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Transcriptional regulation of human mucin MUC4 by bile acids in oesophagus cancer cells is promoter dependent and involves activation of phosphatidylinositol 3-kinase signalling pathway

Christophe Mariette^{1,2}, Michaël Perrais¹, Emmanuelle Leteurtre^{1,3}, Nicolas Jonckheere¹,
Brigitte Hémon¹, Pascal Pigny¹, Surinder Batra⁴, Jean-Pierre Aubert¹, Jean-Pierre Triboulet²,
and Isabelle Van Seuningen¹

¹Unité INSERM 560, Place de Verdun, 59045 Lille Cedex, France. ²Service de Chirurgie digestive et générale et ³laboratoire d'Anatomie et Cytologie pathologiques, Hôpital C. Huriez, CHRU, 59037 Lille Cedex, France. ⁴Department of Biochemistry and Molecular Biology, UNMC/Eppley Cancer Center, University of Nebraska Medical Center, 984525 Nebraska Medical Center, Omaha, NE, USA.

Short title : MUC4 regulation by bile acids in oesophagus cancer cells

To whom correspondence should be sent :

Isabelle Van Seuningen, Ph.D.

Unité INSERM 560, Place de Verdun, 59045 Lille Cedex, France

Phone : 33. 320.29.88.67

FAX : 33.320.53.85.62

E-mail : isabelvs@lille.inserm.fr

Abbreviations

C : cholic acid

CDC : chenodeoxycholic acid

CME : cholic methyl ester

DC : deoxycholic acid

DHC : dehydrocholic acid

EDTA : ethylene diamine tetraacetic acid

GAPDH : glyceraldehyde-3-phosphate dehydrogenase

GC : glycocholic acid

GNA : sodium glycocholate

LC : lithocholic acid

MAPK : mitogen activated protein kinase

PCR : polymerase chain reaction

PI3K : phosphatidylinositol 3-kinase

PKA : protein kinase A

PKC : protein kinase C

RT : reverse transcriptase

TC : taurocholic acid

TDC : taurodeoxycholic acid

TCDC : taurochenodeoxycholic acid

Synopsis

Abnormal gastro-oesophageal reflux and bile acids have been linked to the presence of Barrett's oesophagus premalignant lesion associated with an increase in mucin-producing goblet cells and *MUC4* mucin gene overexpression. However the molecular mechanisms underlying the regulation of *MUC4* by bile acids are unknown. Since total bile is a complex mixture we undertook to identify which bile acids are responsible for *MUC4* up-regulation by using a wide panel of bile acids and their conjugates. *MUC4* apomucin expression was studied by immunohistochemistry both in patient biopsies and OE33 oesophagus cancer cell line. *MUC4* mRNA levels and promoter regulation were studied by RT-PCR and transient transfection assays, respectively. We show that among the bile acids tested, taurocholic-, taurodeoxycholic-, taurochenodeoxycholic-, glycocholic acids and sodium glycocholate are strong activators of *MUC4* expression and that this regulation occurs at the transcriptional level. By using specific pharmacological inhibitors of mitogen activated protein kinase, phosphatidylinositol 3-kinase, protein kinase A and protein kinase C kinases, we demonstrate that bile acid-mediated up-regulation of *MUC4* is promoter-specific and mainly involves activation of phosphatidylinositol 3-kinase. This new mechanism of regulation of *MUC4* mucin gene points out an important role for bile acids as key molecules in targeting *MUC4* overexpression in early stages of oesophagus carcinogenesis.

Keywords : *MUC4*, oesophagus, cancer, transcription, mucins, bile acids, PI3K, MAPK

Introduction

About 5,000 new cases of oesophagus cancer are diagnosed each year in France with high incidence in Northwestern regions [1]. Although epidermoid carcinoma represents the main histological type, adenocarcinoma incidence has been rising lately and represents nowadays half of the oesophagus cancers [2-4]. Most of the adenocarcinomas arise from Barrett mucosa with a prevalence of 10 to 15%, that is a risk of cancer 30 to 50 times more frequent than in the general population [5]. Barrett oesophagus corresponds to an abnormal repair process of lesions induced by gastro-oesophageal reflux and leads to metaplasia in which epidermoid mucosa is replaced by a glandular-like mucosa in a multistep process characterized by the metaplasia-dysplasia-adenocarcinoma sequence [1,6]. Gastro-oesophageal reflux of acid and bile are the predominant initiating factors in Barrett's metaplasia and its degeneration into oesophagus adenocarcinoma by acting on the apical membrane of metaplastic cells [8-12], although the precise mechanism of cytotoxicity is unclear. Gillen *et al.* have shown an increase in intragastric bile acid concentration in patients with Barrett oesophagus [13] 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 [10]. Moreover, duodeno-oesophageal bile reflux has recently been identified as a predominant and sufficient carcinogen to induce oesophageal adenocarcinoma on Barrett oesophagus in a rat model [14,15]. Dysplasia is the only biological tumoral marker for Barrett mucosa surveillance. However, it remains unsatisfactory due to the absence of endoscopic translation and regular distribution, because of complex histological diagnosis and difficulty to predict its evolution [7]. Identification of new markers of early stages of oesophagus carcinogenesis is thus still an active area of research.

Human mucins are secreted in normal oesophagus to protect the underlying mucosa against potential injuries such as reflux of gastroduodenal contents including acid and bile acids [16]. Two main families of mucins are distinguished : secreted mucins that participate or not in mucus gel formation (*MUC2*, *MUC5AC*, *MUC5B*, *MUC6*, *MUC7*) and membrane-bound mucins that are involved in cell signalling and are thought to play important roles in tumour cell biology (*MUC1*, *MUC3*, *MUC4*) [17,18]. It is important to note that *MUC4* is also thought to participate in mucus formation and mucosal defence after proteolytic cleavage of its extracellular domain [17]. In normal oesophagus, *MUC1* and *MUC4* are the main mucin genes expressed in the stratified squamous epithelium whereas *MUC5B* is expressed in the submucosal glands [19,20]. Barrett's oesophagus corresponds to an abnormal healing process in which the normal squamous mucosa is replaced by a columnar epithelium that can be of 3 types : intestinal, cardial and fundic [21]. In high grade dysplasia and adenocarcinoma of Barrett's oesophagus, down-regulation of *MUC2*, *MUC5AC* and *MUC6* are observed whereas expression of *MUC1* and *MUC4* mucin genes are dramatically increased [16,19]. In consequence, altered expression/secretion of mucins will have dramatic impact on the mucus composition, on the biological properties of cancer cells and consequently on the maintenance of epithelium integrity. From these studies, several authors have proposed that mucin genes may be considered as reliable phenotypic markers to follow metaplastic process that leads to Barrett's adenocarcinoma [19-21]. Since *MUC4* is expressed at the surface of epithelial cells and possesses a soluble form, two mechanisms are proposed for *MUC4* function in cancer progression : first, as an anti-adhesive or anti-recognition barrier at the epithelial surface and second as an actor in ErbB2 signalling [18]. However, at this time nothing is known about the molecules and the downstream intracellular signalling cascades that are responsible for the overexpression of *MUC4* mucin gene observed in oesophagus cancer resulting from persistent gastro-oesophageal reflux.

Previous studies carried out in gallbladder and colon cancer cells have shown that bile acids activate mucin secretion *via* protein kinase A (PKA), protein kinase C (PKC) and intracellular Ca^{2+} -dependent signalling pathways [22-26]. It is however not known which mucin gene is activated and if regulation at the transcriptional level is involved. In relation with *MUC4* overexpression in oesophagus adenocarcinoma and the role of bile acids in Barrett's metaplasia and its consequent degeneration into oesophagus adenocarcinoma, we undertook to identify which bile acids were responsible for *MUC4* up-regulation.

