Cancer Research

Combinatorial Chemoprevention Reveals a Novel Smoothened-Independent Role of GLI1 in Esophageal Carcinogenesis

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Abstract

Reflux-induced injury promotes esophageal adenocarcinoma, one of the most rapidly increasing, highly lethal cancers in Western countries. Here, we investigate the efficacy of a combinatorial chemoprevention strategy for esophageal adenocarcinoma and characterize the underlying molecular mechanisms. Specifically, our approach involves the use of ursodeoxycholic acid (Urso) due to its ability to decrease injury-inducing bile salts in combination with Aspirin to mitigate the consequences of injury. We find that Urso-Aspirin combination reduces the risk of adenocarcinoma *in vivo* in animals with reflux, decreases the proliferation of esophageal adenocarcinoma cells, and downregulates a key cell cycle regulator, *CDK2*. Mechanistically, using cell growth, luciferase reporter, expression, and chromatin immunoprecipitation assays, we identify *GLI1*, a Hedgehog-regulated transcription factor, as a novel target of Urso-Aspirin combination. We show that *GLI1* is upregulated during esophageal carcinogenesis, and GLI1 can bind to the *CDK2* promoter and activate its expression. Although the Urso-Aspirin combination downregulates *GLI1*, the GLI1 overexpression not only abrogates the effect of this combination on proliferation but it also restores *CDK-2* expression. These findings support that the chemopreventive effect of the Urso-Aspirin combination occurs, at least in part, through a novel *GLI1-CDK2*–dependent mechanism. To further understand the regulation of *CDK2* by *GLI1*, both pharmacologic and RNAi-mediated approaches show that *GLI1* is a transcriptional activator of *CDK2*, and this regulation occurs independent of Smoothened, the central transducer of the Hedgehog canonical pathway. Collectively, these results identify a novel *GLI1*-to-*CDK2* pathway in esophageal carcinogenesis, which is a bona fide target for effective combinatorial chemoprevention with Urso and Aspirin. *Cancer Res; 70(17); 6787–96. ©2010 AACR.*

Introduction

Chronic injury and inflammation play a central role in several gastrointestinal cancers including Barrett's-associated esophageal adenocarcinoma, a highly lethal and rapidly increasing cancer (1–4). It is well recognized that chronic injury induces an inflammatory response and activates procarcinogenic pathways in injured tissue (1–3, 5, 6). Although combinatorial approaches have been successfully used in HIV and tuberculosis, and are proposed in carcinogenesis (7–9), the usual approach in cancer prevention involves targeting either the cause of injury or its consequences (2, 10, 11). We hypothesized that during carcinogenesis in Barrett's esophagus, targeting both the cause (bile composition) and the consequence of injury (inflammation-associated pathways) will be an optimal chemoprevention strategy.

To address combinatorial chemoprevention in esophageal adenocarcinoma, we evaluated the effect of low-dose Aspirin and ursodeoxycholic acid (Urso) on the development of this cancer. Interestingly, patients who use Urso for cholestatic liver disease are at lower risk of colon cancer (12). Although the effect of Urso in injury-induced carcinogenesis remains unknown, it does lower the levels of bile salts that are strongly implicated as the cause of injury and carcinogenesis in Barrett's esophagus (13). The rationale to combine Urso with Aspirin was that patients who chronically use anti-inflammatory drugs including Aspirin are less likely to be diagnosed with esophageal adenocarcinoma (14). Although there is no *in vivo* experimental evidence that Aspirin by targeting the effect of injury could prevent esophageal adenocarcinoma, anti-inflammatory agents such as cyclooxygenase-2 inhibitors have been shown to reduce the risk of this cancer in animals with reflux (15). We elected to use Aspirin over these anti-inflammatory agents because unlike cyclooxygenase-2 inhibitors, Aspirin does not increase the risk of cardiovascular mortality (16).

In this study, using a battery of *in vitro* and *in vivo* experiments, we show that combinatorial chemoprevention using low-dose Urso-Aspirin reduced the risk of reflux-induced

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doi: 10.1158/0008-5472.CAN-10-0197

esophageal adenocarcinoma, whereas these agents were not effective in preventing cancer when used individually. The key cellular mechanism involved in this chemopreventive effect is the inhibition of cell proliferation, and that the molecular target of this combination is the downregulation of *CDK2*, an important cell cycle regulator. Interestingly, further steps to resolve the molecular mechanisms revealed that *CDK2* is regulated at transcriptional level through a previously unknown *GLI1*-mediated mechanism. Typically, *GLI1*, a known effector molecule of the oncogenic Hedgehog pathway, exerts transcriptional regulation upon its activation by Smoothened receptor; however, here, we show that *CDK2* upregulation by *GLI1* is Smoothened independent. The importance of *GLI1* in context of combinatorial chemoprevention is further supported by our findings that *GLI1* is overexpressed during injury-induced carcinogenesis in Barrett's mucosa, and the Urso-Aspirin combination downregulates *GLI1*. Finally, we show that *GLI1* overexpression not only relieves the *CDK2* repression caused by the Urso-Aspirin combination but also abrogates the effect of this combination on cell proliferation. Therefore, these novel findings expand our knowledge of mechanisms involved in chemoprevention, a relatively underappreciated field of research.

Materials and Methods

Reagents and cell cultures

Unless specified, all reagents were from Sigma. BAR-T (Dr. Jerry Shay, UT Southwestern, Dallas, TX), CPC-A, and CPC-C (Dr. P. Rabinovitch, University of Washington, Seattle, WA) cells were maintained in Barrett's Plus media (17–19). Human Barrett's–associated adenocarcinoma cell lines SKGT4 and FLO-1 (Dr. David Schrump, National Cancer Institute, Bethesda, MD and Dr. David Beer, UMich, Ann Arbor, MI) were maintained in DMEM (Life Technologies) with 1%-10% fetal bovine serum. Cell lines were authenticated with short tandem repeat and DNA fingerprinting within the last 6 months.

Rat model of Barrett's esophagus, interventions, and monitoring

Esophagojejunostomy was performed on 100 rats to cause reflux injury, Barrett's esophagus, and adenocarcinoma (15). The Mayo Clinic Institutional Animal Care and Use Committee approved this animal study. Eight week postoperatively, 86 surviving rats were kept in individual cages and randomized (2:2:2:3) to a diet containing 1% Urso (n = 19), 0.3% Aspirin (n = 19), 1% Urso+0.15% Aspirin (n = 19), or control (n = 29). The dose selection was based on the available literature as well as Barrett's mucosal tissue from 40 patients who received 80 to 325 mg of Aspirin daily for 3 months. The published range for Aspirin dose was 0.03% to 1.2%. We elected to use 0.15% Aspirin as we found that a dietary supplement of 0.1% to 0.2% Aspirin could achieve similar effect on biochemical and molecular markers of injury and inflammation as was achieved by 80 to 325 mg of Aspirin in patients with Barrett's esophagus. The published doses of Urso range from 0.1% to 1%, and we found that 1% Urso supplementation resulted in similar bile salt profile as we have noted in Barrett's esophagus patients who received 250 mg Urso thrice daily for 3 months (20–23). Animals were euthanized 8 months after randomization for evaluation of end points as outlined below. Autopsy was performed as we have previously described (15).

