Minichromosomal maintenance component complex 5 (MCM5) as a marker of Barrett's oesophagus related neoplasia – a feasibility study

M Everson[1,2], C Magee[2], D Alzoubaidi[1,2], S Brogden[2], D Graham[1,2], LB Lovat[1,2], M Novelli[3], R Haidry[1,2]

[1]Division of Surgery & Interventional Science, University College London; [2]Department of Gastroenterology, University College Hospital NHS Foundation Trust, London; [3]Department of Pathology, University College Hospital NHS Foundation Trust, London

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All correspondence to:
Dr Rehan Haidry, Consultant Gastroenterologist, Director of Endoscopy, 235 Euston Road, London, United Kingdom, NW1 2BU
Email: rehan.haidry@nhs.net

Abstract

Background

The endoscopic detection of oesophageal cancer is suboptimal in both patients referred with dyspeptic symptoms and those enrolled in Barrett's surveillance programs. MCM5 expression in cells collected from gastric fluid may be correlated with the presence of dysplasia or adenocarcinoma. Analysis of this biomarker may improve the detection of cancer.

Methods

61 patients were enrolled at a single UK referral centre. 5-10ml of gastric fluid was aspirated endoscopically from each patient. Patients were categorised according to their histology; normal, non-dysplastic Barrett's (NDBE), high grade dysplastic Barrett's (HGD), adenocarcinoma (OAC). All histology was confirmed by Seattle Protocol biopsies or endoscopic mucosal resection. Samples were centrifuged and the cell pellet lysed. MCM5 expression levels were quantified using a proprietary immunofluorometric assay. The mean MCM5 expression was compared between groups by Kruschal Wallis testing. ROC curves were also used to assess diagnostic utility.

Results

The mean expression of MCM5 increases as patients progress from a normal oesophagus to NDBE, HGD and OAC (14.4; 49.8; 112.3 and 154.1 respectively). There was a significant difference in the MCM5 expression of patients with a normal oesophagus compared to those with OAC (p=0.04). There was a trend towards higher MCM5 expression in patients with OAC compared to those with NDBE (p=0.34). MCM5 expression was a fair discriminator (AUC 0.70 [95% CI: 0.57 – 0.83]) between patients without neoplasia (normal and NDBE) and those with early neoplasia (HGD and OAC).

Conclusion

MCM5 expression in gastric fluid samples can differentiate patients with a histologically normal oesophagus compared to those with early adenocarcinoma. Larger, powered studies are needed to assess if it can be used to differentiate those with HGD from NDBE

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Introduction

Access to upper gastrointestinal endoscopy is now commonplace in the developed world; patients are typically referred to services with either symptoms suggestive of upper GI pathology, or as part of a surveillance program for Barrett's oesophagus (BE). One of the major pathologies to be excluded at upper GI endoscopy in both of these patient cohorts is oesophageal cancer.

Screening endoscopies for symptomatic patients

Patients with dysphagia, anaemia, new or refractory dyspeptic symptoms are often referred for screening gastroscopy to rule out oesophageal pathology. In the UK the demand on endoscopy services is increasing and access to urgent endoscopy is subject to ever expanding waiting lists; only 55% of patients referred for urgent endoscopy in 2017 were seen within a two week target[1]. Furthermore, at screening gastroscopy cancers are frequently missed. A meta-analysis by Menon et al suggests that up to 11.3% of upper GI cancer is not detected despite the patient undergoing an endoscopy within the 3 years preceding diagnosis[2,3]. The studies comprising this analysis also determined that in 75% of the cases of missed cancers the root cause was endoscopist error; ranging from lesions going undetected, lesions detected but not biopsied or false negatives due to insufficient biopsies being taken from lesions.

Given the inherent deficiencies in endoscopy for the detection of cancer, it is arguable that biomarkers may provide an adjunct to the endoscopist, by offering objective evidence on whether or not neoplasia is present. A validated biomarker could alert physicians to the presence of cancer that has been missed; thereby prompting a second interval endoscopy or a reassessment of any lesions identified. Conversely such a biomarker could be used to 'rule out' the presence of neoplasia; as such preventing patients undergoing further unnecessary endoscopic assessment and reducing the cost and time burden on endoscopy services.

