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# Original communication

## Activation of MUC1 mucin expression by bile acids in human esophageal adenocarcinomatous cells and tissues is mediated by the phosphatidylinositol 3-kinase

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[Q1]

**Background.** In esophageal adenocarcinoma, MUC1 mucin expression increases in early stages of the carcinogenetic sequence, during which bile reflux has been identified as a major carcinogen. However, no link between MUC1 overexpression and the presence of bile acids in the reflux has been established so far, and molecular mechanisms regulating MUC1 expression during esophageal carcinogenetic sequence are unknown. Our aim was to identify (1) the bile acids able to upregulate MUC1 expression in esophageal cancer cells and mucosal samples, (2) the regulatory regions in MUC1 promoter responsive to bile acids, and (3) the signaling pathway(s) involved in this regulation.

**Methods.** MUC1 mRNA and mucin expression were studied by the means of real-time reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry, both in the human esophageal OE33 adenocarcinoma cell line and in an *ex vivo* explant model. MUC1 promoter was cloned and transcription regulation was studied by transient cell transfection to identify the bile acid-responsive regions. Signaling pathways involved were identified using specific pharmacologic inhibitors and siRNA approach.

**Results.** Taurocholic, taurodeoxycholic, taurochenodeoxycholic, glycocholic, sodium glycocholate, and deoxycholic bile acids upregulated MUC1 mRNA and protein expression. The highest induction was obtained with deoxycholic and taurocholic acids in both cellular and explant models. The bile acid-mediated upregulation of MUC1 transcription occurs at the promoter level, with responsive elements located in the -1472/-234 region of the promoter, and involves the phosphatidylinositol 3-kinase signaling pathway.

**Conclusions.** Bile acids induce MUC1 mucin overexpression in human esophageal adenocarcinoma cells and tissues by activating its transcription through a process involving phosphatidylinositol 3-kinase. (Surgery 2007;■:■-■.)

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ADENOCARCINOMA OF THE ESOPHAGUS, the incidence of which has profoundly increased during the past few decades,<sup>1,2</sup> has one of the poorest outcomes of all human malignancies. The major risk factors for the development of esophageal adenocarcinoma are gastroesophageal reflux disease and its sequela. Barrett's esophagus is a condition in which metaplastic columnar epithelium replaces the normal stratified squamous epithelium.<sup>3-5</sup> Increased intragastric concentrations of bile acids

have has been observed in patients with Barrett's esophagus, and it has been suggested that incomplete intestinal-type metaplasia may be a response to reflux of gastroduodenal contents and, in particular, to bile acids.<sup>6,7</sup> Moreover, duodeno-esophageal bile reflux has recently been identified as a predominant and sufficient carcinogen to induce esophageal adenocarcinoma in Barrett's esophagus in a rat model.<sup>8,9</sup>

Endoscopic surveillance of patients with known Barrett's esophagus is the current strategy to manage the risk of cancer formation, targeted at the detection of dysplasia. Unfortunately, data suggest that dysplasia is an imperfect predictor of cancer risk in Barrett's esophagus.<sup>10</sup> Thus, an earlier indicator of cancer risk would be detection of DNA damage itself before the histologic manifestations of dysplasia are even apparent. The identification of molecular biomarkers may offer easy reproducibility, standardization, and truly early detection of neoplastic progression. Among them, mucins appear as good candidates to evaluate, because their expression is altered dramatically in Barrett's esophagus.<sup>5,11,12</sup>

In normal esophagus, mucins protect the underlying mucosa against potential injuries such as reflux of gastroduodenal contents including bile acids.<sup>13</sup> Among the members that compose the family of mucins, membrane mucins are thought to play important roles in tumor cell biology, cell proliferation, tumor progression, and metastasis.<sup>11</sup> MUC1 is the best-characterized membrane mucin.<sup>14</sup> It is a large O-glycoprotein with an extended, heavily glycosylated extracellular domain that protrudes far away from cellular membrane (200 to 500 nm). This structural feature confers to MUC1 the ability to participate in cell-cell and cell-extracellular matrix recognition processes.<sup>11,15</sup> Consequently, overexpression of MUC1 membrane mucin at the cell surface provides the cancer cell with new biologic properties by altering its interacting properties with immune, epithelial, or endothelial cells or the extracellular matrix.<sup>14,15</sup> Moreover, MUC1 is known to interact with the EGF-receptor and participate in intracellular signaling.<sup>14</sup> These peculiar structural features and biologic activities put forward mucins as important molecules to study in order to better understand their role in carcinogenesis and biology of the cancer cell.<sup>11,16,17</sup>

In normal esophagus, *MUC1* and *MUC4* are the main mucin genes expressed in the stratified squamous epithelium.<sup>12,18</sup> In the preneoplastic Barrett's esophagus, secreted mucins are expressed mainly (*MUC2*, *MUC5AC*, *MUC5B*, *MUC6*) associated with *MUC1* and *MUC4*.<sup>5,18-20</sup> In high-grade dysplasia and adenocarcinoma of Barrett's esophagus, downregulation of secreted mucins is observed,

whereas expression of *MUC1* and *MUC4* mucin genes is sustained at high levels.<sup>12,13,20,21</sup> From these results, several groups<sup>18,19</sup> have been considering *MUC1* as a valuable phenotypic marker to distinguish between Barrett's esophagus patients with different origins and those at risk to develop neoplasia in Barrett's esophagus. Finally, bile acids can activate the expression of 2 mucins found in Barrett's esophagus, the secreted mucin *MUC2*<sup>22</sup> and the membrane mucin *MUC4*,<sup>21,2</sup> suggesting a relation between increased mucin expression and progression of esophageal cancer associated with bile reflux; however, at this time, no data are available to explain the upregulation of *MUC1* in Barrett's esophagus associated with reflux.

In relation with *MUC1* overexpression during the development of esophageal adenocarcinoma in Barrett's esophagus and the role of bile acids in Barrett's metaplasia and its consequent degeneration into esophageal adenocarcinoma, we undertook in this work to identify (1) the bile acids responsible for the upregulation of *MUC1* mucin in esophageal cancer cells and explants, (2) the regulatory regions responsive to bile acids in *MUC1* promoter, and (3) the signaling pathway(s) involved in this regulation.

