

Intestinal Metaplasia of Human Stomach Displays Distinct Patterns of Mucin (MUC1, MUC2, MUC5AC, and MUC6) Expression¹

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ABSTRACT

Intestinal metaplasia is a well-established premalignant condition of the stomach that is characterized by mucin carbohydrate modifications defined by histochemical methods. The purpose of the present study was to see whether the expression of mucin core proteins was modified in the different types of intestinal metaplasia and to evaluate the putative usefulness of mucins as “molecular markers” in this setting. We used a panel of monoclonal antibodies with well-defined specificities to MUC1, MUC2, MUC5AC, and MUC6 to characterize the expression pattern of mucins. In contrast to normal gastric mucosa, the complete form or type I intestinal metaplasia ($n = 20$) displayed little or no expression of MUC1, MUC5AC, or MUC6 in the metaplastic cells and strong expression of the intestinal mucin MUC2 in the goblet cells of all cases. The incomplete forms of intestinal metaplasia, type II ($n = 25$) and type III ($n = 16$), expressed MUC1 and MUC5AC in every case, both in goblet and in columnar cells. MUC6 was also expressed in 16 cases of type II intestinal metaplasia and in 11 cases of type III intestinal metaplasia. The intestinal mucin MUC2 was expressed in every case of incomplete intestinal metaplasia, mostly in goblet cells. The mucin expression profile in the different types of intestinal metaplasia allows the identification of two patterns: one defined by decreased levels of expression of “gastric” mucins (MUC1, MUC5AC, and MUC6) and expression of MUC2 intestinal mucin, which corresponds to type I intestinal metaplasia, and the other defined by coexpression of “gastric mucins” (MUC1, MUC5AC, and MUC6) together with the MUC2 mucin, encompassing types II and III intestinal metaplasia. Our results challenge the classical sequential pathway of intestinal metaplasia (from type I to type III via a type II intermediate step).

INTRODUCTION

Mucins are heavily glycosylated glycoproteins that are the major components of the mucous viscous gel covering the surface of epithelial tissues (1). To date, nine distinct epithelial mucin genes (*MUC1*, 2, 3, 4, 5AC, 5B, 6, 7, and 8) have been identified (2–11). *In situ* hybridization and immunohistochemical studies have shown that these mucins are differentially expressed in epithelia with cell type specificity (12–15). The normal gastric mucosa shows cell type specific expression of MUC1, MUC5AC, and MUC6, with the first two mucins found in the superficial epithelium and MUC6 in the deep glands (13, 16, 17). At variance with MUC1 and MUC5AC, which are expressed in many epithelia, MUC6 is mainly expressed in gastric mucosa. The normal gastric mucosa does not express MUC2 (16–18).

Changes in the expression levels and glycosylation patterns of mucins have been associated with several diseases, including carcinomas (18–22). In gastric cancer, alterations in mucin polypeptide

expression have been reported: loss of expression of MUC5AC (14, 17, 23), increased mucin heterogeneity (14), and glycosylation changes, including exposure of simple mucin-type carbohydrates (24, 25). These observations suggest that mucin alterations can be regarded as “molecular” markers of malignant transformation of gastric mucosa.

The majority of gastric carcinomas, particularly the “intestinal” type (26), which is the most common in populations at high risk, is preceded by a precancerous stage, characterized by the following sequential steps: superficial gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia (27–29).

Intestinal metaplasia consists in the replacement of the gastric mucosa by an epithelium that resembles histologically the intestinal mucosa. Histopathological and histochemical studies allowed the identification of two main types of intestinal metaplasia: (a) the complete type, also designated type I, which is characterized by the presence of absorptive cells, Paneth cells, and goblet cells secreting sialomucins and corresponds to the small intestine phenotype; and (b) the incomplete type, encompassing types II and III, which is characterized by the presence of columnar and goblet cells secreting sialo and/or sulfomucins. Type II differs from type III intestinal metaplasia regarding the mucins produced by columnar cells: neutral and acid sialomucins in type II and sulfomucins in type III (30, 31).

