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Proanthocyanidins mitigate bile acid-induced changes in GSTT2 levels in a panel of racially diverse patient-derived primary esophageal cell cultures

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Abstract

Persistent and symptomatic reflux of gastric and duodenal contents, known as gastroesophageal reflux disease (GERD), is the strongest risk factor for esophageal adenocarcinoma (EAC). Despite similar rates of GERD and other risk factors across racial groups, EAC progression disproportionately impacts Caucasians. We recently reported that elevated tissue levels of the detoxification enzyme GSTT2 in the esophagi of Blacks compared to Caucasians may contribute protection. Herein, we extend our research to investigate whether cranberry proanthocyanidins (C-PAC) mitigate bile acid-induced damage and GSTT2 levels utilizing a racially diverse panel of patient-derived primary esophageal cultures. We have shown that C-PACs mitigate reflux-induced DNA damage through GSTT2 upregulation in a rat esophageal reflux model, but whether effects are recapitulated in humans or differentially based on race remains unknown. We isolated normal primary esophageal cells from Black and Caucasian patients and assessed GSTT2 protein levels and cellular viability following exposure to a bile acid cocktail with and without C-PAC treatment. Constitutive GSTT2 levels were significantly elevated in Black (2.9-fold) compared to Caucasian patients, as were GSTT2 levels in Black patients with GERD. C-PAC treatment induced GSTT2

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AUTHOR CONTRIBUTIONS

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Howell, Andrew C. Chang, and Laura A. Kresty. *Performed the research:* Katherine M. Weh. *Data analysis:* Katherine M. Weh and Jennifer L. Clarke. *Wrote the paper:* Katherine M. Weh and Laura A. Kresty. *Data interpretation an critical revisions:* Katherine M. Weh, Danielle K. Turgeon, Joel H. Rubenstein, Jennifer L. Clarke, Amy B. Howell, Andrew C. Chang and Laura A. Kresty. All authors read and approved the final manuscript.

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levels 1.6-fold in primary normal esophageal cells. GSTT2 induction by C-PAC was greatest in cells with constitutively low GSTT2 expression. Overall, C-PAC mitigated bile-induced reductions of GSTT2 and subsequent loss of cell viability regardless of basal GSTT2 expression or race. These data support that C-PAC may be a safe efficacious agent to promote epithelial fitness through GSTT2 induction and in turn protect against bile acid-induced esophageal injury.

Keywords

cranberry proanthocyanidins; gastroesophageal reflux disease (GERD); glutathione s-transferase theta 2 (GSTT2); patient derived primary esophageal cultures; race

1 | INTRODUCTION

Gastroesophageal reflux disease (GERD) is a chronic condition whereby bile and injurious stomach acids back up into the esophagus and burden over 60 million Americans.[1,2] The repeated mucosal epithelium exposure to gastric refluxate in patients with GERD can lead to epithelial barrier disruption, immune cell migration, and inflammation.[3] Recent epidemiological studies report that Black and Caucasian patients have similar prevalence of GERD and other risk factors yet progression to Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) disproportionately impacts Caucasians.[4–6] Our research group recently explored whether there may be protective factors that contribute to this phenomenon and glutathione s-transferase theta 2 (GSTT2) was identified as differentially expressed in the esophagus based on race.[7]

GSTT2 is a member of the GST family of phase II detoxification enzymes with specific roles in detoxification of xenobiotics and protection against oxidative stress, particularly lipid peroxidation reactions.[8–13] GSTT2 is significantly reduced at both the transcript and protein level in the normal esophageal epithelium of Caucasians compared to Black patients with GERD.[7] These findings support that GSTT2 contributes toward cellular protection in Black patients with reflux and may influence the racial disparity in EAC progression. We confirmed that two previously identified genomic variants at the GSTT2 locus, a 37-kb deletion and a 17-bp promoter duplication were associated with reduced expression of GSTT2.[7,14,15] Using immortalized cell lines, siRNA knockdown of GSTT2 showed increased susceptibility to DNA damage, as indicated by elevated phospho-H2AX^{Ser139} levels upon hydroperoxide challenge.[7]