Materials and methods

Cell culture

The oesophagus cancer cell line OE33 was purchased from ECACC (European Collection of Cell Cultures). The cell line was established from the adenocarcinoma of the lower oesophagus [27]. Cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine and 10% foetal calf serum (Roche diagnostics) and maintained in a 37°C incubator with 5% CO₂. Cells were treated with bile acids and their conjugates for 24h with following concentrations of bile acids. Chenodeoxycholic acid (CDC) : 0.1 mM, cholic acid (C) : 0.5 mM, deoxycholic acid (DC) : 0.05 mM, taurochenodeoxycholic acid (TCDC) : 0.5 mM, sodium glycocholate (GNA) : 0.5 mM, glycocholic acid (GC) : 0.5 mM, taurocholic acid (TC) : 0.5 mM, taurodeoxycholic acid (TDC) : 1 mM, dehydrocholic acid (DHC) : 1 mM, cholic methyl ester (CME) : 0.05 mM, lithocholic acid (LC) : 0.1 mM. Toxicity of each bile acid was evaluated, under the same experimental conditions, by trypan blue exclusion measurement on a hemocytometer. In inhibition studies, pharmacological inhibitors were incubated with the cells for 30 min, prior addition of bile acids, at the following final concentrations : PD98059 (30 μ M, inhibitor of MAPK), KT5720 (1 ng/ml, inhibitor of PKA), GF109203X (10 μ M, inhibitor of PKC), wortmannin (2.5 nM, inhibitor of PI3K). All reagents were from Sigma unless otherwise indicated.

RT-PCR

Total RNAs from OE33 cells were prepared using the RNeasy mini-kit from Qiagen. Cells were harvested at 100% of confluence and 1.5 μ g of total RNA was used to prepare cDNA (AdvantageTM RT-for-PCR kit, Clontech) as described before [28]. PCR was performed on 1 μ l of cDNA using specific pairs of primers (MWG-Biotech, Germany) for

MUC4 mucin gene. Forward primer : 5'-CGCGGTGGTGGAGGCGTTCTT-3', reverse primer : 5'-GAAGAATCCTGACAGCCTTCA-3' (accession number AJ242546). PCR reactions were carried out in 50 μ l final solutions as described in [29]. Annealing temperature was 60°C. PCR products were analysed on 2% agarose gels run in 1X Tris-borate-EDTA buffer. 100 basepair DNA ladder was purchased from Amersham Biosciences. GAPDH (forward primer : 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' ; reverse primer : 5'-CATGTGGGCCATGAGGTCCACCAC-3') was used as the internal control. Expected size for *MUC4* and *GAPDH* PCR products are 596 and 980 basepairs, respectively. RT-PCR were carried out on cDNAs from four different sets of experiments. Densitometric analysis of DNA bands was carried out using the GelAnalyst-GelSmart software (Clara Vision).

pGL3-*MUC4* promoter constructs

pGL3-*MUC4* deletion mutants covering both promoters of *MUC4* were previously described [30]. Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

Transfections

Transfections were performed using Effectene[®] reagent (Qiagen) as previously described [31]. Cells were passed at 0.5×10^6 cells per well the day before the transfection. Total cell extracts were prepared after a 48h incubation time at 37°C using 1X Reagent Lysis Buffer (Promega) as described in the manufacturer's instruction manual. Total protein content in the extract was measured using bicinchoninic acid protein assay (Pierce). The relative luciferase activity is expressed as fold induction of the test plasmid activity compared with that of the corresponding empty vector (pGL3 basic, Promega). Each plasmid was assayed in triplicate in three separate experiments.

Immunohistochemistry

Confluent OE33 cells were trypsinised, centrifuged, washed once with 1X phosphate-buffered saline, the pellet was fixed in 4% paraformaldehyde, then embedded in paraffin and 3 μm sections were prepared. Morphological analysis was done after alcian blue staining. Section pretreatment before immunostaining was performed as described before [32]. MUC1 and MUC4 apomucin expression was also analysed on tissue obtained from patients with oesophageal adenocarcinoma. The surgical specimen was quickly immersed in 10% neutral formaldehyde solution (pH 7.4) in phosphate buffer. Samples from normal oesophageal mucosa and adenocarcinoma were processed for paraffin embedding. The diagnosis was assessed by two pathologists after staining of sections (4 μm) with hematoxylin-eosin-saffron. Immunohistochemistry was performed on serial sections of the same blocks using an automated immunostainer (ES, Ventana medical system, Strasbourg, France). The pretreatment was realised in a microwawe for 20 min in citrate buffer (pH 6.0). The immunohistochemistry method used a 3-step indirect process based on the biotin-avidin complex. Slides were counterstained with hematoxylin. Negative controls were run by omission of the primary antibody. Monoclonal anti-MUC4 antibody [33] was used at a 1:20,000 dilution. Monoclonal MUC1 antibody (LICR-LON-M8, 1:50 dilution) was a kind gift from Dr. D. Swallow (London, England). Polyclonal anti-MUC5B LUM5B-2 antibody (1:50 dilution) was a kind gift from Dr. I. Carlstedt, (Lund, Sweden).

Results

MUC1 and MUC4 apomucin expression in human oesophagus mucosa and adenocarcinoma

MUC1 and *MUC4* mRNAs were previously detected in oesophageal adenocarcinoma tissues by *in situ* hybridization [19,20] but apomucin expression was never studied. In Figure 1 is shown the expression of MUC1 and MUC4 apomucins in normal oesophagus mucosa (Figures 1B and 1C) and in oesophagus adenocarcinoma (Figures 1E and 1F), respectively. In normal oesophageal epithelium (Figure 1A), expression of MUC1 (Figure 1B) and MUC4 (Figure 1C) is moderate and restricted to the cytoplasm. Hematoxylin-eosin-saffron staining of the tissue section from a patient with oesophageal adenocarcinoma shows that it is moderately differentiated with carcinomatous glands dispersed in a stroma reaction (Figure 1D). When the sample was processed for MUC1 (Figure 1E) and MUC4 (Figure 1F), the staining was more intense than in normal mucosa and was found both in the cytoplasm and in the cell membrane.

Regulation of *MUC4* mRNA expression by bile acids in OE33 cancer cells

Regulation of *MUC4* mRNA expression in OE33 cancer cells by bile acids was assessed by RT-PCR. OE33 cells were treated with bile acids or conjugates for 24h as described in the materials and methods section before cell lysis and RNA extraction. The results shown in Figure 2 indicate that *MUC4* mRNA levels are slightly or not modified after cell treatment with chenodeoxycholic acid (CDC, lane 2), cholic acid (C, lane 3) and deoxycholic acid (DC, lane 4). On the other hand, strong increase of *MUC4* mRNA levels are observed after cell treatment with taurochenodeoxycholic acid (TCDC, lane 5), sodium glycocholate (GNA, lane 6), glycocholic acid (GC, lane 7), taurocholic acid (TC, lane 8) and taurodeoxycholic acid

(TDC, lane 9). Finally, treatment with dehydrocholic acid (DHC, lane 10), cholic methylester (CME, lane 11), and lithocholic acid (LC, lane 12) induced a slight decrease of *MUC4* mRNA levels in OE33 cells. These results indicate that bile acids and their conjugates can be classified in three families depending on their effect on *MUC4* mRNA expression : no effect (CDC, C and DC), activators (TCDC, GNA, GC, TC and TDC) or inhibitors (DHC, CME and LC).

Regulation of *MUC4* promoter activity by bile acids in OE33 cancer cells

The 5'-flanking region of *MUC4*, that we previously characterized, contains two transcriptional units [30]. Among the constructs covering the proximal promoter, the highest activity was obtained with fragments -219/-1 and -461/-1 (10 fold activation) (Figure 3) whereas the shortest fragment -144/-1 had no activity. Addition of longer DNA segments of *MUC4* promoter in the constructs (-1187/-220) did not lead to any significant changes in luciferase activity. This indicates that the -219/-145 region of the proximal promoter contains positive regulatory elements that confer maximal activity to *MUC4* proximal promoter in these cells. This is confirmed by the absence of luciferase activity of the fragment -1708/-387, that lacks the first 386 nucleotides of the proximal promoter. The distal promoter is also active and essential elements are present in the -3135/-2572 region (5 fold activation) since the activity of that promoter drops to 2-fold activation when the -3135/-2781 region is not present.

Having shown that *MUC4* endogenous mRNA expression was up-regulated by TC, TDC, TCDC, GC and GNA, we focused our next studies on these bile acids. Bile acid-responsive regions within *MUC4* promoters were identified after performing transient transfection experiments in which transfected cells were treated with bile acids or conjugates under the same conditions as for RT-PCR experiments. Among the pGL3-*MUC4* constructs, we chose

the five deletion mutants covering the two promoters of *MUC4* possessing the highest luciferase activity (see Figure 3). Among the tauric conjugates, TC induced a two-fold transactivation of the distal fragment -2781/-2572 (Figure 4). TDC, the tauric conjugate of DC, transactivates both promoters of *MUC4* in the same proportion (3 fold activation of proximal region -461/-1 (white bar) and 3 fold activation of distal region -3135/-2572 (grey bar)). TCDC, the tauric conjugate of CDC, strongly transactivates the -219/-1 region of *MUC4* proximal promoter (4 fold activation, black bar).

