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REGULATION OF MUCIN AND GLYCOCONJUGATE EXPRESSION: FROM NORMAL EPITHELIUM TO GASTRIC TUMORS

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1. ABSTRACT

Gastric epithelium is protected by a mucus layer rich in MUC5AC and MUC6 mucins synthesised by the superficial epithelium and the glands, respectively. These cell populations also express specific fucosyltransferases that determine the glycosylation pattern of these gastric mucins. The maintenance of the structure and properties of the gastric mucus has been related to the degree of glycosylation and the oligomeric forms of the mucins. In gastric tumors, and in early preneoplastic lesions such as intestinal metaplasia, the glycosylation pattern detected in normal stomach is lost and, intestinal mucins, MUC2 and MUC4, can be ectopically detected in the gastric epithelium. These changes are biologically relevant because the binding of *Helicobacter pylori* to the gastric mucosa is mediated by blood group-related antigens. In vitro and animal models allowing the study of the gastric ecological niche and the requirements for its maintenance are essential for an understanding of the role of bacterial-mucosal interactions in pathological processes such as inflammation and cancer.

2. INTRODUCTION

The mucus layer covering the surface of the gastric epithelium protects it from mechanical aggressions and acid pH. This mucus is mainly composed by mucins, large and highly glycosylated molecules that contribute to the physico-chemical properties of the mucus layer which is also implicated in the bacterial colonisation of the gastric mucosa. Among the human mucins, two groups of genes have been characterised: genes coding for secreted mucins are mainly the products of the gene cluster located in chromosome 11p15, with cysteine-rich regions with homology to the von Willebrand factor, that form oligomeric structures (1); and genes coding for membrane-bound mucins that participate in several physiological processes including protection and intracellular signalling pathways (2-6). Among the secreted mucins, MUC5AC and MUC6 have been cloned from stomach cDNAs and are expressed at high levels in normal gastric mucosa. For this reason in this work we will refer to them as gastric mucins.

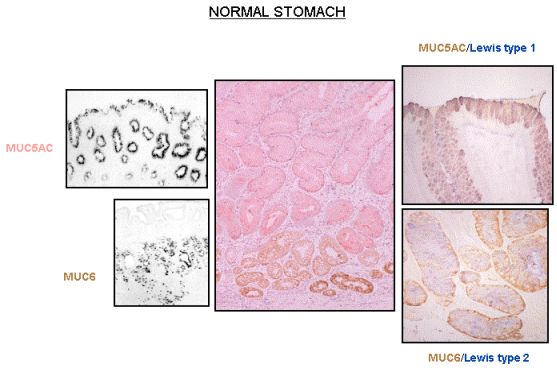

Figure 1. Expression of gastric mucins MUC5AC and MUC6 and association with Lewis antigens in the normal gastric epithelium. MUC5AC and MUC6 are detected by double labelling immunohistochemical assay and by in situ hybridisation in the superficial epithelium and antral glands, respectively. MUC5AC and Lewis b are coexpressed in the superficial epithelium, and MUC6 and Lewis y are coexpressed in the antral glands.

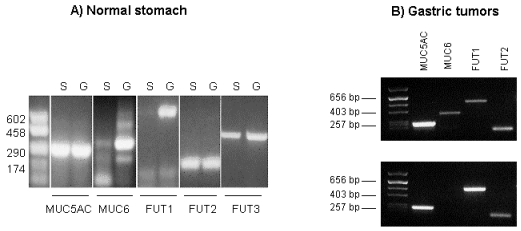

Figure 2. RT-PCR analysis of fucosyltransferase and mucin transcripts in: A) normal gastric mucosa scrapings (S, superficial epithelium scrapings; G, deep gland scrapings). MUC6 and FUT1 are detected exclusively in the deep glands scrapings, whereas MUC5AC and FUT2 were detected in both superficial epithelium and deep glands, suggesting that cells from the superficial epithelium contaminated the deep glands fraction. B) In two independent gastric tumors there was no association between the presence of gastric mucins and fucosyltransferases.

The specific pattern of expression of mucins and Lewis antigens in the gastric mucosa constitutes a good model to study the contribution of glycosyltransferases to mucin glycosylation. This is especially relevant in this organ because the glycoconjugates present in the gastric epithelial cells are ligands for *Helicobacter pylori*, a pathogen that can induce preneoplastic lesions that imply an increased risk for intestinal-type gastric cancer. The process from normal stomach to intestinal type of gastric cancer is well characterised at the histopathological level and alterations detected in the intestinal tumors can be detected early during the carcinogenesis process.