Excitingly, GSTT2 was induced in immortalized normal (Het-1A) and BE-derived (CP-A) cell lines utilizing a cranberry proanthocyanidin-rich extract (C-PAC). C-PACs are promising nontoxic compounds that exert pleiotropic antibacterial, antioxidant, anti-inflammatory, and anticancer activities.[16–22] In immortalized dysplastic BE and EAC cell lines, C-PAC modulates reactive oxygen species inducing cell death[22]; however, C-PAC effects in patient-derived primary esophageal cell cultures isolated from normal or GERD patients without BE had not been assessed. We previously demonstrated that a rat model of acid-induced reflux induced a 4.4-fold decrease in GSTT2 level and that low-level C-PAC treatment (0.69 mg/day) in the drinking water induced GSTT2 levels 1.9-fold in parallel with reductions in DNA damage based on P-H2AX inhibition.[7] Taken together, these data

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support that CPACs have the capacity to mitigate reflux induced damage in a preclinical model and immortalized esophageal cell lines, but whether these results may translate into positive results in GERD patients or model systems that better capture patient heterogeneity, including patient-derived primary cell cultures is unknown. In this study, we sought to determine if constitutive levels of GSTT2 in Black and Caucasian derived primary cell cultures, were like those observed in esophageal tissue biopsies and whether C-PAC could induce GSTT2 in primary esophageal cell cultures leading to mitigation of bile-acid induced cellular damage.

2 | MATERIALS AND METHODS

2.1 | Protection of human subjects and tissue collection

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the University of Michigan. Patients scheduled for upper gastrointestinal biopsies in the Medical Procedures Unit at the University Hospital at the University of Michigan consented for 2–4 extra biopsies of healthy esophageal squamous epithelial tissue from the upper esophagus. Informed consent was obtained from all subjects involved in the study. Tissues were transported to the laboratory in media on ice and immediately processed into cellular suspensions. A total of 17 patients contributed tissues for primary cell culture generation, including 7 Black and 10 Caucasian patients. Patients self-identified their race as either Black or Caucasian. GERD status was extracted from the medical records.

2.2 | Generation of primary esophageal cell cultures

Primary esophageal cell cultures were generated as previously described.[23,24] Cultures were maintained on irradiated J2–3T3 mouse fibroblasts (30 Gy; 1E5 cells/well; Kerafast) and passaged once confluency reached 70%–80%. Mouse fibroblasts were removed using MACS® magnetic separation with feeder removal microbeads (Miltenyi Biotec). Experiments were performed using cell cultures from passage 7 or below and confirmed to be mycoplasma negative.

2.3 | Evaluation of GSTT2 protein levels

To assess GSTT2 levels, approximately 5E5 cells were lysed in RPPA buffer as previously described[7] and evaluated via Western blot. Following protein quantitation with the Bradford DC assay (Bio-Rad), 15 μg of protein was loaded into 4%–20% Criterion gels (Bio-Rad) and transferred to PVDF. Commercially available antibodies from Santa Cruz Biotechnology for GSTT2 (1:500; sc-514667), HSP60 (1:2000; sc-13115), and GAPDH (1:20,000; sc-47724) were evaluated. Protein levels were normalized to housekeeping proteins as determined using chemiluminescent immunodetection.

2.4 | C-PAC preparation, treatment, and bile acid challenge of primary esophageal cells for viability assays and Western blot analysis

C-PAC was purified using published procedures[19] and dissolved in 100% ethanol before diluting in cell media. For viability assays, cells were plated at 15E3 cells per well into 96-well black walled clear bottom plates (Grenier Bio-one) coated with 8.5% growth