Cell proliferation and apoptosis

Proliferation was assessed by bromodeoxyuridine (BrdUrd), and apoptosis was detected using Annexin-5–positive cell on immunostaining using fluorescence microscope (19). MTS assay for metabolically active, viable cells and morphologic features of apoptosis through Hoechst staining were also examined. All experiments were repeated thrice in triplicates.

RNA extraction and reverse transcription-PCR

Total RNA isolated from patient samples, rat tissue, and cell lines (using Trizol reagent, Invitrogen) were purified with RNeasy columns (Qiagen). Using OneStep reverse transcription-PCR (RT-PCR) kit (Qiagen), with primers specific for *GLI1* and *CDK2*, PCR was performed (primers and conditions available upon request). The amplified products were analyzed on a 2% agarose gel.

Luciferase reporter assays

Approximately 60% confluent cells in six-well plates were incubated with 1 mL serum-free Opti-MEM (Invitrogen) containing 12 μL of Lipofectamine (Invitrogen) and 1.2 μg of DNA. After 6 hours, the medium was replaced with DMEM containing 10% fetal bovine serum. Luciferase activities were measured using the Dual-Luciferase Reporter assay (Promega) and normalized by protein quantification. Each data point represents an average of three independent transfections (24).

Chromatin immunoprecipitation assay

Cells were transfected with *GLI1* or parental vector. Samples were immunoprecipitated using a GLI antibody (R&D Systems; ref. 25). Immunoprecipitated DNA was amplified by PCR using primer sets for the four areas containing GLI biding sites in *CDK2* promoter sequence (please see supplemental file for primers).

Plasmids constructs

The *CDK2* promoter-*Luciferase* reporter (8x*GLI*) was kindly provided by Dr. van Wijnen (University of Massachusetts, Worcester, MA). The *GLI* reporter expression constructs were kindly provided by Dr. Chi-chung Hui (Research Institute, Toronto, Ontario, Canada). The cDNA for *GLI1* was cloned in pCMV-Tag2B vector (Stratagene), and short hairpin RNA (shRNA) were designed and cloned into pFRT vector (Invitrogen) using standard recombinant DNA methods as previously described (the targeted sequences are in the supplemental file; ref. 24).

Statistical methods

The statistical analyses were performed using the SAS software. All tests were two sided, and a P value of <0.05 was considered statistically significance (Bonferroni adjustments were made when indicated). The Student's t tests (or when

appropriate, the Wilcoxan rank-sum tests) were used to compare the groups. All experiments performed in triplicate were repeated at least thrice.

Results

Urso-Aspirin combination decreases the rate of esophageal adenocarcinoma *in vivo* and inhibits Barrett's epithelial cell proliferation

To test the hypothesis that targeting both the cause and consequence of chronic reflux injury will be an optimal chemoprevention strategy during carcinogenesis in Barrett's esophagus, we used Urso and Aspirin in an established animal model. We found that the incidence of esophageal adenocarcinoma was significantly lower in animals treated with Urso-Aspirin compared with controls ($P < 0.05$; Fig. 1A). In the combination group, 26% animals (5 of 19) developed esophageal cancer, whereas 62% (18 of 29) developed cancer in the control group (an absolute risk reduction of 58%, with 95% confidence interval, 45–69%). There was no significant difference in the risk of esophageal adenocarcinoma between the Urso alone–treated group (8 of 19, $P = 0.28$) or Aspirin alone–treated group (9 of 19, $P = 0.48$), compared with the control group.

To examine the cellular processes by which Urso-Aspirin combination exerts this tumor-inhibitory effect, premalignant BAR-T cells were treated for 48 hours with either 150 μmol/L Urso, 1.5 mmol/L Aspirin, or 150 μmol/L Urso+1.5 mmol/L Aspirin. Urso-Aspirin combination resulted in a robust reduction in proliferation (91 ± 6.7% reduction compared with control,

