Aberrant expression of minichromosome maintenance proteins 2 and 5, and Ki-67 in dysplastic squamous oesophageal epithelium and Barrett’s mucosa

J J Going, W N Keith, L Neilson, K Stoeb, R C Stuart, G H Williams

**Background:** Minichromosome maintenance (Mcm) proteins are essential for eukaryotic DNA replication, and their expression implies potential for cell proliferation. Expression is dysregulated in dysplastic states but data for oesophageal squamous mucosa and Barrett’s mucosa have not been published.

**Aim:** To test the hypothesis that Mcm proteins are downregulated together with the proliferation marker Ki-67 in differentiating epithelial compartments of non-dysplastic squamous and Barrett’s epithelium, and that this process does not occur in dysplastic mucosae.

**Methods and cases:** Forty five patients with Barrett’s oesophagus included 20 with glandular dysplasia (10 low grade, eight high grade, two both, and four with invasive adenocarcinoma). Twenty five other patients included 12 with oesophageal squamous dysplasia (three low grade, six high grade, three both, and four with invasive squamous carcinoma). Formalin fixed paraffin embedded tissue sections from biopsy series and resections were immunostained using antibodies to Mcm2, Mcm5, and Ki-67. Percentage of nuclei positive for Mcm2, Mcm5, and Ki-67 was estimated and scored from 0 to 6 as: 0, none +; 1, <10%; 2, 10–30%; 3, 30–70%; 4, 70–90%; 5, >90%; 6, all+. Four separate epithelial strata were scored: in squamous epithelium the basal layer and thirds to the surface, in Barrett’s mucosa the luminal surface, upper and lower crypt, and deep glands.

**Results:** In non-dysplastic squamous epithelium and Barrett’s mucosa, high level expression of Mcm2, Mcm5, and Ki-67 proteins was largely confined to the proliferative compartments and downregulated in differentiated compartments. Expression persisted up to the mucosal surface in dysplastic squamous epithelium and Barrett’s mucosa.

**Conclusions:** Persistent expression of Mcm2, Mcm5, and Ki-67 proteins in luminal compartments of dysplastic oesophageal squamous epithelium and dysplastic Barrett’s mucosa may be diagnostic markers and imply disruption of cell cycle control and differentiation in these dysplastic epithelia.
without dysplasia, and in their dysplastic and neoplastic counterparts.

MATERIALS AND METHODS
Production of antibodies
Anti-Mcm2 monoclonal antibody (mAb) (mouse IgG1 isotype) was raised against a fragment of human Mcm2 (amino acids 725–888; BM28, Transduction Laboratories, Lexington, Kentucky, USA). The specificity of the anti-Mcm2 mAb was established by immunoblot, immunofluorescence, and immunoprecipitation assays. Rabbit polyclonal antibodies were raised against a fragment of human Mcm5 (amino acids 372–590) and purified by affinity chromatography over a column prepared by linking the immunogen to Sulfolink Gel (Pierce, Rockford, Illinois, USA). Specificity of anti-Mcm2 mAb and anti-Mcm5 polyclonal antibody was established by immunoblot, immunofluorescence, and immunoprecipitation assays. The anti-Ki-67 mAb MIB1 was supplied by Dako (Ely, Cambridge, UK).

Immunohistochemistry
Preliminary testing of six different antibodies at different dilutions and antigen retrieval schedules including enzymatic digestion and microwave heating in citrate and EDTA buffer was performed on formalin fixed paraffin embedded tissue sections. Good results were obtained with antibodies against Mcm proteins 2 and 5 using EDTA buffer and microwave heating antigen retrieval. Both Mcm antibodies were used diluted 1:4000 and conventional three stage streptavidin/biotin and peroxidase with diaminobenzidine/H2O2 detection. Antibody MIB1 was applied diluted 1:100 following microwave antigen retrieval and detected as for Mcm2/Mcm5.

Cases
Seventy patients were studied. Twenty five patients without Barrett’s oesophagus included 13 with no squamous dysplasia, three with low grade squamous dysplasia, six with high grade squamous dysplasia, and three with both. Four patients in this group also had invasive squamous carcinoma. Forty five patients with Barrett’s oesophagus included 25 without dysplasia, 10 with low grade glandular dysplasia, eight with high grade dysplasia, and two with both. Four patients in this group also had invasive adenocarcinoma.