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factor reduced Matrigel (Corning) and incubated at 37°C and 5% CO_2 overnight. Cells were treated with VEH (0.001% ethanol) or C-PAC (50 μg/ml) for 48h. Cell lysates were made by scraping eight wells of each treatment into RPPA buffer, processing for soluble lysates, and assessed by Western blot as described above. Bile acid treatment (BAC) was performed as previously described.[19] Briefly, a bile acid cocktail is prepared from three primary bile salts (taurocholic, glycocholic, glycochenodeoxycholic) and two secondary bile acids (deoxycholic, glycodeoxycholic) and acidified to pH 3.5 to mimic human refluxate. Cells were exposed to 5, 10, or 15 min of BAC to optimize conditions. In C-PAC pre- and/or posttreatment experiments, cells were treated with VEH (0.001% ethanol) or C-PAC (50 μg/ml) for 48h, subsequently treated for 10 min with 0.2 mM BAC pH 3.5 and either received VEH or C-PAC (50 μg/ml). Calcein-AM (Invitrogen) was used to assess cellular viability as previously reported[25] and fluorescence imaging was conducted using SpectraMax® MiniMax™ Imaging Cytometer with excitation/emission wavelengths of 460/535 nm. Data were analyzed using the SoftMax® Pro 7.1 Software with a minimum of 6 wells per treatment; data are expressed as a mean viability percentage ± standard error. Lysates were generated from wells used for the viability assay and GSTT2 protein levels were assessed.

2.5 | Statistical analysis

GraphPad Prism software was used to evaluate statistical differences by condition or treatment. A Studen s *t*-test was applied for pairwise comparisons. Constitutive GSTT2 levels stratified by race and GERD status, as well as viability data were evaluated for statistical significance using one-way ANOVA with Tukey's post hoc test where multiple conditions were assessed. p-values of <0.05 were considered significant. Fisher's exact test was utilized for determining differences in proportions of high and low GSTT2 expressors.

3 | RESULTS AND DISCUSSION

3.1 | Constitutive levels of esophageal GSTT2 differ based on race

We recently reported that constitutive expression of the detoxification enzyme Glutathione S-Transferase Theta 2 (GSTT2) was significantly lower in normal esophageal tissues from Caucasians compared to Black patients, potentially contributing to the increased risk of EAC in Caucasians.[7] Herein, we generated primary normal esophageal cell lines from seventeen patients to evaluate whether patient-derived primary esophageal cultures reflect tissue level results, in turn providing a valuable and translationally relevant model to research patient-level changes in response to risk factors and protective agents while still capturing unique patient characteristics.

Constitutive GSTT2 protein levels in the seventeen cell cultures are presented in Figure 1. GSTT2 levels varied among 7 Black (Figure 1A) and 10 Caucasian derived-cultures (Figure 1B), with constitutive GSTT2 levels significantly 2.9-fold higher among Black compared to Caucasian derived-cultures (Figure 1C; $p = 0.003$, Studen s *t*-test). In addition, 86% of Black patients were high expressors compared to 30% of Caucasian patients based upon being above the median for this population value ($p = 0.03$; Fisher's exact test). These data confirm that GSTT2 protein levels in primary normal esophageal cell cultures from Black

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patients are significantly higher compared to Caucasians which aligns with our previous tissue-based results in a different patient cohort.[7] Cell cultures isolated from Black patients with GERD have significantly 3.79-fold and 4.42-fold higher levels of GSTT2 compared to cells derived from Caucasians with or without GERD, respectively (Figure 1C; $p < 0.01$, one-way ANOVA, Tukey's post hoc test), again aligning with our earlier report in tissues.[7] Although nonsignificant, cultures derived from Blacks with GERD had a 1.98-fold increase in GSTT2 level over cultures from Blacks without GERD; whereas, Caucasian cultures did not differ. These results lead us to hypothesize that Black patients with GERD mount a strong detoxification response via GSTT2 while Caucasians do not. This in turn may increase susceptibility to reflux-induced esophageal damage and risk for progression to BE and EAC. It is also conceivable that a differential capacity to activate a metabolic response upon exposure to refluxate contributes more than constitutive differences in GSTT2 between Black and Caucasian patients, but additional mechanistic research targeting this question will need to be conducted to address such relative contributions.