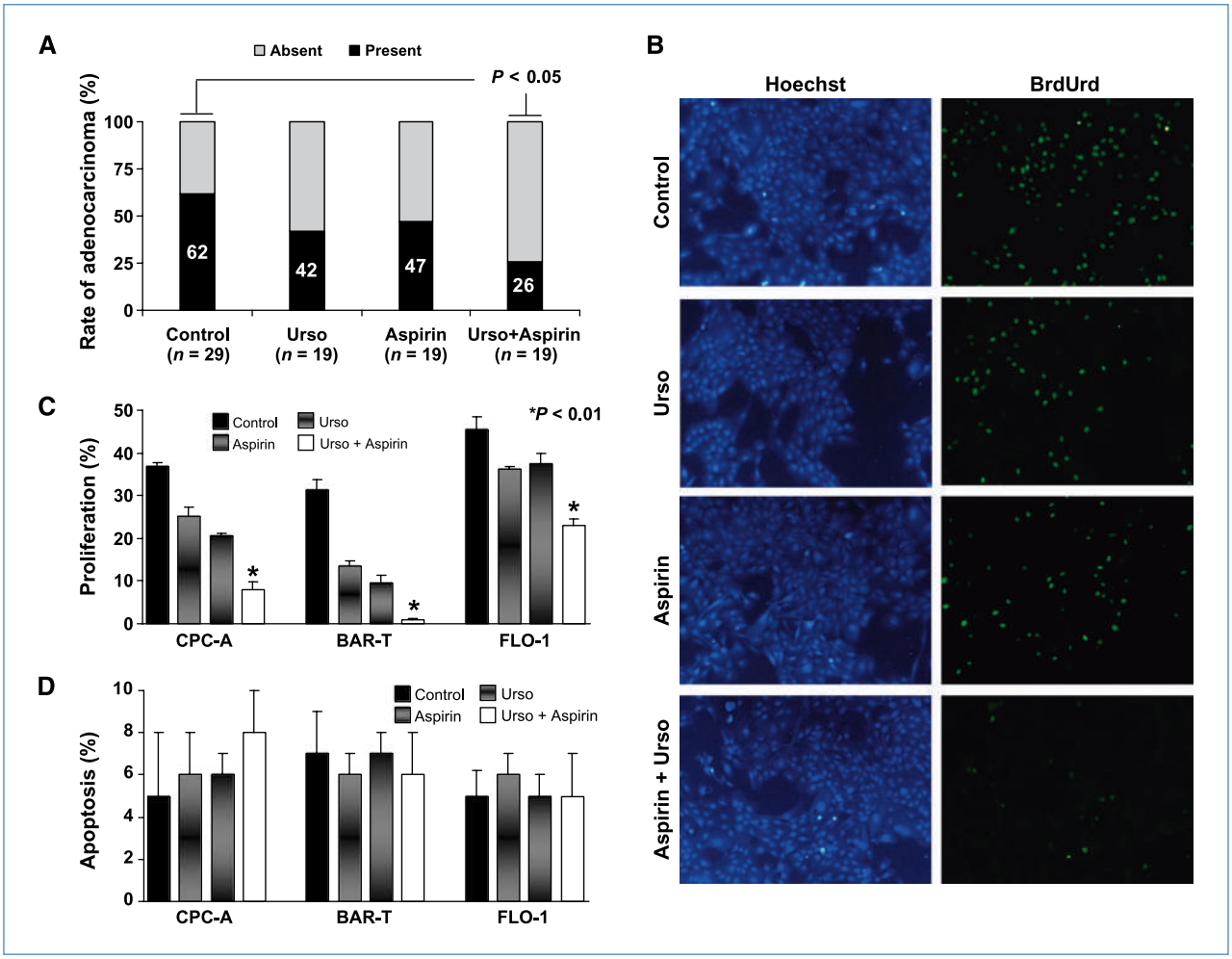

Figure 1. Combination treatment with Urso and Aspirin reduces tumor incidence *in vivo* and inhibits cell proliferation *in vitro*. A, the esophageal cancer risk reduction was noted only when Urso and Aspirin were combined ($P < 0.05$, compared with control). B, at 48 h, compared with control, BAR-T cell proliferation was reduced by 91 ± 6.7% with Urso-Aspirin (150 μmol/L Urso and 1.5 mmol/L Aspirin), 42 ± 3.3% with Urso-alone (150 μmol/L) and 44 ± 4.2% with Aspirin-alone (1.5 mmol/L). The Urso-Aspirin combination treatment had more robust inhibition of proliferation compared with either Urso or Aspirin alone ($P < 0.05$). C and D, a 48-h treatment with Urso-Aspirin (150 μmol/L Urso and 1.5 mmol/L Aspirin) significantly decreased ($P < 0.01$, compared with control) the BrdUrd-positive cells in CPC-A by 77 ± 19.9%, BAR-T by 97 ± 55%, and FLO-1 cells by 51 ± 3.02%; however, compared with control, the rate of apoptosis (Annexin-5 and Hoechst staining) was not different in CPC-A (5 ± 3% versus 8 ± 2.1%), BAR-T (7 ± 2% versus 6 ± 1%), or FLO-1 cells (5 ± 1.2% versus 6 ± 2%).

$P < 0.01$), which was significantly enhanced over Urso alone (42 ± 3.3% reduction) or Aspirin alone (44 ± 4.2% reduction; $P < 0.01$). To further confirm this finding, premalignant (CPC-A, BAR-T) and malignant (FLO-1) esophageal epithelial cell lines were treated with either 150 μmol/L Urso + 1.5 mmol/L Aspirin or vehicle. At 48 hours, the Urso-Aspirin combination decreased BrdUrd-positive cells in CPC-A by 77 ± 19.9%, BAR-T by 97 ± 55%, and FLO-1 cells by 51 ± 3.02% compared with control ($P < 0.05$; Fig. 1B and C). However, the proportion of cells undergoing apoptosis with Urso-Aspirin were not different compared with vehicle-treated cells (Fig. 1D). Together, these findings show that Urso-Aspirin is effective in preventing Barrett's-associated neoplasia, and the cellular process that it targets is proliferation (not apoptosis). Although both Aspirin and Urso can target distinct regulatory proteins that are involved in the cell cycle (26, 27), the mechanism underlying the downregulation of proliferation by their combination remains unknown.

Urso-Aspirin combination downregulates *CDK2*, an important cell cycle regulator, both *in vitro* and *in vivo* in Barrett's esophagus

Having determined the efficacy of Urso-Aspirin in preventing esophageal adenocarcinoma, we conducted a pathway-specific gene expression profile to identify molecular targets of this combination. As Urso-Aspirin downregulated proliferation in Barrett's epithelium, it was interesting to find cell cycle regulator, *CDK2*, as a promising target from this profiling (28). We therefore examined *CDK2* expression in esophageal cell lines treated with the Urso-Aspirin and found that this combination downregulated *CDK2* expression (Fig. 2A). These results were further confirmed *in vivo* in which animals treated with Urso-Aspirin showed a reduction in *Cdk2* expression in Barrett's mucosa compared with controls (Fig. 2B). To determine whether the effect of Urso-Aspirin on *CDK2* expression occurs at the transcriptional level, FLO-1 cells were initially transfected with *CDK2* promoter-luciferase reporter constructs; twenty-four hours posttransfection, cells were further treated with Urso-Aspirin for 24 hours. The protein normalized luciferase activity showed that Urso-Aspirin caused a 4-fold reduction in *CDK2* promoter activity compared with control (100 ± 20 versus 22.2 ± 6, $P < 0.01$; Fig. 2C). These results provide evidence, for the first time, that *CDK2*, a cell cycle regulator known to play an important role in proliferation, is a target of Urso-Aspirin and can be regulated at the transcriptional level by this combination. These novel findings led us to further examine *CDK2* regulation in context of combinatorial chemoprevention in Barrett's esophagus.

Urso-Aspirin combination downregulates *CDK2* by antagonizing a GLI1-mediated, Smoothened-independent mechanism in Barrett's epithelial cells

Because Urso-Aspirin downregulated *CDK2* expression and repressed its promoter activity, we next conducted bioinformatics sequence analysis of the *CDK2* promoter using the TRANSFAC Public database along with the functional screening. We found GLI proteins, particularly GLI1, as promising candidate regulators of *CDK2* promoter (Fig. 3A). To confirm this prediction, we first examined whether GLI1 binds to endogenous *CDK2* promoter. We transfected Barrett's epithelial cells with either control vector or a *GLI1* construct. Chromatin immunoprecipitation using a GLI1 antibody showed that *CDK2* promoter sequence was enriched in cells transfected with *GLI1* (Fig. 3B), suggesting that GLI1 binds to the endogenous *CDK2* promoter, which is therefore a direct target of *GLI1*. To further solidify these findings and to determine functional relevance, we co-transfected Barrett's epithelial cell lines with a *CDK2* promoter-luciferase reporter construct along with either control vector or *GLI1* expression constructs. At 48 hours, *GLI1*-transfected cells had up to 5-fold increase in *CDK2* promoter activity compared with