Several studies have shown that type III intestinal metaplasia is associated with an increased risk of malignant transformation (32–35), whereas the putative value of type I and type II intestinal metaplasia remains controversial.

Altered mucin expression patterns have been reported previously in intestinal metaplasia, including underexpression of MUC1, MUC5AC, and MUC6 (14) and *de novo* expression of MUC2 (14, 36, 37). However, there is not on record a systematic study on the putative relationship between mucin changes and the different types of intestinal metaplasia.

In the present study, we further characterized the pattern of mucin expression in intestinal metaplasia of the stomach in an attempt to evaluate whether there is a counterpart, at the mucin expression level, of the different types of intestinal metaplasia as defined by histological and histochemical criteria. For this purpose, we have used a comprehensive panel of monoclonal antibodies, with well-characterized specificities, directed to MUC1, MUC2, MUC5AC, and MUC6 mucins.

MATERIALS AND METHODS

Tissue Samples and Histochemistry. Endoscopic gastric biopsies were obtained from individuals with nonulcer dyspepsia. Gastric mucosae adjacent to carcinomas were obtained from individuals undergoing surgery in Hospital S. João, Medical Faculty (Porto, Portugal). Every specimen was fixed in 10% formalin and routinely embedded in paraffin wax. Serial sections were cut and used for histochemistry and immunohistochemistry.

Alcian blue (pH 2.5)/periodic acid-Schiff and the high iron diamine-alcian blue technique (38) were used to identify neutral, sialomucins, and sulfomucins. Intestinal metaplasia was classified according to Filipe (31) as follows: type I, mature absorptive cells and goblet cells, the latter secreting sialomucins; type II, few or absent absorptive cells, presence of columnar “intermediate” cells in various stages of differentiation secreting neutral and acid sialomucins and goblet cells secreting sialomucins or, occasionally, sulfomucins, or both; and type III, columnar “intermediate” cells secreting predominantly sulfomucins and goblet cells secreting sialomucins or sulfomucins, or both. We studied 20 cases of intestinal metaplasia type I (10 biopsies and 10 surgical speci-

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mens), 25 cases of type II (15 biopsies and 10 surgical specimens), and 16 cases of type III intestinal metaplasia (7 biopsies and 9 surgical specimens).

Monoclonal Antibodies. Monoclonal antibodies used in this study, their specificity, and their references (17, 37, 39) are listed in Table 1.

Immunohistochemistry. Sections designed for single staining were immunostained by the avidin-biotin-complex method (40). Paraffin sections were dewaxed and rehydrated. Sections designed for neuraminidase treatment were washed two times in Tris-buffered saline (TBS; pH 7.6) and incubated with neuraminidase from *Clostridium perfringens* type VI (Sigma Chemical Co., St. Louis, MO) diluted in 0.1 M sodium acetate buffer (pH 5.5) to a final concentration of 0.1 unit/ml. The incubation was carried out for 2 h at 37°C and followed by three washings in ice-cold water. Sections were treated with 0.5% H₂O₂ in methanol for 30 min, followed by a 20-min incubation with rabbit nonimmune serum. Sections were rinsed and incubated with primary antibody (Table 1) overnight at 4°C. Sections were rinsed and incubated with biotin-labeled rabbit anti-mouse serum diluted 1:200 in TBS for 30 min, rinsed with TBS, and incubated with avidin-biotin-peroxidase complex for 1 h. Sections were rinsed in TBS and developed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride freshly prepared in 0.05 M TBS containing 0.1% H₂O₂. Sections designated for single staining were stained with hematoxylin, dehydrated, and mounted.

Although single staining was used to analyze the expression of mucins in all of the cases, double and triple staining were used for documentation purposes only. Sections designated for sequential double staining were stained for 15 min with 0.05% 3,3'-diaminobenzidine tetrahydrochloride containing 0.1% H₂O₂ and 1 µg/ml nickel. For the second incubation, sections were treated with normal goat nonimmune serum for 20 min, followed by overnight incubation with primary antibody. Incubation with goat anti-mouse secondary antibody, diluted 1:100 in TBS, for 30 min was followed by alkaline phosphatase anti-alkaline phosphatase, diluted 1:50, for 30 min. Sections were then stained for 20 min with 0.01% Fast Red in freshly prepared substrate (naphthol AS-MX-phosphate, dimethylformamide, 0.1 M Tris, and 1 M levamisole).