## MATERIAL AND METHODS

**Cell culture.** The esophageal cancer cell line OE33 was purchased from the European Collection of Cell Cultures (ECACC) and cultured as described previously.<sup>21</sup> Cells were treated with bile acids and their conjugates for 24 hours with the following concentrations as previously described<sup>21</sup>: taurocholic acid (TC), 0.5 mM; taurodeoxycholic acid (TDC), 1 mM; taurochenodeoxycholic acid (TCDC), 0.5 mM; glycocholic acid (GC), 0.5 mM; sodium glycocholate (GNa), 0.5 mM; and deoxycholic acid (DC), 0.05 mM. In inhibition studies, pharmacologic inhibitors were incubated with the cells for 30 minutes prior to the addition of bile acids at the following final concentrations: wortmannin (2.5 nM, inhibitor of PI3K), PD98059 (30 μM, inhibitor of MAPK; Calbiochem, VWR International S.A.S., France), U0126 (10 μM, inhibitor of MEK; Promega, Charbonnières, France), GF109203X (10 μM, inhibitor of PKC; Calbiochem), and KT5720 (1 ng/ml, inhibitor of PKA; Calbiochem). All reagents were from Sigma (St Louis, Mo, USA) unless otherwise indicated. [Q2]

Toxicity of each bile acid was evaluated under the same experimental conditions by trypan blue exclusion measurement on a hemocytometer. Effects of bile acids on cell proliferation and apoptosis were studied by immunochemistry, using

monoclonal anti-Ki67 (dilution of 1:50; Dako/Dakocytomaton, Trappes, France) and polyclonal anticleaved caspase-3 (dilution of 1:150; Cell Signaling, Ozyme, France) antibodies, respectively. Level of caspase-3 mRNA was also evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) using a specific pair of primers (MWG-Biotech, Germany) for caspase-3 gene: forward primer: 5'-TATGGTTTTGTGATGTTTGTCC-3', reverse primer: 5'-TAGATCCAGGGGCATTGTAG-3' (GenBank accession no. G10724). Annealing temperature was 55°C. Expected size for caspase-3 PCR product is 195 basepairs (bp).

**RT-PCR.** Total RNA from OE33 cells or esophageal tissues were prepared using the RNeasy mini-kit or midi-kit (Qiagen, Courtaboeuf, France), respectively. To prepare cDNA ((Advantage RT-for-PCR kit; Clontech, Saint-Germain-en-Laye, France), 1.5 µg (0.4 µg for tissues) of total RNA was used, as described previously.<sup>24</sup> PCR was performed on 2 µl (5 µl for tissues) of cDNA using a specific pair of primers (MWG-Biotech, Germany) for MUC1 mucin gene (GenBank accession no. J05582): forward primer : 5'-GAACTACGGG CAGCTGGACATC-3' (nucleotides 3639-3661), reverse primer : 5'-GCTCTCTGCGCCAGTCCTCC TG-3' (nucleotides 4065-4086).<sup>25</sup> PCR reactions were carried out in 50 µl final solutions as described previously.<sup>26</sup> Annealing temperature was 60°C. PCR products were analyzed on 1.5% agarose gels containing ethidium bromide run in 1× Tris-borate-ethylenediamine tetraacetic acid (EDTA) buffer. A 100 bp DNA ladder was purchased from Amersham Biosciences. Ribosomal 18S subunit (forward primer: 5'-GGACCAGAGGC AAAGCATTGGCC-3'; reverse primer: 5'-TCAATCT CGGCTGGCTGAACGC-3') was used as the internal control. In siRNA experiments, GAPDH (forward primer: 5'- TGAAGGTCCGAGTCAACGGAT TTGGT-3'; reverse primer: 5'- CATGTGGGCCATG AGGTCCACCAC-3') was used as the internal control. A specific pair of primers for PI3K p110 subunit (forward primer: 5'-ACAATGCCTCCAAGACC ATCATC-3'; reverse primer: 5'-CATACATTGCTC TACTATGAGGTG-3') was used to evaluate effectiveness of PI3K knockdown.<sup>27</sup> Expected sizes for MUC1, 18S, GAPDH, and PI3K p110 PCR products are 447, 496, 980, and 499 bp, respectively. RT-PCR was carried out on cDNAs from 3 different sets of experiments.

**Immunohistochemistry.** After treatment with bile acids, confluent OE33 cells were trypsinized, centrifuged, washed once with 1x phosphate-buffered saline (PBS). The final pellet was fixed in 4% (w/v) paraformaldehyde and embedded in

paraffin; 3-µm sections were prepared and processed for immunostaining, as described before.<sup>21</sup> Positive controls for MUC1 were run on normal alveolar pulmonary epithelium. Negative control was run by omission of the primary antibody. Cell counting for MUC1, caspase-3, and Ki67 positivity was performed by 2 independent pathologists. The monoclonal anti-MUC1 antibody (LICR-LON-M8, 1:50 dilution) recognizes the DTR epitope within the tandem repeat region of MUC1 and was a kind gift from Dr D. Swallow (MRC, London, England).<sup>28</sup>

#### Tissue sample collection and explant studies.

Surgical biopsy specimens were collected from patients who underwent curative esophagectomy by 1 surgeon (C.M.) for Barrett's adenocarcinoma without neoadjuvant treatment. Macroscopic adenocarcinoma mucosal samples of 1-mm thickness and weighing more than 20 mg were removed from the surgical specimen under sterile conditions using a frozen scalpel immediately after the vascular section. Consent was obtained from each patient included in this experimental study.

For the explant studies, tissues were placed immediately in supplemental medium and cultured under the same conditions used for the cell culture experiments. Tissues were exposed to each bile acid for 30 minutes at the concentrations used in cell culture studies. In all experiments, a control sample was included by treating the explant with an identical volume of sterile water. Samples were then divided into 2 parts using aseptic technique. One part was immersed in 5 ml of a 4% neutral formaldehyde solution (v/v, pH 7.4) in phosphate buffer and processed for paraffin embedding. The diagnosis of adenocarcinoma mucosa was confirmed by 2 pathologists after staining the section (4 µm) with hematoxylin-eosin-saffron. The second part was immersed overnight in 5 ml of RNA later solution (Qiagen) and processed for RNA extraction as described above.

**Western blotting.** OE33 total cellular extract preparation, protein electrophoresis, and Western blotting were carried out as described previously.<sup>23</sup> Standard immunodetection procedure was then performed using the following specific antibodies: anti-p38 MAPK (1:1000 dilution), anti-stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK, 1:1000 dilution) rabbit polyclonal antibodies (Cell Signaling), anti-active p38 (1:2000 dilution), and anti-active JNK (1:5000 dilution) rabbit polyclonal antibodies (Promega). Secondary antibody consisted of antirabbit IgG alkaline phosphatase-conjugate (Promega). Blots were then incubated with Nitro Blue Tetrazolium Chloride and

**Table I.** Sequences of the pairs of primers used in PCR to produce deletion mutants of *MUC1* promoter in pGL3 basic vector

Position on the promoter	Primer sequence (5' → 3')	Orientation
-233/+33	CGC <u><i>GGT ACC</i></u> CAG GGA GTG GTT GGT GA	Sense
	CGC <u><i>GGT ACC</i></u> GAT TCA GGC AGG CGC TGG	Antisense
-616/+33	CGC <u><i>GGT ACC</i></u> CAA GGA GGG AAC CCA GGC	Sense
	CGC <u><i>GGT ACC</i></u> GAT TCA GGC AGG CGC TGG	Antisense
-1472/+33	CGC <u><i>GGT ACC</i></u> CTG CTC CCC AAA GGA TAG	Sense
	CGC <u><i>GGT ACC</i></u> GAT TCA GGC AGG CGC TGG	Antisense

KpnI cloning site is italicized and underlined.

5-bromo-4-chloro-3-indolyl phosphate substrate (Life Technologies), and the reaction was stopped [Q7] in water.

