Diagnosis of oesophageal cancer by detection of minichromosome maintenance 5 protein in gastric aspirates

GH Williams1,7, R Swinn2,7, AT Prevost3, P de Clive-Lowe4, I Halsall5, JJ Going5, CN Hales2, K Stoeberr,1 and SJ Middleton6

1Wolfson Institute for Biomedical Research and Department of Histopathology, University College London, The Cruciform Building, Gower Street, London WC1E 6BT, UK; 2Department of Clinical Biochemistry, University of Cambridge, Level 4, Laboratory Block, Box 2.32, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 2QR, UK; 3Department of Public Health and Primary Care, Centre for Applied Medical Statistics, University of Cambridge, University Fennie Site, Robinson Way, Cambridge CB2 2SR, UK; 4Department of Urology, Box 4.3, Addenbrooke’s Hospital National Health Service Trust, Hills Road, Cambridge CB2 2QQ, UK; 5Department of Pathology, Glasgow University, Glasgow Royal Infirmary, Castle Street, Glasgow G4 0SF, UK; 6Department of Gastroenterology, Addenbrooke’s Hospital National Health Service Trust, Hills Road, Cambridge CB2 2QQ, UK

The incidence of oesophageal cancer in Western societies is increasing rapidly (Blot et al, 1991) and currently stands at approximately 6 per 100 000 in England and Wales (Office for National Statistics England and Wales, 1999). It accounts for about 6700 cancer-related deaths per year in the UK (Cancer Research Campaign, 1998, 1999). Most patients are not candidates for curative treatment as the symptoms associated with oesophageal cancer predominantly arise when the tumour is at an advanced stage. Without curative treatment, the overall mean survival of patients with oesophageal cancer is 6 months, even including those patients deemed suitable for curative treatment, the overall survival is poor, about 5% at 5 years (Newnham et al, 2003).

However, when applied to patients with very early disease, the latest chemotherapy regimes offer excellent results, and 5-year survival for T1–2N0 disease is now greater than 80% (Urschel and Vasan, 2003). Therefore, at present, the key to successful treatment of oesophageal cancer is early diagnosis and there is consequently great interest in the development of a screening test that will identify patients with asymptomatic oesophageal malignant or premalignant disease. Patients with Barrett’s oesophagus (Miros et al, 1994), known to have a high risk of developing oesophageal carcinoma, can be screened endoscopically, but this is time consuming and of questionable effectiveness. An extension of current endoscopic screening to include patients with gastro-oesophageal reflux disease would have even less cost effectiveness, even though this group is at increased risk of developing oesophageal cancer (Iftikhar et al, 1992). Certain risk factors such as cigarette smoking (Brown et al, 1994; Hu et al, 1994; Rolon et al, 1995), alcohol (Brown et al, 1994; Hu et al, 1994; Rolon et al, 1995) and age (Office for National Statistics England and Wales, 1999) are also associated with higher risk but again lack suitable specificity to be useful as screening tools.

Population screening for squamous oesophageal carcinoma is only practised where the incidence is high such as Japan and China using techniques such as abrasive cytology, barium oesophagography and fiberoptic oesophagoscopy (Riddell, 1996). This is a labour-intensive approach requiring skilled cytopathologists, gastroenterologists and radiologists. Dysplasia and cancer

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surveillance by endoscopy and biopsy of Barrett’s patients in Western populations is also undertaken, but with uncertain benefit (Macdonald et al., 2000).