Sections designated for triple staining were incubated for 30 min in TBS at 60°C for denaturation of any available alkaline phosphatase. Sections were then incubated overnight with primary antibody, followed by alkaline phosphatase anti-alkaline phosphatase under the same conditions described above. Sections were then stained for 20 min with 0.01% Fast Blue in freshly prepared substrate as described above. All double and triple stained sections were mounted in Glycergel.

All series included positive and negative controls. Multiple staining was always compared with serial sections stained with one antibody per section as control.

RESULTS

Expression Pattern of MUC1, MUC2, MUC5AC, and MUC6 Mucins in Normal Gastric Mucosa. MUC1 was expressed in the foveolar epithelium and in a few mucous gland cells of the antrum. The glands of the oxyntic region also showed expression of MUC1 in principal and parietal cells. MUC5AC was highly expressed in foveolar epithelium and mucous neck cells of both antrum and body regions. Expression of MUC6 was detected in the glands of the antrum and the mucopeptic cells of the neck zone of the normal gastric body region (Fig. 1). MUC2 was not detected in normal gastric mucosa, except for an occasional weak staining of the foveolar cells in the Golgi region.

Immunodetection of Mucins in Type I Intestinal Metaplasia. There was no expression of MUC1 in goblet and columnar cells but for the staining of few superficial columnar cells in a single case (Fig. 1C). Most of the cases showed no expression of MUC5AC and MUC6, either in the goblet or in the columnar cells (Fig. 1D). However, six cases

showed expression of MUC5AC in rare goblet cells. Four cases showed MUC5AC immunoreactivity in a few superficial columnar cells. Four cases showed expression of MUC6 in rare goblet cells. In 7 cases, expression of MUC6 in rare columnar cells was also observed. In all of the cases, MUC2 was expressed in most goblet cells (Fig. 1D). No immunoreactivity for MUC2 was observed in columnar cells.

The overall profile of mucin expression in type I intestinal metaplasia is characterized by absence or markedly decreased levels of mucins normally expressed in the gastric mucosa (MUC1, MUC5AC, and MUC6) and *de novo* expression of the MUC2 intestinal mucin (Fig. 2).

Immunodetection of Mucins in Type II Intestinal Metaplasia. There was expression of MUC1 in all of the 25 cases analyzed, both in goblet and in columnar cells. Most of the cases expressed MUC1 in >75% of the goblet cells (19 cases) and of the columnar cells (20 cases). There was expression of MUC5AC in all of the cases, both in goblet and in columnar cells. The percentage of stained cells varied from case to case; in 14 cases, MUC5AC was expressed in >75% of the goblet and columnar cells. In most cases, there was a trend for a higher number of cells expressing MUC5AC in the superficial part of the metaplastic glands than in the deep part. Expression of MUC6 was detected in goblet cells (12 cases) and in columnar cells (16 cases), and it was restricted to a low percentage of the cells. In all of the cases, MUC2 was expressed in goblet and columnar cells. Most cases ($n = 17$) showed expression of MUC2 in >50% of the goblet cells. In contrast, MUC2 was expressed in <25% of the columnar cells in all cases.

The overall profile of mucin expression in type II intestinal metaplasia is characterized by maintenance of expression of MUC1 and MUC5AC mucins, which are normally expressed in the gastric mucosa, decreased levels of expression of the MUC6 gastric mucin, and *de novo* expression of the intestinal mucin MUC2 (Fig. 2).

Immunodetection of Mucins in Type III Intestinal Metaplasia. All of the cases expressed MUC1 in >75% of the goblet and columnar cells (Fig. 1G). All of the cases also expressed MUC5AC both in goblet and columnar cells (Fig. 1H). Most cases ($n = 10$) showed MUC5AC in >75% of goblet and columnar cells. In most cases, there was also a trend for a higher number of cells expressing MUC5AC in the superficial part of the metaplastic glands. Expression of MUC6 was detected in goblet cells ($n = 7$) and in columnar cells ($n = 10$), and it was restricted to a low percentage of the cells. MUC2 was expressed *de novo* in goblet and columnar cells in every case (Fig. 1H). Most cases expressed MUC2 in >75% of the goblet cells and in <25% of columnar cells (10 and 14 cases, respectively).

