Lack of mucin MUC5AC field change expression associated with tubulovillous and villous colorectal adenomas

R J Longman, J Douthwaite, P A Sylvester, D O'Leary, B F Warren, A P Corfield, M G Thomas

Abstract

Background—MUC5AC is a secreted mucin aberrantly expressed by polyoid colorectal adenomas. It has been hypothesised that the “normal” surrounding colorectal mucosa expresses MUC5AC as a field change phenomenon that can be used to predict adenoma recurrence following resection.

Aim—To determine if there is a field change of de novo MUC5AC expression in histologically normal rectal mucosa adjacent to villous and tubulovillous adenomas, and thus whether MUC5AC expression can be used as a marker of early tumour recurrence.

Methods—In a prospective cohort study paired mucosal biopsies of adenomatous and macroscopically “normal” mucosa were obtained from 11 patients with villous and 11 patients with tubulovillous adenomas who underwent primary resection for purpose of cure. The tissues were studied to determine MUC5AC gene expression by immunohistochemistry and in situ hybridisation. Patients were followed up by flexible sigmoidoscopy to detect the presence of early local recurrence.

Results—10 villous adenomas showed mature MUC5AC glycoprotein and all 11 expressed MUC5AC mRNA. Five tubulovillous adenomas showed mature MUC5AC glycoprotein and 10 expressed MUC5AC mRNA. Neoexpression of the MUC5AC mucin gene was not detected in any of the mucosal biopsies taken adjacent to either villous or tubulovillous adenomas, even in three patients with early, locally recurrent disease.

Conclusions—Aberrant MUC5AC gene expression is not a “field change” in the colorectal mucosa in patients with rectal adenomas and therefore cannot be used to predict local recurrence of villous and tubulovillous adenomas.

(J Clin Pathol 2000;53:100–104)

Keywords: mucin; colorectal adenoma; gene expression; field change

There are three commonly recognised histological types of polyoid colorectal adenomas: tubular, tubulovillous, and villous. Villous and tubulovillous adenomas are more prone to malignant transformation, and this is related to the size of the adenoma and the degree of epithelial dysplasia. In lesions greater than 2 cm in size, 46% of villous adenomas contain atypical epithelial changes amounting to severe dysplasia. The size and position of the tumour largely dictate treatment of adenomas in the rectum. Resection can be achieved by surgical means or endoscopically. Recurrence rates following excision of villous adenomas vary from 8.7% to 27.3%. High rates of recurrence may be biased by incomplete excision, and although precise data are difficult to obtain, complete excision of the tumour is unlikely to decrease recurrence rates below those quoted by Buess and Mentego. Complete histological excision of benign rectal tumours is still associated with at least a 5% early recurrence rate, suggesting that histologically confirmed resection of macroscopic disease does not guarantee protection from further disease. This suggests that it may not be inadequate resection that causes the high recurrence rate but instability of the mucosa. Macroscopically normal tissue close to colorectal carcinomas shows subtle abnormalities in histological appearance, mucin biology, and cell proliferation that may indicate a wider field change in the epithelium. This “transitional mucosa” possesses elongated crypts with enlarged goblet cells, an increased proportion of non-sulphated sialomucin in the goblet cells, and altered glycosyltransferase activity. There is also acceleration in the rate of cell proliferation and a proliferative compartment shift within the colonic crypts in this transitional mucosa.

The mucus layer, also known as the supramucosal defence barrier, is composed of large glycoprotein mucin molecules that are synthesised in a site specific fashion by the mucosa along the length of the gastrointestinal tract. The nine recognised human epithelial mucins (MUC1–4, 5B, 5AC, 6–8) can be broadly classified into two groups: secreted mucins and membrane bound mucins. There is a cluster of genes on chromosome 11p15 coding for four secreted mucins (MUC2, MUC5B, MUC5AC, and MUC6). MUC5AC is usually expressed in the stomach and bronchus. The large bowel normally expresses the mucins MUC1, MUC2, MUC3, and MUC4, of which MUC2 is the predominant secreted mucin glycoprotein. Aberrant expression of the MUC5AC and MUC6 genes has recently been demonstrated in colorectal adenomas. In situ hybridisation studies using cDNA probes have shown MUC5AC gene mRNA expression in villous...
adenomas from 22 patients. MUC5AC gene expression and mature glycoprotein production increase with the size of adenoma and degree of villous architecture. Interestingly, in one study, MUC5AC expression was found in histologically normal colonic mucosa that was obtained approximately 5 cm from villous adenomas in four of 22 cases. It has been proposed that this field effect of MUC5AC expression in histologically normal mucosa adjacent to colorectal adenomas may be useful in predicting recurrence of disease.