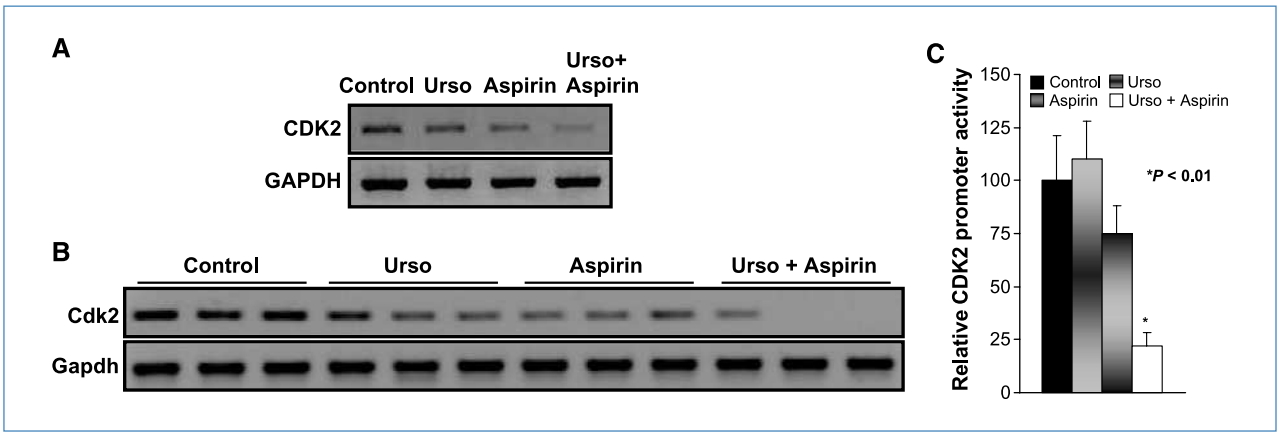

Figure 2. Urso-Aspirin combination decreases *CDK2* expression and promoter activity both *in vitro* and *in vivo* in Barrett's esophagus. A, RT-PCR showed that FLO-1 cells treated with Urso-Aspirin combination (150 μmol/L + 1.5 mmol/L) for 24 h had a marked reduction in *CDK2* expression compared with control cells as well as cells treated with either Urso or Aspirin alone. B, a similar reduction in *Cdk2* expression is seen in esophageal tissue derived from rats receiving the combination therapy but not when these agents were used individually. C, FLO-1 cells transfected with *CDK2* promoter-luciferase reporter constructs were treated 24 h posttransfection with either vehicle, Urso, Aspirin, or a combination of Urso-Aspirin (150 μmol/L + 1.5 mmol). Twenty-four hours later, compared with control, the Urso-Aspirin combination markedly reduced *CDK2* promoter activity (100 ± 20 versus 22.2 ± 6, $P < 0.01$).

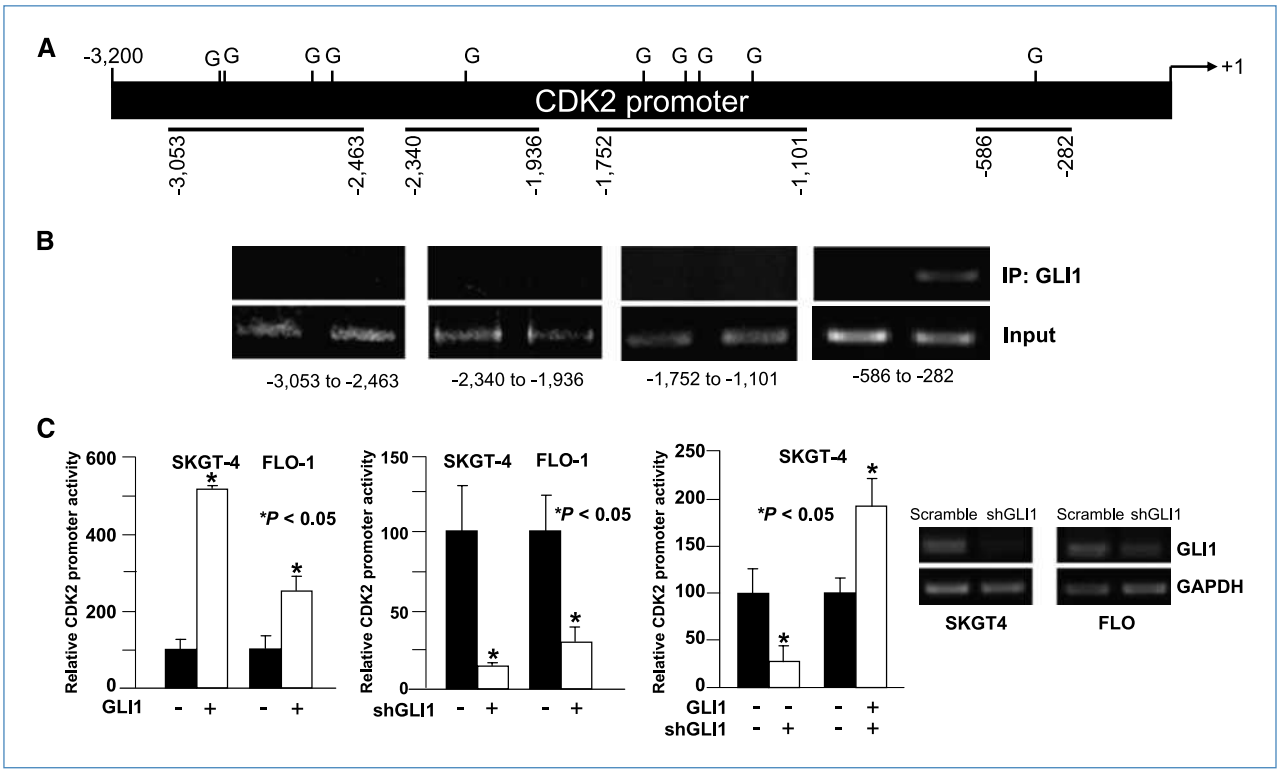

Figure 3. GLI1 binds to endogenous *CDK2* promoter and activates *CDK2* promoter. A, outline of *CDK2* promoter with putative GLI binding site (G). B, chromatin immunoprecipitation (IP) assay shows that GLI1 could directly binds to endogenous *CDK2* promoter in the core promoter region. C, SKGT4 and FLO-1 cells were cotransfected with *GLI1* and *CDK2*. In both cell lines, there was an increase in *CDK2* activity in the *GLI1*-transfected cells when compared with control ($P < 0.05$), and this increase is prevented by the transfection of *shGLI1*. The latter effect is rescued by overexpression of shRNA-resistant *GLI1* construct, which does not contain the 3′end of *GLI1* mRNA that is the target of shRNA.

control (Fig. 3C), suggesting that not only did GLI1 bind the *CDK2* promoter but also acted as a transcriptional activator of *CDK2*. To further substantiate these findings, esophageal cells were co-transfected with a *CDK2* promoter-luciferase reporter construct along with either shRNA against *GLI1* (shGLI1) or scrambled shRNA control. Congruent with the above data, the *shGLI1*-transfected cells had up to a 5-fold reduction in *CDK2* promoter activity when compared with control.