The overall profile of mucin expression in type III intestinal metaplasia is thus similar or almost identical to that of type II intestinal metaplasia (Fig. 2).

Immunocytolocalization of Mucin Expression in the Different Cell Types of Intestinal Metaplasia. MUC1 was expressed in columnar cells with a distinct pattern, depending on the antibody that was used: diffuse cytoplasmic staining with HMFG2, and cytoplasmic as well as apical membrane staining with HMFG1. Both MUC1 antibodies showed a supranuclear/Golgi pattern in goblet cells.

MUC5AC and MUC6 displayed a similar cytolocalization profile, with a diffuse cytoplasmic expression pattern in columnar cells and a supranuclear/Golgi pattern in goblet cells.

MUC2 immunodetection before and after neuraminidase treatment was similar, although in some cases the staining intensity and the number of stained cells increased after neuraminidase treatment; we used, for quantification purposes, the results obtained after neuraminidase. MUC2 expression displayed a diffuse cytoplasmic pattern in columnar cells and usually a vacuolar staining in goblet cells. In a few cases, some goblet cells showed cytoplasmic staining restricted to the perinuclear area.

DISCUSSION

Mucins are expressed with a cell- and tissue-specific pattern in normal tissues. Alterations of the expression pattern of mucins have been described in carcinomas as well as in their precursor lesions; in

Table 1 Antibodies: Their specificity and references

Monoclonal antibody	Specificity	Reference
HMFG1	MUC1	Taylor-Papadimitriou <i>et al.</i> (39)
HMFG2	MUC1	Taylor-Papadimitriou <i>et al.</i> (39)
PMH1	MUC2-GalNAc	Reis <i>et al.</i> (37)
CLH2 ^a	MUC5AC	Reis <i>et al.</i> (17)
CLH5	MUC6	Reis <i>et al.</i> , unpublished results

^a Code number MAB2011 from Chemicon International, Inc. (Temecula, CA).