Glycine conjugate of cholic acid (GC), transactivates the -1187/-1 region of *MUC4* proximal promoter (2 fold). Since the shortest fragments do not show any transactivation by GC, one can conclude that GC-responsive elements are located within the -1187/-461 region of the proximal promoter. GNA strongly transactivates the distal region -3135/-2572 of *MUC4* promoter (3 fold activation).

In conclusion, the reporter assays show that bile-acid responsive elements are present in both promoters of *MUC4* and that bile acid-mediated up-regulation of *MUC4* transcription is promoter dependent.

Effect of bile acids on MUC4 apomucin expression in OE33 cells

Having shown that *MUC4* promoters contain responsive elements for bile acids that control *MUC4* expression in OE33 cells, we undertook to check whether that transcriptional control was having an impact on the expression of MUC4 apomucin. To this aim, cells were treated with bile acids for 24h under the same conditions as for RT-PCR and reporter assays, they were then trypsinised and processed for immunostaining with a specific antibody against MUC4 (Figure 5). Bile acid treatment under the conditions used in this study did not induce any change in the morphology of the cells (compare Figures 5E and 5F to 5D) nor did it induce cell death as trypan blue exclusion measurements were identical both in untreated and

bile acid-treated cells. Untreated cells show a relatively low degree of differentiation as no vacuoles and a few grains of secretions were detected (Figure 5A). To better characterize OE33 cells, expression of MUC1 and MUC5B apomucins was also studied. A few cells express MUC1 and the expression is found both in the cytoplasm (cyt) and in the membrane (mb) (Figure 5B, arrows) whereas there is no expression of MUC5B (Figure 5C). When immunostained with anti-MUC4 antibody, 30% of the cells were positively labelled (Figure 5D). The staining was seen both in the membrane and cytoplasm. The labelling became very intense and 100% of the cells were positively stained for MUC4 when cells were treated with TC (Figure 5E) or TDC (Figure 5F). Cell treatment with TCDC, GNA and GC also resulted in a dramatic increase of MUC4 apomucin expression with 100% of the cells labelled. In conclusion, these studies indicate that TC, TDC, TCDC, GC, and GNA are strong activators of MUC4 apomucin expression in OE33 cells.

Identification of the signalling pathways involved in bile acid-mediated up-regulation of MUC4 in OE33 cells

Having identified TC, TDC, TCDC, GC, and GNA as the bile acids responsible for MUC4 up-regulation in OE33 cells, we undertook to identify the intracellular signalling pathways responsible for that phenomenon. To this aim, specific inhibitors of MAPK (PD98059), PKC (GF109203X), PKA (KT5720) and PI3K (wortmannin) were used and preincubated for 30 min with the cells before the 24h incubation with each bile acid. In Figure 6A, are shown the results obtained with bile acids previously shown to activate the proximal promoter of *MUC4* (see Figure 4). Inhibition of PKC (GF, hatched bars) did not alter the transactivating effect of GC, TC, TCDC and TDC on *MUC4* promoter activity. Inhibition of PKA (KT, white bars) had no effect on the transactivating effect of GC, TC and TCDC, except for TDC for which it induced a mild transactivating effect (1.8 fold). Inhibition of

PI3K by wortmannin (W, black bars) led to a dramatic inhibition of bile acid-mediated up-regulation of *MUC4* proximal promoter. GC effect was inhibited by 50% whereas 70-75% inhibition was observed for TC, TDC and TCDC. Inhibition of MAPK (PD, grey bars) did not inhibit but surprisingly activated the transactivating effect of GC, TC and TCDC by two-fold. No effect was seen on TDC transactivation. These results indicate that GC, TC, TCDC and TDC transactivation of *MUC4* proximal promoter is mediated by PI3K signalling pathway whereas PKA (TDC) and MAPK (GC, TC, TCDC) cascades are negatively controlling these effects.

The bile acids that were transactivating the distal promoter of *MUC4* are also triggering the PI3K signalling pathway since pretreatment with wortmannin (W, black bars) inhibited TDC, GNA, and TCDC transactivating effect by 75-80% (Figure 6B). MAPK is also involved in TDC-mediated up-regulation of *MUC4* distal promoter as 50% of the activity is lost when cells are preincubated with PD98059 inhibitor (grey bars). Pretreatment with inhibitor of MAPK induced mild activation of the transactivation by TCDC (1.8 fold activation, grey bars). Inhibition of PKA (KT, white bars) and PKC (GF, hatched bars) induced activation of the transactivating effect of TDC (approx. 2 fold activation) and, to a lower extent, of GNA (1.5-1.75 fold activation).

Importance of the PI3K signalling pathway in the bile acid-mediated regulation of *MUC4* expression in OE33 cells

To confirm the importance of the PI3K signalling pathway in mediating bile acid effects on *MUC4* expression in OE33 cells, RT-PCR and immunohistochemistry were performed on cells pretreated with the PI3K inhibitor (wortmannin) before bile acid incubation. RT-PCR studies indicate that, as previously shown in Figure 2, TDC and TC increase *MUC4* mRNA levels in the cells (Figure 7A, lanes 2 and 4, respectively). Pretreatment with wortmannin

inhibited TDC- and TC-mediated increase of *MUC4* mRNA levels which then returned to basal levels (Figure 7A, lanes 3 and 5, respectively). Immunostaining of the cells with anti-MUC4 antibody confirmed the involvement of PI3K in TDC-mediated up-regulation of MUC4 apomucin expression as the labelling went from 72% in TDC-treated cells (Figure 7B) to 16% in wortmannin-pretreated cells (Figure 7C). The same conclusion was drawn regarding TC-mediated up-regulation of MUC4 apomucin expression as 64% of TC-treated cells are positive for MUC4 (Figure 7D) whereas only 29% remain labelled when cells were pretreated with wortmannin (Figure 7E).

Discussion

Human mucins are secreted in normal oesophagus to protect the underlying mucosa [16]. In normal oesophagus, *MUC1* and *MUC4* are the main mucin genes expressed in the surface epithelial cells whereas *MUC5B* is expressed in the submucosal glands [19,20]. In high grade dysplasia and adenocarcinoma of Barrett's oesophagus, expression of *MUC1* and *MUC4* mucin genes are dramatically increased [16,19]. Progression of oesophageal cells to cancer follows a multistep process characterized by the metaplasia-dysplasia-adenocarcinoma sequence [1]. Genetic defects have been identified such as loss of p16 and p53 expression or gain of cyclin D1 expression [34,35] but gastro-oesophageal reflux of acid and bile are the predominant initiating factors in Barrett's metaplasia, although the precise mechanism of cytotoxicity is unclear [36]. Lately, much attention has focused on MUC4 for it could represent a new therapeutic target in epithelial cancers as it is often strongly overexpressed in carcinomas, is a transmembrane protein that interferes with tumor cell properties and participate to ErbB2 signaling [17,18]. It is also thought that MUC4 may participate in mucus formation after proteolytic cleavage of its extracellular domain and its release in the lumen [18]. However, at this time not much is known about MUC4 regulation at the transcriptional level in oesophagus cancer. Recently, some evidence suggested that bile reflux may be considered as an important carcinogen in the development of oesophageal adenocarcinoma on a Barrett oesophagus [14,15]. For that reason and because it was previously shown that (i) most bile acids are active in the refluxate, (ii) increased exposure to total bile correlates with worsening mucosal damage, (iii) bile acids promote goblet cells-containing metaplasia in the oesophagus as well as in other epithelia, (iv) bile acids induce mucin secretion and (v) *MUC4* mucin gene is overexpressed in oesophagus adenocarcinoma, we undertook to study the

regulation of MUC4 by bile acids and conjugates both at the transcriptional and translational levels in an oesophagus cancer cell line.

