Research Article

GSTT2, a phase II gene induced by apple polyphenols, protects colon epithelial cells against genotoxic damage

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The potential protective effect of a polyphenol-rich diet for colon carcinogenesis is of great scientific and medical interest. Apples are a main source of polyphenols, and apple juice has been shown to attenuate chemically induced colon carcinogenesis in animal models. In addition to an antioxidant and antiproliferative activity, apple polyphenols have been shown to elevate expression of the phase II gene glutathione S-transferase T2 (*GSTT2*) in colon epithelial cells. We hypothesized that apple polyphenols may thereby provide protection against oxidant-induced DNA damage. Using *GSTT2* promoter constructs and luciferase reporter assays, we found that polyphenolic apple extracts (AE) can directly enhance GSTT2 promoter activity. Comet assays demonstrated that the genotoxicity of the GSTT2 substrate cumene hydroperoxide (CumOOH) was significantly reduced when HT29 colon epithelial cells were pretreated with AE. Overexpression of GSTT2 in HT29 cells significantly reduced CumOOH induced DNA damage, whereas shRNA mediated knockdown of *GSTT2* gene expression resulted in higher damage. Our results causally link GSTT2 levels with protection from genotoxic stress, and provide evidence that the antigenotoxic effects of apple polyphenols *in vitro* are at least in part due to an induction of *GSTT2* expression. Induction of phase II genes may contribute to primary chemoprevention of colon cancer by apple polyphenols.

Keywords: Apple polyphenols / Chemoprevention / Cumene hydroperoxide / Genotoxic stress / GSTT2

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1 Introduction

Malignant alterations in the colon, caused frequently by the typical diet and lifestyle in Western countries [1], can possibly be prevented by a diet rich in vegetables and fruits [2, 3]. These beneficial effects have been partially attributed to secondary plant products, notably polyphenols, which are among the most abundant antioxidant compounds. Apples and the corresponding apple juices, favoured in the Western diet, contain high amounts of phenolic compounds such as hydroxycinnamates, flavan-3-ols, flavonols, dihydrochalcones and anthocyanins [4, 5], and their intake may therefore be beneficial with respect to prevention of colorectal cancer. In animal models of colon carcinogenesis, apple juice and apple polyphenol extracts had indeed a protective effect [6, 7], however, the underlying mechanisms of protection are yet incompletely understood. *In vitro* studies with colon adenoma and carcinoma cell lines have revealed different potential mechanisms relating to the chemopreventive properties of apple polyphenols. Antioxidant activity of apple polyphenols has been shown in several studies, and this activity could also be demonstrated with reconstituted mixtures of pure compounds [8, 9]. Apple polyphenol extracts have also been shown to inhibit proliferation of colon adenoma and colon carcinoma cells, and to induce apoptosis [10, 11]. The antiproliferative activity may be partially mediated by induction of the *PTPRJ* gene (*protein tyrosine*

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Abbreviations: AE, apple extract; **CumOOH,** cumene hydroperoxide; **FCS,** fetal calf serum; **GFP,** green fluorescent protein; **GST,** glutathione S-transferase; **H_2O_2,** hydrogen peroxide

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phosphatase receptor type J), a candidate tumour suppressor gene encoding the protein-tyrosine phosphatase DEP-1 [12]. In addition to the antiproliferative, pro-apoptotic and antioxidative properties of apple ingredients, an induction of phase II gene expression was shown as a further potential mechanism of chemoprevention. Among the phase II enzymes, the glutathione S-transferases (GSTs) comprise one of the most efficient detoxifying enzyme systems in humans. GSTs catalyse the conjugation of electrophiles with glutathione to more hydrophilic and less reactive derivates [13]. The human GSTs of the theta-class GSTT1 and GSTT2, possess activity against a wide range of compounds, including known carcinogens, implicating a possible role in prevention of carcinogenesis [14]. Previous work from our and other laboratories has indicated that GSTT2 may be important for chemoprevention by apple polyphenols. Expression of *GSTT2* was significantly up-regulated in HT29 cells *in vitro* after pre-treatment with an apple polyphenol extract [15]. Moreover, recent data indicated an interaction between a reduced genotoxicity, enhanced antigenotoxicity and modulation of *GSTT2* gene expression in HT29 cells using ileostomy samples collected from human volunteers after intervention with apple juice [16].