Despite advances in the molecular pathology of oesophageal neoplasia, no useful clinical biomarkers have yet been identified for diagnostic and screening applications. The initiation of DNA replication represents a final and critical step in growth regulation and lies downstream at the convergence point of growth regulatory pathways (Williams and Stoebel, 1999). Proteins of the minichromosome maintenance (MCM) family (minichromosome maintenance protein 2–7, Mcm2–7) play a critical role (DNA replicative helicase) in the initiation of DNA replication (Bell and Dutta, 2002). We have previously demonstrated that dysregulation of MCM proteins is an early event in epithelial carcinogenesis, resulting in exfoliation of MCM-positive tumour cells, and have used these novel biomarkers of growth in diagnostic screening applications for cervical and genital neoplasia (Williams et al., 1998; Stoebel et al., 1999; Stoebel et al., 2002). Moreover, we have shown that dysregulation of the DNA replication initiation pathway is an early event in oesophageal carcinogenesis with aberrant expression of MCM proteins occurring in both squamous dysplasia and glandular dysplasia complicating Barrett’s oesophagus (Going et al., 2002).

These data suggest that detection of MCM proteins in exfoliated tumour cells might provide a potentially sensitive indicator of oesophageal neoplasia. Here, we describe an evaluation of this approach using a liquid phase immunofluorometric assay to measure quantitatively Mcm5 levels in gastric luminal samples obtained from patients undergoing gastroscopy for upper gastrointestinal symptoms. Gastric luminal secretions can be obtained without the need for endoscopy, and can be collected in health centres as a screening tool.

MATERIALS AND METHODS

Study subjects

Gastric aspirates were obtained from 40 patients undergoing gastroscopy at Addenbrookes Hospital National Health Service Trust (Cambridge, UK) for suspected or known oesophageal carcinoma or symptoms of dyspepsia. All patients gave full consent and the study was approved by the Local Research Ethics Committee. Aspirates were obtained through the endoscope suction channel. A full endoscopic examination of the oesophagus, stomach and duodenum was performed. Endoscopies were undertaken with conscious sedation using Midazolam. Punch biopsies were taken from regions that looked abnormal to identify any underlying pathological process. Aspirates were analysed in a blinded manner for immunofluorometric Mcm5 detection. On completion of the study, patient data were decoded and the immunofluorometric signals compared with endoscopy and biopsy histology results.

Gastric aspirates collection and storage

Prior to biopsy sampling, an endoscope was passed carefully down the oesophagus, which was inflated with air thus minimising any contact with the oesophageal surface. Gastric juice was then aspirated immediately to reduce instrument-related trauma. Gastric aspirates were kept on ice until processing for storage. Storage buffer (10 × phosphate-buffered saline (PBS), 5% bovine serum albumin (BSA), 1 m sucrose, 0.2% Na3) containing one complete EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics Ltd, Lewes, East Sussex, UK) per 10 ml of buffer was added to gastric aspirates at one-tenth aspirate volume and carefully mixed with the sample. Gastric aspirates in storage buffer were transferred into 5 ml cryovials and stored in liquid nitrogen (LN2) for cryopreservation. The aspirates were stored in LN2 within 5 h of the samples being collected.

Processing of standards and gastric aspirates

Standards for the immunofluorometric Mcm5 assay were prepared, and standards and gastric samples processed as described previously (Stoebel et al., 2002). Briefly, standards and clinical samples were thawed, and the cells were isolated by centrifugation at 1500 g for 5 min at 4 °C. The supernatants were discarded, and the cell pellets were washed three times with 500 μl of PBS. Cell pellets were resuspended in 250 μl (for those pellets with a volume less than approximately 200 μl) or 500 μl (for those pellets with a volume greater than approximately 200 μl) of processing buffer (PBS, 0.4% sodium dodecyl sulphate (SDS), 0.02% NaN3). Cell lysates were prepared by incubating the resuspended samples at 95 °C for 45 min. The DNA in each sample was sheared by passing the lysates through a 21-gauge needle (Becton Dickinson UK Ltd, Cowley, Oxford, UK), and nucleic acids were digested with DNase I (20 U ml−1; Roche Diagnostics) and RNase A (1 μg ml−1; Roche Diagnostics) for 2 h at 37 °C. The samples were centrifuged at 15 000 g for 10 min to pellet the cell debris, the supernatants were collected and 50 μl of each was directly used in the immunofluorometric assay.