In this work, we have identified TC, TDC, TCDC, GC, and GNA as strong inducers of MUC4 mucin expression and showed that the regulation occurs at the transcriptional level. Consequently, these conjugates may be considered as the most important factors in the bile to mediate MUC4 up-regulation in oesophagus cancer associated with bile acid refluxate. However, dramatic induction of MUC4 apomucin expression in immunohistochemistry studies also suggests that post-transcriptional mechanisms may also occur, which is not an uncommon feature for mucins. Since bile acids are known to directly act on the apical membrane of metaplastic epithelial cells and because MUC4 is a transmembrane mucin expressed at the surface of those same cells, that has a particularly long extracellular domain (2.12 μm) [37], that protrudes far away from cell membrane and glycocalyx, one can envision that altered expression/secretion of MUC4 will have dramatic consequences on the biological properties of the oesophageal epithelial cancer cells.

Previous reports had essentially focused on studying the role of bile acids on mucin secretion in primary gallbladder cells or colon cancer cells in relationship with gallstone formation and colon cancer pathogeny, respectively [22-26]. In an isolated vascularly perfused rat colon model, authors showed that mucin secretion and mucus discharge was induced by DC, CDC whereas C, ursodeoxycholate or detergent tween-20 had no effect [38]. Interestingly, these authors noticed that tauric conjugates of these bile acids were not as efficient. In our study we found that tauric conjugates were quite efficient on MUC4 up-regulation. This may indicate that bile acid action differs or that the composition of the bile is different in oesophageal and colonic or gallbladder epitheliums. This hypothesis seems to be the most probable as these authors showed in another study that the bile acid content of bile influenced its capacity to induce mucin secretion [23]. They concluded that a bile composed

of hydrophobic bile acids like (TCDC, TDC) favors mucin secretion when compared to a bile composed of more hydrophilic bile acids like TC and tauroursodeoxycholic acid [23]. Our results also suggest a major role for TDC and TCDC in mucin and more particularly MUC4 overexpression.

Bile salts mediate their biological effects *via* several signalling cascades (PI3K, MAPK, PKC, PKA) [38-43]. TC-, TDC-, TCDC-, GC-, and GNA-mediated up-regulation of MUC4 mainly goes through activation of PI3K. However, in some cases inhibition of PKA, PKC or MAPK resulted in the activation of MUC4 expression by bile salts. Bile salts are thus able to activate more than one signalling pathway with a different output on the expression of *MUC4*. Moreover, bile salt effects on *MUC4* expression are promoter-dependent and most likely involve different combinations of transcription factors. Bile acids are known to interact with farnesoid X receptor (FXR) and pregnane X receptor (PXR) to regulate transcription of their target genes [44,45]. Future experiments aiming at deciphering the molecular mechanisms used by TC, TDC, TCDC, GC, and GNA and more particularly at exploring the role of FXR and PXR transcription factors in the regulation of *MUC4* expression will be carried out in order to show their direct or indirect involvement. We hypothesize that the diversity of activated pathways and transcription factors binding to *MUC4* promoter in a sequence specific manner may explain the differences seen in the responses between the different bile salts. Consequently, the balance between PI3K and MAPK, PKA and PKC pathways will be important and will result either in MUC4 activation (PI3K) or MUC4 inhibition (MAPK, PKA, PKC) and will determine the specific outcome of each bile acid on MUC4 expression.

A better understanding of MUC4 regulation in Barrett's oesophagus will allow to demonstrate whether MUC4, as a target of bile acids, which are deleterious factors in Barrett's oesophagus associated with gastro-oesophageal reflux and its consequent evolution

into oesophagus adenocarcinoma, may be considered as a possible marker in the early steps of oesophagus carcinogenesis.

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References

1. Jankowski, J. A., Harrison, R. F., Perry, I., Balkwill, F. and Tselepis, C. (2000). Barrett's metaplasia. *Lancet* **356**, 2079-2085
2. Endo, T., Tamaki, K., Arimura, Y., Itoh, F., Hinoda, Y., Hareyama, M., Irimura, T., Fujita, M. and Imai, K. (1998). Expression of sulfated carbohydrate chain and core peptides of mucin detected by monoclonal antibodies in Barrett's esophagus and esophageal adenocarcinoma. *J. Gastroenterol.* **33**, 811-815
3. Falk, G. W. (2002). Barrett's esophagus. *Gastroenterology* **122**, 1569-1591
4. Labouvie, C., Machado, J. C., Carneiro, F., Sarbia, M., Vieth, M., Porschen, R., Seitz, G. and Blin, N. (1999). Differential expression of mucins and trefoil peptides in native epithelium, Barrett's metaplasia and squamous cell carcinoma of the oesophagus. *J. Cancer Res. Clin. Oncol.* **125**, 71-76
5. Mariette, C., Castel, B., Tourse, H., Fabre, S., Balon, J.-M and Triboulet, J.-P. (2002). Surgical management of and long-term survival after adenocarcinoma of the cardia. *Br. J. Surg.* **89**, 1156-1163
6. Sagara, M., Yonezawa, S., Nagata, K., Tezuka, Y., Natsugoe, S., Xing, P. X., McKenzie, I. F., Aikou, T. and Sato, E. (1999). Expression of mucin 1 (MUC1) in esophageal squamous-cell carcinoma: its relationship with prognosis. *Int. J. Cancer* **84**, 251-257
7. Mariette, C., Balon, J.-M., Piessen, G., Fabre, S., Van Seuning, I. and Triboulet, J.-P. (2003). Pattern of recurrence following complete resection of esophageal carcinoma and factors predictive of recurrent disease. *Cancer* **97**, 1616-1623
8. Debruyne, P. R., Bruyneel, E.A., Li, X., Zimber, A., Gespach, C. and Mareel, M. M. (2001). The role of bile acids in carcinogenesis. *Mutat. Res.* **480-481**, 359-369

9. Garewal, H. S. (2000). Recent developments in Barrett's esophagus. *Curr. Oncol. Rep.* **2**, 271-276
10. Jankowski, J. A. (1993). Gene expression in Barrett's mucosa: acute and chronic adaptive responses in the oesophagus. *Gut* **34**, 1649-1650
11. Jankowski, J. A., Wright, N. A., Meltzer, S. J., Triadafilopoulos, G., Geboes, K., Casson A. G., Kerr, D. and Young, L. S. (1999). Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am. J. Pathol.* **154**, 965-973
12. Sharma, P. (2001). An update on strategies for eradication of Barrett's mucosa. *Am. J. Med.* **111**, 147-152
13. Gillen, P., Keeling, P., Byrne, P. J., Healy, M., O'Moore, R. R. and Hennessy, T. P. (1994). Proliferating cell nuclear antigen in the assessment of Barrett's mucosa. *Br. J. Surg.* **75**, 540-543
14. Sato, T., Miwa, K., Segawa, M. and Hattori, T. (2002). The sequential model of Barrett's esophagus and adenocarcinoma induced by duodeno-esophageal reflux without exogenous carcinogens. *Anticancer Res.* **22**, 39-44
15. Miwa, K., Sahara, H., Segawa, M., Kinami, S., Sato, T., Miyazaki, I. And Hattori, T. (1996). Reflux of duodenal or gastro-duodenal contents induces esophageal carcinoma in rats. *Int. J. Cancer* **67**, 269-274
16. Corfield, A. P., Myerscough, N., Longman, R., Sylvester, P., Arul, S. and Pignatelli, M. (2000). Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut* **47**, 589-594
17. Dekker, J., Rossen, J. W., Buller, H. A. and Einerhand, A. W. C. (2002). The MUC family: an obituary. *Trends Biochem. Sci.* **27**, 126-131
18. Carraway, K. L., Ramsauer, V. P., Haq, B. and Carraway, C. A. (2003). Cell signaling through membrane mucins. *Bioessays* **25**, 66-71