Our aim in this prospective study was to determine whether there is a field change of de novo MUC5AC expression in histologically normal rectal mucosa adjacent to villous and tubulovillous adenomas, and therefore to determine whether MUC5AC expression could be used as a marker of early tumour recurrence.

**Methods**

**TISSUE SPECIMENS**

Formalin fixed, paraffin embedded tissues from 11 patients with villous and 11 with tubulovillous rectal adenomas undergoing surgical resection were studied. There were 10 female and 12 male patients with a median age of 72 years (range 49 to 88). The adenomas were excised by open surgical resection, endoscopic excision and the absence of invasion was histologically confirmed. Tissues from main bronchus (from Whipple’s procedures for carcinoma of lung), colorectum (colectomy specimens for adenocarcinoma), colorectum (from Whipple’s procedures for carcinoma of the head of the pancreas) were used as controls.

All the patients underwent routine follow up with endoscopic review by flexible sigmoidoscopy at three monthly intervals for the first six months and subsequently six monthly for two years following resection.

**IMMUNOHISTOCHEMISTRY**

All immunohistological staining was performed on 5 µm tissue sections cut onto poly-L-lysine coated slides. Tissue sections were dewaxed in xylene and rehydrated through decreasing concentrations of alcohols.

Formalin fixed, paraaffin embedded tissues were dewaxed in xylene and rehydrated through increasing concentrations of alcohols, washed with xylene and rehydrated through decreasing concentrations of alcohols to water, and finally air dried.

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water. The primary antibody, 21M1, was a mouse monoclonal antibody directed against a low glycosylated, non-variable tandem repeat domain (non-VNTR) region of the peptide core of human mucin MUC5AC. The primary antibody was added to the tissues as a neat supernatant, and incubated for 16 hours at 4°C. Phosphate buffered saline (PBS) was substituted for the primary antibody on a duplicate of all tissues as a negative control. The second layer horseradish peroxidase conjugated rabbit antimouse antibody (Dako) was added as a 1:200 solution in PBS and incubated for one hour at room temperature. Slides were developed with 0.6 mg/ml 3,3'-diaminobenzidine and 0.037% (vol/vol) H$_2$O$_2$ (Sigma) to demonstrate peroxidase activity, and finally counterstained with haematoxylin.

**IN SITU HYBRIDISATION**

In situ hybridisations were performed on 5 µm tissue sections cut onto gelatine coated slides. A 48-mer oligonucleotide probe directed against the variable number tandem repeat (VNTR) sequence of the MUC5AC gene (AGG GGC AGA AGT TGT TGT GGG AGC AGA GGT TGT GCT TGT AGC AGA GGT TGT GCT GGT TGT) was end labelled with 35S-ATP (Amerham Life Sci) using a terminal deoxynucleotidyldtransferase kit (Boehringer Mannheim). The probe was purified using a QIAquick nucleotide removal kit (Qiagen), and eluted with sterile, RNase-free water.

Slides were dewaxed in two five minute washes with xylene and rehydrated through decreasing concentrations of alcohols to distilled sterile water. Protein unmasking was performed by incubation for 20 minutes at 37°C with 5 µg/ml proteinase K in 0.05 M Tris HCl, pH 7.5. Following washing in distilled water and PBS, tissues were acetylated in a freshly prepared solution of 0.1 M triethanolamine, 0.25% acetic anhydride (vol/vol), and 0.9% sodium chloride (wt/vol) at pH 8.0 for 10 minutes at room temperature to reduce nonspecific binding. Tissues were dehydrated through increasing concentrations of alcohols, before delipidation with 100% chloroform for five minutes. Tissues were again rehydrated through alcohols to water, and finally air dried at room temperature.