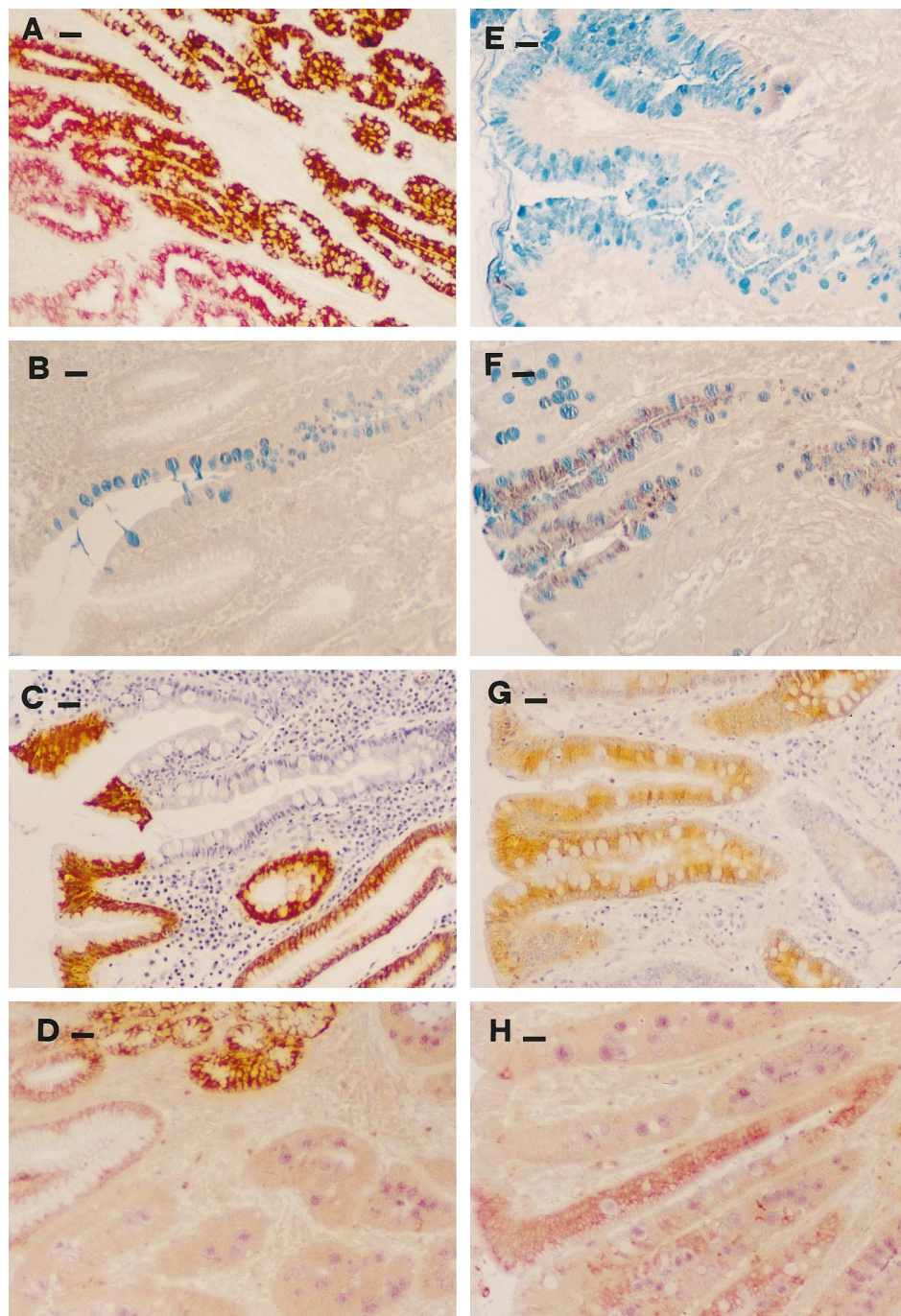


Fig. 1. Histochemical and immunohistochemical (MUC1, MUC2, MUC5AC, and MUC6) staining of normal gastric mucosa and intestinal metaplasia. *A*, normal gastric mucosa immunohistochemically double stained with monoclonal antibody CLH2 (anti-MUC5AC, red) and monoclonal antibody CLH5 (anti-MUC6, brown). *B*, gastric mucosa with intestinal metaplasia type I histochemically stained with alcian blue/high iron diamine. *C*, gastric mucosa with intestinal metaplasia type I immunohistochemically stained with monoclonal antibody HMFG2 (anti-MUC1) showing absence of MUC1 staining in the metaplastic glands. *D*, gastric mucosa with intestinal metaplasia type I immunohistochemically triple stained with monoclonal antibody CLH2 (anti-MUC5AC, red), monoclonal antibody CLH5 (anti-MUC6, brown), and monoclonal antibody PMH1 (anti-MUC2, blue), showing absence of MUC5AC and MUC6 staining in the metaplastic glands and expression of MUC2 in goblet cells. *E* and *F*, gastric mucosa with intestinal metaplasia type II (*E*) and type III (*F*), histochemically stained with alcian blue/high iron diamine showing sialomucins in type II (*E*) and sulfomucins in type III (*F*) intestinal metaplasia. *G*, area of intestinal metaplasia type III immunohistochemically stained with monoclonal antibody HMFG2 (anti-MUC1, brown) showing expression of MUC1 in goblet and columnar cells of metaplastic glands. *H*, area of intestinal metaplasia type III immunohistochemically double stained with monoclonal antibody CLH2 (anti-MUC5AC, red) and monoclonal antibody PMH1 (anti-MUC2, blue) showing expression of MUC5AC and MUC2 in cells of metaplastic glands. Bars, 11.6 μm .

the latter, altered mucin carbohydrate and peptide moieties of mucins may constitute molecular markers of an increased risk of malignant transformation (18, 41–44).