19. Arul, G. S., Moorghen, M., Myerscough, N., Alderson, D. A., Spicer, R. D. and Corfield A. P. (2000). Mucin gene expression in Barrett's oesophagus: an in situ hybridisation and immunohistochemical study. *Gut* **47**, 753-761
20. Guillem, P., Billeret, V., Buisine, M.-P., Fléjou, J.-F, Lecomte-Houcke, M., Degand, P., Aubert, J.-P, Triboulet, J.-P and Porchet, N. (2000). Mucin gene expression and cell differentiation in human normal, premalignant and malignant esophagus. *Int. J. Cancer* **88**, 856-861
21. Warson, C., Van De Bovenkamp, J. H., Korteland-Van Male, A. M., Buller, H. A., Einerhand, A. W. C., Ectors, N. L. and Dekker, J. (2002). Barrett's esophagus is characterized by expression of gastric-type mucins (MUC5AC, MUC6) and TFF peptides (TFF1 and TFF2), but the risk of carcinoma development may be indicated by the intestinal-type mucin, MUC2. *Hum. Pathol.* **33**, 660-668
22. Dray-Charier, N., Paul, A., Combettes, L., Bouin, M., Mergey, M., Balladur, P., Capeau, J. and Housset, C. (1997). Regulation of mucin secretion in human gallbladder epithelial cells: predominant role of calcium and protein kinase C. *Gastroenterology* **112**, 978-990
23. Klinkspoor, J. H., Kuver, R., Savard, C. E., Oda, D., Azzouz, H., Tytgat, G. N., Groen, A. K. and Lee, S. P. (1995). Model bile and bile salts accelerate mucin secretion by cultured dog gallbladder epithelial cells. *Gastroenterology* **109**, 264-274
24. Klinkspoor, J. H., Yoshida, T. and Lee, S. P. (1998). Bile salts stimulate mucin secretion by cultured dog gallbladder epithelial cells independent of their detergent effect. *Biochem. J.* **332**, 257-262
25. Klinkspoor, J. H., Mok, K. S., Van Klinken, B. J., Tytgat, G. N., Lee, S. P. and Groen, A. K. (1999). Mucin secretion by the human colon cell line LS174T is regulated by bile salts. *Glycobiology* **9**, 13-19

26. Shekels, L. L., Lyftogt, C. T. and Ho, S. B. (1996). Bile acid-induced alterations of mucin production in differentiated human colon cancer cell lines. *Int. J. Biochem. Cell Biol.* **28**, 193-201
27. Rockett, J. C., Larkin, K., Darnton, S. J., Morris, A. G. and Matthews, H. R. (1997). Five newly established oesophageal carcinoma cell lines: phenotypic and immunological characterization. *Br. J. Cancer* **75**, 258-263
28. Van Seuning, I., Perrais, M., Pigny, P., Porchet, N. and Aubert, J.-P. (2000). Sequence of the 5'-flanking region and promoter activity of the human mucin gene MUC5B in different phenotypes of colon cancer cells. *Biochem. J.* **348**, 675-686
29. Mesquita, P., Jonckheere, N., Almeida, R., Ducourouble, M.-P., Serpa, J., Silva, E., Pigny, P., Santos Silva, F., Reis, C., Silberg, D., Van Seuning, I. and David, L. (2003) Human MUC2 mucin gene is transcriptionally regulated by Cdx homeodomain proteins in gastrointestinal carcinoma cell lines. *J. Biol. Chem.*, in press
30. Perrais, M., Pigny, P., Ducourouble, M.-P., Petitprez, D., Porchet, N., Aubert, J.-P. and Van Seuning, I. (2001). Characterization of human mucin gene MUC4 promoter: importance of growth factors and proinflammatory cytokines for its regulation in pancreatic cancer cells. *J. Biol. Chem.* **276**, 30923-30933
31. Perrais, M., Pigny, P., Copin, M.-C., Aubert, J.-P. and Van Seuning, I. (2002). Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. *J. Biol. Chem.* **277**, 32258-32267
32. Copin, M.-C., Devisme, L., Buisine, M.-P., Marquette, C.-H., Wurtz, A., Aubert, J.-P., Gosselin, B. and Porchet, N. (2000). From normal respiratory mucosa to epidermoid carcinoma: expression of human mucin genes. *Int. J. Cancer* **86**, 162-168

33. Swartz, M. J., Batra, S. K., Varshney, G. C., Hollingsworth, M. A., Yeo, C. J., Cameron, J. L., Wilentz, R. E., Hruban, R. H. and Argani, P. (2002). MUC4 expression increases progressively in pancreatic intraepithelial neoplasia. *Am. J. Clin. Pathol.* **117**, 791-6
34. Jenkins, G. J. S., Doak, S. H., Parry, J. M., D'Souza, F. R., Griffiths, A. P. and Baxter, J. N. (2002). Genetic pathways involved in the progression of Barrett's metaplasia to adenocarcinoma. *Br. J. Surg.* **89**, 824-837
35. Wijnhoven, B. P. L., Tilanus, H. W. and Dinjens, W. N. M. (2001). Molecular biology of Barrett's adenocarcinoma. *Ann. Surg.* **233**, 322-337
36. Richter, J. E. (2001). Hepatocellular carcinoma in southern Germany: epidemiological and clinicopathological characteristics and risk factors. *Dig. Dis.* **18**, 208-16
37. Moniaux, N., Escande, F., Porchet, N., Aubert, J.-P. and Batra, S. K. (2001). Structural organization and classification of the human mucin genes. *Front. Biosci* **6**, 1192-1206
38. Barcelo, A., Claustre, J., Toumi, F., Burlet, G., Chayvialle, J.-A., Cuber, J.-C. and Plaisancie P. (2001). Effect of bile salts on colonic mucus secretion in isolated vascularly perfused rat colon. *Dig. Dis. Sci.* **46**, 1223-1231
39. Beuers, U. (1997). Effects of bile acids on hepatocellular signaling and secretion. *Yale J. Biol. Med.* **70**, 341-346
40. Brady, L. M., Beno, D. W. A. and Davis, B. H. (1996). Bile acid stimulation of early growth response gene and mitogen-activated protein kinase is protein kinase C-dependent. *Biochem. J.* **316**, 765-769
41. Qiao, D., Stratagouleas, E. D. and Martinez, J. D. (2001). Activation and role of mitogen-activated protein kinases in deoxycholic acid-induced apoptosis. *Carcinogenesis* **22**, 35-41
42. Rust, C., Karnitz, L. M., Paya, C. V., Moscat, J., Simari, R. D. and Gores, G. J. (2000). The bile acid taurochenodeoxycholate activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. *J. Biol. Chem.* **275**, 20210-20216

43. Takikawa, Y., Miyoshi, H., Rust, C., Roberts, P., Stiegel, R., Mandal, P. K., Millikan, R. E. and Gores, G. J. (2001). The bile acid-activated phosphatidylinositol 3-kinase pathway inhibits Fas apoptosis upstream of bid in rodent hepatocytes. *Gastroenterology* **120**, 1810-1817
44. Chawla, A., Repa, J. J., Evans, R. M. and Mangelsdorf, D. J. (2001). Nuclear receptors and lipid physiology: opening the X-files. *Science* **294**, 1866-1870
45. Goodwin, B. and Kliewer, S. A. (2002). Nuclear receptors. I. Nuclear receptors and bile acid homeostasis. *Am. J. Physiol.* **282**, G926-G931

Legends to the figures

Figure 1 : Expression of MUC1 and MUC4 apomucins in human oesophagus mucosa and adenocarcinoma. Immunohistochemistry was performed as described in the materials and methods section. **(A-C)** : normal oesophagus mucosa, **(D-F)** : oesophagus adenocarcinoma. **(A)** : Hematoxylin-eosin-saffron staining of normal oesophagus mucosa, **(B)** : MUC1 staining in normal oesophagus mucosa, **(C)** : MUC4 staining in normal oesophagus mucosa, **(D)** : Hematoxylin-eosin-saffron staining of adenocarcinomatous oesophagus mucosa, **(E)** : MUC1 staining in oesophagus adenocarcinoma, **(F)** : MUC4 staining in oesophagus adenocarcinoma. Magnification x 250.

Figure 2 : Study of the effect of bile acids and their conjugates on the expression of *MUC4* mRNA in OE33 cancer cells by RT-PCR. Cells were treated with the indicated bile acids for 24h as described in the materials and methods section. **(A)** : *MUC4* and *GAPDH* PCR products (8 μ l and 2 μ l, respectively) were separated on a 2% agarose gel containing ethidium bromide after electrophoresis in 1X Tris-borate-EDTA buffer. **(B)** : Densitometric analysis of the DNA bands.

Figure 3 : Characterization of the promoter activity of *MUC4* in OE33 cancer cells by transient transfection. 1 μ g of each pGL3-*MUC4* deletion mutants was transfected in OE33 cells as described in the materials and methods section. Ref. refers to the transfected cell with the empty vector pGL3 basic. Standard deviation represents the means of values obtained in triplicate in three separate experiments.