Tissue hybridisation was performed with the application of 60 to 90 µl of hybridisation buffer containing 2 × 10$^4$ cpm of [35S] deoxy-ATP labelled probe to each section. The hybridisation buffer was composed of 50% deionised formamide (vol/vol) containing 4× Denhardt’s solution, 10% dextran sulphate (vol/vol), 0.05 M dithiothreitol (DTT), 0.25 mg/ml yeast tRNA, 0.5 mg/ml sheared single stranded salmon sperm DNA, and 4× SSC solution (0.12 M sodium chloride; 0.012 M sodium citrate; pH 7.0). The sections were hybridised for 16 hours at 45°C in humidity chambers. Hybridisation with a 48-mer oligonucleotide probe to β actin (ATG TCC ACG TCG CAC TTC ATG ATC GAG TTA AAG GTC GTC TCG TGG AGT) was used as a control to detect tissue mRNA. Further controls consisted of a prehybridisation digestion with 50 µg/ml...
RNAse A (Boehringer Mannheim) in 0.4 M NaCl, 5 mM EDTA, and 10 mM Tris HCl at pH 7.5 for 30 minutes at 37°C, followed by five stringent washes in ×2 SSC at 65°C for 10 minutes each.

Post-hybridisation washes were as follows: (1) five brief washes in ×1 SSC at room temperature; (2) four washes in ×1 SSC at 57°C for 30 minutes each; (3) two washes in ×1 SSC at room temperature for 30 minutes; and finally (4) one five minute wash in distilled water to remove salts. The slides were then air dried at room temperature.

Slides were laid down on Hyperfilm MP radiographic film (Amersham Life Science) for 2 weeks before developing. Slides were then

Figure 1 (A) (B): Detection of mucin MUC5AC expression in serial sections from a rectal tubulovillous adenoma by immunohistochemistry (A: immunoperoxidase staining (brown) counterstained with haematoxylin, ×42) and by in situ hybridisation (B: light phase (black) image with toluidine blue counterstain, ×42). (C), (D): Absence of mucin MUC5AC expression in serial sections from macroscopically normal rectal mucosa 5 cm proximal to the tubulovillous adenoma in (A) and (B) by immunohistochemistry (C): immunoperoxidase staining counterstained with haematoxylin, ×42), and by in situ hybridisation (D): light phase image with toluidine blue counterstain, ×42).

Figure 2 (A) (B): Detection of mucin MUC5AC expression in serial sections from a rectal villous adenoma by immunohistochemistry (A): immunoperoxidase staining counterstained with haematoxylin, ×42), and by in situ hybridisation (B): light phase image with toluidine blue counterstain, ×42). (C) (D): Absence of mucin MUC5AC expression in serial sections from macroscopically normal rectal mucosa 5 cm proximal to the villous adenoma in (A) and (B) by immunohistochemistry (C): immunoperoxidase staining counterstained with haematoxylin, ×42), and by in situ hybridisation (D): light phase image with toluidine blue counterstain, ×42).
dipped in emulsion (K5 gel emulsion; Ilford) and placed in a desiccation chamber for three weeks, after which they were developed (all slide developing solutions from Kodak UK). Localisation of mucin mRNA was performed from radiographic films and from the counterstained slides under light and dark field microscopy.

Results
Mature MUC5AC glycoprotein was demonstrated in 10/11 villous adenomas by intense staining of mucus secreting cells and of secreted mucus in the adenoma tissue (fig 1A). High levels of MUC5AC transcripts were detected in all 11 villous adenomas (fig 1B), while none of the normal mucosa biopsies showed MUC5AC mRNA or glycoprotein expression (fig 1C and D).

Only 5/11 tubulovillous adenomas showed mature MUC5AC glycoprotein in the mucus secreting cells (fig 2A), but 10/11 expressed MUC5AC mRNA (fig 2B). None of the normal mucosal biopsies taken 5 cm proximal to the adenomas expressed MUC5AC mRNA or glycoprotein (fig 2C and D).

The staining pattern of MUC5AC and distribution of message transcripts within the adenomas was similar to that previously reported.29 30

The non-VNTR anti-MUC5AC antibody detects both unglycosylated mucin MUC5AC in goblet cell cytoplasm, in the supranuclear peri-Golgi region, as well as mature glycosylated mucin in the goblet vesicles and within the secreted mucus layer overlying the mucosa. The same cellular staining pattern was evident in all 15 adenomas that showed MUC5AC mucin glycoprotein immunoreactivity.

Interestingly, the staining pattern for MUC5AC in both villous and tubulovillous adenomas had a heterogeneous nature. In some areas of the 15 adenomas, whether sparse or near absent staining while adjacent areas showed strong immunoreactivity, despite no histological evidence of a difference in architecture or dysplasia between these areas. This heterogeneity was reflected in the mRNA localisation by in situ hybridisation.

Three of the 22 adenomas had recurred locally within two years of complete excision (two villous, one tubulovillous). The initial adenomas in these three patients all showed MUC5AC gene expression. A further patient subsequently developed a metachronous villous adenoma at the splenic flexure, identified endoscopically, 13 months after initial resection of the rectal villous adenoma. There were, however, no discernible differences in the MUC5AC expression pattern in the adenomas from the patients with recurrences compared with the other villous adenomas in the study cohort.