3.2 | C-PAC induces GSTT2 in normal primary esophageal cells

Given differential GSTT2 protein level by race and GERD status, we next sought to determine if GSTT2 was inducible by C-PAC based on prior positive results in preclinical models.[7,16–22] Primary normal esophageal cultures were pretreated with C-PAC (50 μg/ml) for 48 h and assessed for GSTT2 via Western blot. As shown in Figure 2, C-PAC pretreatment induced GSTT2 in all patient-derived cell cultures, with a significant mean induction of 1.61-fold compared to vehicle treatment ($p < 0.05$; Studen s t-test; range 1.1- to 2.0-fold ± 0.32). C-PAC induced GSTT2 levels in cell cultures with both high and low constitutive levels, derived from Black and Caucasian patients, respectively. However, induction was greatest in cells with low basal or constitutive expression with an average GSTT2 induction of 1.73-fold in C-PAC treated cells compared to vehicle cells (Figure 2; Patients 8, 9, 11, and 15). These results show that GSTT2 is inducible by C-PAC in normal primary esophageal cell cultures, especially in low expressors who may not activate detoxification mechanisms readily. Importantly, the results also support that the patient-derived primary culture system allows for patient heterogeneity to be captured. For example, patient 6 had the highest constitutive level of GSTT2 with C-PAC treatment resulting in a modest 1.12-fold induction suggesting that inducibility may have a threshold or not be needed in individuals with constitutively high GSTT2 levels. Finally, these results are consistent with GSTT2 induction in immortalized normal Het-1A esophageal cells and Barrett's metaplastic CP-A cell line, with similar levels of induction as we previously reported.[7] To date, few agents are documented to modulate GSTT2. Others have shown that apple polyphenols and in vitro-fermented raw and roasted walnuts inhibit growth and induce cell death through GSTT2 expression in colon cancer cells.[26–28] Our study is the first, to our knowledge, to examine induction of GSTT2 protein levels in normal, non-immortalized, patient-derived primary cell cultures and to evaluate C-PAC induction capacity in such a model system. Butyrate has also been reported to induce GSTT2, raising the question of whether polyphenols that alter microbiome profiles may act in a similar manner.[11]

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3.3 | Acidified bile acid exposure modulates GSTT2 levels and viability of normal primary esophageal cells

To mimic GERD in an experimental model, normal primary esophageal cells were treated with an acidified bile acid cocktail (BAC, pH 3.5) for 5, 10, and 15 min and assessed 24 h later for cellular viability and GSTT2 levels. BAC treatment results in significant loss of cell viability linked to cell seeding density and time of BAC exposure (Figure 3A, Patient 8). In agreement with the viability results, GSTT2 protein levels were reduced in a time and cell seeding density manner 24 h following BAC exposure (Figure 3B). Levels of GSTT2 were similar in 15E3 and 20E3 cells/well at 5- and 10-min BAC exposure but decreased dramatically following 15 min BAC exposure. Overall, results show that a bile cocktail mimicking human refluxate reduces GSTT2 and induces cell death. Intriguingly, these results were derived from a GERD patient (Figure 2, Patient 8) with the lowest constitutive level of GSTT2 in the population; yet, C-PAC induced GSTT2 in this patient. Thus, GSTT2 inducing agents may prove efficacious at mitigating bile-induced injury and in turn decrease the risk of progression to Barretts preneoplasia or EAC.

3.4 | C-PAC protects normal primary esophageal cells from bile-induced injury through GSTT2 induction

Considering that C-PAC induces GSTT2, while acute bile acid exposure reduces GSTT2 levels, we next determined if C-PAC treatment could mitigate bile-induced loss of GSTT2 and subsequen cellular damage and death. We utilized histologically normal primary esophageal cultures isolated from two GERD patients, a Caucasian with the lowest GSTT2 levels and a Black patient with the highest GSTT2 levels, Patient 8 and Patient 6, respectively. As shown in Figure 4A, BAC treatment (10 min) reduced GSTT2 levels by 60% and C-PAC strongly mitigated bile-induced inhibition of GSTT2. C-PAC pretreatment followed by BAC exposure restored GSTT2 levels to baseline compared to a 0.4-fold reduction in bile-treated cells. Pre-and posttreatment with C-PAC offered additional gain in GSTT2 levels to 1.4-fold in this low expressor. Figure 4B shows GSTT2 levels from the highest expressor, Patient 6. BAC treatment decreased GSTT2 protein by 30% and pretreatment with C-PAC resulted in 10% mitigation of loss. However, pretreatment with C-PAC, followed by BAC treatment and posttreatment with C-PAC increased GSTT2 levels to pretreatment levels (1.1-fold), even though no additional protective benefit was observed in cellular viability (Figure 4C,D). Finally, pretreatment and posttreatment with C-PAC in the absence of BAC resulted in the highest level of GSTT2 protein levels in both primary cell lines, suggesting that repeated C-PAC exposure increases GSTT2 protein levels, and that while inducible, GSTT2 levels likely reach a threshold after which there is no additional benefit toward viability.