**Cloning of the MUC1 promoter.** The pMAH-*MUC1* vector, which contains 2.8 kb of *MUC1* promoter in pT7-*Luc* vector, was a kind gift from [Q8] Dr M. A. Hollingsworth (UNMC, Omaha, Neb, USA). Internal deletion mutants were generated by PCR using pairs of primers bearing KpnI restriction site at their 5' end (Table I). PCR reaction was [T1] performed as described previously on 0.46 μg of plasmidic DNA with a specific annealing temperature of 70°C. PCR products were digested with KpnI restriction enzyme (Roche Biochemicals, Meylan, France), gel-purified (QIAquick gel extraction kit, Qiagen) and ligated into the promoterless pGL3 Basic vector (Promega), which had been linearized previously with KpnI using a DNA rapid ligation kit (Roche Biochemicals). Positive clones containing the insert were sequenced on both strands on an automatic LI-COR sequencer (ScienceTec, Les Ulis, France) using infrared-labeled RV3 and GL2 primers (Promega). Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

**Transfections.** Transfections were performed using Effectene reagent (Qiagen), as described previously.<sup>29</sup> Cells were passed at  $0.5 \times 10^6$  cells/well the day before the transfection. Luciferase activity was corrected for transfection efficiency by cotransfecting cells with pRL-TK vector (Promega). Total cell extracts were prepared after a 48-hour incubation time at 37°C using 1× Passive Lysis Buffer (Promega), per manufacturer's instructions. Luciferase activities were measured on [Q9] a TD 20/20 luminometer (Turner Design). The relative luciferase activity was expressed as fold induction of the test plasmid activity compared with that of the corresponding empty vector. In bile acids experiments, relative luciferase activity was expressed as fold activation of luciferase activity in bile acid-treated cells compared with untreated

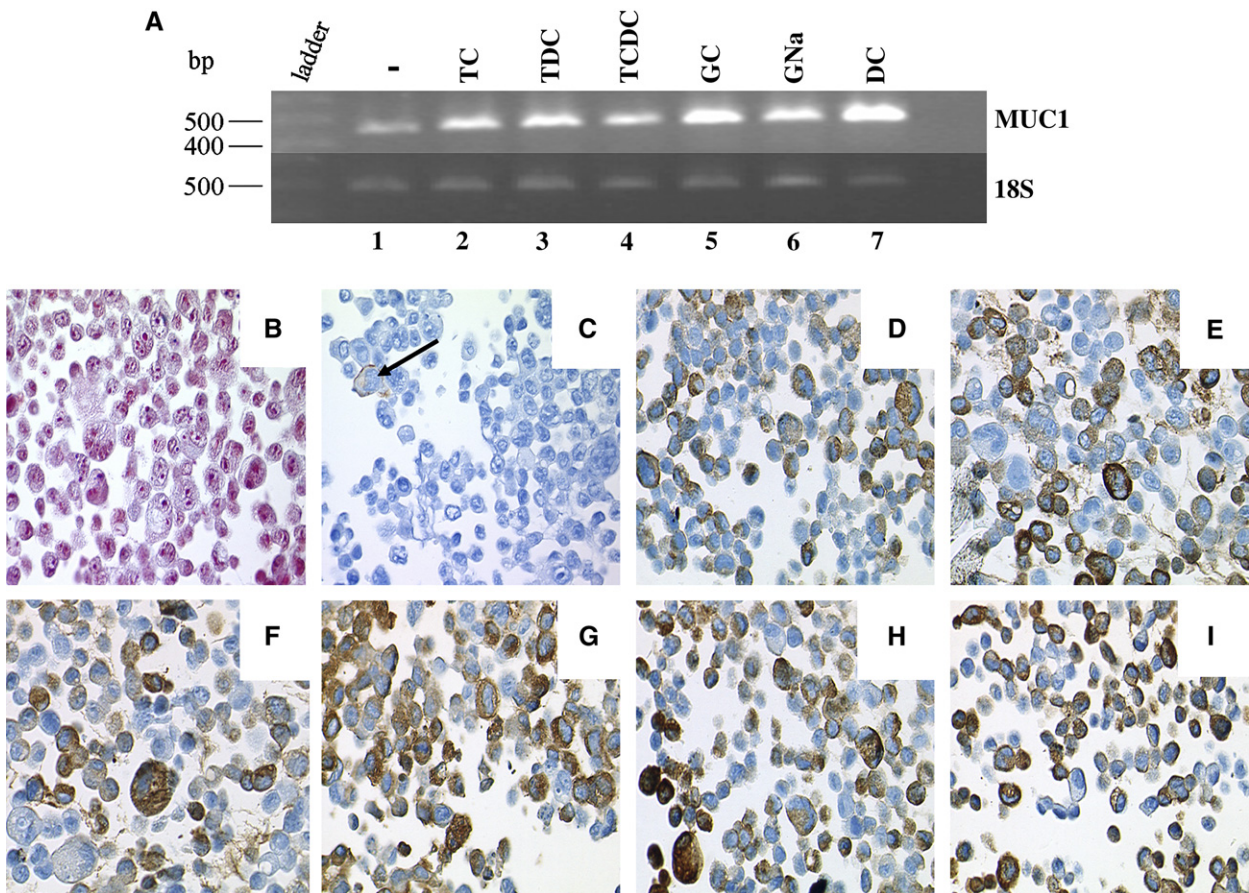
cells. All experiments were performed in triplicate and repeated 3 times.

**Small interfering RNA (siRNA) assays.** OE33 cell seeding ( $15 \times 10^3$  cells/well) and transfections were performed as described previously<sup>23</sup> with 100 nM of PI3K SMARTpool siRNA, using 1 μl of DharmaFECT1 transfection reagent (Dharmacon, Brebières, France). Controls included mock transfected cells, cells transfected with siCONTROL non-targeting siRNA, or siCONTROL GAPD siRNA. Bile acid treatment was performed 24 hours after transfection and lasted 24 hours, as described above. Total RNA was isolated 48 hours after transfection, as described above. Each siRNA was assayed in triplicate in at least 3 separate experiments. PCR was performed on 5 μl of cDNA, as described previously. Densitometric analysis of DNA bands was carried out using the GelAnalyst-GelSmart software (Clara Vision). Results were [Q10] expressed as MUC1/GAPDH and PI3K p110/GAPDH ratio.

**Statistics.** When indicated, data were analyzed by the Mann-Whitney *U* test with differences of  $P < .05$  considered significant.

## RESULTS

**Regulation of MUC1 mRNA expression by bile acids in OE33 cancer cells.** The level of MUC1 mRNA expression in OE33 cancer cells treated with different bile acids was assessed by RT-PCR (Fig 1, A). The level of MUC1 mRNA in untreated [F1-4/C] cells (lane 1) was increased substantially after cell treatment with taurocholic acid (TC, lane 2), taurodeoxycholic acid (TDC, lane 3), taurochenodeoxycholic acid (TCDC, lane 4), glycocholic acid (GC, lane 5), sodium glycocholate (GNa, lane 6), and deoxycholic acid (DC, lane 7). The highest induction (4.5-fold) was obtained with DC. Other bile acids (cholic acid, chenodeoxycholic acid, dehydrocholic acid, and cholic methylester) previously shown to activate MUC4 mucin expression<sup>21</sup> were studied but had no effect on MUC1 (data not shown).