3. NORMAL GASTRIC EPITHELIUM

3.1. Mucin genes expression and glycosylation patterns in normal stomach

The normal gastric epithelium displays two distinct populations of mucus-secreting cells according to their cytochemical characteristics: cells in the superficial epithelium are stained by periodic-acid Schiff and secrete neutral mucins, whereas cells in the deep glands are stained by Alcian Blue (pH:2.5) and produce acid mucins.

These two populations of gastric epithelial cells display specific patterns of mucin gene and Lewis antigen expression: PAS-positive cells in the superficial epithelium express MUC5AC and Lewis type 1 antgens, whereas deep gland cells express MUC6 and Lewis type 2 antigens (7-9) (Figure 1). Biochemical studies using extracted and purified mucins from the surface epithelium, have demonstrated that the reactivity with anti-Le b antibodies followed the MUC5AC distribution; by contrast, reactivity with antibodies against Le y coincided with the reactivity of anti-MUC6 in mucins extracted from gland tissue (H. Nordman, submitted). Studies of mucin biosynthesis in the gastric mucosa using metabolic labelling and immunoprecipitation, support the above results showing that MUC5AC and MUC6 precursors are detected in antrum homogenates (8,9). Also, the mucin precursors of the mucin genes cluster in 11p15, oligomerized in to a single species oligomer by a common mechanism involving dimerization. This process takes place in the RER and precedes O-glycosylation, whereas N-glycosylation is necessary for efficient dimerization (10).

FUT1, FUT2, and FUT3 are the fucosyltranferases implicated in the synthesis of the epithelial Lewis antigens. FUT1 and FUT2 are responsible for the synthesis of Lewis type 2 and type 1 antigens by the addition of alpha1-2 fucose to a type 2 or type 1 precursor chain, respectively. In normal stomach scrapings, we have found that FUT1 transcripts are exclusively detected, by RT-PCR, associated to the expression of MUC6, whereas FUT2 mRNA is only expressed when MUC5AC is detected (Figure 2A) (14). All these data suggest that the mucin glycosylation pattern found in normal gastric epithelium is dictated by the specific set of fucosyltransferases expressed by each cell type.

Regarding the expression of other mucin genes in normal gastric mucosa, MUC1 is strongly detected in the mucus-producing cells in the superficial epithelium and neck of the normal stomach (15,16); MUC2, MUC3, MUC4, MUC7 and MUC8 are generally absent from the normal gastric mucosa, although MUC2 apomucin has been found focally in some stomach samples, and MUC3 transcripts have been detected in antral samples (14-19, J. Bara personal communication). Controversial data have been reported on MUC5B: some studies report MUC5B expression in the superficial epithelial cells and in the submucous glands whereas in other studies MUC5B has not been detected in the gastric mucosa cells (15, 17, De Bolós, Bara unpublished data). These differences can be due to the specificity of the antibodies used for the detection.

The trefoil factors (TFF) are small secreted proteins that also display specific expression pattern along the gastrointestinal tract. In the stomach, TFF1 or pS2 and MUC5AC are co-detected in the superficial epithelium,

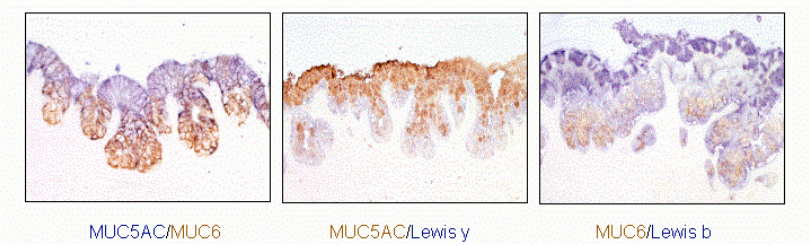

Figure 3: Mucin and Lewis antigen association in fetal stomach samples: Double labelling immunohistochemical detection of MUC5AC and MUC6 in one sample of 21 weeks; MUC5AC and Lewis y and; MUC6 and Lewis b in a fetal sample of 22 weeks.

whereas TFF2 or SP and MUC6 are co-expressed in the glands (11). The hypothesis that TFFs participate in mucus formation and structure maintenance is supported by the fact that these peptides display conserved Cys residues that can promote homo and heterodimerization through the interaction with the Cys residues in the Von Willebrand factor D domain of the mucins (12). Recently, using the 2-hybrid system, the clones interacting with TFF1 have been identified as Muc2 and Muc5AC. Overall, the specificity of these interactions seems to be only attributable to the simultaneous expression of TFFs and mucins (13).