Exposure of BAC for 10 min resulted in a 63.2% decrease in viability (Figure 4C). Importantly, pretreatment with C-PAC alone did not affect cell viability supporting that is not toxic at the concentration tested which is a behaviorally achievable level from dietary sources. Moreover, C-PAC protects cells from death due to BAC as evidenced by significantly increasing viability from 34.8% to 71.6% ($p < 0.0001$; ANOVA, Tukey's post hoc test). Pretreatment with C-PAC, followed by BAC treatment and posttreatment with

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C-PAC did not have any additional viability benefit, paralleling Western blot results for GSTT2 levels.

In summary, our data support that C-PAC is efficacious for mitigating bile-induced inhibition of GSTT2 and subsequent death of histologically normal patient-derived primary esophageal cell cultures. Moreover, our results parallel earlier tissue-based findings reporting that esophageal tissue levels of GSTT2 are elevated in Black patients compared to Caucasians, both in GERD and non-GERD patients.[7] Our results extend these findings to patient-derived primary esophageal cell cultures which reveal that the protective effects of GSTT2 likely extend beyond constitutive expression differences to include differential sensitivity to bile acids and activation of detoxification mechanisms via GSTT2. Primary esophageal cells derived from Black patients with GERD showed significantly elevated GSTT2 levels suggesting they mount a stronger toxicological response when faced with the insult of reflux and our in vitro data supports this finding. Epidemiological studies report similar levels of GERD between the two groups; yet, Caucasians have higher levels of erosive esophagitis, higher rates of cancer progression, and thus may have a weaker response to injury or insult or an inability to repair the damage.[5,29] Further research employing larger panels of primary cell lines and genetic approaches are warranted to comprehensively understand the requisite role GSTT2 plays in esophageal detoxification and protection. Patient-derived esophageal cell cultures show promise for modeling complex patient responses, including phase II enzyme induction to protect esophageal cells against bile acid injury. This platform captures patient heterogeneity and allows for prospectively evaluating various risk factors as well as protective agents in the context of race or other patient characteristics. In conclusion, results support that C-PAC may be a safe efficacious agent to promote epithelial fitness through GSTT2 induction and in turn protect against bile acid-induced esophageal injury and progression to premalignancy or cancer.

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DATA AVAILABILITY STATEMENT

The data that support the study findings are available from the corresponding author upon reasonable request.