**Fig 1.** Effect of bile acids and their conjugates on expression of *MUC1* mRNA and of MUC1 apomucin in OE33 cancer cells. **A**, Cells were treated with indicated bile acids for 24 hours, as described in Material and Methods. *MUC1* and *18S* PCR products (7.5  $\mu$ l and 4  $\mu$ l, respectively) were separated on a 1.5% agarose gel containing ethidium bromide after electrophoresis in 1X Tris-borate-EDTA buffer. Immunohistochemistry was performed as described in Material and Methods. **B-C**, Untreated cells. **B**, Alcian blue staining; **C**, MUC1 immunostaining; **D-I**, MUC1 immunostaining after treatment with TC (**D**), TDC (**E**), TCDC (**F**), GC (**G**), GNa (**H**) and DC (**I**). Original magnification  $\times 200$ .

**Effect of bile acids on MUC1 apomucin expression in OE33 cancer cells.** Having shown that *MUC1* mRNA expression was upregulated by bile acids, we examined whether that regulation had an impact on the expression of MUC1 apomucin [T2] (Figs 1, B-I, and Table II). Untreated OE33 cells showed a relatively low degree of differentiation, because no vacuoles and a few grains of secretions were detected (Fig 1, B). When immunostained [Q11] with anti-MUC1 antibody, 1% to 5% of the cells were labeled positively (Fig 1, C). The staining was seen in the membrane and cytoplasm (Fig 1, C, arrow). The labeling became very intense and up to 90% of the cells were stained positively for MUC1 when they were treated with TC (Fig 1, D), TDC (Fig 1, E), TCDC (Fig 1, F), GC (Fig 1, G), GNa (Fig 1, H), and DC (Fig 1, I).

Bile acid treatment under the same conditions did not induce any change in the morphology of

the cells (compare Figs 1, B and C [untreated cells] with Fig 1, D-I [bile acid-treated cells]) nor did it induce cell death, because trypan blue exclusion measurements were similar in untreated and bile acid-treated cells (0% to 1% dead cells). Absence of effect on apoptosis was also observed by studying caspase-3 expression at the mRNA and protein levels. No induction of caspase-3 mRNA (Fig 2, A) [F2-4/C] or cleaved caspase-3 protein level (Figs 2, B-D and Table II) was observed after bile acid treatment. Finally, under our experimental conditions, bile acids did not alter cell proliferation because the number of cells immunostained for the proliferation marker Ki67 was similar in untreated cells (Fig 2, E) and bile acid-treated cells (Fig 2, F and Table II).

**Effect of bile acids on the expression of MUC1 mRNA in adenocarcinomatous esophageal mucosa.** MUC1 regulation by bile acids was also

**Table II.** Effect of bile acids and their conjugates on MUC1 apomucin expression, cell death (trypan blue), apoptosis (caspase-3), and proliferation (Ki67) in OE33 cancer cells

	<i>Control</i>	<i>TC</i>	<i>TDC</i>	<i>TCDC</i>	<i>GC</i>	<i>GNa</i>	<i>DC</i>
MUC1	1-5	89	85	91	90	82	92
Trypan blue	0-1	0-1	0-1	0-1	0-1	0-1	0-1
Caspase-3	<1	<1	<1	<1	<1	<1	<1
Ki67	90	85	93	94	86	85	85

Cells were treated with the indicated bile acids for 24 hours and processed for immunostaining with anti-MUC1, anticleaved caspase-3, and anti-Ki67 antibodies (Material and Methods). Percentage of positive cells was calculated by 2 independent pathologists.

studied in an ex vivo organ culture model. To this aim, surgical specimens of adenocarcinomatous esophageal mucosa from 9 patients were cultured and exposed to bile acids. Expression of MUC1 was assessed by RT-PCR and immunochemistry. The means ratio of MUC1 mRNA expression over 18S [F3] from the 9 samples is shown in Fig 3, A. A 2-fold induction of MUC1 mRNA level was observed in [F4] explants treated with TDC (Fig 4, B, lane 3), TCDC (lane 4), GC (lane 5), and GNa (lane 6). When treated with TC (lane 2) and DC (lane 7) a 4- to 4.5-fold induction was obtained. The increase of MUC1 mRNA could not be correlated to an increase of MUC1 apomucin expression by immunochemistry because of an already high level of MUC1 expression in the control samples (90% to 100% of cells were MUC1-positive [data not shown]). These results, however, indicate that bile acids are able to activate MUC1 expression at the mRNA level in explants and that TC and DC are the most potent activators in OE33-cultured cancer cells and in mucosal explants.

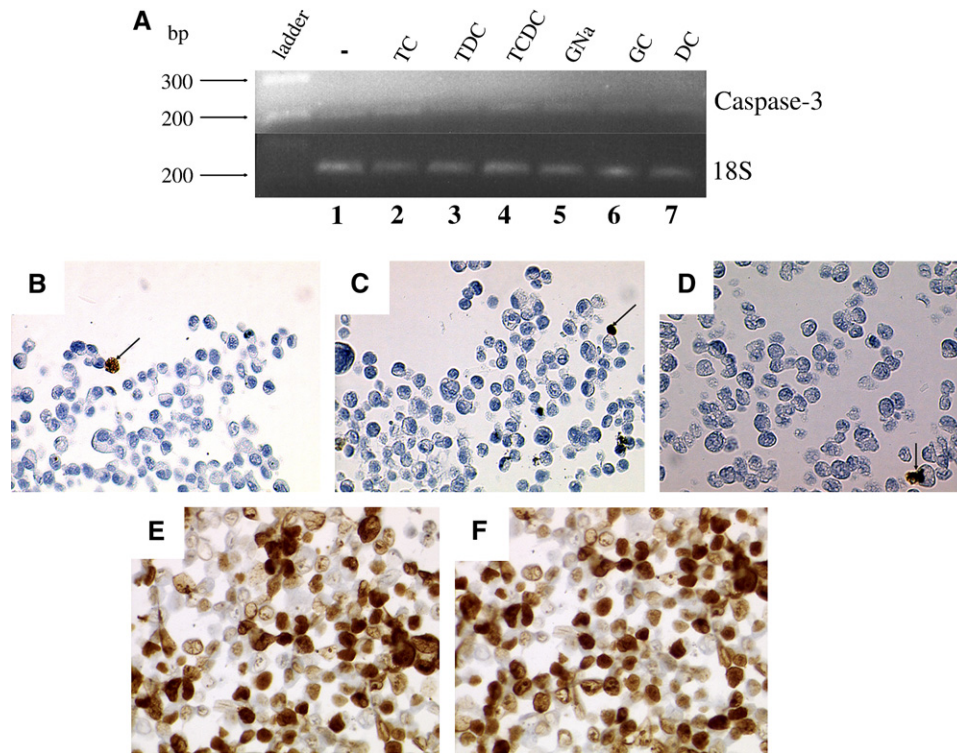
**Identification of bile acid-responsive regions in the MUC1 promoter.** To identify bile acid-responsive regions responsible for MUC1 transcriptional upregulation, 1.47 kb of the MUC1 promoter was cloned into promoterless pGL3 basic vector (Fig 4, A). The 3 constructs used in this study were active in OE33 cells (Fig 4, B). The fragment that covers the first 233 nucleotides of the promoter was already very active (15-fold activation). The activity increased to 40-fold activation when the intermediate -616/+33 deletion mutant was transfected. With the longest fragment (-1472/+33), the activity went up to 50-fold, indicating that all the elements necessary for maximal activity of MUC1 promoter were present in the 1.47 kb region.