3.2. Mucin gene expression in developing stomach

The stomach develops from the foregut at approximately 4 weeks gestation. The primitive stomach is initially lined by a stratified or pseudostratified layer of epithelial cells that becomes replaced by cuboidal cells. Gastric pits are apparent by 6 to 9 weeks and gastric glands begin to develop at 11 to 14 weeks (20).

In the primitive gut (from 6.5 to 9 weeks) only MUC3 and MUC4 mRNA are consistently detected, MUC2 and MUC5AC mRNA are expressed variably, whereas MUC5B and MUC6 are not detected (21). The expression of mucin genes in fetal stomach has been reported in samples from 15 to 41 weeks gestational age. Gastric mucins, MUC5AC and MUC6, are always expressed at high levels and, unlike in the adult gastric epithelium, they are coexpressed at the single cell level (Figure 3). Regarding the intestinal mucins, MUC2 is variably expressed, showing no correlation with the gestational age, and MUC4 is not detected (22,23). These results do not correlate with the pattern of mucin gene expression displayed by gastric tumors, in which the expression of MUC4 is highly detected (see section 5).

In the fetal stomach, gastric apomucins and Lewis antigens are expressed following a complex pattern in which all the combinations can be detected. Overall, MUC5AC is preferentially associated with Lewis type 1 structures although coexpression with Lewis type 2 antigens is also detected; MUC6 is also preferentially, but not exclusively, associated with Lewis type 2 structures (Figure 3). Despite the variability observed, there is a trend toward the establishment of the adult expression pattern (22).

4. MUCIN GENE EXPRESSION AND GLYCOSYLATION PATTERNS DURING GASTRIC CARCINOGENESIS

The most common gastric tumors are the intestinal and diffuse types. The intestinal type has been related epidemiologically to external aggressions, mainly *Helicobacter pylori* infection, whereas the diffuse type is not associated to external environmental factors. The multistep process of carcinogenesis from normal gastric mucosa to the intestinal type tumor has been well characterised. It is initiated by a multifocal atrophic gastritis, followed by intestinal metaplasia and dysplasia, the latter considered to be identifiable precursor lesions of this type of gastric cancer (24). By contrast, no identifiable precursor lesions have been reported for the diffuse type.

4.1. Chronic atrophic gastritis

Chronic atrophic gastritis is mainly characterised histologically by mucosal surface erosion and acute or chronic inflammation. The expression of mucin genes in this disorder is usually similar to that observed in normal gastric mucosa, although in some cases MUC4 can be detected in the superficial epithelium (14). No changes in the expression of the carbohydrate structures have been found (14, 25).

4.2. Intestinal metaplasia: complete and incomplete

Intestinal metaplasia is a well-characterised gastric disorder defined by the replacement of the stomach mucosa by an epithelium that resembles the intestinal mucosa. On the basis of morphology and mucin staining, two main types of intestinal metaplasia have been identified: a) the complete type, also designated type I, which is characterised by the presence of absorptive cells, Paneth cells, and goblet cells secreting sialomucins; and b) the incomplete type, including types II and III, with columnar and goblet cells secreting sialo (type II) and/or sulfomucins (type III) (26). Regarding mucin gene expression, MUC5AC and MUC6 gastric mucins are down-regulated in metaplastic cells whereas expression of MUC2 and MUC4 intestinal mucin gene is detected. Furthermore, gastric and intestinal apomucins can be co-expressed in the same metaplastic cell (14, 27). An up-regulation of MUC3 in metaplastic cells has also been reported (18). These changes are more evident in the complete than in the incomplete type of intestinal metaplasia (14, 17, 27).