Patients were from cohorts undergoing diagnostic endoscopy for upper gastrointestinal symptoms, enrolled in a yearly surveillance of Barrett’s oesophagus, or having surgical resection of oesophageal carcinoma. Dysplastic changes were
assessed on haematoxylin and eosin stained sections of paraffin embedded endoscopic biopsies and tissue blocks from resection specimens which were chosen from pathology department archives to represent a range of morphologies from normal (non-dysplastic) oesophageal squamous epithelium through low and high grade squamous dysplasia to invasive squamous carcinoma. Similarly, examples of Barrett’s mucosa without dysplasia, low grade and high grade dysplasia in Barrett’s mucosa, and invasive Barrett’s adenocarcinoma were selected for study, and 4µm sections were immuno-stained as described.

Scoring immunocytochemistry

A semiquantitative scoring scheme was designed to describe the immunostaining observed. Cell nuclei were positive or negative for Mcm2, Mcm5, or Ki-67. All scoring was done by one specialist upper gastrointestinal pathologist (JJG). Within each separate mucosal compartment, the estimated percentage of positive cells was allocated to scoring bands as follows: 0, none+; 1, <10%+; 2, 10–30%+; 3, 30–70%+; 4, 70–90%+; 5, >90%+; and 6, all+. Four compartments were recognised in oesophageal squamous epithelium: the most basal single layer of cells, and the thickness of the epithelium above that divided into parabasal, middle, and luminal thirds. In Barrett’s mucosa, four strata again were defined: the surface epithelium between “crypts”, the underlying “crypts” or “pits” divided into upper and lower halves, and the deepest layer, a differentiated glandular zone. These compartments correspond to those defined by Lauwers and colleagues in their study of cell proliferation in Barrett's mucosa.

**Reproducibility of scoring and statistical analysis**

Reproducibility of scoring was evaluated by “blind” re-scoring by JJG of all sections stained for Mcm2 after six months. Weighted kappa $\kappa_w$ was calculated for duplicate scores from 562 separate cellular populations; $\kappa_w = 0.65$ implied acceptable agreement. Univariate statistical significance of differences between immunostaining was tested using the non-parametric Mann-Whitney test for two independent samples, two tailed, with correction for ties. Kappa and Mann-Whitney calculations were performed in Analyse-It for Microsoft Excel (Analyse-It Software, Leeds, UK).

**RESULTS**

Immunostaining with the Mcm2 antibody yielded predominantly nuclear staining. The Mcm5 antibody stained nuclei but also cell membranes in glandular mucosae and tumours. Qualitatively, nuclear staining was similar with the two antibodies. Ki-67 staining was purely nuclear.

In non-dysplastic squamous epithelium and Barrett’s mucosa, strong Mcm2, Mcm5, and Ki-67 staining of most to...
DISCUSSION

Our data document spatial organisation of cell proliferation in normal oesophageal squamous epithelium and non-dysplastic Barrett’s mucosa. In differentiated compartments—that is, the surface of squamous epithelium and Barrett’s mucosa—and in the small differentiated deep glands of Barrett’s mucosa, expression was downregulated. In dysplastic squamous epithelium and dysplastic Barrett’s mucosa there was persistence of Mcm2, Mcm5, and Ki-67 expression in compartments in which they are normally absent or sparse, especially towards the surface of squamous epithelium and Barrett’s mucosa. Downregulation of Mcm2 and Mcm5 expression in the deep (glandular) mucosal compartment of Barrett’s mucosa was also significantly reduced in high grade dysplasia. These relationships are illustrated in figs 1 and 2 which plot median, quartiles, and range for Mcm2, Mcm5, and Ki-67 staining scores for the various compartments of oesophageal squamous (fig 1) and Barrett’s mucosa (fig 2). Photomicrographs illustrate these staining patterns (fig 3A–C for Barrett’s mucosa and fig 4A, B for squamous epithelium). Statistical testing confirms that overexpression of these markers in the surface and subsurface compartments of dysplastic squamous and Barrett’s mucosa is significant at a high level of probability (table 1).

Although abnormally persistent expression of Mcm2, Mcm5, and Ki-67 is clearly associated with premalignant dysplasia in oesophageal squamous epithelium and Barrett’s mucosa, variant patterns were identified. The dysplastic squamous epithelium in fig 5A clearly downregulates Mcm2 expression towards the surface, and in the same case Mcm2 downregulation occurs in invasive squamous carcinoma towards the centre of cell nests—that is, in areas of differentiation (fig 5B). Similarly, viable but Mcm2 and Mcm5 negative cells were present in invasive adenocarcinomas (fig 6).