Surveillance endoscopies for patients with known Barrett's oesophagus

BE is a premalignant condition in which the normal oesophageal squamous mucosa is replaced with columnar epithelium with intestinal metaplasia. There is a well characterised linear progression from non-dysplastic BE, to dysplastic BE and eventually adenocarcinoma[4,5,6] and hence patients with known BE are enrolled into interval endoscopic surveillance programs[7].

Endoscopic eradication therapy (EET) through endoscopic mucosal resection (EMR) and radiofrequency ablation (RFA) now affords patients with early BE associated neoplastic lesions high rates of cure, particularly if lesions are confined to the mucosa[8–10]. In order to identify such lesions at a stage amenable to therapy, patients undergo interval endoscopic surveillance with a systematic protocol of biopsies taken throughout the BE segment – the Seattle Protocol[11,12]. While it remains the gold standard, the Seattle Protocol is suboptimal, with the sensitivity of neoplasia detection estimated between 28-85%[13]. Furthermore, an estimated 25.3% (95%CI: 16.4-36.8%) of BE associated adenocarcinoma (OAC) is missed during an endoscopic assessment in the preceding year[14]. There is a mixture of evidence regarding the cost effectiveness of Barrett's surveillance programs[15]. There is significant time and financial expenditure as patients require a longer endoscopic procedure with four biopsies taken every two centimetres for histopathologic analysis. With an incidence rate of only 0.2-2% in one study[16] and one cancer diagnosis for every 1/52[17] to 1/285[15] patient years under surveillance reported in others, the process of random biopsies may not be the most effective in health systems with resource constraints. With such variation in practice and a high miss rate of potentially treatable lesions, there is an unmet need to identify patients with potentially missed lesions – biomarker analysis may serve as an adjunct for physicians undertaking endoscopy to exclude cancer

Minichromosomal maintenance complex component 5 (MCM5) as a biomarker of neoplasia

Minichromosomal maintenance complex component 5 (MCM5) is a cell cycle protein which forms part of the DNA replicative helicase[18]. MCM proteins are upregulated during the transition from G_0 to G_1 of the eukaryotic cell cycle and so are believed to be implicated in DNA replication and cell cycle regulation[18,19,20]. Previous work has shown that dysregulation of MCM5 expression is associated with the development of cancer in epithelial tissues – including cervical[21] and urothelial cancers[22,23]. Such epithelial tissue can shed cells intraluminally which can be easily harvested and used for laboratory analysis of MCM5 expression. Going et al investigated the expression of MCM5 proteins in immunostained, formalin-fixed histological specimens taken from Barrett's oesophagus and squamous mucosa. They demonstrated that the failure of MCM5 expression to downregulate, a feature seen in non-dysplastic tissue, was observed in cells classified as dysplastic. This phenomenon was observed in both glandular Barrett's associated dysplasia, and in squamous dysplasia[24]. Importantly this study also demonstrated that cells with raised MCM5 expression were present

up to the luminal surface of the oesophageal mucosa[24], suggesting that they could be exfoliated into the alimentary tract and collected endoscopically through fluid aspiration.