The characteristic pattern of apomucin/Lewis antigen association detected in the normal gastric epithelium is not observed in metaplasia. Metaplastic cells co-express gastric and intestinal mucin genes in association with both types of Lewis antigens. The altered patterns detected in intestinal metaplasia indicate that these changes are early events in the gastric carcinogenesis process (14,27) (Figure 4). Regarding specific carbohydrate

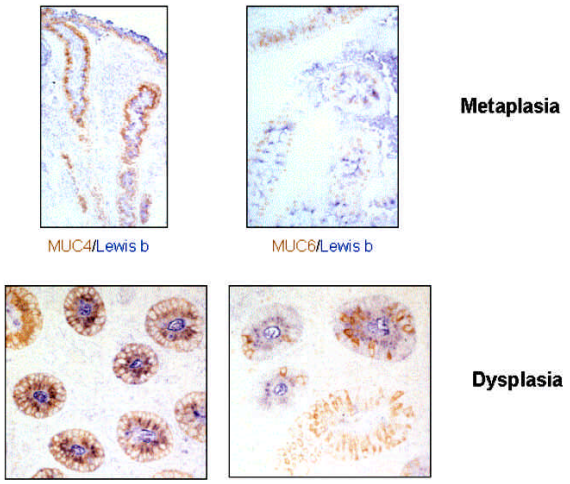

Figure 4. Detection of mucin genes and Lewis antigens in intestinal metaplasia and dysplasia. In these gastric disorders the ectopic expression of MUC4, and co-expression between MUC6 and Lewis type 1 antigens are detected. In these lesions the association between gastric mucin and fucosyltransferase genes is lost.

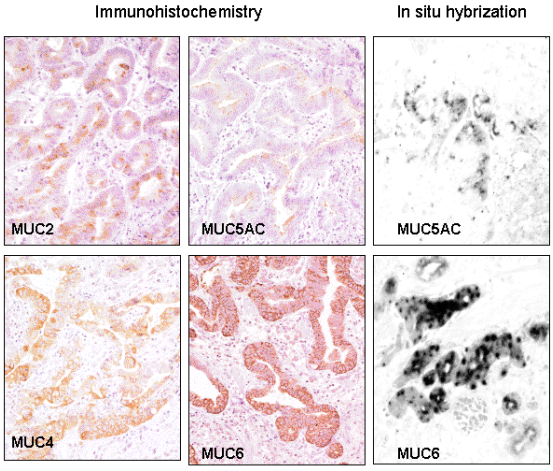

Figure 5. Expression of gastric (MUC5AC and MUC6) and intestinal (MUC2 and MUC4) mucin genes in the same intestinal gastric tumor detected by immunohistochemistry and in situ hybridisation.

structures, an ectopic expression of Lewis antigens has been reported in metaplastic cells, for example the presence of Lewis a in intestinal metaplasia from individuals with the secretor phenotype (28,29). Also, the accumulation of precursor structures, T, Tn and sialyl-Tn, can be detected in metaplastic cells; for example, the expression of T antigen, detected after neuraminidase treatment, is more prevalent in the incomplete than in the complete type of intestinal metaplasia (25).

4.3. Dysplasia

The dysplastic gastric epithelium is characterised by nuclear abnormalities and an irregular cell-shape. These dysplastic changes may represent cytological alterations alone or may be associated with a disorganised architecture of the epithelium. Frequently, dysplasia is present in biopsies with chronic atrophic gastritis and/or intestinal metaplasia. The pattern of mucin gene expression in these cells is similar to that observed in incomplete intestinal metaplasia: intestinal and gastric mucins are usually detected in the same cell and are associated to both, type 1 and type 2, Lewis antigens (14) (Figure 4). In this preneoplastic disorder one of the most distinctive feature on the glycosylation pattern is the higher expression of sialyl-Tn antigen (25).

5. GASTRIC CANCER

Differences in the incidence of the intestinal and the diffuse types of gastric cancer exist between countries with high rates of these tumors. In western countries a decrease in the mortality due to gastric cancer has occurred and it is associated with a decrease in the incidence of the intestinal type.

5.1. Intestinal tumors

The intestinal type of gastric cancer is histologically characterised by the presence of cohesive cells forming glandular and papillary structures that expand into the gastric wall. Regarding mucin gene expression, a general feature of this tumor type is the down-regulation of gastric mucin genes (MUC5AC and MUC6) and the up-regulation of intestinal mucin genes (MUC2 and MUC4) (14,30) (Figure 5). Also, MUC1 is highly expressed and MUC3 is ectopically detected (17). No data about the expression of other mucin genes are, at this moment, available.