To determine if the decrease in the *CDK2* promoter activity by *shGLI1* could be relieved by *GLI1* overexpression, we co-transfected esophageal cells with a *CDK2* promoter reporter construct along with *shGLI1* or scrambled shRNA together with a shRNA-resistant *GLI1* expression construct. We found that under these experimental conditions, *GLI1* restored *CDK2* promoter activity (Fig. 3C).

GLI1 is a downstream effector of the Hedgehog pathway (29). To determine the involvement of this cascade in the modulation *CDK2* expression and promoter activity, esophageal cancer cell lines FLO-1 and SKGT4 were transfected with *CDK2* promoter-luciferase reporter constructs. At 24 hours, these cells were treated with either vehicle or cyclopamine (5 μmol/L), which blocks Hedgehog pathway at the level of Smoothened, a central transducer of canonical Hedgehog pathway (30). Protein normalized luciferase activity showed that cyclopamine failed to decrease the *CDK2* promoter activity compared with control (Fig. 4A). Moreover, there was no change in *CDK2* expression with cyclopamine treatment (Fig. 4B). The failure of cyclopamine to decrease *CDK2* activity suggests that the *GLI1*-dependent increase in *CDK2* activity occurs in a Smoothened-independent manner (non-canonical), or suppression of *GLI1* by cyclopamine is insufficient to block *CDK2* expression. To confirm these pharmacologic experiments, FLO-1 cells were cotransfected with *CDK2* promoter-reporter with either empty vector or a constitutively active Smoothened (Ca-SMO) construct. At 48 hours, Smoothened-transfected cells had no significant change in *CDK2* promoter activity compared with empty vector (100 ± 14 versus 78.5 ± 8.9, $P > 0.05$; Fig. 4C). These findings further support the observation that *GLI1*-dependent activation of *CDK2* promoter is Smoothened independent. Finally, FLO-1 cells were co-transfected with *CDK2* promoter reporter along with empty vector or Ca-SMO. The next day, cells were treated with either vehicle or Urso-Aspirin. Twenty-four hours later, as anticipated, compared with control, Urso-Aspirin combination reduced *CDK2* promoter activity by 78% (100 ± 21.7 versus 21.74 ± 4, $P < 0.05$), and *Ca-SMO* failed to rescue the *CDK2* promoter inhibition by the chemoprevention combination (Fig. 4C, right). Together, these findings show that Urso-Aspirin combinatorial therapy downregulated *CDK2*

through a *GLI1*-mediated, Smoothened-independent mechanism.

GLI1 is overexpressed during carcinogenesis in Barrett's esophagus and can be downregulated by Urso-Aspirin

To determine translational relevance of *GLI1* to carcinogenesis in Barrett's esophagus, its expression was examined *in vitro* in cell lines, biopsy samples from Barrett's esophagus patients, and animal tissue. The esophageal adenocarcinoma (FLO-1 and SKGT4) cell lines had a higher *GLI1* mRNA expression by RT-PCR compared with the normal squamous and Barrett's cell lines (BAR-T and CPC-A). We also noted increased *GLI1* expression in patients with adenocarcinoma compared with squamous and Barrett's tissue (Fig. 5A). Corroborating these findings, we examined *Gli1* expression in rat esophageal tissue and found that there was increased *Gli1* expression in esophageal adenocarcinoma compared with Barrett's and normal squamous samples (Fig. 5B). To apply our understanding of the role of *GLI1* in the context of the chemopreventive effect of Urso-Aspirin, FLO-1 cells were treated with either control or Urso-Aspirin for 48 hours. Compared with control, Urso-Aspirin significantly reduced *GLI1* expression (Fig. 5C). In agreement with these data, when *Gli1* expression was examined *in vivo*, there was marked reduction in *Gli1* expression in animals that received the Urso-Aspirin compared with animals that received the control diet (Fig. 5C). Finally, to investigate if Urso-Aspirin–dependent decrease in *GLI1* expression has an effect on its transcriptional activity, FLO-1 cells were transfected with