Based on these results, we hypothesized that apple polyphenols may provide protection against oxidant-induced DNA damage by increasing expression of GSTT2. By reporter assays we could demonstrate a direct effect of apple polyphenol treatment on *GSTT2* promoter activity. Apple polyphenole extracts (AE) were protective against the genotoxic activity of cumene hydroperoxide (CumOOH), a model substrate of GSTT2 [17, 18]. Further, GSTT2 expression in genetically modified HT29 cell lines was inversely correlated with susceptibility to CumOOH-induced DNA damage.

2 Materials and methods

2.1 Preparation and analysis of the apple polyphenol extracts (AE)

The apple polyphenol extract AE02 was produced from a mixture of apple varieties from the cultivar year 2002. For the AE05, clear apple juice was produced from a composition of cider apples from the crop year 2005, representing a commercially available cider apple juice. The juice production, preparation of a polyphenol extract as well as the analysis of polyphenol composition have been described before [19, 20].

2.2 Cell culture conditions

The human colon adenocarcinoma cells HT29 (American Tissue Culture Collection, Rockville, MD), established in 1964 by Fogh and Tempe [21], were grown in tissue culture flasks with Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) and maintained under sterile conditions in a humidified incubator (37°C, 5% CO_2, 95% humidity). Cells were routinely checked for mycoplasma contaminations using the MycoAlert® Detection Assay (Lonza Biosciences, Verviers, Belgium), and were cultured without antibiotics. For the experiments in this study, cells in passages 15 – 35 were used.

Phoenix amphotropic packaging cells (kindly provided by Dr. Gary Nolan, Stanford, CA, USA) were maintained in DMEM supplemented with 10% FCS. HEK293 cells were grown in DMEM-F12 medium (Invitrogen, Karlsruhe, Germany) containing 10% FCS.

2.3 Luciferase reporter assay for GSTT2 promoter activity

GSTT2 promoter activity in response to AE treatment was determined with a dual luciferase reporter assay. pGL3 constructs containing the firefly luciferase gene under control of the *GSTT2* promoter, were kindly provided by Dr. Paul R. Buckland, University of Cardiff, U.K. [22]. For the experiments in this study, the most prevalent polymorphic variant A was used. The promoterless pGL3 Basic Vector (Promega, Mannheim, Germany) served as control for basal luciferase activity. A plasmid encoding *Renilla* luciferase under control of the thymidine kinase promoter (pRL-TK, Promega, Mannheim, Germany) served for normalization to cell count and transfection efficiency.

HT29 cells were transfected with the *GSTT2* promoter construct (or pGL3-Basic for control) and pRL-TK (ratio 10:1) using Lipofectamine 2000, with a DNA:Lipofectamine ratio of 1:6. After 5 h of transfection, cells were trypsinized and 2×10^4 cells were seeded per well of a 96 well plate (flat bottom, clear, Greiner Bio-One, Frickenhausen, Germany, cat.No 655098). AE treatment was started the next day, either in presence or absence of 100 U/mL catalase (Sigma – Aldrich, Taufkirchen, Germany). After treatment, cells were washed twice with PBS, and lysed in 20 µl Passive Lysis Buffer (Promega, Mannheim, Germany). Luminescence was measured in a LumiStar Galaxy reader (BMG Labtechnologies, Durham, NC, USA) as described in ref. [23]. The firefly luciferase values were normalized to *Renilla* luciferase activity. Reporter activity was measured in at least three independent experiments, with at least four replicates each.

2.4 Formation of H_2O_2 by apple polyphenols

H_2O_2 production in the HT29 culture medium was measured by the ferrous ion oxidation-xylenol orange (FOX) assay [24]. Different concentrations of AE02 and AE05 (0 – 850 µg/mL) were incubated in DMEM for 24 and 48 h in a humidified incubator. Afterwards, 40 µL of each concentration was added to 360 µL of FOX reagent [250 µM ammo-

nium ferrous sulphate, 100 μM xylenol orange, 25 mM H_2SO_4, 4 mM butylated hydroxytoluene (BHT) in 90% v/v methanol], followed by 30 min incubation at room temperature. Finally, the samples were centrifuged (10 min, 10 000 × *g*) to eliminate flocculated material and the absorbance of the supernatant was then read at 550 nm. Concentrations of H_2O_2 were calibrated using a standard curve of H_2O_2 (0 – 300 μM), performed in parallel.