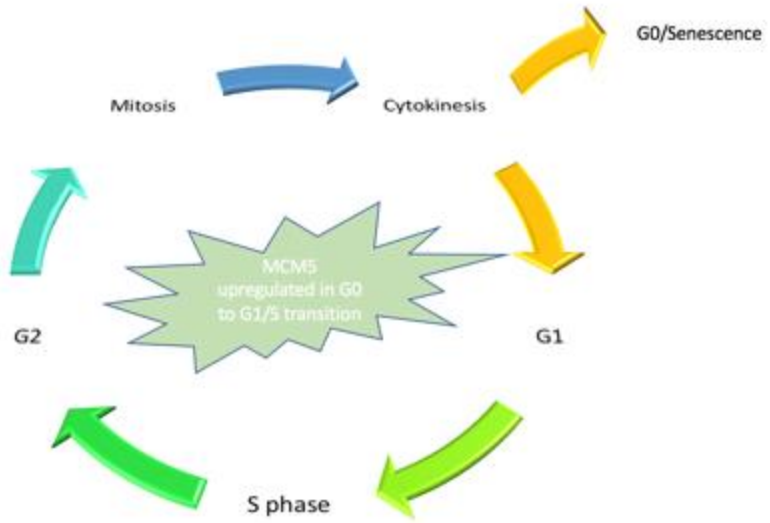

Fig 1: Schematic of the cell cycle. MCM5 in a complex with other MCM proteins has been shown to upregulate during the G0-G1/S transition and is proposed to have a role in DNA replication[20].

Williams et al. have previously demonstrated that MCM5 expression in shed oesophageal cells harvested from gastric fluid, as measured by an immunofluorometric assay, was significantly raised in individuals with adenocarcinoma or squamous cell carcinoma of the oesophagus compared those with a normal oesophagus[25]. Interestingly, almost half of the non-cancer cohort in this study comprised patients with non-dysplastic Barrett's oesophagus, but the relationship between the presence of Barrett's dysplasia and MCM5 expression was not characterised. In the age of EET, early detection of BE dysplasia is integral to allow prompt intervention and reduce the number patients with progression to inoperable OAC. We propose that MCM5 expression may be a biomarker indicative of BE associated neoplasia and as such could be used as an adjunct to endoscopic assessment to improve its detection.

In this prospective cohort feasibility study, we investigate the differential MCM5 expression in shed oesophageal epithelial cells collected from gastric aspirates, between patients with a macroscopically normal oesophagus, non-dysplastic BE, high grade dysplastic BE and adenocarcinoma. We aim to investigate if there is an incremental expression of MCM5 following the metaplasia to mucosal neoplasia and then invasive cancer sequence. Given the previous reported work that MCM5 expression is raised in oesophageal adenocarcinoma as

well as dysplastic BE, we aim to characterise the utility of MCM5 as a potential biomarker and adjunct to conventional random biopsies for the progression to dysplastic Barrett's.

Methods

Patient recruitment, inclusion and exclusion criteria

Patients with all grades of BE and known OAC were recruited from a single tertiary referral centre in the UK between August 2017 and April 2018. Patients were recruited into one of four groups, depending on the histology results taken at the index endoscopy from which the gastric aspirates were obtained.. The four subgroups were; macroscopically normal squamous oesophagus (NS), non-dysplastic Barrett's oesophagus (NDBE), high grade dysplastic (HGD) Barrett's oesophagus, oesophageal adenocarcinoma (OAC). All histology was verified by two expert G.I pathologists for all these patients.

Patients were excluded if they had concomitant systemic inflammatory conditions or active sepsis or infection; other solid organ malignancy or active oesophageal ulceration at endoscopy. Patients were also excluded if they had previously been found to have low grade dysplasia or had biopsies indeterminate for dysplasia during a previous endoscopy. This decision was taken given the high inter-observer variability in the diagnosis of LGD[26,27]. Patients were also excluded if they had previously received chemo-radiotherapy or ablative endoscopic therapies for dysplasia (radiofrequency ablation, argon plasma coagulation, photodynamic therapy). Patients with food contamination or no gastric fluid to aspirate were also excluded.

Our study had full ethical approval (IRAS 214612) and participants were required to give written consent prior to enrolment.

Endoscopic procedures

All patients underwent upper gastrointestinal endoscopy with conscious sedation. Prior to the procedure the working channel of the endoscope was flushed with air to prevent water contamination of the gastric fluid. The endoscope was carefully passed down the oesophagus

to avoid mucosal trauma and no suction was applied . A sterile plastic catheter was then passed down the working channel and used to aspirate up to 10ml of gastric fluid. The oesophagus was then cleaned with a solution of 2% simethicone and carefully inspected.