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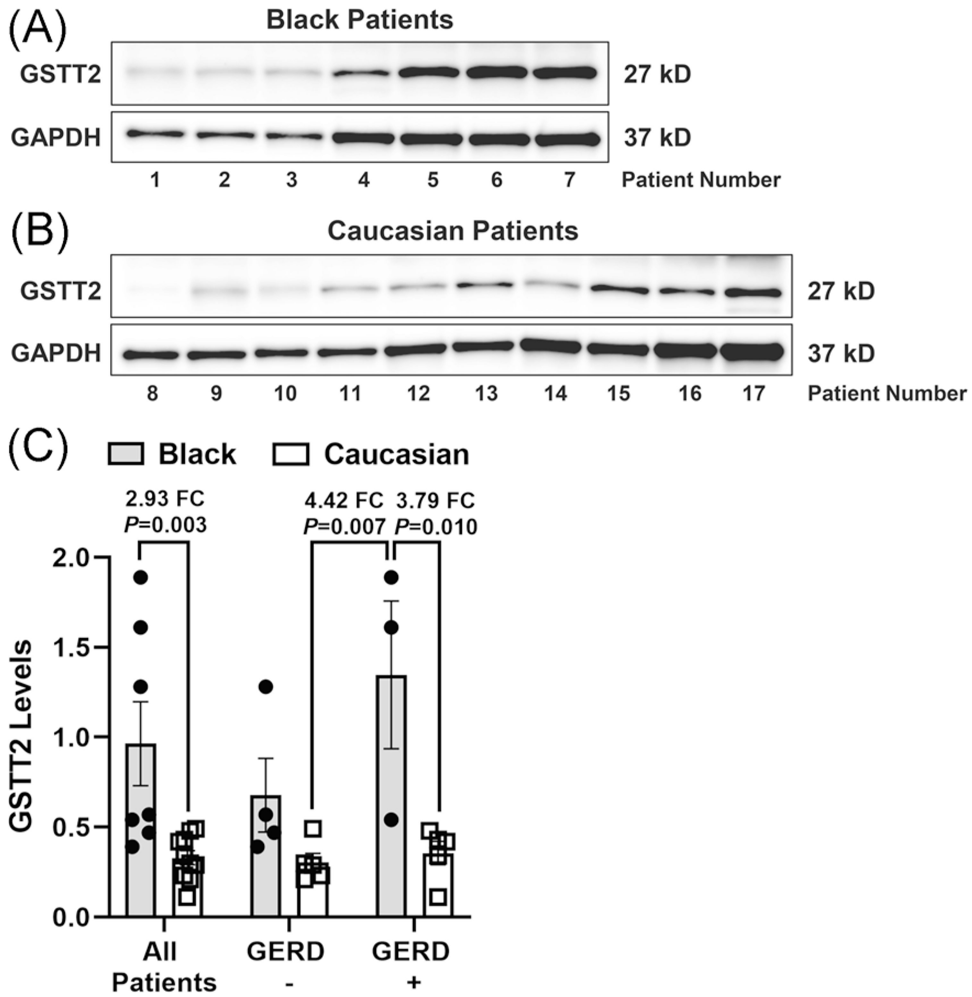

FIGURE 1.

Constitutive GSTT2 levels in primary normal esophageal cells are elevated in Black compared to Caucasian patients. Seventeen cultures were examined for constitutive GSTT2 protein expression via Western blots. (A) Seven Black and (B) 10 Caucasian derived primary normal cell cultures were evaluated with GAPDH used as the loading control. (C) GSTT2 levels for all patients and patients stratified by GERD status are reported as individual patient-level data. The fold change (FC) calculation is based on the mean expression of GSTT2 in Black compared to Caucasian patients and statistical significance determined based on Students *t*-test, $p < 0.05$ considered significantly different for GSTT2. ANOVA with Tukey's post hoc test was utilized to determine statistical significance between Black and Caucasian patients stratified by GERD status with p-values reported in the graphic

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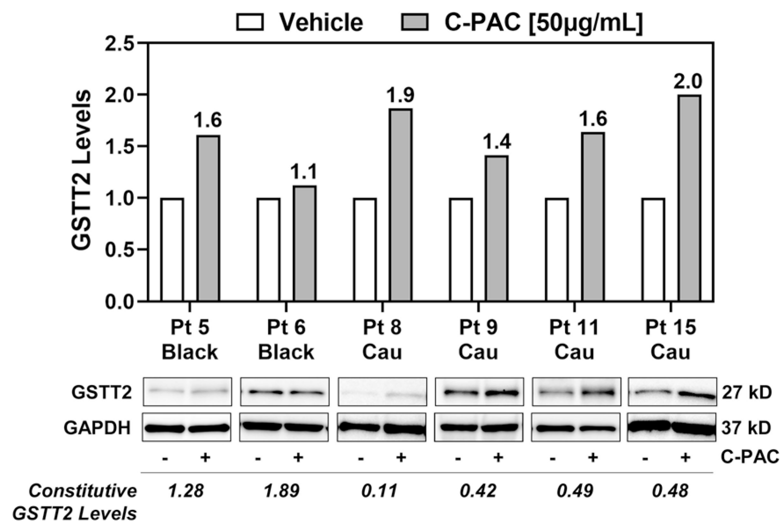

FIGURE 2.