Discussion
The concept of a “field change” that increases susceptibility to the development of colorectal neoplasia has been hypothesised for over two decades.31 Although familial adenomatous polyposis (FAP) is a model for colorectal adenoma formation, the lack of any phenotypic markers in areas of apparently normal mucosa of patients with FAP, and indeed in hereditary non-polyposis colorectal cancer (HNPCC), would suggest that the concept of a mucosal field change is unfounded.32 35 In sporadic colorectal adenomas and carcinomas, Boland et al have demonstrated an absence of genotypic, and hence phenotypic, alterations of recognised tumour suppressor genes in surrounding normal mucosa.36 Changes in mucin histochemistry, however, have been suggested as supportive of evidence for a mucosal field change.37 38 The presence of non-sulphated sialomucin in mucosa adjacent to up to 15% of colorectal cancer resection specimens has been reported,39 40 as has the altered expression of non-specific mucin epitopes.41 Changes in mucin biology across the length of the colorectal epithelium are unlikely to be a result of aberrant gene expression, but may possibly reflect altered translation of gene message, alterations in glycation patterns, or other variations in mature mucin processing. The presence of a transitional zone surrounding neoplastic changes within the colorectal mucosa is substantiated by changes in cellular proliferation but this does not support a wider field change.41 42 Alterations in MUC2 subcellular localisation within epithelial cells are seen in this transitional zone but return to that of normal colorectal mucosa within a few colonic crypts in distance from the polyps (authors’ personal observations; unpublished data).

This study included large, moderately dysplastic sessile adenomas that are prone to recurrence and malignant change, and is thus reflected by the relatively high local recurrence rate (3/22) in this small cohort of 11 villous adenomas. We have confirmed previous reports that a high proportion (more than 90%) of such adenomas express the MUC5AC gene.10 The level of expression of MUC5AC, whether as MUC5AC transcripts or mature glycoprotein, varied according to the nature of the adenoma. Mature MUC5AC glycoprotein was identified more often in villous than in tubulovillous adenomas, perhaps reflecting differing patterns of tumour differentiation. The heterogeneous nature of MUC5AC gene expression within individual adenomas cannot be explained by this study, but may be a result of clonality within early colorectal neoplasia. This is supported by the evidence that fewer severely dysplastic colorectal adenomas and adenocarcinomas express MUC5AC when compared with lesions showing moderately dysplastic features,10 39 and this might be a reflection of the process of clonal selection within colorectal neoplasia. While nearly all the adenomas in this study (21/22) showed MUC5AC mRNA expression, only 15 displayed MUC5AC glycoprotein immunoreactivity. Bartman and colleagues found similar results.42 However, this may have been a result of using a VNTR directed antibody, whereby glycosylation of this central region may mask the peptide epitope detected by their antibody. Enhanced or reduced glycosylation of mucin MUC5AC might explain, as proposed, the differences in immunoreactivity seen between adenomas of...
different size, histological type, and degree of dysplasia. This does not, however, explain the varying levels of mRNA expression between different subtypes of adenomas. In our study, the use of a non-VTR antibody that is directed against a relatively low glycosylated peptide epitope of the mucin core protein would be expected to negate any differences in post-translational glycosylation. This is, however, not the case, and differences in mRNA expression patterns and MUC5AC mucin immunoreactivity patterns cannot easily be explained. With the data from our study and those of Bartman and colleagues, it might be concluded that glycosylation of mucin MUC5AC may not be the only post-translational, or indeed post-transcriptional, modification process occurring in colorectal neoplasia.

MUC5AC gene expression has previously been reported at a distance from some colorectal adenomas, raising the possibility that it might be a marker for future adenoma formation or recurrence. Our study refutes this. We found no evidence of MUC5AC gene expression by macroscopically normal colonic mucosa, as either MUC5AC mRNA or mature glycoprotein.

The biological significance of aberrant mucin gene expression by colorectal neoplasia is unclear. This study indicates that aberrant expression of the MUC5AC gene is not a general field change in the colon mucosa of patients with colorectal adenomas and cannot indicate risk of adenoma formation or recurrence.

We thank Professor J Bara (INSERM, Paris, France) for the gift of the 21M1 anti-MUC5AC antibody. This work is supported by the Mason Medical Research Foundation of the United Kingdom.