2.5 Comet assay

To study the antigenotoxic potential of apple polyphenols, DNA damage after incubation with CumOOH was measured using the alkaline version of single cell micro gel electrophoresis (Comet assay). HT29 cells were seeded in cell culture flasks (25 cm^2) and plated for 24 h. A pre-treatment with 510 μg/mL of AE05 in absence or presence of 100 U/mL of catalase (Sigma – Aldrich, Taufkirchen, Germany) for 24 or 48 h followed. After incubation, cells were harvested, washed and then treated with CumOOH in concentrations ranging from 18.8 to 150 μM for 15 min at 37°C. The Comet assay was performed as described by Tice *et al.* [25]. Cells were embedded into low melting agarose on precoated microscope slides, lysed for 60 min, before dissected DNA was unwound in alkaline electrophoresis buffer. After unwinding, DNA fragments caused by CumOOH *via* strand breaking activities were electrophoretically separated at 1.25 V/cm, 300 mA for 20 min, washed three times for 5 min with PBS, and stained with 30 μL of fluorescence dye SYBR Green per slide (Sigma – Aldrich, Taufkirchen, Germany). DNA damage was microscopically quantified using a ZEISS Axiovert 25 microscope (Carl Zeiss Jena GmbH, Jena, Germany) and the Komet 4.0 image analysis system (Kinetic Imaging Corp., Liverpool, UK). 3 × 50 cells were scored per experiment and the mean of tail intensity (TI; percentage of fluorescence in the tail) was calculated in percentage relative to the control treated with the highest CumOOH concentration for normalization. For analysis of DNA damage in HT29 cells with genetically modified *GSTT2* expression levels, the AE pre-incubation step was omitted. DNA damage was induced by incubation with 75 μM CumOOH for 15 min at 37°C. Otherwise, the assay was performed as described above.

2.6 Generation of GSTT2-overexpressing HT29 cells

The *GSTT2* cDNA with N-terminal His-tag was subcloned from a bacterial expression vector (pQEHT1-GSTT2, kindly provided by Dr. P. G. Board, Canberra) [26] into the eukaryotic expression vector pcDNA3.1 using standard methods. In order to test whether the expression construct is functional, we transfected HEK293 cells with the plasmid using polyethylenimine (Sigma – Aldrich, Taufkirchen, Germany) [27]. Control cells were transfected with a green fluorescent protein (GFP) expression plasmid. Twenty-four hours after transfection, cells were lysed in lysis buffer containing 1% NP40, 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 10% glycerol and protease inhibitors. Lysates were cleared by centrifugation, boiled in reducing sample buffer and separated on a 13% SDS – PAGE gel. Proteins were then transferred to a PVDF membrane (Millipore). Detection of the His-tagged GSTT2 protein was performed with anti-hGSTT2 antibody (Abnova, Heidelberg, Germany) and subsequent incubation with horseradish peroxidase (HRP)-coupled secondary antibody or with HRP-coupled anti-penta His antibody (Qiagen, Hilden, Germany), respectively.

To stably overexpress *GSTT2*, HT29 cells were transfected with the expression vector pcDNA3.1-GSTT2, or the empty pcDNA3.1 vector using Lipofectamine 2000. Selection of stably transfected cells with 600 μg/mL G418 (PAA Laboratories, Cölbe, Germany) was started 48 h after transfection. Single cell clones were obtained by the dilution method.

2.7 GSTT2 knock down in HT29 cells

Stable *GSTT2* knock down in HT29 cells was achieved with retrovirally delivered *GSTT2*-targeting shRNA expression constructs. Phoenix amphotrophic packaging cells were transfected with pRS plasmids encoding either one of four non validated *GSTT2* targeting shRNAs (only the sense strand of the targeting sequence is given; sequence 1: GTCTACATCTTCGCCAAGAAGAATGGCAT; sequence 2: CCGACTGCATCCGTGGCACCTTTGGTATA; sequence 3: GAGAAGGTGGAACGCAACAGGACTGCCAT; sequence 4: ATCATCTTGAGCATCCTGGAACAGGCGGC) or a non targeting shRNA (Origene, Rockville, USA) with the polyethylenimine (PEI) method. Twenty-four and 48 h after transfection, the medium containing the replication-deficient retrovirus particles was collected and used for infection of HT29 cells. The incubation with the virus was performed for 5 h in the presence of 8 μg/mL polybrene (1.5-dimethyl-1.5-diazaundecamethylene polymethobromide, AL-118, Sigma – Aldrich, Taufkirchen, Germany). Selection of stably transfected cells was started 48 h after the second infection with 1.5 μg/mL puromycin (Sigma – Aldrich, Taufkirchen, Germany).

