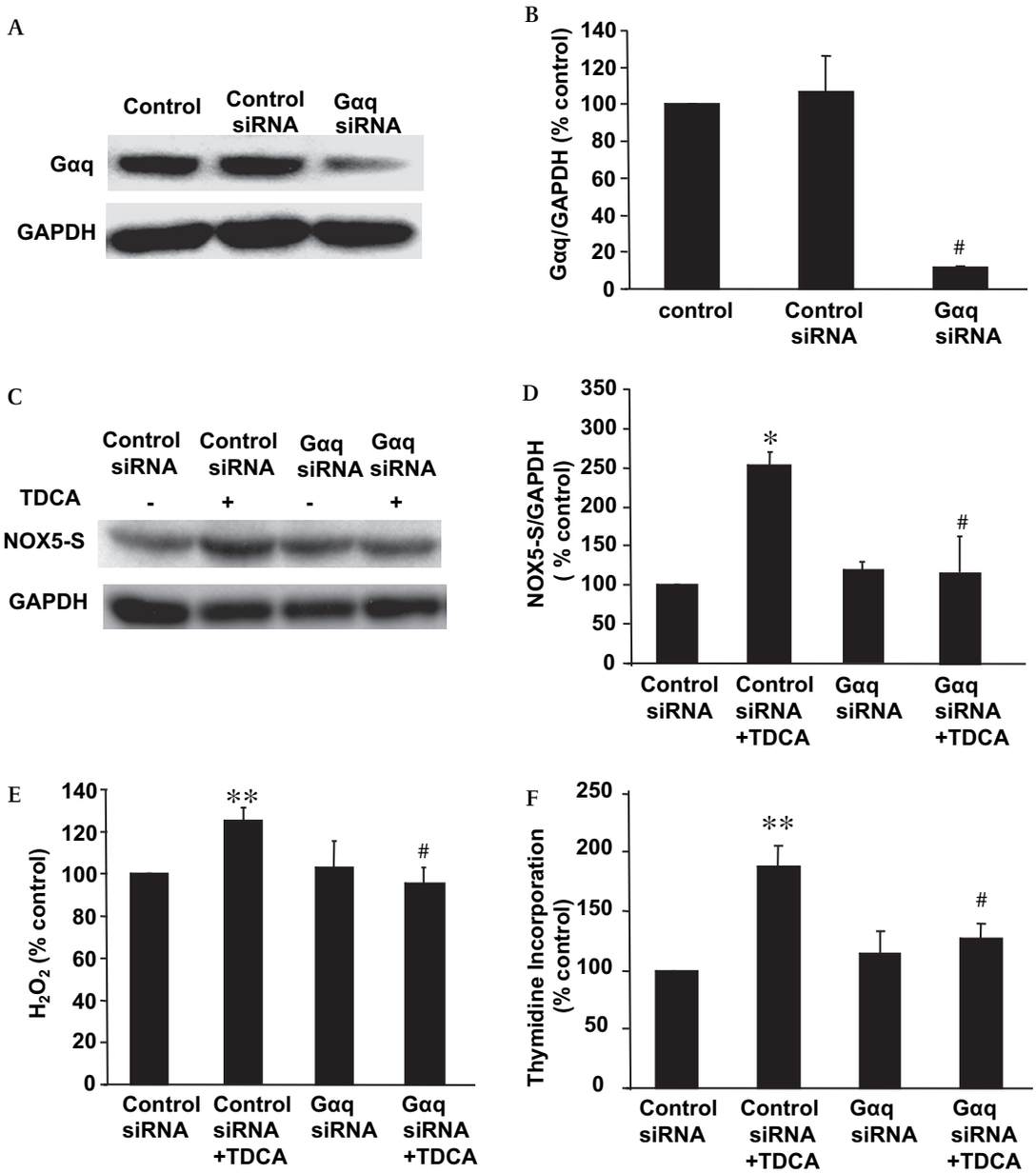

Figure 9 NOX5-S expression after downregulation of Gαq protein. (A) Western blot analysis and (B) summarised data showed that transfection with Gαq small interfering RNA (siRNA) significantly decreased Gαq protein expression in FLO cells, indicating that Gαq siRNA effectively knocked down Gαq protein (n=3). (C) Western blot analysis and (D) summarised data showed that knockdown of Gαq protein significantly decreased taurodeoxycholic acid (TDCA)-induced NOX5-S expression, (E) H_2O_2 production and (F) thymidine incorporation. The data suggest that Gαq proteins may contribute to TDCA-induced increase in NOX5-S expression, H_2O_2 production and cell proliferation in FLO oesophageal adenocarcinoma cells. n=3, differences between different groups were tested using ANOVA. *$p<0.001$, **$p<0.05$, compared with control siRNA group; #$p<0.01$, compared with control siRNA+TDCA group.

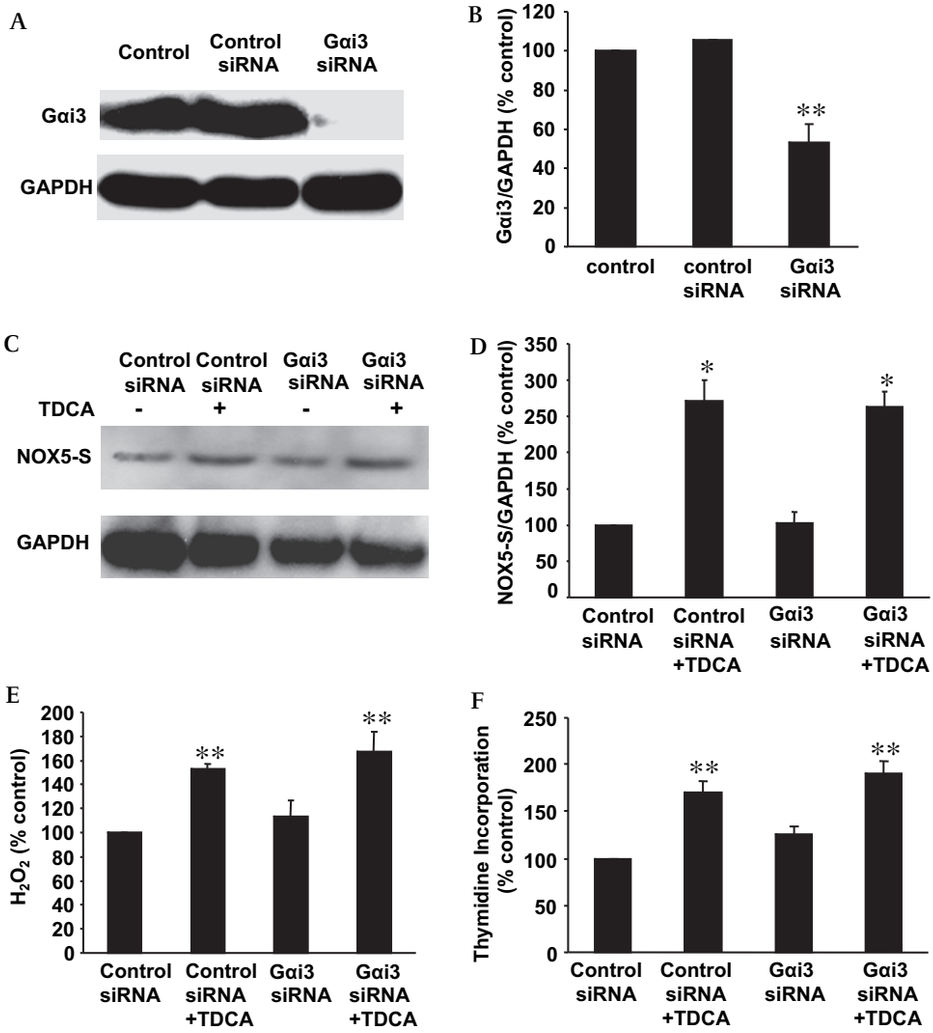

Figure 10 NOX5-S expression after down-regulation of Gαi3 protein. (A) Western blot analysis and (B) summarised data show that transfection with Gαi3 small interfering RNA (siRNA) significantly decreased Gαi3 protein expression in FLO oesophageal adenocarcinoma (OA) cells, indicating that Gαi3 siRNA effectively downregulated Gαi3 protein expression (n=3). (C) Western blot analysis and (D) summarised data showed that knockdown of Gαi3 protein did not significantly affect TDCA-induced increase in NOX5-S expression, (E) H_2O_2 production and (F) thymidine incorporation. The data suggest that TDCA-induced increase in NOX5-S expression, H_2O_2 production and cell proliferation may not be mediated by activation of Gαi3 in FLO OA cells. n=3, differences among different groups were tested using ANOVA. *$p<0.05$, **$p<0.01$, compared with control siRNA group. There was no statistically significant difference between the control siRNA+TDCA group and the Gαi3 siRNA+TDCA group.