In agreement with previous studies reporting the distribution of mucins in normal stomach, we found expression of MUC1 in mucous cells of foveolar epithelium and glands of the antrum and in parietal and principal cells of the body (18, 23, 43, 45). We observed, moreover, that MUC5AC is highly expressed in foveolar cells of both body and antrum, as reported previously (13, 14, 17). We also found that MUC6 is expressed in mucous cells of the neck zone of the body and in pyloric glands of the antrum with a similar pattern to that described previously with polyclonal sera for MUC6 (13, 14). Expression of MUC2 was usually not detected in normal gastric mucosa (16, 18, 37).

Intestinal metaplasia is one of the lesions identified in the cascade of events that precedes the development of gastric carcinoma (27). By histology, intestinal metaplasia is identified by the substitution of the gastric mucosa by an epithelium that resembles the intestinal mucosa. Histochemical methods showed that the glycosylation pattern in intestinal metaplasia is different from that of normal gastric mucosa (30, 31). Recently, immunohistochemical studies using monoclonal antibodies directed to several carbohydrates confirmed the modified glycosylation pattern of intestinal metaplasia, with accumulation of simple mucin-type carbohydrate antigens (25, 46, 47) and abnormal expression of Lewis antigens, *i.e.*, aberrant expression of Lewis^a (48, 49).

In normal gastric mucosa, Lewis^a, Lewis^b, and sialyl-Lewis^a are colocalized in superficial cells expressing MUC5AC mucin (13).

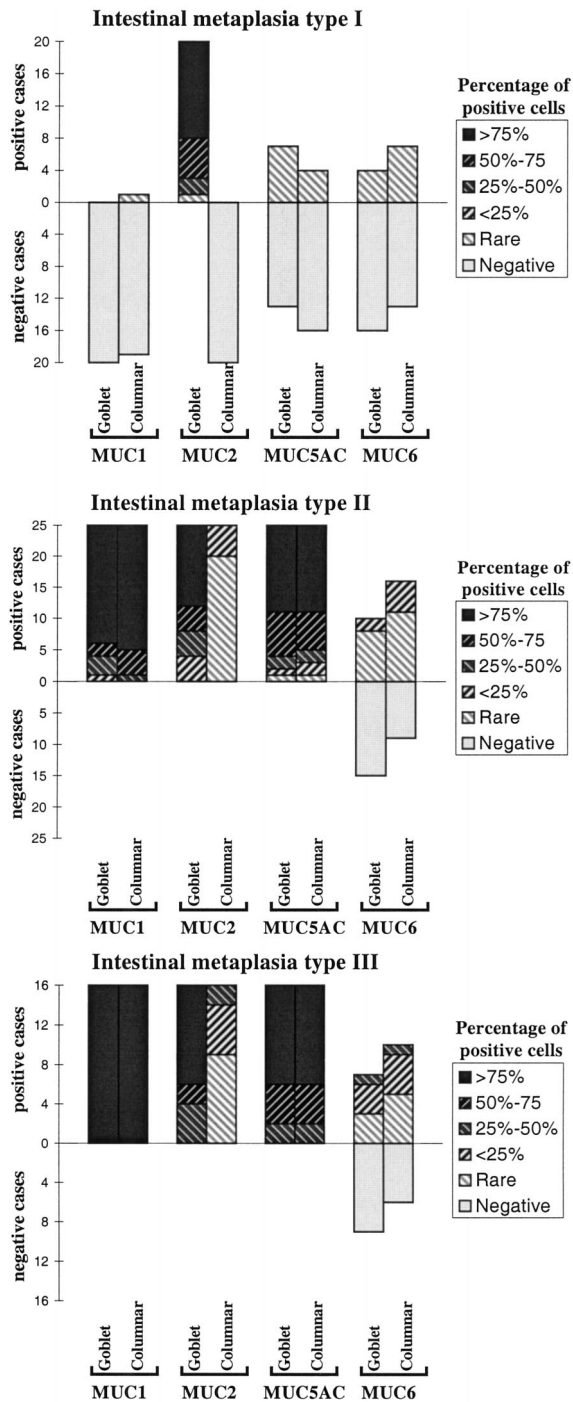


Fig. 2. Diagram with the expression results of mucins in intestinal metaplasia type I, type II, and type III.