In this type of tumor, the association between gastric mucin genes, fucosyltransferase genes, and Lewis antigen expression is lost and several combinations can be detected supporting the notion that the glycosylation of MUC5AC and MUC6 is not due to the intrinsic characteristics of the peptide and is rather determined by the specific set of glycosyltransferases (Figure 2B) (14). All these alterations are already detected in intestinal metaplasia, suggesting that this disorder can be considered as a precursor for the intestinal type of gastric cancer.

As it has been reported for other epithelial tumors, aberrant carbohydrate epitopes can also be detected in gastric cancer. The core structures T, Tn, sTn are expressed in the intestinal type gastric cancer (31-33) indicating that in these tumoral cells an underglycosylation takes place: for example, the expression of T antigen has been related to a poorer prognosis (34). Also, altered patterns of terminal carbohydrate structures can be detected and it has been reported that the up-regulation of sialyl-Lewis x is related to an increased risk of gastric cancer metastasis (35).

5.2. Diffuse tumors

The diffuse type of gastric cancer is characterised histologically by discohesive cells and the common presence of signet ring cells. This tumor is widely infiltrative and histologic precursor lesions have not been identified. Possible familial factors have been associated to

Table 1. Mucin gene expression detected by RT-PCR and IHC. Fucosyltransferase transcripts detected by RT-PCR. Lewis antigens detected by IHC

Cell line	MUC2	MUC4	MUC5AC	MUC6	FUT1	FUT2	FUT3	Lewis type 1*	Lewis type 2**
COLON									
HT-29	-	-	-	-	-	+/-	+/-	+	+/-
HT-29MTX^{-3}	-	-	+	-	-	+	+/-	+/-	+/-
HT-29MTX^{-6}	+/-	-	+	-	+	+	+	+	+/-
LS174T	+		-	+	-	+	-	+	+
WiDr			+/-	-	+	+	+	+	+/-
SW480			+/-	-	+	+	-	-	+/-
COLO201			-	-	+	+	+	+	+
STOMACH									
MKN45	-	-	+	-	+	+	-	+	+/-
NUG4			+	+	+	+	+	-	+/-
KATOIII	+	+	+	-	+	+	+	+	+
St-3051	-	-	+	-	+	+	+	+	+
St-23132	-	-	+	-	+	+	+	+	+
St-2957	-	-	+	-	+	+	+	+	+
GP220	-	-	+	-	+	+	+	+	+

*Lewis a, Lewis b, sialyl-Lewis a. ** Lewis x, sialyl-Lewis x, Lewis y.

this type of gastric cancer. Diffuse tumor cells express high levels of gastric mucins, and relatively low levels of intestinal mucin genes (14,30). As in the intestinal type, no specific association between Lewis antigens and apomucins has been found in the diffuse type (19).

6. GASTRIC MUCOSA AND COLONISATION BY *HELICOBACTER PYLORI*

The gastric mucosa can be colonised by *H. pylori,* a Gram-negative spiral bacterium that is the most common cause of histological gastritis, causes most gastric ulcers, and is associated with an increased risk of gastric adenocarcinoma and lymphoma (36,37). In developing countries, the rate of infection increases with age and 50% of people are infected by the bacterium at the age of 60. However, only a minority of infected people develops symptomatic gastric pathology (38).

The presence of H. *pylori* in human gastric mucosa has been associated with the expression of specific ABO structures (39) and the binding of the bacteria to the gastric epithelium has been described to be mediated by the Lewis b antigen (40). Moreover, structural data on the bacterial glycoconjugates, allow the classification of the *H. pylori* strains into six glycotypes regarding the presence of Lewis antigens carried by the bacterial LPS, thus mimicking the human gastric cell surface with Lewis type 1 and/or type 2 antigens. This suggests that each strain may have a different gastric ecological niche depending on the Lewis structures displayed on their surface glycoconjugates (41). Studies on gastric colonisation of the different *H. pylori* strains indicate that those which strongly express Le x/y cause a higher colonisation density than strains with lower expression (42). These data suggest that the enhanced colonisation associates with the expression of Lewis x and could be mediated through an increased adherence to a gastric Lewis x-binding lectin.