To identify bile acid-responsive regions within the MUC1 promoter, transfected cells were treated with bile acids for 24 hours (Fig 1) before measurement of luciferase activity. Promoter activity was then compared between bile acid-treated cells and untreated cells. As shown in Fig 4, C, a 2- to 2.5-fold activation of the -616/-234 promoter region (gray bars) indicated that TC- and GC-

responsive elements were present in that region of the promoter. For TDC and DC, similar activations (2-fold) of -1472/+33 and -616/+33 regions were observed, indicating that responsive elements for these 2 bile acids are present in the -1472/-234 region of the promoter. In contrast, responsive elements for TCDC and GNa were present within the more distal region -1472/-762 (2.5- and 2.2-fold activation [black bars]).

**PI3K signaling pathway mediates upregulation of MUC1 by bile acids in OE33 cells.** Having shown that bile acids regulate MUC1 expression at the transcriptional level in OE33 cells, we worked to identify the signaling pathway(s) responsible for that regulation. To this aim, OE33 cells were pretreated for 30 minutes with specific pharmacologic inhibitors of PI3K, MAPK, PKC, and PKA, which are known to be activated by bile acids,<sup>21,30-33</sup> before adding bile acids to the cells for another 24 hours. Inhibition of MAPK (PD98059, U0126), PKC (GF109203X), or PKA (KT5720) did not alter the level of MUC1 mRNA in the cells, indicating that these pathways were not involved (data not shown). We also used Western blotting analysis to determine whether the other 2 MAPK pathways (p38 MAPK and SAPK/JNK) were activated by bile acids. No variation in [Q12] the level of phosphorylation of these 2 kinases, however, was visualized, indicating that these pathways are not triggered by bile acids under these conditions (data not shown). In contrast, inhibition of PI3K pathway with wortmannin (w) before cell treatment with TC, TDC, TCDC, and DC induced a substantial decrease of MUC1 mRNA level, which then returned to the basal level (Fig 5, A, [F5-4/C] lanes 3, 5, 7, and 13 vs lanes 2, 4, 6, and 12). Inhibition of PI3K signaling pathway before cell treatment with GC and GNa had no effect on MUC1 mRNA level (compare lanes 9 and 11 to lanes 8 and 10).

The direct implication of the PI3K signaling pathway in bile acid regulation of MUC1 endogenous expression was then assessed by performing knockdown assays with specific siRNA for PI3K



**Fig 2.** Effect of bile acids and their conjugates on apoptosis and proliferation in OE33 cancer cells. A, Level of caspase-3 mRNA by RT-PCR in untreated cells (lane 1) and bile acid-treated cells (lanes 2-7) as described in Material and Methods. *Caspase-3* and *18S* PCR products (15  $\mu$ l and 4  $\mu$ l, respectively) were analyzed on a 1.5% agarose gel. Cleaved caspase-3 immunostaining in untreated cells (B) and after treatment by TC (C) and TCDC (D). Original magnification  $\times 200$ . Ki67 immunostaining in untreated cells (E) and after treatment by TDC (F). Original magnification  $\times 200$ .

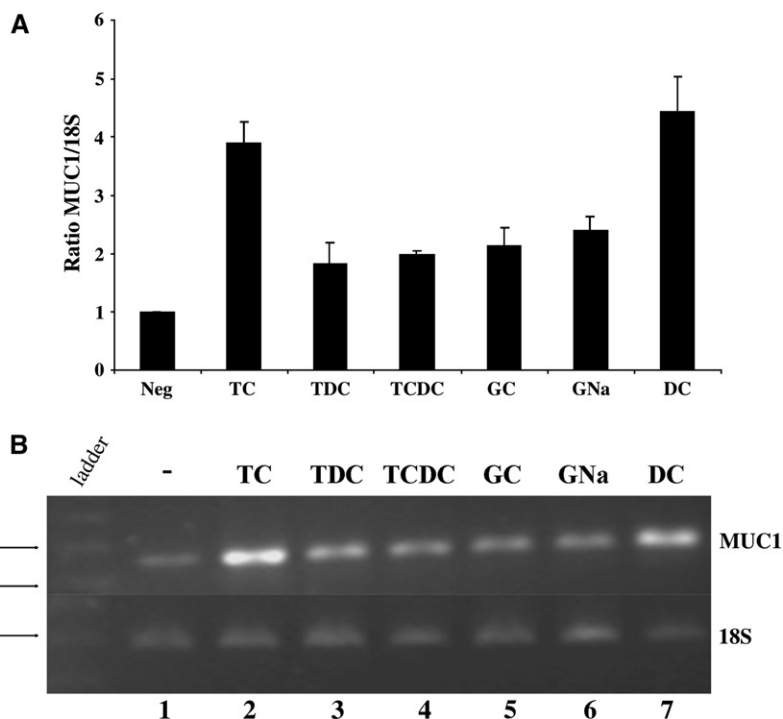
(Fig 5, B). The results showed that knockdown of PI3K significantly ( $P < .05$ ) inhibited TC-, TDC-, TCDC-, and DC-mediated increase of *MUC1* mRNA level ( $73\% \pm 11\%$ ,  $52\% \pm 19\%$ ,  $44\% \pm 7\%$ , and  $84\% \pm 2\%$  inhibition, respectively). This indicates that PI3K mediates *MUC1* endogenous expression in response to these bile acids. Treatment of cells with GC and GNa enhanced the basal expression of *MUC1* mRNA, but knockdown of PI3K did not significantly alter *MUC1* mRNA level ( $P > .05$ , data not shown).

Immunostaining of OE33 cells with a specific anti-MUC1 antibody confirmed the involvement of the PI3K pathway in bile acid-mediated upregulation of MUC1 apomucin expression (Figs 5, C-H). The labeling decreased from 82% in TC-treated cells (Fig 5, C) to 43% in wortmannin-pretreated cells (Fig 5, F) and from 62% in TDC-treated cells [F6] (Fig 6, D) to 21% in wortmannin-pretreated cells (Fig 5, G). The same conclusion was drawn regarding DC-mediated upregulation of MUC1 apomucin expression because 63% of DC-treated cells were positive for MUC1 (Fig 5, E), whereas only 29% remained labeled when cells were pretreated with wortmannin (Fig 5, H).

We then confirmed the involvement of the PI3K signaling pathway in mediating bile acid upregulation of *MUC1* at the promoter level (Fig 6). OE33 cells were transfected with responsive constructs to bile acids then pretreated with wortmannin for 30 minutes before adding the bile acids for another 24 hours. The results indicated that 75% to 80% of the transactivation of the -616/+33 region of the *MUC1* promoter by TC and TDC (Fig 6, gray bars) was lost when cells were pretreated with wortmannin. The same decrease was observed on the -1472/+33 region of the promoter when cells were pretreated with wortmannin before incubation with TDC, TCDC, and DC bile acids (Fig 6, black bars).