2.8 cDNA synthesis and quantitative real time PCR

The extent of *GSTT2* overexpression or depletion in the stably transfected HT29 cells was examined with quantitative real time PCR. Total RNA was isolated from the stably transfected HT29 cells using the RNeasy kit (Qiagen, Hilden, Germany). cDNA was synthesized from 4 μg RNA by Super Script First-Strand Synthesis System for RT-PCR with oligo$(dT)_{12\text{-}18}$ primers according to the instructions of the manufacturer (Invitrogen, Karlsruhe, Germany). Real time PCR

was carried out with Quantitect SYBR Green PCR Kit (Qiagen, Hilden, Germany) using primers specific for human *GSTT2* (Hs_GSTT2_2_SG QuantiTect Primer Assay; Qiagen, Hilden, Germany) and *GAPDH* for normalization (GAPDH for 5′ ACCCACTCCTCCACCTTTGAC 3′; GAPDH rev 5′ TCCACCACCCTGTTGCTGTAG 3′; Sigma-Genosys, Germany). Samples were measured in duplicates.

2.9 Statistical analysis

Statistical analysis was performed using the GraphPad® Prism software Version 4.0 (GraphPad® Software Inc., San Diego, USA). All experiments performed *in vitro* were repeated at least three times independently. All values are presented as mean ± SD. The two-tailed *t*-test was chosen to compare reporter activity of medium-treated and AE-treated cells as well as Comet assay data obtained for cells with genetically modified GSTT2 expression levels. FOX assay data were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test. Significant differences between medium-treated cells and AE-treated cells after damage with CumOOH were calculated using two-way ANOVA and Bonferroni's multiple comparison test. Differences with p values <0.05 are indicated as significant.

3 Results

3.1 Polyphenolic apple extracts induce a GSTT2 reporter

We have previously shown that endogenous *GSTT2* expression in HT29 colon carcinoma cells is enhanced upon treatment with an apple polyphenol extract (here referred to as AE02) [15]. To establish a direct effect of apple polyphenols on *GSTT2* gene regulation, we employed reporter assays with a *GSTT2* promoter-regulated firefly luciferase expression construct. HT29 cells were transiently transfected with the reporter, and subsequently treated with AE02 under conditions which caused endogenous *GSTT2* expression regulation (510 µg/mL for 24 h). As depicted in Fig. 1, reporter activity was increased 2.3-fold by AE02. An even more pronounced effect (3.1-fold induction) was obtained upon treatment with AE02 for 48 h. We also tested an AE from a different crop year (AE05) for its capacity to activate the reporter expression. As shown in Fig. 1, AE05 was also capable of inducing the reporter in a time dependent manner, albeit to a somewhat lesser extent than AE02. Luciferase activity was 1.5-fold increased after 24 h and 2.5-fold after 48 h of incubation with AE05, respectively. Thus, in addition to the previously described increase in endogenous *GSTT2* transcript levels, we confirmed the *GSTT2* inducing activity of apple polyphenols at the level of promoter activity. This regulation was not related to the known hydrogen peroxide (H_2O_2) production during the

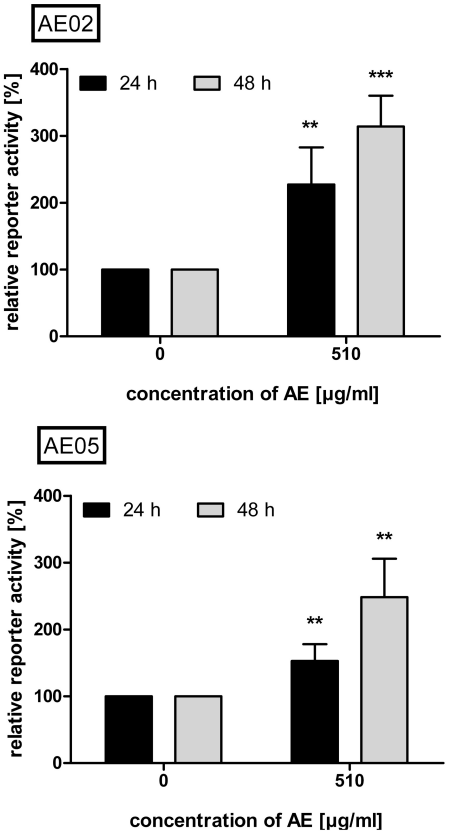

Figure 1. Induction of GSTT2 promoter activity by apple polyphenols. HT29 colon carcinoma cells were transiently transfected with a *GSTT2* promoter construct driving expression of firefly luciferase. Cells were treated with AE02 or AE05 for the indicated times. Cells were lysed and luciferase activity was measured and normalized to activity of co-transfected *Renilla* luciferase. Results are shown as mean ± SD. Significant differences to medium control were calculated by two-tailed student *t*-test (**$p < 0.01$, ***$p < 0.001$; $n = 5$ for 24 h and $n = 6$ for 48 h measurement).

interaction of apple polyphenols with cell culture media [28, 29]. While AE02 and AE05 indeed caused H_2O_2 production in cell-free culture medium (Figure S1 of Supporting Information), addition of catalase completely decomposed the generated H_2O_2 (data not shown). The induction of the *GSTT2* reporter was undistinguishable in absence or presence of catalase (Figure S2 of Supporting Information). Nevertheless, further experiments were carried out in the presence of catalase to exclude possible effects of H_2O_2.