C-PAC induces GSTT2 in primary normal esophageal cells. GSTT2 inducibility in six primary normal esophageal cell cultures by Western blot analysis is displayed for each patient. Fold change (FC) from vehicle values is presented above each gray C-PAC bar. GSTT2 induction was evaluated following a 48 h treatment with C-PAC (50 μg/ml). Expression values were normalized to GAPDH and an FC from VEH was presented. Constitutive GSTT2 levels are denoted below each patient and are based on results obtained in Figure 1

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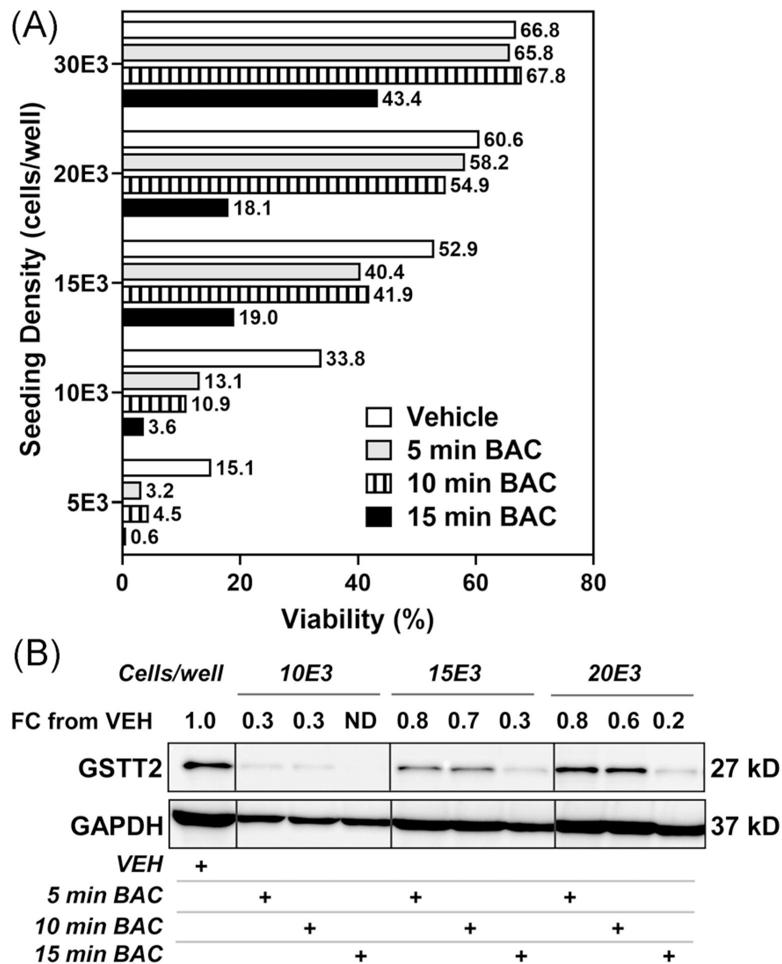

FIGURE 3.

Exposure to an acidified bile acid cocktail inhibits cell viability and reduces GSTT2 levels in a time and cell-density-dependent manner. (A) Viability results of Caucasian Patient 8 primary normal esophageal cells plated at varying densities (5E3, 10E3, 15E3, 20E3, and 30E3 cells/well) and exposed to either vehicle or acidified bile acid cocktail (BAC, pH 3.5) for 5, 10, or 15 min. Viability via Calcein-AM staining occurred 24 h post BAC exposure. Mean viability values are reported to the right of each bar. (B) Lysates were generated from the wells used for the viability assay and evaluated for GSTT2 and GAPDH protein levels by Western blot analysis. Expression values were normalized to GAPDH and a fold change (FC) from VEH was calculated with ImageLab software. Bands with no detectable expression are denoted as ND