It has been postulated that the interaction of *H. pylori* with the gastric epithelium is associated with an abnormal niche: *H pylori*-bound cells express MUC6 and Lewis x in the superficial epithelium (43). However, we have not replicated these findings (14, unpublished data). Recently, Van den Brink, using triple immunohistochemical staining, reported the colocalisation of MUC5AC and *H. pylori*, whereas no colocalisation with MUC6 was found (44). Furthermore, the adherence of the pathogen to metaplastic cells can only be detected in cells displaying incomplete metaplasia that are positively stained by Alcian blue, and related to the levels of MUC5AC detected with the M1 antibody (45). Also, the direct binding of *H. pylori* to the stomach mucus has been analysed in fractions of pig gastric mucins (46). In these mucins, the binding of the bacteria depends on the *H. pylori* strain and is influenced by the pH of the different mucin fractions suggesting, again, that the nature of the carbohydrate structures is determinant for the binding.

Several adhesins have been proposed to mediate the attachment of *H. pylori* to the gastric epithelial cells. Following the binding, several signalling pathways can be activated in the host cells including cytoplasmic and nuclear events. Some of them are related to the *H. pylori* strains that contain the *cag* DNA fragment that induces the activation of NF-kappaB with the consequent secretion of proinflamatory cytoquines such as IL-8 (47). Moreover, the bacterial antigen CagA is delivered into the gastric epithelial cells where is phosphorylated by a host cell kinase (48). Recently, this kinase has been identified as PAK1, that mediates the activation of NF-kappaB (49). More research is necessary to better elucidate the interactions between the bacteria and the host gastric epithelial cells during the infection that precedes gastric preneoplastic diseases.

7. *IN VITRO* AND ANIMAL MODELS

The generation of in vitro and in vivo systems is a helpful tool to directly analyse the mechanisms that instruct the glycosylation patterns, and to study the biological activities of specific glycoconjugates. In this context, we have analysed the expression of mucin and

fucosyltransferase transcripts, as well as apomucins and carbohydrate epitopes, in a panel of epithelial cell lines (Table 1) (50, López-Ferrer unpublished data). The availability of these well characterised cell lines will be useful in the generation of in vitro models. Selected cell lines expressing a specific set of fucosyltranferases will allow to directly analyse the glycosylation pattern of a specific apomucin molecule. The de novo expression of specific glycoconjugates, by transfection of a glycosyltransferase cDNA, will help in the analysis of their biological activities. For example, carbohydrate epitopes may be important for the adhesion of microorganisms, especially *H. pylori*, to the gastric mucosa. Similarly, it has been extensively reported that the fucosylated structures sialyl-Lewis a and sialyl-Lewis x are the ligand molecules for selectins that are implicated in migration and adhesion processes. In these cell lines, the de novo expression of specific fucosylated products alters their biological properties and may be useful as a potential tool to analyse their direct implication in these processes.

Animal models are useful to identify the bacterial and host molecules that produce the attachment and induce gastric pathologies, as well as other biological activities of the carbohydrate epitopes. Several data have been reported, pointing to the relationship between the colonisation of the gastrointestinal epithelium by bacteria and the presence of fucosylated structures. In the mouse, the activation of the alpha-1,2-fucosyltransferase gene and the expression of the corresponding fucosylated products in the small intestine epithelium, requires the presence of the normal microflora (51). Also, Lewis a and Lewis b are expressed in the small intestine of the FUT3 transgenic mice, whereas the localisation of Lewis x is restricted to the crypt cells (52). In this mouse, the infection with a clinical isolate of the bacteria resulted in attachment of *H. pylori* to both normal and transgenic gastric epithelium indicating that the receptor (Lewis b) is not necessary to establish and maintain the infection (53). However, in the FUT3 mice auto-antibodies against Lewis x and chronic gastritis are detected (54).

8. PERSPECTIVE

The transformation of gastric epithelium from normal stomach to the intestinal type of gastric tumor provides a useful model to study gastric differentiation. This multistep process has been well characterised histologically, but the genetic events are not fully established. The fact that in intestinal metaplasia, and also in dysplasia and tumors, gastric and intestinal mucin genes are expressed, suggests that two differentiation programs are activated in these cells. Furthermore, the identification of the specific set of glycosyltransferases present in a determined gastric cell type will provide a useful tool to biochemically analyse the glycoproteins implicated in several processes like bacterial binding, adhesion, and invasion. The binding and signalling pathways of *H. pylori* in the gastric mucosa should be elucidated in order to characterise the specific biological phenomena implicated in the sequential steps of the gastric carcinogenesis. For this purpose, in vitro and especially animal models are needed to analyse all the processes implicated in the pathogenesis of this bacteria.

9. ACKNOWLEDGEMENTS

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