3.2 Treatment of HT29 cells with AE protects from CumOOH-induced DNA damage

Comet assays were carried out to evaluate the antigenotoxic potential of apple polyphenols and the possible role of *GSTT2* expression. To induce oxidative DNA damage, the genotoxic compound CumOOH was selected as test sub-

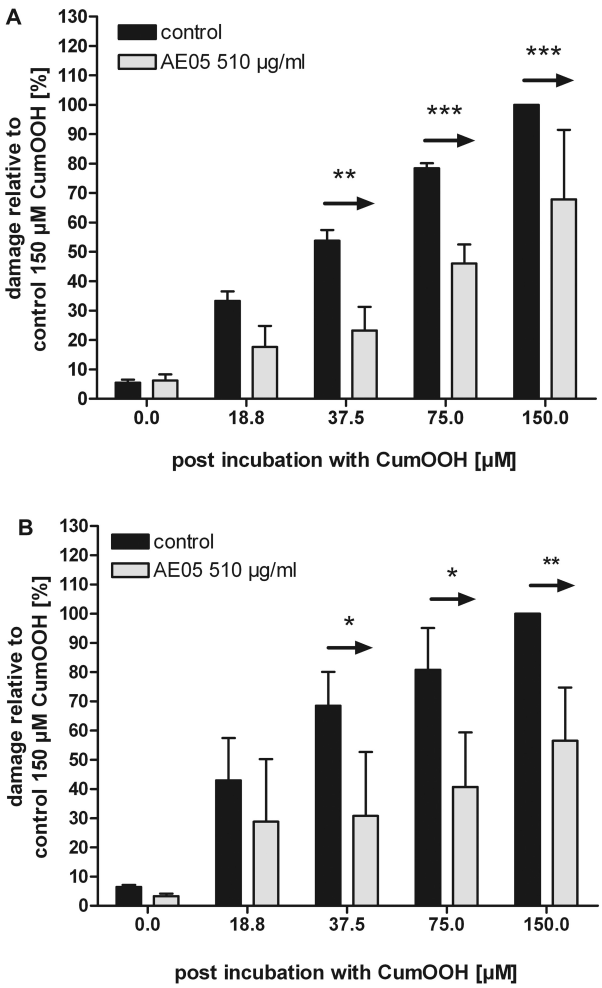

Figure 2. Impact of apple polyphenols on CumOOH-induced DNA damage. Levels of DNA damage in HT29 colon carcinoma cells induced by 18.8 – 150 µM CumOOH (15 min at 37°C) after pre-incubation with AE05 (510 µg/mL, grey bars) or DMEM medium control (black bars) for 24 (A) and 48 h (B) in presence of catalase were measured with an alkaline Comet assay. Tail intensities relative to 150 µM CumOOH-treated control are shown as mean ± SD. Significant differences to untreated medium control were calculated by two-way ANOVA, including Bonferroni's multiple comparison test (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$; $n = 3$).