Immunofluorometric measurement of Mcm5 levels in gastric aspirates

Monoclonal antibodies (MAbs) 12A7 and 4B4 raised against His-tagged human Mcm5 were protein A purified from hybridoma supernatants as described previously (Stoebel et al., 2002). Protein A-purified MAb 4B4 was labelled with europium using a DELFIA® Eu-labelling kit (Perkin-Elmer Life Science, Wallac Oy, Turku, Finland) according to the manufacturer’s instructions. The assay was standardised using HeLa cells as described previously (Stoebel et al., 2002), and one fluorescence unit was defined as the signal generated by the Mcm5 contents of one proliferating HeLa S3 cell, approximately 106 Mcm5 molecules (Kearsey and Libib, 1998). DELFIA® research reagents were obtained from Perkin-Elmer Life Science. All other reagents were obtained from Sigma-Aldrich.

Immunofluorometric measurements of Mcm5 levels in gastric aspirates were performed as described previously (Stoebel et al., 2002). Standard curves were constructed from fluorescence values generated by the blank and standard wells, and the fluorescence values of the gastric aspirate samples were calculated with the MultiCalc Advanced Immunoassay Data Management package (Perkin-Elmer Life Science). For immunofluorometric measurement of Mcm5 levels, assay standards, control samples and gastric aspirate samples were run as duplicates and the mean of the duplicate results reported. For acceptance of immunofluorometric measurements in the assay, the following coefficients of variations were required: CV <20% for results between 1500 and 5000 cells well−1 standard curve points; CV <15% for results between 5000 and 15 000 cells well−1; and CV <10% for results >15 000 cells well−1.

Immunohistochemistry

Formalin-fixed, paraffin-embedded surgical biopsy material from tumour-positive cases was selected for immunohistochemical
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Table 1  Patient demographics and sample characteristics at endoscopy and biopsy

| Patient characteristics (n = 40 patients) | N (%) or median (interquartile range) |
|-----------------------------------------|--------------------------------------|
| Sex                                     |                                       |
| Male                                    | 24 (60%)                              |
| Female                                  | 16 (40%)                              |
| Age (years)                             | 74 (58–82)                            |
| Sample characteristics (n = 47 samples)*|                                       |
| Gastric aspirate volume (ml)            | 4 (4–4.5)                             |
| Endoscopy and/or biopsy findings        |                                       |
| Tumour absent                           | 27 (57%)                              |
| Normal oesophagus                       | 2                                    |
| Diverticulum                            | 1                                    |
| Shatzki ring                            | 1                                    |
| Chemical gastropathy                    | 1                                    |
| Oesophagitisb                          | 9                                    |
| Barrett’s oesophagus without dysplasia  | 13                                   |
| Tumour present                         | 20 (43%)                              |
| AdCa                                    | 10                                   |
| SCC                                     | 10                                   |
| Tumour stage                            |                                       |
| T2N1M00                                 |                                      |
| T3N1M00                                 | 9                                    |
| T3N1M11                                 |                                      |
| T3N1M1                                 |                                      |
| T4N1M00                                 |                                      |

AdCa = adenocarcinoma; SCC = squamous cell carcinoma. *Two samples from one patient with AdCa, four samples from one patient and two samples from two patients with SCC, and two samples from one patient with Barrett’s oesophagus with associated inflammation and ulceration were included in the analysis. †Three AdCa’s had associated severely atypical Barrett’s oesophagitis.

Analysis. Automatic immunostaining for Mcm2 and Mcm5 was performed on a DAKO Techmate™ 500 as described previously (Stoeger et al, 2001). Primary antibodies were omitted in negative controls and in addition appropriate tissue sections were used as positive and negative controls. Microscopic images were acquired with an Olympus BX51 light microscope/CCD camera set-up and ANALysis image capturing software (Soft Imaging Systems GmbH, Münster, Germany). A semi-quantitative determination of the extent of staining was obtained by calculating a labelling index for each protein stained. At least 200 nuclei were assessed per case. Results were expressed as a percentage of positively stained nuclei out of the total number of nuclei counted in representative microscopic fields. The median and range of labelling indices were calculated.