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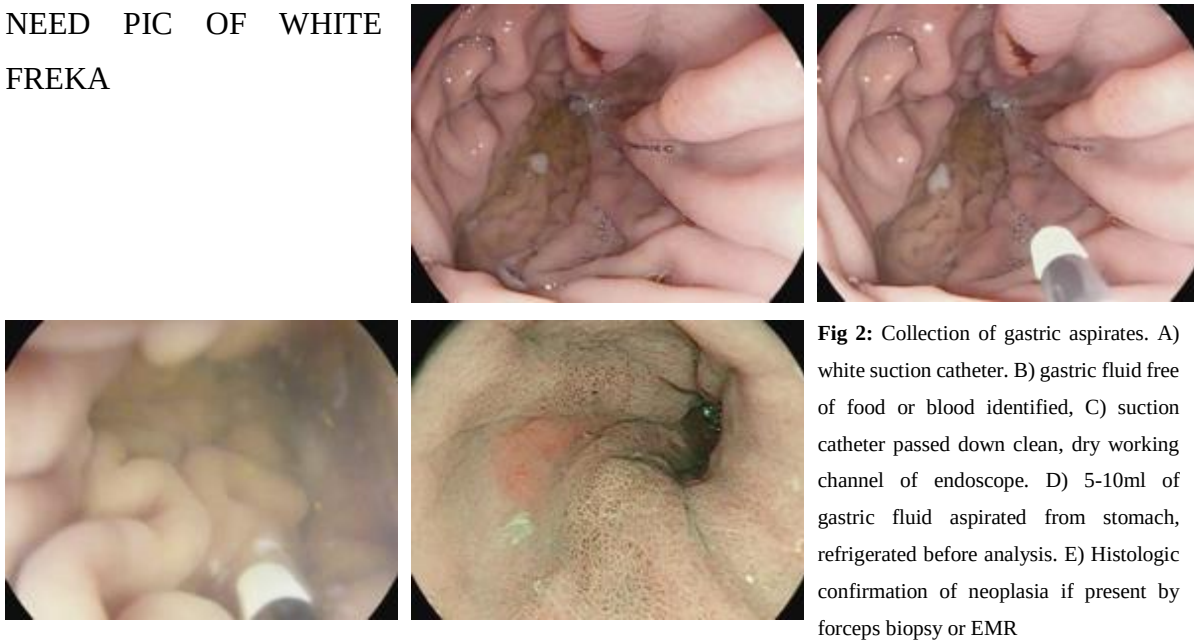

Fig 2: Collection of gastric aspirates. A) white suction catheter. B) gastric fluid free of food or blood identified, C) suction catheter passed down clean, dry working channel of endoscope. D) 5-10ml of gastric fluid aspirated from stomach, refrigerated before analysis. E) Histologic confirmation of neoplasia if present by forceps biopsy or EMR

Sample preparation and storage

Samples of gastric fluid were stored in sterile vials and refrigerated at 4°C immediately after collection. All samples were processed within 4 hours of collection. The fluid was centrifuged at 1500g for 5 minutes to form a cell pellet. The supernatant was then carefully aspirated by pipette and discarded. The cell pellet was then fully resuspended using 500µl of cell lysis buffer (Arquer). The lysed cell suspension was then stored for up to 3 weeks frozen at -80°C prior to analysis. To prevent degradation of MCM5 cells were transferred to an external lab for processing using dry ice to prevent thawing.

Determining MCM5 expression

MCM5 expression levels were calculated using a proprietary assay (Arquer) and reported in pg/ml. Expression levels for each subject were normalised according to the volume of gastric aspirate acquired at the index endoscopy. Since cell numbers could not be quantified prior to

cell lysis, the volume of gastric fluid from which the cells were acquired for each patient was recorded. The level of MCM5 expression could then be calculated per mL of gastric fluid acquired.