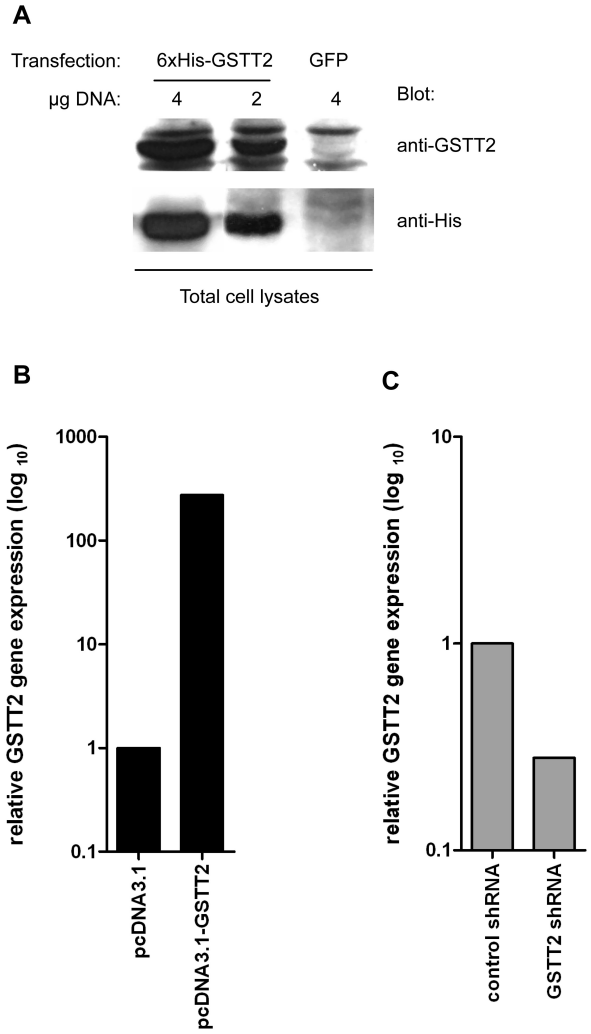

Figure 3. Modulation of GSTT2 expression levels in HT29 colon epithelial cells. (A) Functionality of a GSTT2 expression construct in HEK293 cells. The *GSTT2* cDNA with N-terminal His tag was subcloned into pcDNA3.1. Expression of the His-GSTT2 was tested in HEK293 cells transiently transfected with the construct, or a GFP expression vector as control. Cells were lysed 24 h after transfection and analysed by immunoblotting using anti-GSTT2 or anti-penta His antibody, as indicated. The lanes of the anti-His blot were on the same membrane with identical exposure and image processing, but were rearranged for better clarity. (B) Stable overexpression of GSTT2 in HT29 colon epithelial cells. Cells were transfected with the expression construct analysed in (A), or pcDNA3.1 control vector, and selected with G418. *GSTT2* mRNA expression was analysed by qRT-PCR. A clone with high-level overexpression was chosen for functional analysis. (C) Stable downregulation of GSTT2 expression in HT29 colon epithelial cells. Cells were retrovirally transduced with different non-validated shRNAs directed against *GSTT2*, and stable cell pools were selected with puromycin. Downregulation of *GSTT2* mRNA was validated by qRT-PCR and is shown for cells transduced with the best targeting construct 2, which were used for further analysis.

stance. Cells were pre-treated with apple polyphenols (AE05) at a concentration, which is known to induce expression of *GSTT2*. As shown in Fig. 2, a 24 h (2A) or 48 h (2B) pre-incubation with 510 µg/mL AE05 significantly reduced genotoxicity of CumOOH in HT29 cells. Pre-treatment with AE05 resulted in a decrease of DNA damage by 30% after 24 h and by ~40% after 48 h compared to the medium control, respectively. These results indicate that apple polyphenols protect from genotoxic damage generated by a substrate of GSTT2 enzymes.

3.3 Genetic modulation of GSTT2 expression affects susceptibility to CumOOH-induced DNA damage

We next addressed the question of whether the protective effect of apple polyphenols may be attributed to the induction of *GSTT2* gene expression. To analyse this, we generated HT29 cells with genetically modified *GSTT2* expression levels by either overexpressing *GSTT2* or knocking down endogenous *GSTT2* expression. To overexpress *GSTT2*, an expression construct in the eukaryotic expression vector pcDNA3.1 was employed. Functionality of this construct was tested by transient transfection of HEK293 cells with the expression plasmid (or a GFP encoding plasmid as control) and analysis of corresponding cell lysates by immunoblotting. As shown in Fig. 3A, GSTT2 protein was detectable both with a GSTT2- or a His-tag specific antibody, indicating that the construct effectively drives GSTT2 protein expression. We next stably transfected HT29 cells with the *GSTT2* expression construct or the empty pcDNA3.1 vector as control. Since endogenous GSTT2 protein expression was not detectable by immunoblotting, presumably due to lower expression levels compared to transiently transfected HEK293 cells, and expression of His-tagged GSTT2 in stably transfected cell lines was only weakly detectable (hampered by technical problems, data not shown), we measured *GSTT2* mRNA expression levels by quantitative RT-PCR, and selected a cell line with high *GSTT2* expression level (Fig. 3B) for comparison with empty vector transfected cells for testing the effect of GSTT2 overexpression on CumOOH-induced DNA damage. As depicted in Fig. 4A, HT29 cells overexpressing GSTT2 showed significantly reduced DNA damage compared with cells transfected with pcDNA3.1. To further corroborate a correlation between GSTT2 expression levels and susceptibility to DNA damage, we also analysed the genotoxic effect of CumOOH in HT29 cells with reduced *GSTT2* levels. In order to knock down endogenous *GSTT2* expression, cells were virally transduced with expression plasmids encoding either one of four non validated *GSTT2* targeting shRNAs or a control shRNA. *GSTT2* expression in stably transfected cells was measured by quantitative RT-PCR. Of the four *GSTT2* targeting shRNA sequences, only sequence 2 considerably reduced *GSTT2* expression to 28% of the control cell level (Fig. 3C). This *GSTT2* depleted cell line and cells stably transduced with an expression construct for non-targeting shRNA (control cells) were now compared for their susceptibility to CumOOH-induced DNA damage. As apparent from Fig. 4A, *GSTT2* knockdown significantly increased tail intensity compared to the control cells. From these results, it can be concluded that endogenous GSTT2 protects cells from oxidative DNA damage by CumOOH. Taken together, *GSTT2* expression levels were inversely correlated with susceptibility to genotoxic stress.