Statistical analysis

Sensitivity and specificity characteristics of the immunofluorometric Mcm5 test for the detection of oesophageal cancer are presented as a receiver operating characteristics (ROC) curve. The area under the nonparametric ROC curve was used to assess the overall accuracy of the test (McNeil and Hanley, 1984; Altman and Bland, 1994). Three cut points were used to demonstrate test performance under different circumstances as follows: at the lower detection limit of the assay (fluorescence signal generated from 1500 proliferating HeLa S3 cells well⁻¹), where sensitivity of the test was maximal; at the point where the false-positive and false-negative rates of the test were equal (5000 cells well⁻¹), and where specificity exceeded 95% (i.e. 7500 cells well⁻¹). An exact 95% confidence interval (CI) for each proportion, including sensitivity, specificity and predictive values of Mcm5 and cytology, was derived assuming a binomial distribution using StatXact software, Version 4.0 (Cytel Software Corporation, Cambridge, MA, USA). Unless otherwise stated, statistical tests were performed using SPSS software, Version 11.5 (SPSS Inc., Chicago, IL, USA). The level of the signal was compared between patient groups using the Kruskal–Wallis test and for pairs of groups using the Mann–Whitney U-test. All statistical tests were two-tailed, and a 5% level was used to indicate statistical significance.

RESULTS

The patient characteristics, clinical symptoms on presentation, endoscopy findings and histopathological diagnoses derived from the 47 gastric aspirate samples were obtained for analysis (Table 1).
the test had 85% (17 of 20) sensitivity and 81% (17 of 21) positive predictive value. At the 7500-cell cut point, the test had 75% (15 of 20) sensitivity and 94% (15 of 16) positive predictive value.

Table 3 and Figure 2 show the performance of the immunofluorometric Mcm5 test according to the diagnosis made at clinical follow-up using endoscopy findings and histopathological diagnosis as the gold standard. The level of signal in the four
subgroups was significantly different \( (\text{Kruskal–Wallis test,} \ P < 0.001) \). Interestingly, the Mcm5 immunofluorometric signal was lower than for other patients without malignancy, where the median signal was below the lower detection limit of 1500 cells well\(^{-1}\) \( (\text{Mann–Whitney } U\text{-test,} \ P = 0.002) \). The level of signal was not significantly different \( (\text{Mann–Whitney } U\text{-test,} \ P = 0.16) \) between AdCas \( (\text{median 11 210}) \) and SCCs \( (\text{median 25 036}) \). The largest difference \( (\text{Mann–Whitney } U\text{-test,} \ P < 0.001) \) was between those samples from patients without tumour \( (\text{median 1718}) \) and those with tumour \( (\text{median 16 401}) \).

The elevated levels of the Mcm5 DNA replication licensing protein found in gastric aspirates from patients with oesophageal cancer is consistent with our previous immunohistochemical findings demonstrating aberrant expression of Mcm2 and Mcm5 proteins in dysplastic and malignant oesophageal lesions \( (\text{Going et al, 2002}) \). Immunohistochemical analysis of the tumours detected in this study confirms our previous findings showing high levels of MCM protein expression, with the majority of tumour cells expressing the Mcm2 and Mcm5 replication licensing factors \( (\text{SCCs: Mcm2 (92 – 98%, mean: 95%), Mcm5 (93 – 100%, mean: 96%); AdCas: Mcm2 (83 – 99%, mean: 94%), Mcm5 (90 – 98%, mean: 94%)}) \); Figure 3).