Statistical analysis

Differences in the expression of MCM5 based on histological subgroup were assessed using Kruskal-Wallis testing. To assess for differences in the medians between individual subgroups Dunns tests of multiple comparisons was used. The diagnostic performance of the assay was assessed by calculating the sensitivity and specificity. ROC curves were used to calculate the AUC to assess the accuracy of the assay. The AUC was categorised to assess how good a discriminator MCM5 expression was to detect patients with neoplastic compared to non-neoplastic oesophageal mucosae. The AUC was categorised according to the following scale; 1-0.9 (very good), 0.9-0.8 (good), 0.8-0.7 (fair), 0.7-0.6 (poor), 0.6-0.5 (fail). Formal power calculations were not undertaken as this was a feasibility study – we aimed to recruit 60 patients across all four subgroups.

Results

Patient demographics

In total 61 patients were included in this study. The mean BE segment length by histologic subgroup and biopsy results taken during the index endoscopy are displayed in table 1. The mean age of study participants was 67 years (range 26 – 89years). There was no significant difference in the mean length of Barrett's segments (where recorded) in patients of each of the histologic subgroups (NDBE vs Cancer p >1; NDBE vs HGD p = 0.4; HGD vs Cancer p = 0.28). Patients were recruited into one of four histological subgroups; normal oesophagus/acid reflux only; non-dysplastic BE; high-grade dysplastic BE; and adenocarcinoma. Of the cancer subgroup all patients had adenocarcinoma confirmed after EMR or ESD with variable invasion depths; M1 (3); M2 (4); M3 (5) and SM1 (3). The histologic characteristics of patients with histologically confirmed OAC is summarised in table 2.

Patient demographics

Age	67 (26-89)	
Mean Barrett's length (total C+M in cm)	Normal	0
	NDBE	6.4
	HGD	5.8
	Cancer	9
Histology (by subgroup)	Normal	14
	NDBE	14
	HGD	18
	Cancer	15

Table 1: Summary table showing patient demographics and histological subgroups.

Characteristics of OAC subgroup		
Invasion depth	M1	3
	M2	4
	M3	5
	SM1	3
Differentiation	Good	3
	Moderate	8
	Poor	4
Lymphovascular invasion	Present	1
	Absent	14
Metastatic disease	Present	1
	Absent	14

Table 2: Summary table showing histological characteristics of patients with confirmed adenocarcinoma. (M1 – intraepithelial invasion, M2 – lamina propria invasion (M1 – intraepithelial invasion, M2 – lamina propria invasion, M3 – muscularis mucosa contact or invasion, SM1 – upper third of submucosal layer

MCM5 expression is raised in patients with high grade dysplastic Barrett's or adenocarcinoma compared to a macroscopically normal oesophagus

Our results demonstrate that MCM5 expression is significantly raised in patients with oesophageal adenocarcinoma compared to patients with a macroscopically normal oesophagus (NS) (figure 1; mean expression 123.9 vs 14.5, p = 0.03). When all four subgroups were analysed individually there was a significant difference in MCM5 expression between patients with adenocarcinoma and a normal oesophagus only. There was no significant difference in MCM5 expression between patients with NDBE and those with a normal oesophagus, HGD or cancer. Similarly, there was no significant difference in MCM5 expression between patients with a macroscopically normal oesophagus and those with HGD or cancer. The mean expression and differences between MCM5 expression levels across the histological subgroups is summarised below in figure 1 and table 2. However, there is a trend towards increased MCM5 expression as one moves form the NS to NDBE to HGD and then cancer sub-groups. This suggests there may some association but clearly a study powered with more patients in each sub group will verify this association and hypothesis.