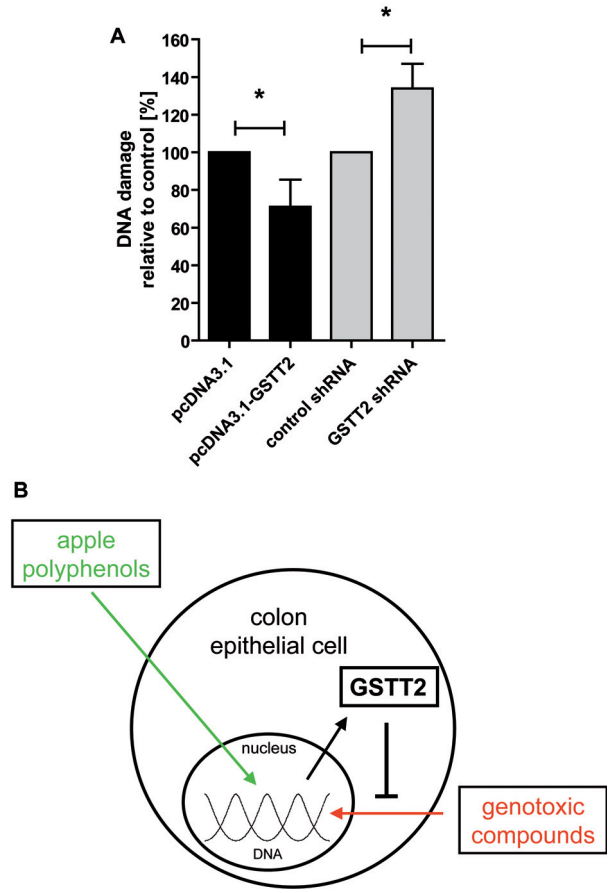

Figure 4. Modulation of CumOOH-induced DNA damage by alteration of GSTT2 expression levels. (A) GSTT2-overexpressing, GSTT2-depleted and corresponding control HT29 cell lines were incubated with 75 μM CumOOH for 15 min and analysed by Comet assay. Data were cumulated from four independent experiments. Tail intensities were normalized to intensities of the respective control cell line and shown as mean ± SD. Significance of differences were calculated by two-tailed student *t*-test (*$p < 0.05$; $n = 4$). (B) Schematic representation of the proposed role of GSTT2 regulation by apple polyphenols for protection against genotoxic damage.

4 Discussion

Several epidemiological studies have linked the consumption of apple with a reduced risk for colorectal cancer. One mechanism by which apple compounds may exert a protective function is the modulation of phase I and phase II carcinogen metabolism (reviewed in [30]). We have previously shown that the transcript levels of *GSTT2* and other genes involved in biotransformation of xenobiotics are modulated by the treatment with a polyphenolic AE [15]. With this study we demonstrate that apple polyphenols protect from oxidant-induced DNA damage and provide evidence that this effect may be directly caused by the increased expression of GSTT2.

To establish a direct effect of apple polyphenols on *GSTT2* gene regulation, we used a reporter assay to study *GSTT2* promoter activity in response to AE treatment in HT29 cells. For two different apple polyphenol extracts we found a time-dependent increase in promoter activity. This finding demonstrates that the previously found enhancement of endogenous *GSTT2* mRNA levels was at least partially mediated by activation of transcription.