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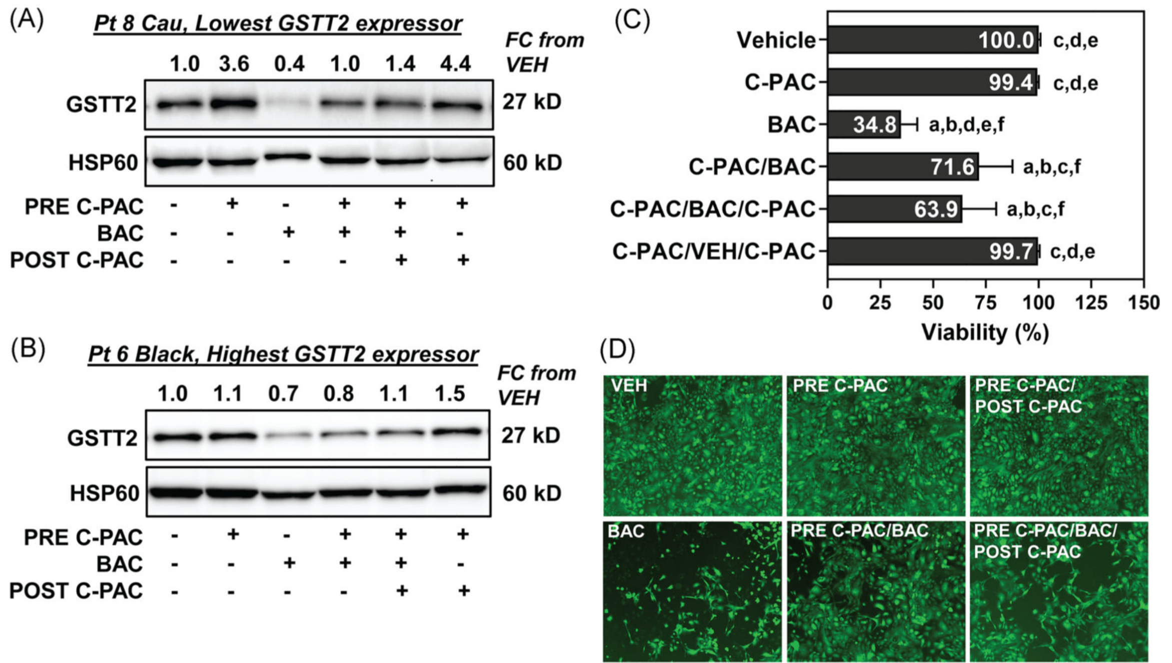

FIGURE 4.

C-PAC protects primary normal esophageal cells from bile acid-induced injury through modulation of GSTT2. (A) Western blot analysis for GSTT2 of lysates generated from Patient 8 (Caucasian; lowest GSTT2 level) following a 48 h pretreatment with C-PAC (50 μg/ml; PRE C-PAC) and subsequently treated for 10 min with BAC, with either media or C-PAC posttreatment (50 μg/mL; POST C-PAC). (B) Western blot analysis of GSTT2 of lysates generated from Patient 6 (Black; highest GSTT2 level) treated the same as in (A). Expression values were normalized to HSP60 and a fold change (FC) from VEH was calculated with ImageLab software. (C) Viability of cells from Patient 6 was assessed using Calcein-AM staining 24 h post BAC treatment. Mean viability of eight wells per treatment is denoted in black bars with error bars for the standard error of the mean. Viability was analyzed by ANOVA with Tukey's post hoc test ($p < 0.05$) where multiple comparisons were assessed. Treatments were significantly different from a = vehicle, b = C-PAC pretreatment (PRE C-PAC), c = BAC, d = C-PAC pretreatment and BAC (PRE C-PAC/BAC), e = C-PAC pretreatment and posttreatment with BAC (PRE C-PAC/BAC/POST C-PAC) and f=C-PAC pretreatment and posttreatment (PRE C-PAC/VEH/POST C-PAC). (D) Representative fluorescent images of Calcein-AM stained Patient 6 primary normal esophageal cells