## DISCUSSION

Bile reflux has been identified as a predominant and sufficient carcinogen to induce esophageal adenocarcinoma on Barrett's esophagus.<sup>7,9</sup> The MUC1 mucin pattern of expression is altered profoundly during the esophageal carcinogenetic sequence, but no link between MUC1 overexpression during early stages of Barrett's esophagus and the presence of bile acids in the reflux has been established to date. In the present study, we found



**Fig 3.** Effect of bile acids and their conjugates on expression of *MUC1* mRNA and of MUC1 apomucin in human explants of adenocarcinomatous esophageal mucosa by RT-PCR. Tissues were treated with indicated bile acids for 30 minutes, as described in Material and Methods. **A**, Diagram shows MUC1/18S ratio calculated from compilation of the results obtained from 9 different surgical specimens. **B**, RT-PCR profile for 1 patient. *MUC1* and *18S* PCR products (7.5  $\mu$ l and 4  $\mu$ l, respectively) were analyzed on a 1.5% agarose gel.

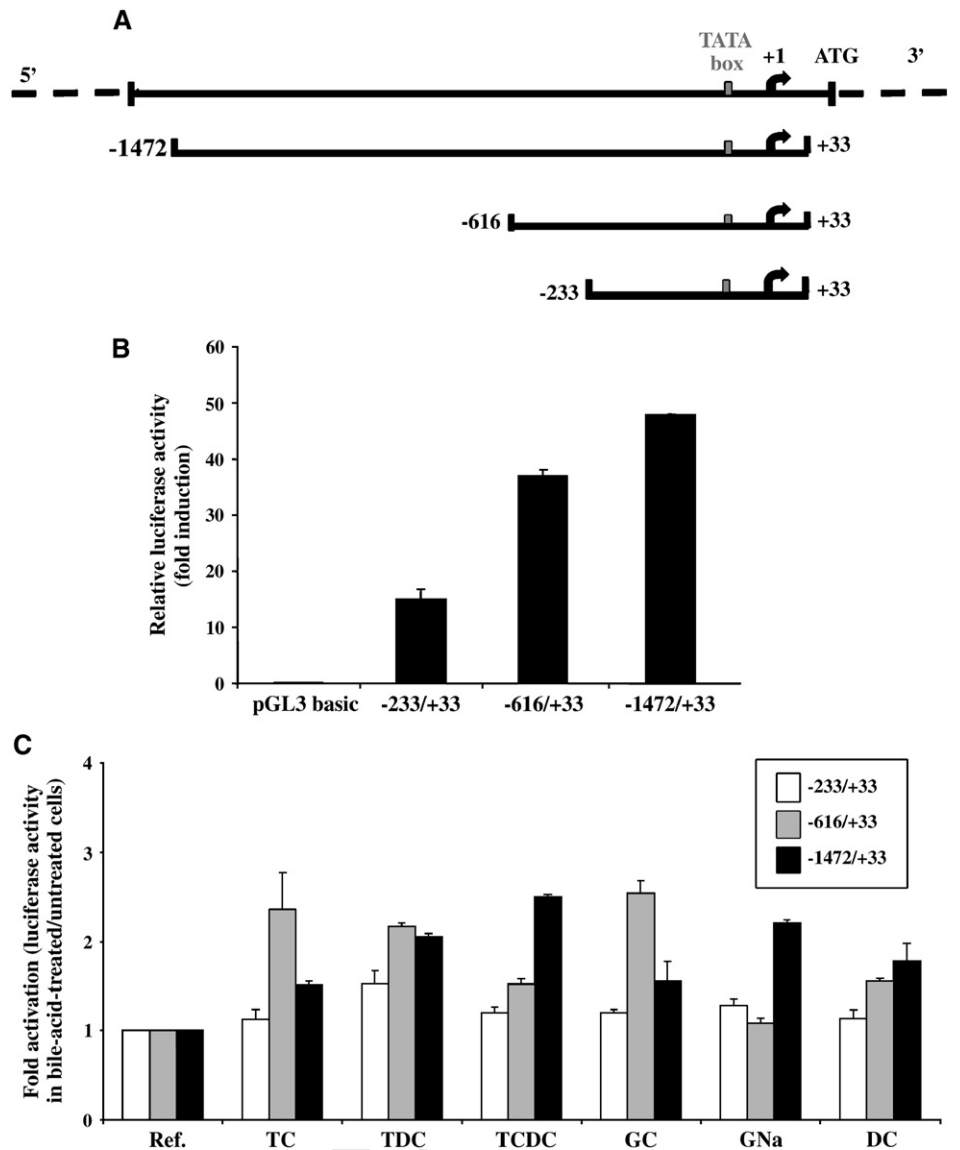
that bile acids are strong inducers of MUC1 mucin expression in human esophageal adenocarcinoma cells and in human tumoral tissues. We showed that the regulation occurs at the transcriptional level and is mediated by phosphatidylinositol 3 kinase. We also showed that, among the bile acids tested, DC and TC are the strongest inducers.

Activation of PI3K by bile acids is well-documented<sup>30-33</sup> and may be one of the mechanisms of bile acid-induced carcinogenesis. We found that PI3K inhibitor wortmannin and PI3K siRNA efficiently blocked MUC1 induction by bile acids, indicating that PI3K is involved in the bile acid-dependent induction of MUC1. Pharmacologic inhibition of MAPK, PKC, and PKA did not alter the level of *MUC1* mRNA in the cells, indicating that these pathways are not involved in this upregulation. Moreover, at this time, we have not been able to identify the signaling pathway that could mediate GC and GNa effects on the mRNA expression of MUC1, suggesting indirect regulation for these bile acids. This result, and previous results in which we demonstrated that PI3K also was involved in the bile acid-mediated upregulation of the membrane mucin MUC4,<sup>21</sup> suggest strongly that PI3K is a major and common signaling

pathway used to mediate bile acids effects on membrane-bound mucins. Bile acids could induce the transcription of another mucin gene that encodes the secreted mucin MUC2<sup>22</sup>; in that case, however, the mechanism implied activation of an AP-1 complex. From these results and ours, we hypothesize that during esophageal carcinogenesis associated with reflux and the presence of bile acids in esophageal lumen, mucin expression is activated via different mechanisms: PI3K activation for membrane mucins and AP-1 activation for secreted mucins. This hypothesis suggests that the esophageal mucosa is able to respond to a chronic aggression by using specific intracellular mechanisms to drive either the secretion of MUC2, which participates in mucus formation and epithelium defense/integrity, or expression at the cell surface of MUC1 and/or MUC4 membrane mucins.