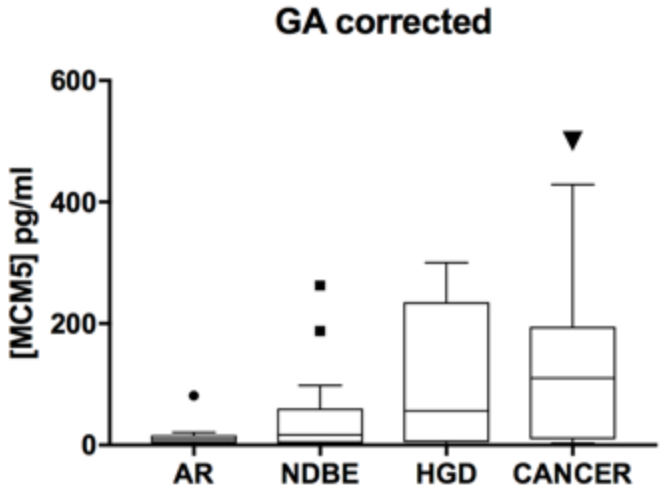

Figure 1: Comparison of MCM5 expression levels in gastric aspirate samples (pg/ml) between patients of each histological subgroup (AR: macroscopically normal/acid reflux only, NDBE: non-dysplastic Barrett's oesophagus, HGD: high grade dysplasia and cancer).

Histologic subgroup	Mean MCM5 expression (pg/ml)

Normal	14.4 [0 – 81]
NDBE	49.8 [0.4 – 262.9]
HGD	112.3 [0 – 300]
Cancer	154.1 [3.3 – 500]

Difference in MCM5 expression	Significance (p value)
Normal vs NDBE	>0.99
Normal vs HGD	0.33
Normal vs Cancer	**0.04***
NDBE vs HGD	>0.99
NDBE vs Cancer	0.34
NDBE vs Normal	>0.99

Table 2: Comparison of differences between mean MCM5 expression levels and histological subtypes

Performance characteristics of the MCM5 assay for detection of dysplastic or neoplastic tissue

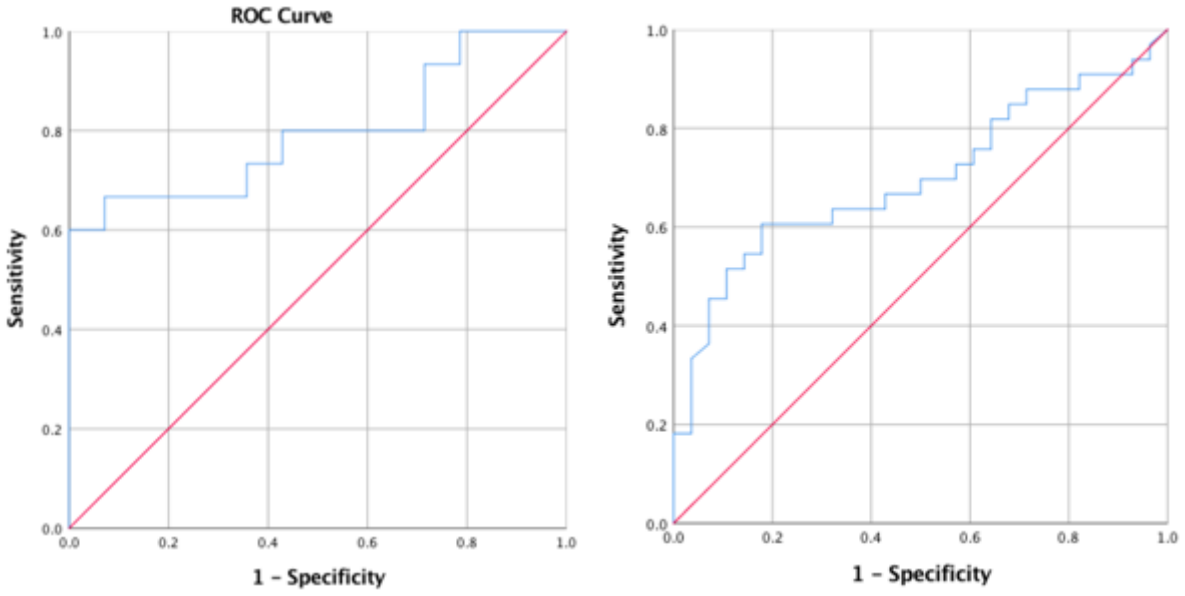

Figure 2: (Left) ROC curve (blue) for the diagnostic performance of our MCM5 expression assay for the characterisation of either a macroscopically normal oesophagus or adenocarcinoma compared to the null hypothesis reference line (red). **(Right)** ROC curve (blue) for the diagnostic performance of our MCM5 expression assay for the characterisation of patients as having neoplastic histology (adenocarcinoma or HGD) compared to non-neoplastic histology (NDBE or normal histology). The null hypothesis reference line is shown in red.