Figure 4 : Effect of bile acids and their conjugates on the transcriptional activity of *MUC4* promoters in OE33 cancer cells. Effect on constructs representing the proximal promoter of *MUC4* : -219/-1 (black bars), -461/-1 (white bars), -1187/-1 (hatched bars), and the distal promoter : -3135/-2572 (grey bars), and -2781/-2572 (speckled bars). 1 μ g of each pGL3-*MUC4* deletion mutants was transfected in OE33 cells as described in the materials and methods section. Cells were then treated with bile acids for 24h with indicated concentrations. Ref. refers to the transfected cell without bile acid treatment (this value was defined as 1). Standard deviation represents the means of values obtained in triplicate in three separate experiments.

Figure 5 : Effect of bile acids on *MUC4* apomucin expression in OE33 cancer cells. Immunohistochemistry was performed as described in the materials and methods section. **A-D** : untreated cells. **(A)** : Alcian blue staining, **(B)** : MUC1 immunostaining, **(C)** : MUC5B immunostaining, **(D)** : MUC4 immunostaining, **(E)** : MUC4 immunostaining after TC treatment, **(F)** : MUC4 immunostaining after TDC treatment. Magnification is x 400. Staining of the membrane (mb) or cytoplasm (cyt) is indicated by arrows.

Figure 6 : Identification of the signalling pathways triggered by bile acids to regulate *MUC4* promoter activity in OE33 cancer cells. **(A)** : bile acids transactivating the proximal promoter of *MUC4*, **(B)** : bile acids transactivating the distal promoter of *MUC4*. 1 μ g of each pGL3-*MUC4* deletion mutants was transfected in OE33 cells as described in the materials and methods section. Before bile acid treatment for 24h, cells were pretreated for 30 min with the pharmacological inhibitor as indicated (W : wortmannin, KT : KT5720, GF : GF109203X, PD : PD98059). REF. refers to the bile acid-treated transfected cells without inhibitor

pretreatment (that value was defined as 1). Standard deviation represents the means of values obtained in triplicate in three separate experiments.

Figure 7 : Importance of the PI3K signalling in TDC- and TC-mediated regulation of MUC4 expression in OE33 cancer cells. (A) Cells treatment with wortmannin and the indicated bile acids and RT-PCR were performed as described in the materials and methods section. *MUC4* and *GAPDH* PCR products (8 μ l and 2 μ l, respectively) were separated on a 2% agarose gel containing ethidium bromide after electrophoresis in 1X Tris-borate-EDTA buffer. Immunohistochemistry with anti-MUC4 antibody was performed as described in the materials and methods section. (B) : TDC-treated cells, (C) : TDC-treated cells pretreated with wortmannin, (D) : TC-treated cells, (E) : TC-treated cells pretreated with wortmannin. Magnification is x 400.