We showed in this work that MUC1 activation by bile acids in esophageal cancer cells was not accompanied by increased cell proliferation or cell apoptosis. Moreover, MUC1 is known to activate PI3K/Akt and Bcl-x<sub>L</sub> antiapoptotic pathways,<sup>34</sup> a pathway (PI3K) also activated by bile acids to induce cell survival.<sup>32</sup> Having shown that PI3K activates MUC1 expression, our work suggests that esophageal cancer

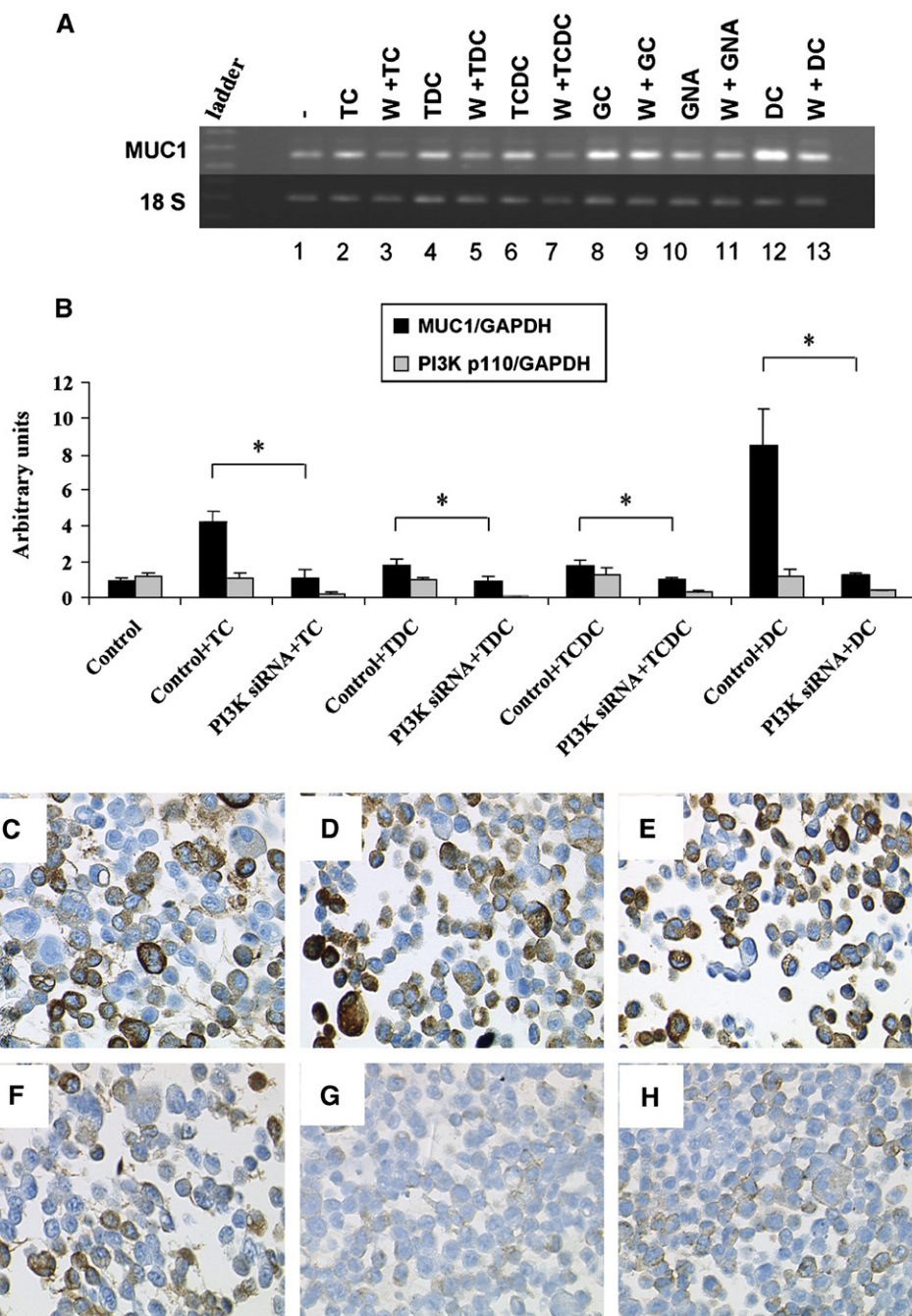
[Q14]



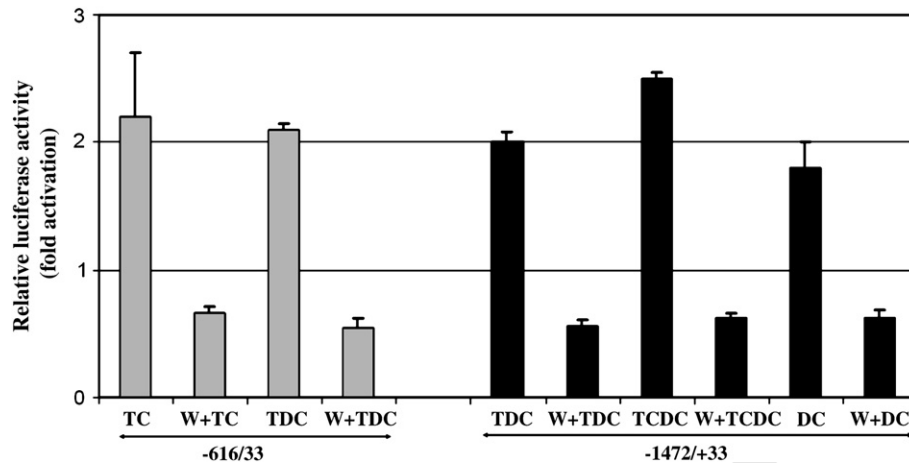
**Fig 4.** Characterization of the promoter activity of *MUC1* in OE33 cancer cells by transient transfection and effect of bile acids on the transcriptional activity of *MUC1* promoter. A, Schematic representation of 3 promoter constructs covering 1.47 kb of the *MUC1* promoter. B, Luciferase diagram showing activity of 3 deletion mutants. Ref., Cells transfected with the empty vector pGL3 basic. C, Diagram representing the ratio of luciferase activity in bile-acid treated cells versus untreated cells on 3 pGL3-*MUC1* promoter constructs. Cells were treated with bile acids for 24 hours. Ref., cells transfected without bile acid treatment (this value was arbitrarily set to 1). Mean  $\pm$  SD values were calculated from the values obtained in triplicate from 3 separate experiments.

cells, when submitted to bile acid aggression, may use an autocrine system to overexpress MUC1 mucin at their surface via PI3K (a pathway implicated in the regulation of genes contributing to tumorigenesis<sup>35</sup>) and thereby promote cancer progression. Our conclusions are in agreement with recent data<sup>11,14-16</sup> that demonstrated that overexpression of MUC1 (a transmembrane protein with a long extracellular glycosylated domain, which plays the role of sensor of the cellular environment) favors tumor progression and metastasis.