The diagnostic performance of the immunoflurometric assay for MCM5 expression as a marker for oesophageal adenocarcinoma was assessed using a ROC curve. Quantifying MCM5 expression was able to discriminate with fair accuracy the presence of oesophageal adenocarcinoma, compared to a macroscopically normal oesophagus (AUC 0.73 [95% CI: 0.62 – 0.96]). Using our assay, in 73% of cases a patient with adenocarcinoma would have a higher MCM5 expression compared to a patient with a macroscopically normal oesophagus ($p = 0.007$).

Our assay performed less favourably in the differentiation of patients with neoplastic histology (defined as HGD or adenocarcinoma), compared to those with non-neoplastic histology (normal or NDBE). Analysis using a ROC curve showed that MCM5 expression is a fair differentiator between patients with non-neoplastic and neoplastic histology (AUC 0.70 [95% CI: 0.57 – 0.83]). Our results show that in 70% of cases a patient with either HGD or adenocarcinoma would have a higher MCM5 expression than a patient with either NDBE or normal histology ($p = 0.008$).

DISCUSSION

MCM5 is a cell cycle protein that is believed to play a role in cell cycle regulation through its involvement in forming the DNA replicative helicase. Previous work has shown that aberrant MCM5 expression and subsequent dysregulation of the cell cycle is found in numerous epithelial cancers – including bladder, cervical and oesophageal cancer. Williams et al have shown that collection of sloughed oesophageal cells from gastric fluid aspirates, when paired with immunofluorometric analysis, is a feasible method of quantifying MCM5 expression[25]. Furthermore, they demonstrated that MCM5 expression was raised in patients with oesophageal squamous cell carcinoma or adenocarcinoma, compared with those with a macroscopically normal oesophagus. In this study, almost half of the patients with a normal oesophagus in fact had Barrett's oesophagus without dysplasia.

Endoscopic assessment of Barrett's oesophagus has a variable sensitivity for the detection of dysplasia (28-85%)[13], furthermore up to 25.3% (95%CI: 16.4-36.8%) of cancers are 'missed' during endoscopic assessment in the year preceding diagnosis[14]. Since the Seattle protocol of random biopsies throughout the BE segment has a high propensity to under sample[28] or miss early, potentially treatable neoplasia the quantification of reliable and easy to replicate biomarkers may assist clinicians in identifying these patients. MCM5 expression, previously shown to be raised in patients with oesophageal cancer, may be used to predict the presence of neoplasia within Barrett's oesophagus.

Our prospective feasibility study has assessed the feasibility of a using a proprietary assay to quantify MCM5 levels in oesophageal cells obtained from gastric aspirates. We also assess whether raised MCM5 expression in gastric fluid is associated with the presence of dysplastic Barrett's oesophagus or oesophageal adenocarcinoma in patients undergoing endoscopy. Our study demonstrates that MCM5 levels are significantly raised in patients with oesophageal adenocarcinoma compared to patients with a macroscopically normal oesophagus (154.1 vs 14.4 [$p = 0.04$]). We observed a stepwise association in the mean MCM5 expression levels between patients with a normal oesophagus, NDBE, HGD and oesophageal adenocarcinoma (14.4, 49.8, 112.3, 154.1 respectively). There was no statistically significant difference in MCM5 expression levels between patients with NDBE, HGD and adenocarcinoma. Using ROC curves to assess the diagnostic performance of our assay, we demonstrate that MCM5 expression is a fair discriminator between patients with a macroscopically normal oesophagus and those with adenocarcinoma (AUC 0.73, $p = 0.007$). The MCM5 expression level was also a fair discriminator (AUC 0.70, $p = 0.008$) between patients with neoplasia (HGD or cancer) compared to those without neoplasia (NDBE or a normal oesophagus).