Figure 1 : Mariette *et al.*

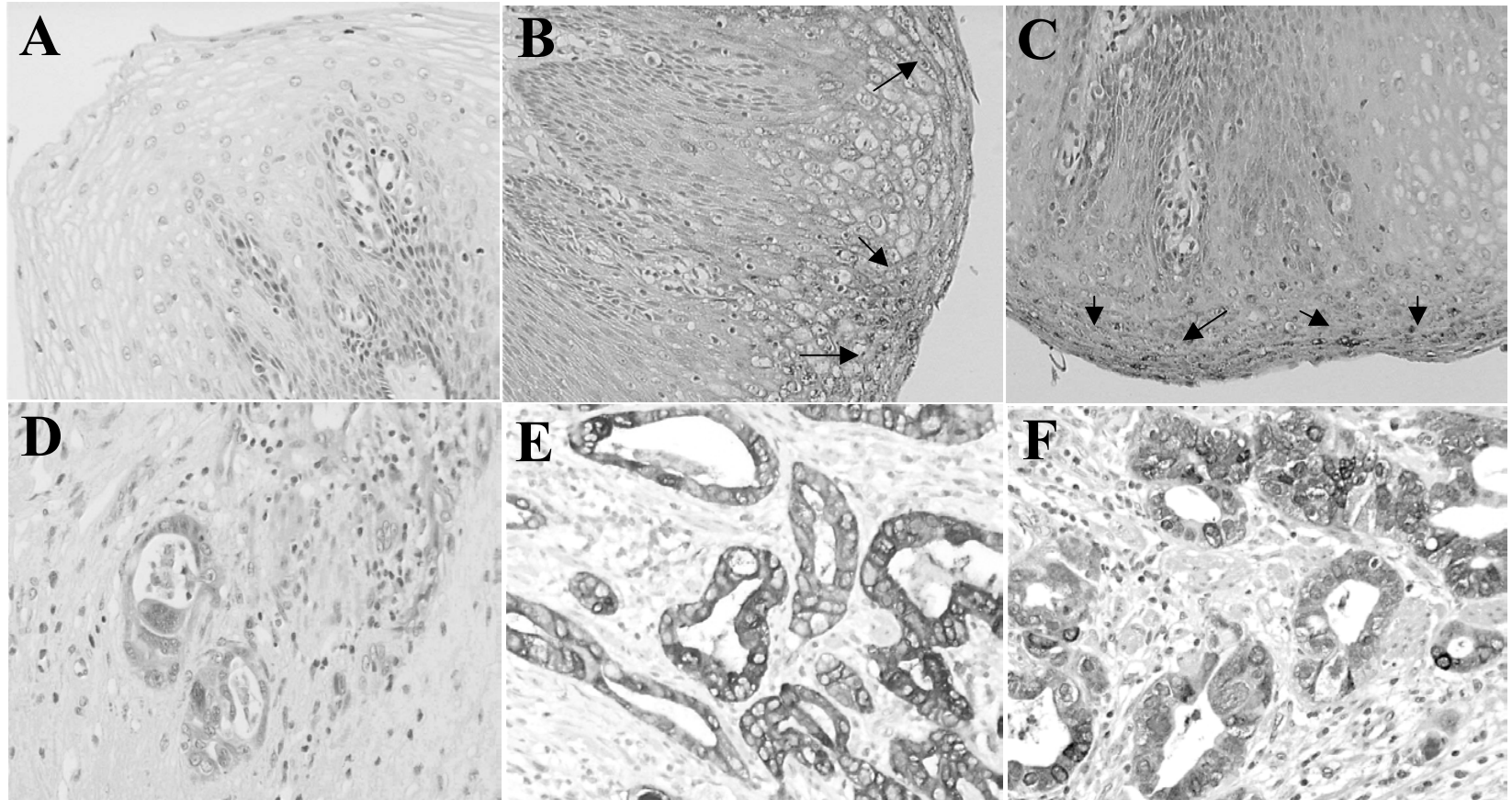


Figure 2 : Mariette *et al.*

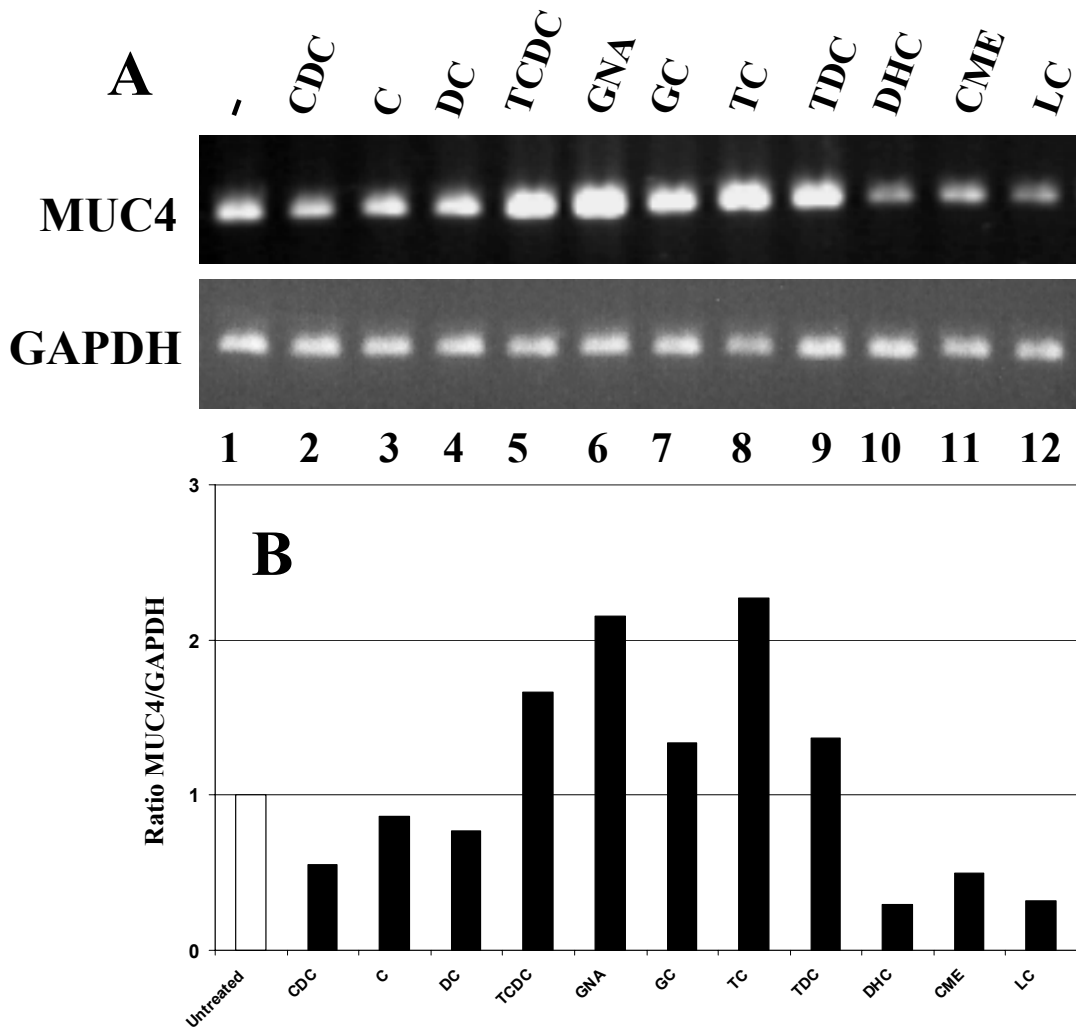


Figure 3 : Mariette *et al.*

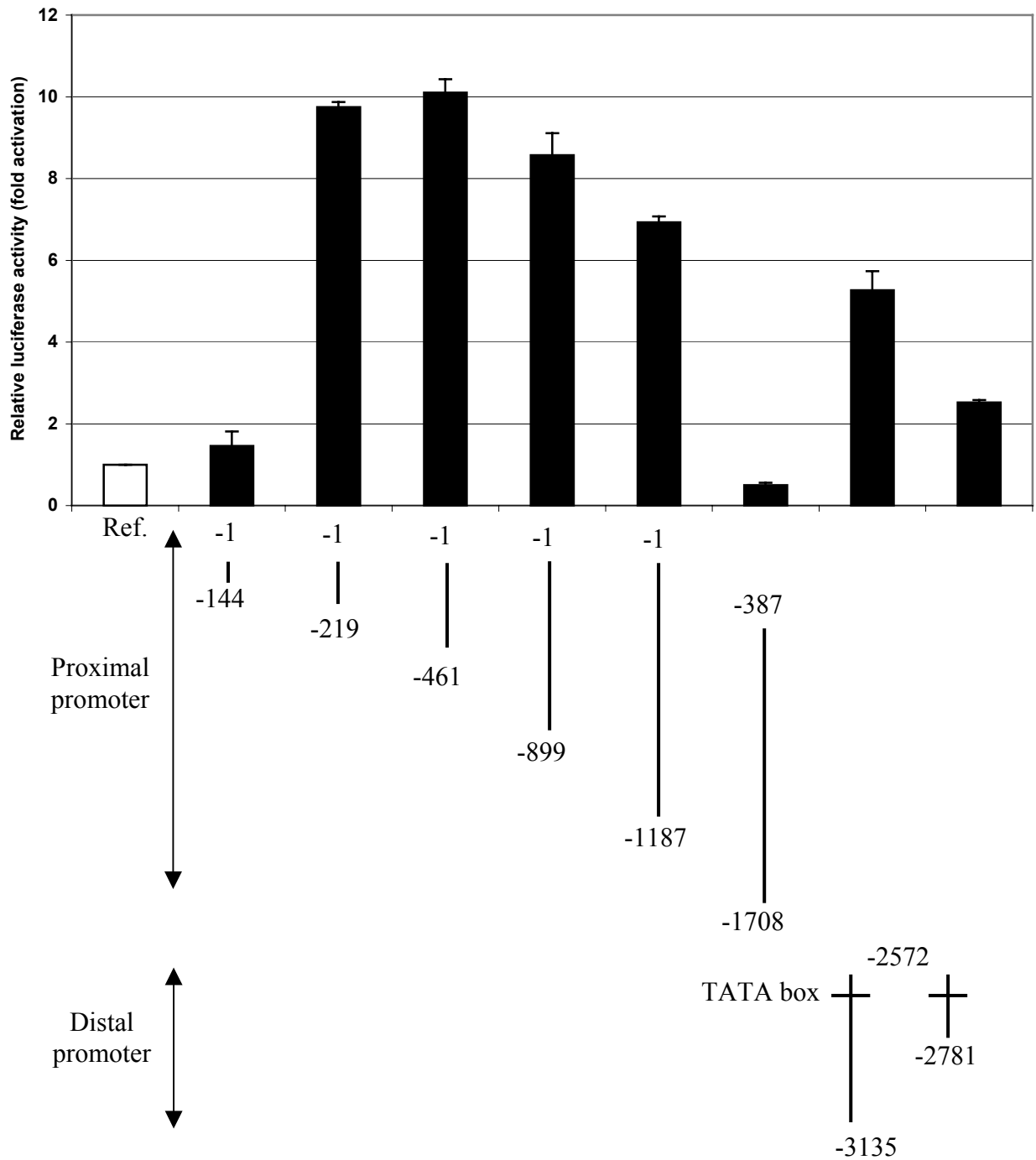