has five isoforms: α, β, δ, γ and NOX5-S.[29 38] NOX5 α, β, δ and γ have EF-hand (a helix-loop-helix structural domain) motifs at its N-terminal,[29] whereas NOX5-S does not.[39]

We have shown that NOX5-S mRNA is significantly higher in Barrett's tissues with high-grade dysplasia than in tissues without dysplasia. In this study we found that NOX5-S is the major isoform of NADPH oxidase in FLO OA cells (figure 1A,B). We have previously shown that acid-induced H_2O_2 production is mediated by the NADPH oxidase NOX5-S and that acid-induced NOX5-S expression depends on an increase in intracellular calcium and activation of cyclic AMP response element binding protein. Overproduction of ROS, derived from upregulation of NOX5-S, increases cell proliferation and decreases apoptosis.[4] In this study, we also found that TDCA significantly increased NOX5-S expression and H_2O_2 production in FLO and BAR-T cells (figures 2B–D and 3A,B). ROS production induced by 24 h treatment with bile acids may be mediated by both activation and upregulation of NOX5-S, whereas ROS production caused by short-term exposure to bile acids may be due to activation of NOX5-S only. The mechanisms of NOX5-S activation are not known and this needs to be further explored.

Since bile acids have been implicated in the development of OA in a rat model of BO,[13 14 40] we studied whether bile acids upregulate NOX5-S expression and increase cell proliferation in OA cells. Bile acids, a group of structurally diverse molecules that are primarily synthesised in the liver, are the major components of bile. Besides their well-established roles in dietary lipid absorption and cholesterol homoeostasis, it has recently emerged that bile acids are also signalling molecules in cell metabolism and signal transduction.[21 41] TDCA has been reported to be one of the major bile acids in the refluxate of patients with BO,[42] and is more toxic than primary bile acids, thus we used TDCA in our studies. Although reflux episodes are usually measured in minutes or hours, particularly in long-segment BO and in the supine position, the percentage of the total time during which the pH is <4 ranges from 10.0% to 46.0% in patients with BO[42]—that is, oesophageal mucosa is exposed to the refluxate for 2.4–11.0 h a day. It is possible that a low concentration of bile acids is present in the mucus layer that covers the surface of metaplastic cells for a much longer time after reflux episodes. In addition, it has been reported that short-term bile acid treatment does not alter cell proliferation in BAR-T cells.[43] Therefore, 24 h treatment with TDCA was used in our studies.

We found that a low dose of TDCA significantly increased cell proliferation in FLO and BAR-T cells (figures 2A, 3C and 6C). This result is consistent with other reports showing that bile acids increase cell proliferation in a rat intestinal epithelial line IEC6.[44 45] The concentrations of bile acids in the micromolar range are commonly found in the refluxate of patients with BO,[42] but metaplastic cells may be exposed to much lower

concentrations of bile acids owing to the mucus layer that covers the surface of these cells. This notion is implicated by an in vivo study showing that the interstitial pH is conserved in normal rat oesophagus when the luminal pH is reduced to 1.0.[46] During the process of oesophageal tumourigenesis, cells become hyperproliferative, independently of exogenous mitogenic stimuli, resistant to growth-inhibitory signals and resistant to apoptosis.[47] Therefore, 10^{-11} M TDCA which increases cell proliferation was used in our study.