**DISCUSSION**

Patients with oesophageal cancer present at an advanced stage, symptoms are usually of recent origin and their period of survival is short. The incidence of oesophageal cancer is increasing and therefore there is an urgent need for reliable cost effective methods for early diagnosis \( (\text{Wang et al, 1997}) \). Abrasive brush cytology as a screening technique for oesophageal cancer has been used for many years in high incidence areas of China \( (\text{Shu, 1983}) \). Although the brush biopsy capsule is inexpensive, the hidden costs including preparation of slides and expert cytopathological assessment are considerable. Moreover, despite the alarming increase in the incidence of AdCa of the oesophagus in North America and Europe, screening for neoplasia in Barrett’s oesophagus is controversial partly due to the invasive nature and expense of introducing endoscopic biopsy surveillance programmes \( (\text{Wright et al, 1996}) \).

Maturation arrest and failure to engage correctly the differentiation programme, the hallmark of dysplastic precancerous lesions, is associated with aberrant expression of the MCM replication initiation factors \( (\text{Williams et al, 1998; Stoeber et al, 1999; Going et al, 2002; Stoeber et al, 2002}) \). Importantly, aberrant expression of MCM proteins was identified in both squamous dysplasia and Barrett’s glandular dysplasia, but not in Barrett’s metastatic oesophagus \( (\text{Figure 3; Going et al, 2002}) \). We have previously shown that detection of MCM proteins in urine sediments is a sensitive and specific test for urothelial neoplasia allowing detection of bladder cancers at all stages and grades including severe dysplasia/carcinoma in situ, the latter corresponding to a similar step in tumour progression represented by dysplastic Barrett’s oesophagus \( (\text{Stoeber et al, 1999, 2002}) \).

Using a similar analytical approach previously applied to the genitourinary tract \( (\text{Stoeber et al, 2002}) \), application of the immunofluorometric Mcm5 test to gastric aspirates has resulted in a strikingly similar performance \( (\text{Tables 2 and 3 and Figure 1}) \). Patients with tumours, including three cases with associated severe dysplastic Barrett’s oesophagus, were detected with high sensitivity \( (85–95% \text{ at the low cut-off point}) \). Importantly, inflammatory conditions including oesophagitis and Barrett’s metaplastic oesophagus were not associated with false-positive results. Interestingly, ulcerative lesions gave a signal, but with an amplitude below that generated by tumours, most likely reflecting reparative growth with exposure of the stem-transit compartment to luminal secretions and the shedding of reactive Mcm5-positive cells. Similar results were found in the urinary tract in relation to renal calculi \( (\text{Stoeber et al, 2002}) \).

The immunofluorometric Mcm5 test provides a new approach to the detection of oesophageal cancer. Given the magnitude in the difference between Mcm5 levels in benign and malignant disease found in this study, it is likely that even small cases at an early stage will be detected. Studies on large unselected populations will now be required to determine whether this novel diagnostic approach can be exploited as a screening tool to detect early curable tumours. Furthermore, our previous studies have shown that aberrant expression of the MCM proteins is a powerful marker of dysplasia in Barrett’s oesopagus \( (\text{Going et al, 2002}) \). These data suggest that the immunofluorometric Mcm5 test could be further refined for screening of Barrett’s oesopagus by employing balloon cytology catheters, increasing the yield of cellular material for biochemical analysis \( (\text{Liu et al, 1994; Sepehr et al, 2000}) \). The sensitivity and specificity data are strikingly similar when comparing oesophageal cancer with genitourinary tract cancers. The areas under the ROC curve for oesopagus, bladder and prostate are all around 0.93 \( (\text{Figure 1; Stoeber et al, 2002}) \). It is likely that similar results will be obtained for other cancers arising from self-renewing tissues using this approach.

These pilot data, including our previous studies examining the expression profile of MCM proteins during oesophageal carcinogenesis, suggest that immunofluorometric detection of Mcm5 in gastric aspirates provides a new approach for the detection of oesophageal neoplasia. Its use as a screening and diagnostic tool for oesophageal neoplasia needs to be urgently investigated considering the increasing incidence and high mortality rate associated with this disease.

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