Barrett’s mucosa, and disruption of this highly organised spatial arrangement in premalignant dysplasia. These disturbances are relevant to the identification of dysplasia in oesophageal squamous epithelium and Barrett’s mucosa, both of which are problematic in individuals and populations. Squamous oesophageal cancer is a target for screening in Far Eastern populations. Barrett’s oesophagus and Barrett’s cancer are relatively common in the West. Patients with Barrett’s oesophagus may be subjected to relatively frequent endoscopy and biopsy (for example, yearly). A sensitive and specific test for dysplasia might allow Barrett’s patients to be screened for dysplasia and divided into a cohort without dysplasia, at low risk of oesophageal adenocarcinoma, for whom less intensive follow up would be safe, and a higher risk group, with dysplasia, for whom more frequent endoscopic and biopsy surveillance could be appropriate. A sensitive test for early dysplastic changes in endoscopic biopsies would be useful to the pathologist although to date various tests that have been proposed have been disappointing in practice.

Abnormal expression of Mcm5 protein in dyskaryotic cervical smears is associated with cervical intraepithelial neoplasia, and immunocytochemistry for Mcm5 protein facilitates detection of dyskaryotic cells in such smears, which may otherwise be a problem if they are present in small numbers. Similarly, biochemical assay of these proteins in urine is a sensitive and specific test for the presence of urothelial epithelial neoplasia. These examples and the present

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Table 1  p values by Mann-Whitney testing of the differences between groups

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Ki-67</th>
<th>Mcm5</th>
<th>Mcm2</th>
</tr>
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<tbody>
<tr>
<td>Normal v LGD</td>
<td>&lt;0.0001</td>
<td>0.0059</td>
<td>0.0029</td>
</tr>
<tr>
<td>Normal v HGD</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>Subsurface</td>
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<td></td>
<td></td>
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<tr>
<td>Normal v LGD</td>
<td>&lt;0.0001</td>
<td>0.0016</td>
<td>0.0022</td>
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<tr>
<td>Normal v HGD</td>
<td>&lt;0.0001</td>
<td>&lt;0.0002</td>
<td>&lt;0.0001</td>
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<tr>
<td>Parabasal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal v LGD</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Normal v HGD</td>
<td>NS</td>
<td>NS</td>
<td>0.0216</td>
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<tr>
<td>Basal</td>
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<td></td>
<td></td>
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<tr>
<td>Normal v LGD</td>
<td>0.0039</td>
<td>0.0498</td>
<td>NS</td>
</tr>
<tr>
<td>Normal v HGD</td>
<td>0.0113</td>
<td>0.0118</td>
<td>0.0048</td>
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<tr>
<td>Barrett’s mucosa</td>
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<tr>
<td>Surface</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal v LGD</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal v HGD</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Upper crypt</td>
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<td></td>
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<tr>
<td>Normal v LGD</td>
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<td>0.0002</td>
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<tr>
<td>Normal v HGD</td>
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<td>0.0027</td>
<td>&lt;0.0001</td>
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<tr>
<td>Lower crypt</td>
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<td>Normal v LGD</td>
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<tr>
<td>Normal v HGD</td>
<td>NS</td>
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<td>Deep glands</td>
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<tr>
<td>Normal v HGD</td>
<td>0.0055</td>
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</table>

LGD, low grade dysplasia; HGD, high grade dysplasia.
study support the use of Mcm proteins (and Ki-67) as markers of dysplasia. These studies in cervix, bladder, and esophagus suggest the concept that persistence of Mcm protein expression in dysplastic epithelia is associated with preneoplastic cells locked in the cell cycle (confirmed by persistence of Ki-67 expression) compared with normal epithelial cells that exit the cell division cycle during maturation and differentiation.

A problem in Barrett’s surveillance is that dysplastic changes may be very focal, and biopsy series may not sample dysplasia, even if large biopsy forceps are used. Changes may be very focal, and biopsy series may not sample dysplasia, even if large biopsy forceps are used. 

Although persistence of Mcm2 and 5 proteins in differentiating compartments of dysplastic squamous oesophageal epithelium and Barrett’s mucosa is characteristic, it is not invariably. Downregulation can occur in surface cells overlying clearly atypical cells of squamous and glandular mucosae. These surface cells may themselves appear atypical, or relatively normal in morphology. One interpretation of this appearance is the biologically trivial one that the morphologically normal cells may appear to be related to the underlying cells only as a consequence of vagaries of the plane of section in randomly orientated mucosal biopsies. Histological interpretation of Barrett’s dysplasia routinely requires this to be taken into account. However, our own data show that even in invasive carcinomas, molecular events associated with differentiation appear capable of switching off Mcm protein expression. These findings support the concept of mutual antagonism between the cellular circuits controlling differentiation and proliferation. As this would usually be understood to prevent further cell division, such events may have therapeutic relevance.

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REFERENCES


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