The mechanisms of bile acid-induced increase in cell proliferation are poorly understood. TDCA-induced cell proliferation was significantly decreased by knockdown of NOX5-S expression with NOX5-S siRNA in FLO OA and BAR-T cells (figures 2E and 3C), suggesting that the TDCA-induced increase in cell proliferation may depend on overexpression of NOX5-S and overproduction of H_2O_2 in FLO and BAR-T cells. This is consistent with other reports showing that blockade of NOX5 inhibits cell proliferation and increases apoptosis in the prostate cancer cell line DU145[28] and that blockade of NOX4 by transfection of NOX4 antisense oligo decreases melanoma cell proliferation.[48]

A novel G protein-coupled receptor TGR5 has been shown to mediate the effects of bile acids as a cell-surface receptor.[30] TGR5 receptor is abundantly expressed in human monocytes and macrophages, and participates in the regulation of cell metabolism.[49 50] Primary bile acids (cholic acid, taurocholic acid and glycocholic acid) and secondary bile acids (deoxycholic acid, taurodeoxycholic acid, glycodeoxycholic acid and taurolithocholic acid) have been reported to be present in the refluxate of patients with BO.[42] All these bile acids have been shown to bind to TGR5 receptors.[30] Primary bile acids induce cyclic AMP production via activation of TGR5 much more weakly than secondary bile acids. Deoxycholic acid, taurodeoxycholic acid and glycodeoxycholic acid have similar strength in inducing cyclic AMP production.[30] We found that TGR5 receptors are present in FLO cells (figure 4A) and that TGR5 expression levels were significantly greater in OA tissues than in normal oesophageal mucosa or Barrett's mucosa (figure 4B–D), suggesting that TGR5 may have an important role in the development of OA. In addition, we found that TGR5 receptors may mediate TDCA-induced NOX5-S expression, H_2O_2 production and an increase in cell proliferation since (*a*) knockdown of TGR5 expression with TGR5 siRNA remarkably decreased TDCA-induced increase in NOX5-S expression (figures 5C,D and 6A), H_2O_2 production (figures 5E and 6B) and cell proliferation (figures 5F and 6C) in FLO OA and BAR-T cells; (*b*) TGR5 overexpression significantly increased NOX5-S expression, H_2O_2 production and cell proliferation in response to TDCA treatment in FLO cells (figure 7C–F).

Which G proteins are coupled to TGR5 receptor is not fully understood. In CHO cells, TGR5 receptor may be coupled to Gαs protein.[30] In FLO cells we found that Gαq and Gαi3 proteins are coupled to the TGR5 receptor (figure 8) and that Gαq proteins, but not Gαi3, may mediate TDCA-induced NOX5-S expression and H_2O_2 production. These data suggest that one receptor may be coupled to more than one G protein and that only Gαq is involved in TDCA-induced increase in NOX5-S expression and cell proliferation. This finding is consistent with our previous finding in the lower oesophageal sphincter where leukotriene receptor is coupled to Gαq and Gαi3 proteins, but only the Gαq protein mediates leukotriene D4-induced contraction.[51]

In conclusion, the TGR5 receptor is overexpressed in OA tissues. A TDCA-induced increase in cell proliferation depends on upregulation of NOX5-S expression. TDCA-induced NOX5-S expression is mediated by activation of the TGR5 receptor and Gαq protein in OA cells. Our data may provide potential targets to prevent and/or treat Barrett's OA.

Summary box

- Mechanisms of the progression from Barrett's oesophagus to oesophageal adenocarcinoma are not fully understood. Bile acids may have an important role in this progression.
- In animal models, diversion of duodenal contents into the lower oesophagus leads to oesophageal adenocarcinoma.[12–14]
- Reflux of bile acids into the oesophagus causes short-term damage to the mucosa and also induces long-term oxidative stress and cellular DNA damage.[15 16]
- In this study a novel bile acid receptor TGR5 and a novel NADPH oxidase NOX5-S were identified in the oesophageal adenocarcinoma cell line FLO.
- TGR5 mRNA and protein levels were significantly higher in oesophageal adenocarcinoma tissues than in normal oesophageal mucosa or Barrett's mucosa.
- Bile acid taurodeoxycholic acid-induced increase in cell proliferation may depend on activation of the TGR5 receptor, Gαq protein and NOX5-S.
- Our data may provide potential targets to prevent and/or treat Barrett's oesophageal adenocarcinoma.

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