Transcription factors implicated in *MUC1* upregulation by bile acids after the activation of PI3K are unknown. Activation of gene transcription by PI3K may be mediated by several families of transcription factors. Among those that have been identified are the following: activating transcription factor-1/cAMP-responsive element-binding protein (ATF/CREB),<sup>36</sup> nuclear factor- $\kappa$ B (NF- $\kappa$ B),<sup>37-39</sup> and factors of the hepatocyte nuclear factor family (HNF).<sup>40</sup> Putative binding sites for these factors are present in the *MUC1* promoter, and



**Fig 5.** Importance of PI3K signaling pathway in bile acid-mediated regulation of MUC1 expression in OE33 cancer cells. **A**, Cell treatment with wortmannin (W) and the indicated bile acids (TC, TDC, TCDC, GC, GNa, DC) and RT-PCR were performed as described in Material and Methods. *MUC1* and *18S* PCR products (7.5  $\mu$ l and 4  $\mu$ l, respectively) were analyzed on a 1.5% agarose gel. **B**, siRNA experiments and bile acids treatment were carried out as described in Material and Methods. *MUC1*, *PI3K p110*, and *GAPDH* mRNA levels were assessed by RT-PCR. PCR products (10  $\mu$ l for *MUC1* and *PI3K p110* and 5  $\mu$ l for *GAPDH*) were analyzed on a 1.5% agarose gel. **Control** corresponds to the means value from mock cells and cells transfected with non-targeting siRNA; SD represents mean value obtained in triplicate in at least 3 separate experiments. Diagram showing data are expressed as MUC1/GAPDH and PI3K p110/GAPDH ratio in arbitrary units. \* $P < .05$ . **C-H**, Immunohistochemistry with anti-MUC1 antibody on OE33 cells treated under same conditions as in (A). Cells treated with TC (C), TDC (D), and DC (E). Cells pretreated with wortmannin before TC (F), TDC (G), and DC (H) incubation. Original magnification  $\times 200$ .



**Fig 6.** Inhibition of PI3K signaling pathway impairs *MUC1* promoter activation by bile acids in OE33 cells. pGL3-*MUC1* promoter constructs (-616/+33 and -1472/+33) were transfected in OE33 cells that were then pretreated for 30 minutes with wortmannin (W) before adding bile acid for another 24 hours. Means  $\pm$  SD were calculated from the values obtained in triplicate from 3 separate experiments.

preliminary cotransfection studies indicated that ATF/CREB and HNF-4 $\alpha$  transcription factors were able to transactivate the *MUC1* promoter (Piessen G and Van Seuningen I, unpublished data). Cdx-2 transcription factor was also hypothesized to be a good candidate, because it regulates mucin expression<sup>26</sup>, was shown recently to be activated by bile acids,<sup>41</sup> and is able to transactivate human *MUC1* promoter (Piessen G and Van Seuningen I, unpublished data).

Bile acids, amphiphilic derivatives of cholesterol, have been shown to enhance cellular transformation in vitro and are known promoters of gastrointestinal neoplasia in vivo.<sup>42</sup> Moreover, conjugated bile acids exacerbate esophageal mucosa injury either in combination with acid or alone, both in vitro or in animals<sup>8,43,44</sup> and humans.<sup>45</sup> Wide variations in biologic activity have been reported for individual bile acid fractions, and minor changes in the structure of bile acids dramatically alter their biologic effects.<sup>32</sup> Moreover, the concentrations of the individual bile acids present in bile reflux are modified as a result of the process of reflux itself.<sup>46</sup> All these data make bile acids important molecules to consider in esophageal carcinogenesis associated with bile reflux; identifying their target genes, therefore, will help in a better control their biologic effects on epithelial cells. We showed in the current study that the unconjugated bile acid DC and, in a lower proportion, the taurine conjugate bile acid TC are the major bile acids responsible for *MUC1* upregulation. Consequently, these bile acids may be considered as the most important components of the bile in

mediating *MUC1* upregulation and promoting its role in tumor progression in esophageal cancer associated with bile acid refluxate. From this work and others, DC appears as the major bile acid to activate mucin gene transcription, because it is able to activate *MUC1*, *MUC4*,<sup>21</sup> and *MUC2*.<sup>22</sup> We thus hypothesize that, in esophageal carcinoma associated with bile reflux, mucin activation is under the control of DC.

Previous studies have shown the following: (1) secondary bile acids in patients with Barrett's esophagus may contribute to the metaplastic change, and (2) a significant proportion of the bile acids in patients with extensive mucosal injury were composed of the dehydroxylated taurodeoxycholic acid (TDC) and the unconjugated deoxycholic acid (DC).<sup>46</sup> DC has also been under considerable interest because of excessive free bile acid deconjugation in the small intestine, leading to extensive damage with loss of villi through inhibition of the main active transport systems for glucose, amino acids, and sodium in the jejunum.<sup>47</sup> Finally, DC has been shown to do the following: (1) increase colon tumor incidence in N-methyl-N-nitrosourea-treated rats,<sup>48</sup> indicating that DC is a tumor promoter rather than a complete carcinogen,<sup>48,49</sup> (2) cause apoptosis in metaplastic esophageal cells expressing wild-type p53<sup>7</sup> and in colonic cells, especially in goblet cells,<sup>50</sup> and (3) cause extensive cell death in rat colonic mucosa, which is followed by a period of increased cell proliferation.<sup>51</sup> In contrast, we showed that under our experimental conditions, neither cell proliferation nor apoptosis were altered by bile acid

treatment, suggesting that the molecular mechanisms induced by bile acids are tissue-specific and different in colonic and esophageal cells.

Of the different components of the refluxate, bile acids—either alone or in combination with acid—are probably the most important.<sup>52</sup> The pH of the refluxate has been shown to modify the effect of bile acids on epithelial cells.<sup>42</sup> One explanation is that pH variation can modify the biologic characteristics of bile salts, thus facilitating penetration of certain bile salts in epithelial cells.<sup>46</sup> Other elements of the refluxate include gastric pepsin, cholesterol, and pancreatic juice (trypsin, lipase). Pancreatic juice has been suggested to be to be most potent component of the duodenal reflux in the promotion of esophageal carcinogenesis in a rat model with the use of dimethylnitrosomorpholine as a carcinogen.<sup>53</sup> In one study,<sup>53</sup> biliary reflux exerted a co-carcinogenic effect when combined with pancreatic secretions. The clinical relevance of these findings, however, needs further evaluation.

In conclusion we have shown that treatment of human esophageal adenocarcinomatous cells and tissues with bile acids leads to MUC1 upregulation via a PI3K-mediated molecular transcriptional mechanism. The biologic consequences of the induction of MUC1 expression by bile acids in esophageal cancer cells are still unknown, but our data favor a role in tumor progression and metastasis. Further studies are needed to determine whether the induction of MUC1 by bile acids may increase the invasive and metastatic potential of esophageal cancer cells. The development of a rat model of esophageal carcinogenesis in which esophageal mucosa is exposed to bile or acid reflux or both will be very informative regarding the following: (1) deciphering the precise molecular mechanisms activated by bile acids to induce MUC1 expression, (2) evaluating the consequences of MUC1 overexpression during the carcinogenic sequence on tumor cell behavior and biologic properties, and (3) evaluating PI3K signaling pathway as a therapeutic target in esophageal carcinogenesis.<sup>54-56</sup> This rat model will also be useful in demonstrating the pivotal role of DC as the main inducer of the expression of both membrane and secreted mucins.

This paper is dedicated to Dr Jean-Pierre Aubert, Director of our Laboratory, who died in September 2005. He initiated the collaboration between surgeons and scientists of the Research Unit to stimulate translational research and promote potential clinical implications. We also are indebted to Marie-Paule Ducourouble and Dominique Demeyer for their excellent technical

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