The mechanisms leading to the transcriptional activation of the *GSTT2* promoter, and the promoter elements involved in the process remain to be identified. Antioxidant-response elements (ARE) have been described in promoters of rodent *GST* genes [13]. The *GSTT2*-reporter, which we have used in this study was, however, not induced under conditions causing strong activation of an ARE-containing Nrf2 reporter (not shown), suggesting the absence of ARE elements. Dissection of *GSTT2* promoter regulation would clearly be facilitated by identifying the chemical components in the apple polyphenol extracts, which are responsible for the *GSTT2* induction. We therefore tested a number of known components of the extracts (phloridzin, epicatechin, caffeic acid, quercetin-3-rutinosid, quercetin-3-galactosid, quercetin-3-rhamnosid, quercetin-3-glycosid, phloretin (aglycon), quercetin (aglycon), chlorogenic acid and procyanidines B1 and B2) [20] for their capacity to activate the *GSTT2* promoter. However, none of the compounds significantly induced reporter activity (data not shown). This indicates that either the active compound was not among the tested substances or that compounds are active only when applied as a mixture allowing additive or synergistic effects.

We next addressed the question of whether apple polyphenols might confer protection from genotoxic stress by inducing *GSTT2* expression. To analyse this, HT29 cells were preincubated with an apple polyphenol extract and subsequently challenged with different concentrations of the GSTT2 substrate CumOOH. Our results demonstrate clearly that apple polyphenols reduce the genotoxic activity of CumOOH. This effect is independent of H_2O_2 generation in the medium since the protective effect was insensitive to catalase addition (data without catalase not shown). An obvious explanation for the AE mediated protection is an enhanced detoxification of CumOOH due to the elevated GSTT2 expression. To test this hypothesis we created cell lines with elevated or decreased *GSTT2* expression levels and analysed them for their susceptibility to CumOOH-induced DNA damage. The Comet assay results showed that cells overexpressing GSTT2 were more resistant towards the action of CumOOH while *GSTT2* depleted cells exhibited a higher susceptibility. These findings clearly prove that (I) endogenous levels of GSTT2 protect cells from CumOOH-induced damage and (II) that protection can be enhanced by an upregulation of *GSTT2* expression levels.

CumOOH, which we used to study the GSTT2 mediated antigenotoxicity, is a synthetic hydroperoxide [31]. Among the physiological substrates described for GSTT2 are peroxides generated during lipid acid metabolism and arachidonic peroxidation, such as arachidonic acid 15-hydroperoxide [32], which is involved in DNA-adduct formation. From our results it could be speculated that elevated GSTT2 levels might also protect from oxidative stress caused by endogenously produced peroxides.

Nevertheless, further glutathione transferases, such as GSTA2 and GSTK1 are involved in detoxification of CumOOH [13]. Furthermore, it is not yet known whether these enzymes are modulated by apple polyphenols. Thus, it cannot be excluded that these enzymes could also contribute to the AE-mediated protection against CumOOH-induced DNA damage. However, our experiments with genetically modified HT29 cells suggest that this protection is due at least in part to an enhanced GSTT2 level.

Pool-Zobel *et al.* reported that *GSTT2* expression levels in the colon vary among different individuals [33]. Our results suggest that individuals might therefore be differently susceptible to oxidative DNA damage by agents which are detoxified by GSTT2. An impact of *GSTT2* expression is also suggested by the study of Jang *et al.*, who reported an association of *GSTT2* promoter polymorphisms with colorectal cancer risk [34]. One could speculate that a favourable modulation of *GSTT2* expression in the colon might reduce the risk for oxidative DNA damage and colon cancer.

Our data show that apple polyphenols can induce *GSTT2* expression in HT29 cells *in vitro*. Whether the consumption of apple polyphenols affects *GSTT2* expression level in the colon *in vivo* is currently unknown. Analysis of ileostomy samples collected from human volunteers after intervention with cloudy apple juice revealed that polyphenols are subjected to rapid metabolism and/or absorption upon passage of stomach and small intestine [35]. However, an enhanced *GSTT2* mRNA level and promoter activity as well as a reduced genotoxicity were detected in HT29 cells treated with ileostomy samples from the intervention study [16]. These observations suggest that sufficient amounts of *GSTT2* inducing components in apple juice may arrive in the colon.

In conclusion, our study revealed that apple polyphenols induce *GSTT2* expression in HT29 colon carcinoma cells by enhancing *GSTT2* promoter activity. Furthermore, we demonstrated that AE treatment reduces oxidative DNA damage caused by the GSTT2 substrate CumOOH. By genetically modifying *GSTT2* expression levels we proved that GSTT2 has an antigenotoxic potential, which is likely to mediate at least in part the protective effects of apple polyphenols (Fig. 4B). These results suggest that GSTT2 might be a promising target for dietary chemoprevention.

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The authors have declared no conflict of interest.

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