Figure 4 : Mariette *et al.*

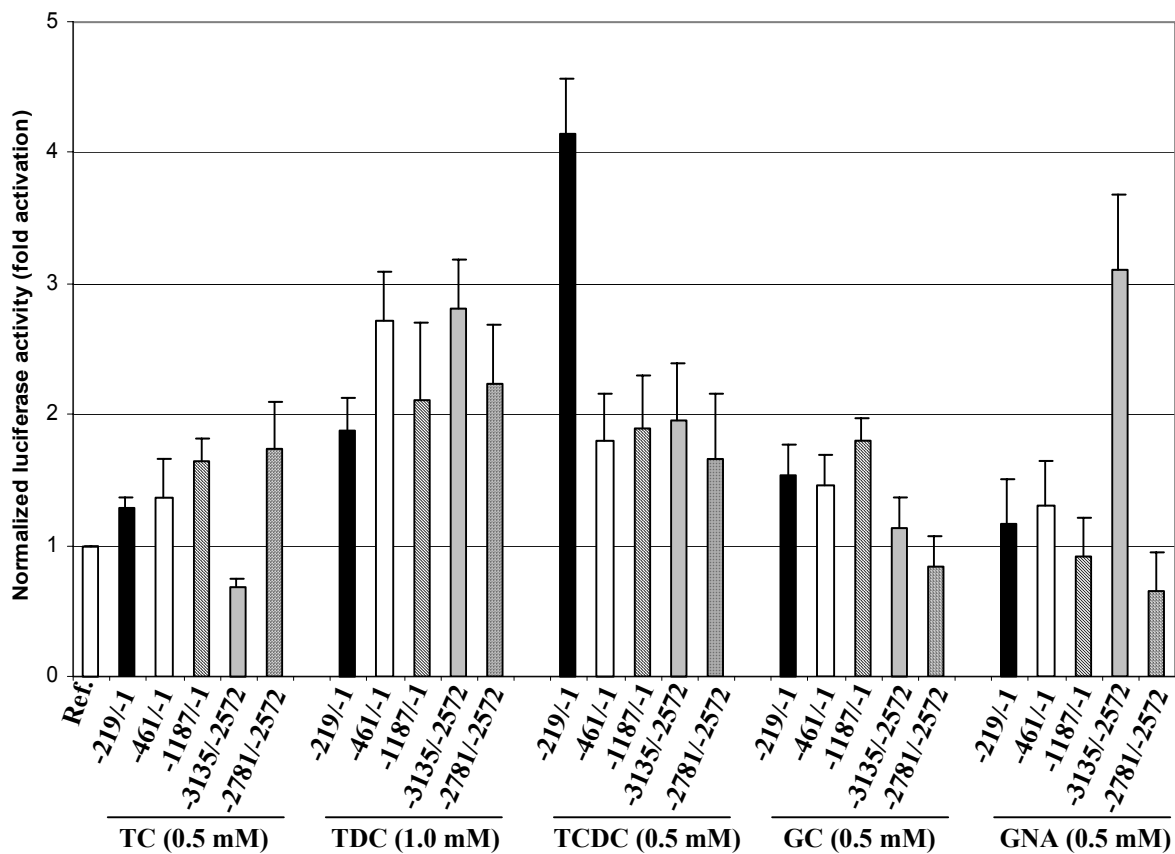


Figure 5 : Mariette *et al.*

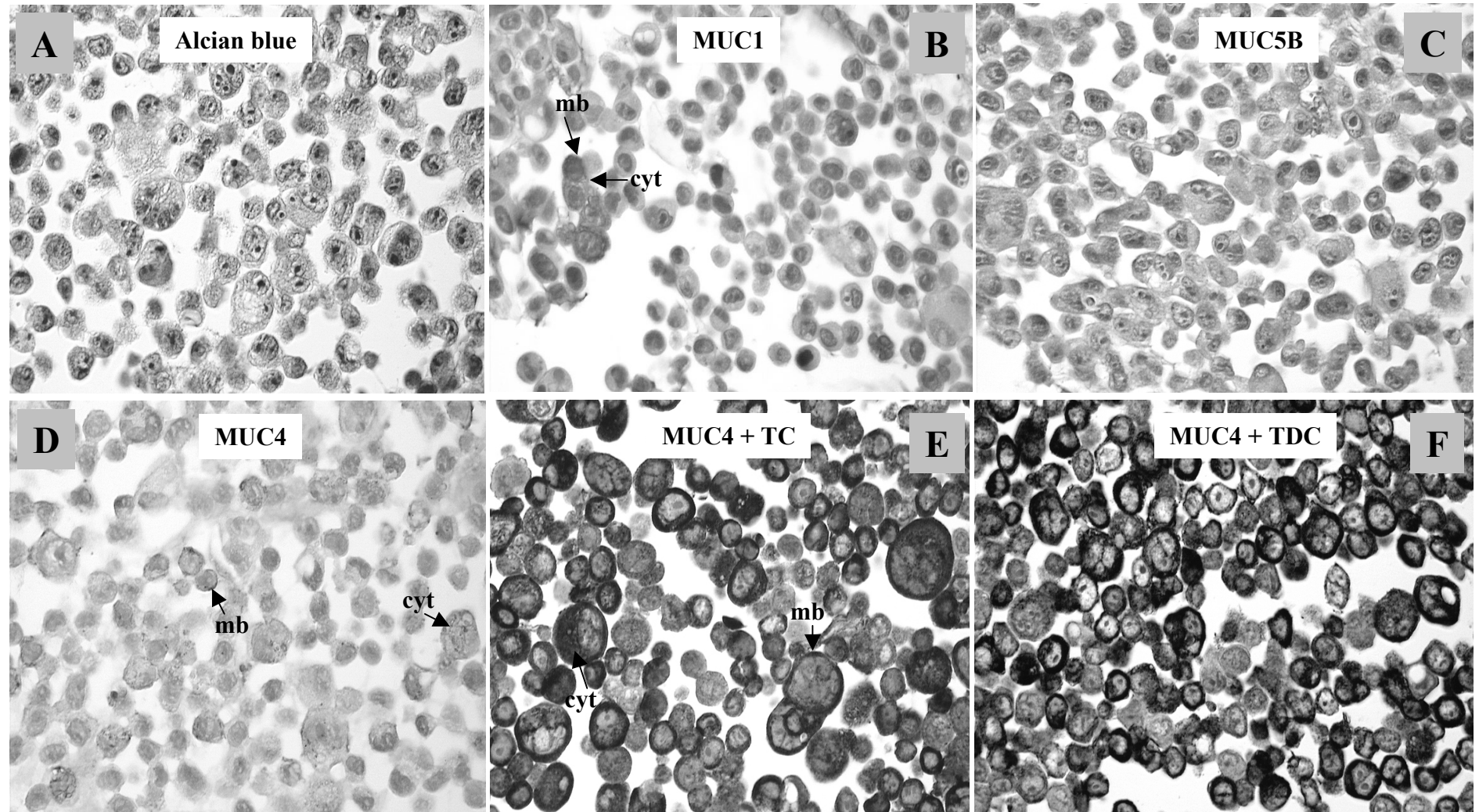


Figure 6 : Mariette *et al.*

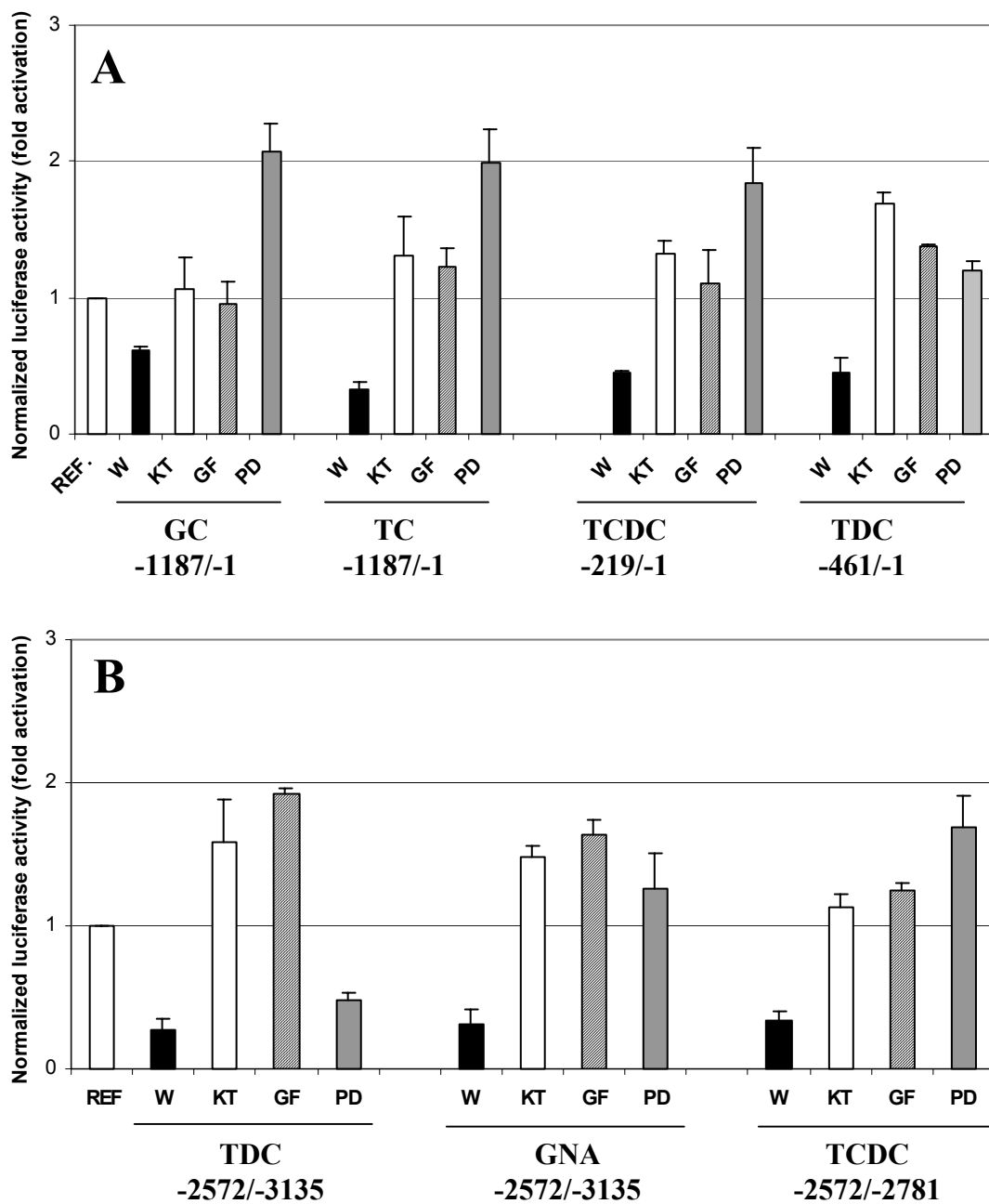


Figure 7 : Mariette *et al.*