Our study used a relatively small number of patients (61), the lack of significant associations may be because the study included a relatively small number of patients and may have been underpowered to detect more subtle differences in MCM5 expression between NDBE patients and those with neoplasia. Larger, multi-centre studies should interrogate MCM5 expression between these histological subgroups to better assess for variations in expression levels. Future studies should also assess for dysregulated MCM5 expression in NDBE and investigate whether increased segment length affects expression levels. We suggest that if MCM5 expression is raised in NDBE prior to the development of neoplasia, longer Barrett's segments may yield higher MCM5 expression and so could give false positive results for dysplasia.

We observed a wide range of MCM5 expression in patients with NDBE, with the difference in mean MCM5 expression in the group not statistically significant compared to the mean expression in patients with HGD or cancer. The gold standard for diagnosis used in this study was histological sampling of the oesophageal mucosa taken at the time of endoscopy. The sensitivity for dysplasia detection on random biopsies taken through a Barrett's oesophagus segment varies widely in reported studies (28-85%), largely because such a sampling technique samples less than 5% of the total mucosal surface area[28]. It should therefore be considered that while patients in this study were categorised as having only NDBE or HGD based on their histology results, in high MCM5 expressors with histologically NDBE, there may be undetected neoplasia missed by random biopsies. Similarly, it may be that the evolution of dysplasia with BE tissue is a stepwise event, aberrant MCM5 may be one of several cellular changes that precede the development of dysplasia – hence raised MCM5 expression in isolation may not be consistently demonstrative of dysplasia. Future studies should consider whether patients with high MCM5 expression, but with no histologic evidence of dysplastic Barrett's, go on to develop neoplasia at a later date with a higher frequency than patients without raised expression.

Due to logistical aspects of this study methodology, cells acquired from gastric aspirates were lysed within four hours of collection at endoscopy. Expression was therefore quantified using a proprietary assay and the expression levels normalised compared to the volume of gastric aspirate acquired from each patient. This method was introduced to allow a fair comparison of expression levels between patients where variable quantities of gastric fluid could be acquired. One limitation of this study is that gastric fluid volumes may not correlate with the number of cells present, for instance a patient with a large volume of gastric aspirate may have a higher proportion of that fluid made up of gastric juice than cells, compared to another patient who may have had a higher concentration of sloughed oesophageal cells despite lower aspirated fluid volume. We suggest that future studies should aim to quantify the cell concentration in aspirated gastric fluid samples; using this figure to normalise MCM5 expression levels between patients. Similarly, a small number of patients in this study recorded an MCM5 expression level of 0pg/ml. This may be because their MCM5 expression level was too low to record, but may also be because too few cells were aspirated within the gastric fluid, quantification of cell numbers prior to processing would allow more accurate identification of these patients, who could then be excluded from analysis. Such a pre-processing step, where cell number needs to

be quantified prior to analysis, could limit the value of calculating MCM5 expression as a diagnostic test to be performed in the outpatient setting – mainly due to the increased workload and potential added cost.

We observed a wide range of MCM5 expression in patients with NDBE, with the difference in mean MCM5 expression in the group not statistically significant compared to the mean expression in patients with HGD or cancer. The gold standard for diagnosis used in this study was histological samples taken from the oesophageal mucosa at the time of endoscopy. The sensitivity for dysplasia detection on random biopsies taken through a Barrett's oesophagus segment varies widely in reported studies (28-85%). It should therefore be considered that while patients in this study were categorised as having only NDBE or HGD based on their histology results, in high MCM5 expressors, there may be undetected neoplasia missed by random biopsies. Similarly, it may be that the evolution of dysplasia with BE tissue is a stepwise event, aberrant MCM5 may be one of several cellular changes that precede the development of dysplasia. Future studies should consider whether patients with high MCM5 expression, but with no histologic evidence of dysplastic Barrett's, go on to develop neoplasia at a later date with a higher frequency than patients without raised expression.

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