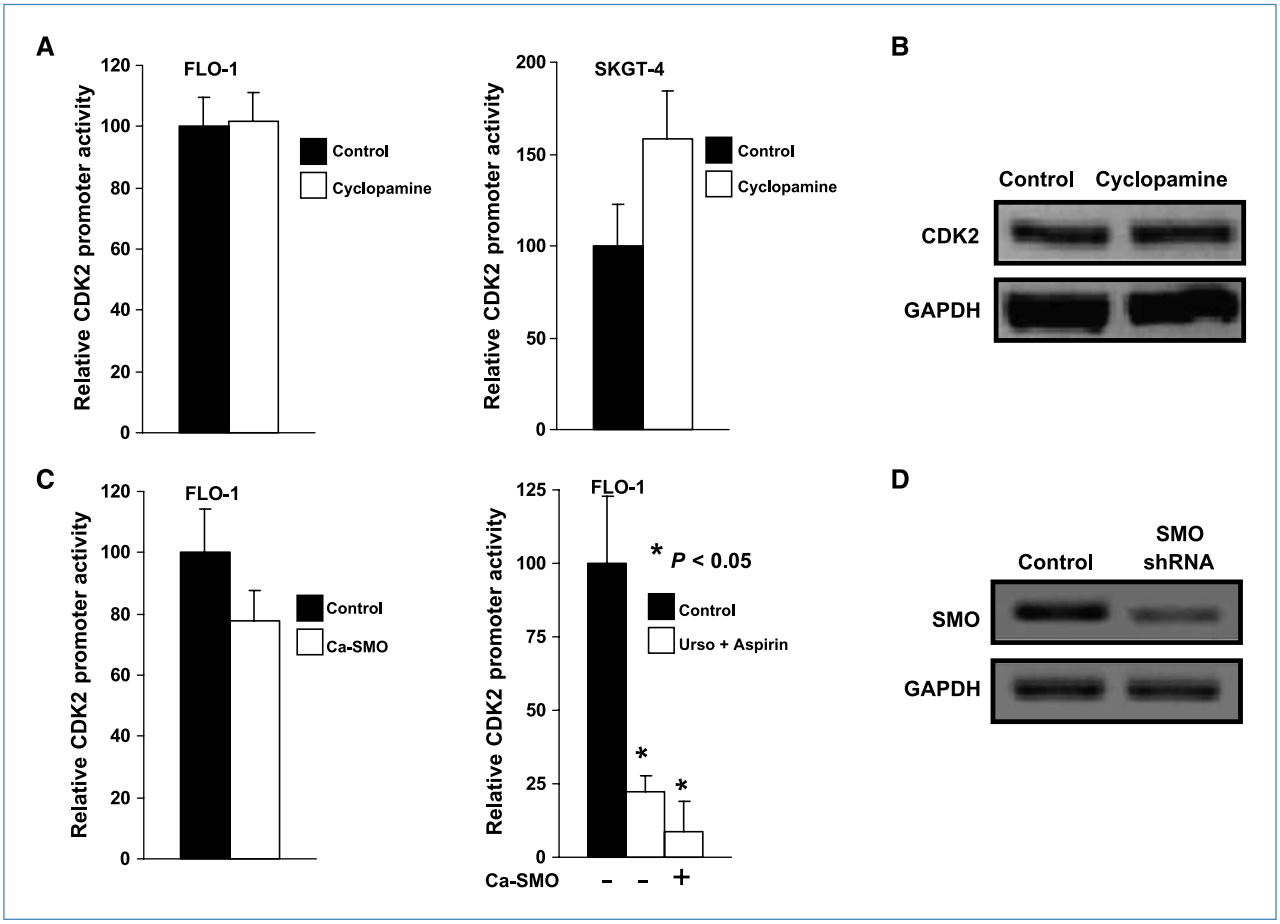

Figure 4. *GLI1* regulates *CDK2* promoter in Barrett's epithelial cells, and this *GLI* activity is independent of upstream canonical Hedgehog pathway. A, FLO-1– and SKGT4-transfected with *CDK2* promoter-reporter constructs were treated with either vehicle or cyclopamine (5 μmol/L to inhibit smoothened in the canonical Hedgehog pathway). Compared with control, cyclopamine-treated cells did not show any change in *CDK2* promoter activity. B, FLO-1 and SKGT4 were treated with either vehicle or cyclopamine (5 μmol/L), and up to 24 h, compared with control, no changes in *CDK2* expression were noted with Cyclopamine treatment. C, FLO-1 cells were cotransfected with *CDK2* promoter reporter along with either empty vector or *Ca-SMO* construct for 48 h. Compared with empty vector, *Ca-SMO*–transfected cells had no significant change in *CDK2* promoter activity (100 ± 14 versus 78.5 ± 8.9, $P > 0.05$). D, FLO-1 cells cotransfected with *CDK2* promoter reporter along with empty vector or *Ca-SMO* construct. Twenty-four hours later, cells were treated either with vehicle or Urso-Aspirin. Compared with control, Urso-Aspirin reduced *CDK2* promoter activity by 78% (100 ± 21.7 versus 21.74 ± 4, $P < 0.05$). A similar repression of *CDK2* promoter activity was also noted in the cells that were cotransfected with *Ca-SMO* (100 ± 21.7 versus 10.5 ± 8, $P < 0.050$), suggesting that *Ca-SMO* failed to release the *CDK2* promoter inhibition by Urso-Aspirin combination.

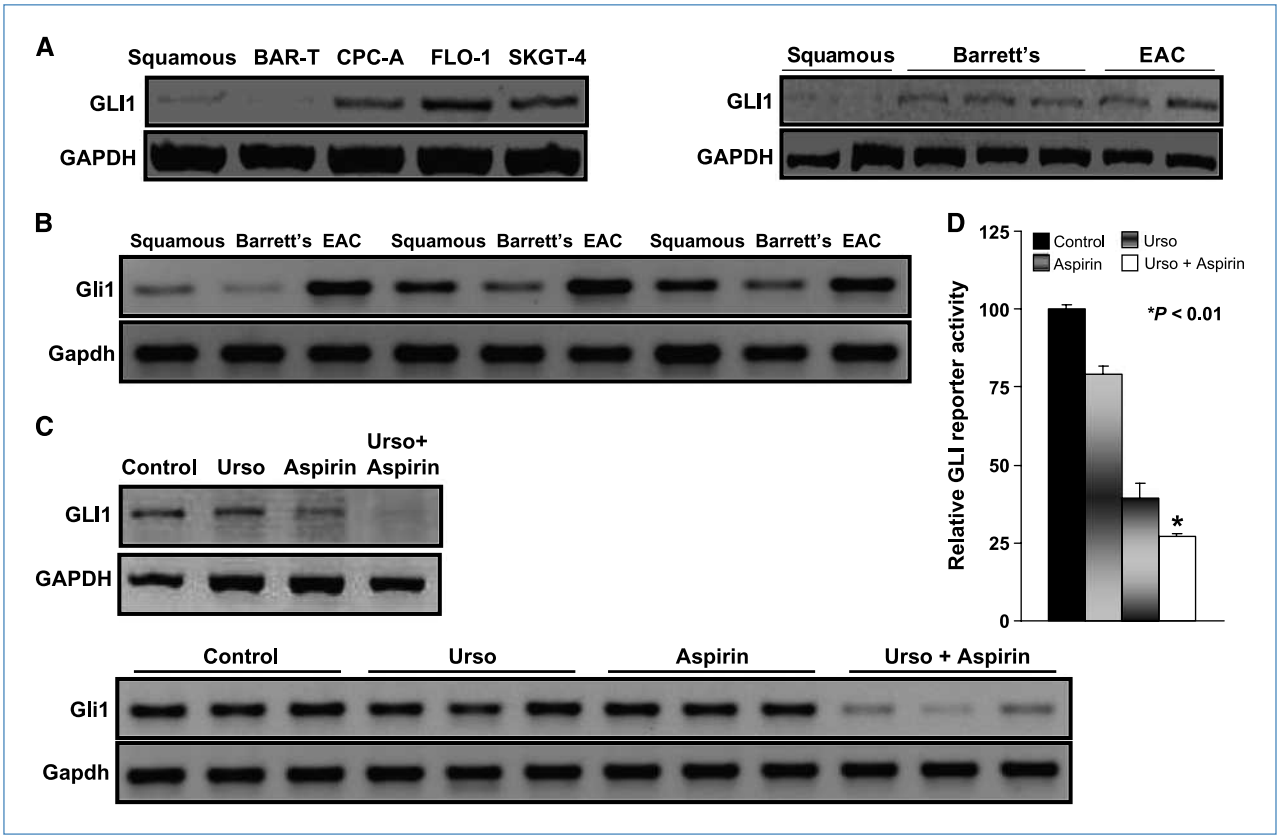

Figure 5. *GLI1* Expression increases during carcinogenesis in Barrett's esophagus and can be targeted by the Urso-Aspirin combination. A, *GLI1* expression increased progressively from esophageal squamous to adenocarcinoma cell lines (left). RT-PCR revealed a progressive increase in *GLI1* expression from squamous to cancer in patient samples (right). B, RNA was extracted from squamous, Barrett's, and adenocarcinoma rat samples from control diet group, and RT-PCR revealed that *Gli1* expression was increased from Barrett's to cancer in rat. C, esophageal cell line (FLO-1) treated with control or Urso, Aspirin, or Urso+Aspirin (150 μmol/L+1.5 mmol/L) for 24 h. RT-PCR revealed a decrease in *GLI 1* expression in the cells treated with the Urso-Aspirin. RT-PCR performed on RNA extracted from rat esophageal tissue showed a similar decrease in *Gli1* expression in the combination-treated group compared with control or when these agents were used individually. D, FLO-1 cells transfected with *GLI* reporter to measure *GLI* activity were treated either with vehicle, Urso, Aspirin, or Urso-Aspirin (150 μmol/L+1.5 mmol/L) for 24 h. The combination treatment significantly decreased *GLI* activity in FLO-1 cells compared with control treatment ($P < 0.01$).