There is also colocalization of Lewis^x and Lewis^y in deep glands expressing MUC6 mucin (13). These observations prompted us to investigate the hypothesis of a coordinated expression of the histochemical patterns that define the different types of intestinal metaplasia and some mucins.

We identified two major profiles of mucin expression in intestinal metaplasia: the one corresponding to complete (type I) intestinal metaplasia and the other corresponding to incomplete (types II and III) intestinal metaplasia.

The mucin expression pattern in type I intestinal metaplasia is characterized by clearly decreased levels of mucins normally expressed in the gastric mucosa, MUC1, MUC5AC, and MUC6, and *de novo* expression

of the intestinal mucin MUC2 in goblet cells. These results support the assumption that type I intestinal metaplasia does represent a complete differentiation of the mucosa toward the small intestinal phenotype (30, 50). In the few cases with expression of MUC1, MUC5AC, and/or MUC6, the staining was observed in rare cells (<5%), and we cannot exclude that some of these cells might represent remaining gastric cells intermingled with cells from metaplastic glands.

At variance with type I intestinal metaplasia, incomplete intestinal metaplasia (types II and III) is characterized by the coexpression of some mucins of gastric type; MUC1 and MUC5AC were expressed in every case, and MUC6 was expressed in the majority of the cases. *De novo* expression of MUC2 was observed both in goblet and columnar cells. The pattern of expression of mucins was similar in the two types (types II and III) of incomplete intestinal metaplasia.

Taking together our data on mucin expression in intestinal metaplasia and the results from the literature on the expression of different markers of gastric and intestinal differentiation (51, 52), we conclude that there are two main types of differentiation in intestinal metaplasia: type I, which shows a nongastric, small intestine phenotype reflecting a complete switch in the differentiation program (30, 52); and incomplete (type II and type III), which shows a mixed gastric and intestinal phenotype, reflecting an aberrant differentiation program that does not reproduce any phenotype occurring in normal adult gastrointestinal epithelia (52).

The distinction between type II and type III intestinal metaplasia is based on the histochemical features of mucous-secreting cells (30, 31). The presence of neutral and/or sialylated mucins in columnar cells characterizes type II, whereas the recognition of type III intestinal metaplasia is dependent on the identification of sulfated mucins in the same cells (30, 31). Our finding of similar mucins in columnar cells of type II and type III intestinal metaplasia suggests that the expression of sialylated or sulfated residues is not dependent upon the protein core of mucins. It remains to be seen if all gastric-type mucins (MUC1, MUC5AC, and MUC6) as well as MUC2 mucin may serve as carriers of the sialylated and/or sulfated carbohydrate structures detected in intestinal metaplasia.

Taking this apparent similarity of the mucin protein expression in type II and type III intestinal metaplasia together with the increased risk of malignant transformation of the gastric mucosa in type III (33, 34), it is tempting to suggest that the putative significance of intestinal metaplasia from a predictive standpoint is related to the processes of mucin glycosylation rather than to the pattern of mucin expression.

Finally, our results may contribute to clarify the sequential evolution of the different types of intestinal metaplasia. Briefly, if one puts our data together with histological and histochemical data reported previously (27, 28, 30, 31), two interpretative hypotheses appear plausible: (a) because complete intestinal metaplasia shows markedly decreased levels of “gastric” mucins, MUC1, MUC5AC, and MUC6, and incomplete (both type II and type III) intestinal metaplasia maintains the expression of “gastric” mucins, it seems conceivable that complete and incomplete intestinal metaplasia represent, *ab initio*, divergent differentiation programs; or (b) incomplete type II intestinal metaplasia may represent a first step in the intestinal metaplasia pathway, which may evolve to complete intestinal metaplasia with loss of expression of the “gastric” mucins, MUC1, MUC5AC, and MUC6, or to incomplete type III intestinal metaplasia by further deregulation of mucin glycans processing with sulfation. Summing up, the data we have obtained challenge the classical sequential pathway of intestinal metaplasia (from type I to type III via a type II intermediate step).

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