GLI-luciferase reporter construct that had eight consecutive GLI binding sites (8x*GLI*), and 24 hours later, they were further treated with either control or Urso-Aspirin for 24 hours. Luciferase activity showed that Urso-Aspirin caused a 59 ± 8% reduction in the *GLI*-luciferase reporter activity compared with control ($P < 0.01$; Fig. 5D). These findings indicate that *GLI1* is relevant to chronic injury–associated carcinogenesis in Barrett's esophagus and can be downregulated by Urso-Aspirin.

GLI1 overexpression antagonizes the chemopreventive effect of Urso-Aspirin combination on cell proliferation and restores *CDK2* expression

Having established that *GLI1* expression increases during esophageal carcinogenesis and Urso-Aspirin decreases *GLI1* expression, we next examined whether the effect of Urso-Aspirin on cell proliferation and *CDK2* is *GLI1* dependent. To address this, FLO-1 cells were treated for 48 hours with either Urso-Aspirin or control. As expected, the combination decreased the proliferation of FLO-1 cells by 39% ($P < 0.05$). However, in FLO-1 cells that were transfected with *GLI1* before treatment with Urso-Aspirin, there was no significant reduction in proliferation compared with control (11%, $P = 0.54$; Fig. 6A). After confirming that the effect of Urso-Aspirin on cell proliferation can be abrogated by *GLI1* overexpression, we investigated whether *GLI1* overexpression could also reverse the Urso-Aspirin–dependent downregulation of *CDK2* promoter activity. FLO-1 cells were co-transfected with *CDK2* promoter-luciferase reporter constructs along with either *GLI1* constructs or control vector for 24 hours. These cells were treated with either Urso-Aspirin or control for 24 hours. A 70% reduction in *CDK2* promoter-Luciferase activity was noted in Urso-Aspirin only–treated cells ($P < 0.05$), which was abrogated in *GLI1*-transfected cells (Fig. 6B). To complement this finding, we investigated whether the effect of Urso-Aspirin on *CDK2* expression was also *GLI1* dependent. FLO-1 cells were transfected with either *GLI1* or control vector, and 24 hours, later they were treated with either

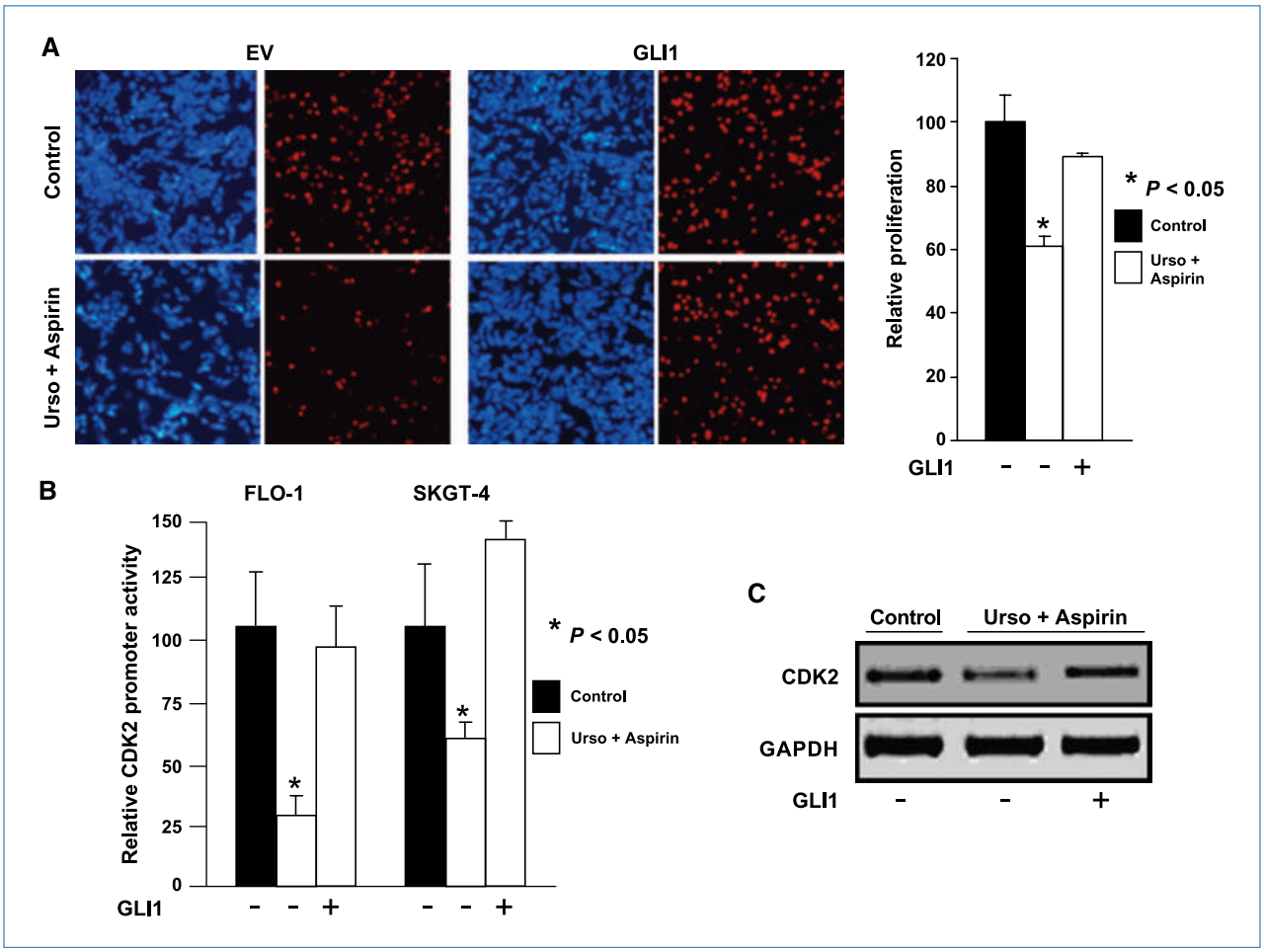

Figure 6. *GLI1* overexpression antagonizes the effect of Urso-Aspirin combination on cell proliferation and *CDK2* expression. A, FLO-1 cells treated with the Urso-Aspirin combination had a significant reduction ($P < 0.05$) in proliferation compared with control. This effect of Urso-Aspirin combination on proliferation of FLO-1 cells was reversed when the cells were transfected with *GLI1* before treatment with the combination ($P = 0.54$). B, FLO-1 cells treated with the combination treatment showed a decrease in *CDK2* expression. When these cells were transfected with *GLI1* and then subsequently treated with the combination treatment, the effect of Urso-Aspirin on *CDK2* expression was abrogated.

Urso-Aspirin or control. Whereas the combination caused a downregulation of *CDK2* expression in the control vector–transfected cells, it failed to do so in the *GLI1*-transfected cells (Fig. 6C). Together, these findings show that the effect of Urso-Aspirin on cell proliferation and *CDK2* expression occurs at least in part through *GLI1* inhibition. These findings, along with prevention of esophageal cancer, inhibition of Barrett's epithelial cell proliferation, as well as downregulation of *CDK2* by Urso-Aspirin, support the observation that *GLI* inhibition is mechanistically linked to the prevention of neoplastic transformation by the Urso-Aspirin.

Discussion

A wealth of experimental and epidemiologic evidence has established the importance of chronic injury inflammation in carcinogenesis (1–3, 15). Given the epidemiologic evidence that patients who use anti-inflammatory agents are at lower risk of developing several cancers (14, 31–34), anti-inflammatory agents have been widely investigated and found to have tumor suppressive effect both *in vivo* and *in vitro* (1, 35). This approach to target the effect of injury, although important, is usually not sufficient in preventing carcinogenesis, possibly because it does not take into account the cause of injury. This notion, along with the evolving concept of combinatorial chemoprevention (36, 37), led us to hypothesize that targeting both the cause and effect of chronic injury will lead to better control of carcinogenesis. We examined the injury-induced carcinogenesis in Barrett's esophagus to address the effectiveness of the combinatorial chemoprevention approach and to examine the underlying mechanisms. A long premalignant phase and the association of Barrett's esophagus with a highly lethal adenocarcinoma makes it an important disease to examine chemoprevention strategy (2, 10). Moreover, the contents of reflux, particularly primary and secondary bile salts, which are implicated in chronic injury during carcinogenesis in Barrett's esophagus (2), can be modified by the tertiary bile salt, Urso (13). Therefore, carcinogenesis in Barrett's esophagus

provides a distinct opportunity to test a combinatorial chemoprevention strategy that involves the modification of bile salts along with the use of anti-inflammatory agents, which minimize the consequence of injury.

Using low-dose Urso to reduce the concentration of injury-inducing bile salts along with a low-dose Aspirin in Barrett's esophagus, we found that this combination significantly reduced the rate of esophageal adenocarcinoma in animals with reflux injury. In contrast, when used individually, both Urso and Aspirin were not effective in reducing the rate of esophageal adenocarcinoma. Our *in vitro* results show that Urso-Aspirin decreases Barrett's epithelial cell proliferation, a key cellular process that is associated with neoplastic progression in Barrett's epithelial cells (38, 39). Although there is epidemiologic data supporting the chemopreventive potential of aspirin in Barrett's esophagus and there is indirect evidence that Urso, by modifying the concentration of injury-inducing bile salts, may help prevent esophageal adenocarcinoma (13, 14, 18, 31–33, 40), this is the first *in vitro* and *in vivo* experimental evidence to support that these agents, when used together, prevent esophageal adenocarcinoma.

To our knowledge, this study, for the first time, provides evidence that *GLI1* is involved in reflux injury–induced carcinogenesis and that it is a key molecular target of combinatorial chemoprevention by Urso-Aspirin. GLI proteins are highly conserved (41, 42) proteins that are emerging as important transcriptional regulators of oncogenic pathways by regulating apoptosis and epithelium to mesenchyme transformation (29, 43–47). In this study, we uncovered an additional mechanism that GLI proteins could use to promote carcinogenesis by showing that GLI1 binds to *CDK2* promoter, upregulates *CDK2* transcription, increases *CDK2* expression, and induces cell proliferation. Furthermore, the translational relevance of this molecular mechanism, in the context of chemoprevention, is supported by several novel findings in this study. First, *GLI1* is upregulated both in patients as well as in animals during injury inflammation–induced carcinogenesis. Second, Urso-Aspirin combination downregulates *GLI1*, represses *CDK2*, decreases proliferation, and prevents cancer development. Finally, *GLI1* overexpression can reverse the effect of Urso-Aspirin combination not only on *CDK2* expression but also on proliferation. Together, these findings provide alternative mechanisms that *GLI1* could use during oncogenesis and reveal the role of *GLI1* in chemoprevention.

This study also provides an additional pharmacologic option to inhibit emerging pro-oncogenic protein GLI1. It is well accepted that the *GLI1* activity can be upregulated either through canonical Hedgehog-Smoothened–dependent signaling or a less well-understood noncanonical pathway upon which several signaling pathways, including transforming growth factor-β (TGF-β) or Ras, could converge (29). At present, cyclopamine is the only available Food and Drug Administration–approved drug that inhibits GLI1 by targeting Smoothened in the canonical pathway (47–49). Our data, which show that Urso-Aspirin combination targets *GLI1* in a Smoothened-independent manner (likely through TGF-β and prostaglandin E2–mediated mechanisms; data not shown) provides an additional novel pharmacologic approach to downregulate *GLI1*. Therefore, under the circumstances, in which both canonical and noncanonical signaling upregulate *GLI1* during carcinogenesis, one can envision a combinatorial strategy involving cyclopamine to target the canonical pathway and Urso-Aspirin to inhibit the noncanonical pathway.

Because this was a proof of principal study, it was beyond the scope of this article to test the efficacy of these agents at the lowest possible doses; however, it remains an important consideration for future animal or clinical study. As outlined in the method section, the doses selected in this study were within the published range and were further refined based on the pharmacokinetic and/or molecular data available from patients who received Urso or Aspirin, and will therefore be achievable *in vivo* in patients. At this fixed, low-dose Urso-Aspirin combination, we did not encounter any side effects in animals. Although both the safety and efficacy of this approach need to be investigated in patients with Barrett's esophagus, we do not anticipate any serious side effects. Urso is well tolerated by patients who take it up to 15 mg/kg/d on long-term basis, and low-dose Aspirin is clinically safe given that the majority of Barrett's patients take proton pump inhibitors, which can prevent gastrointestinal bleeding.

In conclusion, our study contributes several novel observations in the field of chemoprevention that, although discovered while studying carcinogenesis in Barrett's esophagus, may find wider implications to other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Mayo Foundation, Mayo Clinic Cancer Center, NIH grants CA110022-01 and DK076845-01 (N.S. Buttar), and CA102701 and CA136526 to (M.E. Fernandez-Zapico).

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Received 02/18/2010; revised 06/02/2010; accepted 06/15/2010; published OnlineFirst 07/20/2010.

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