ORIGINAL ARTICLE

The metabolic marker tumour pyruvate kinase type M2 (tumour M2-PK) shows increased expression along the metaplasia–dysplasia–adenocarcinoma sequence in Barrett's oesophagus

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Background: Proliferating and tumour cells express the glycolytic isoenzyme, pyruvate kinase type M2 (M2-PK). In tumours cells, M2-PK usually exists in dimeric form (tumour M2-PK), causing the accumulation of glycolytic phosphometabolites, which allows cells to invade areas with low oxygen and glucose concentrations.

Aims: To investigate the expression of tumour M2-PK during the metaplasia–dysplasia–adenocarcinoma sequence of Barrett's oesophagus, and to assess the prognostic usefulness of tumour M2-PK in oesophageal cancer.

Materials/Methods: One hundred and ninety cases selected from the histopathology archives as follows: 17 reflux oesophagitis, 37 Barrett's oesophagus, 21 high grade dysplasia, 112 adenocarcinomas, and three control tumours. Sections were stained immunohistochemically with antibody to tumour M2-PK.

Results: Tumour M2-PK was expressed in all cases, and increased cytoplasmic expression was seen with progression along the metaplasia–dysplasia–adenocarcinoma sequence. All cases of adenocarcinoma showed 100% staining so that tumour M2-PK was not a useful prognostic marker.

Conclusions: Tumour M2-PK is not a specific marker of Barrett's adenocarcinoma, but may be important as a marker of transformed and highly proliferating clones during progression along the metaplasia–dysplasia–adenocarcinoma sequence.

Barrett's oesophagus is the clinical entity of a distal oesophagus lined by columnar mucosa. The columnar epithelium can be of five types, namely: transitional epithelium, pancreatic acinar metaplasia, oxyntic metaplasia, cardiac metaplasia, or intestinal metaplasia, but it is the last type that is most important because it associated with a 30–125 fold increased risk of developing adenocarcinoma compared with normal oesophageal epithelium.[1] In this regard, there is increasing evidence from both epidemiological and molecular studies that Barrett's oesophagus is a premalignant condition, progressing along the metaplasia–dysplasia–adenocarcinoma sequence.[2] Importantly, adenocarcinoma of the oesophagus has become increasingly common in the past 30 years in the Western world, and has become a major health economics issue.[3]

Barrett's oesophagus is thought to arise as a result of reflux of acid and bile from the stomach; that is, gastro-oesophageal reflux disease.[4] Although gastro-oesophageal reflux disease can be minimised or prevented by means of pharmaceutical and/or surgical strategies, it has become apparent that the risk of developing adenocarcinoma is not substantially altered, and that once metaplasia has occurred the neoplastic potential cannot be reversed to that of the normal epithelial type.

> "Adenocarcinoma of the oesophagus has become increasingly common in the past 30 years in the Western world, and has become a major health economics issue"

The glycolytic isoenzyme, pyruvate kinase type M2 (M2-PK) plays a central role in the increased aerobic glycolytic metabolism of tumour cells, and is expressed not only in non-neoplastic proliferating cells but also in a wide range of tumours.[5] During tumorigenesis, the tissue specific isoenzymes of pyruvate kinase (PK), such as L-PK in liver and kidney, M1-PK in muscle and brain, and R-PK in red blood cells,[6] are consistently altered and replaced by M2-PK.[7] Pyruvate kinase isoenzymes normally exist as enzymatically highly active tetramers. However, the tetrameric M2-PK can shift to a nearly inactive dimeric form, which has a low affinity for phosphoenolpyruvate and also interacts with different oncoproteins.[8] In tumour cells, the dimeric form is consistently predominant and has therefore been named tumour M2-PK. The switch between the two forms regulates glycolytic phosphometabolite pools, and the interaction between glycolysis and glutaminolysis leads to an accumulation of all glycolytic phosphometabolites above pyruvate kinase in the pathway. This switch allows tumour cells to invade areas with low amounts of oxygen and glucose. Tumour M2-PK has been detected in normal colonic epithelium, but increases greatly in colonic adenocarcinomas,[5] and plasma concentrations are strongly correlated with tumour load in patients with lung cancer.[6] In addition, raised concentrations of tumour M2-PK correlate with the ability of tumour cells to metastasise.[9] Recently, tumour M2-PK has been shown to be measurable not only in plasma, but also in faeces, leading to an interest in its potential as a metabolic marker for the screening of patients at increased risk of colorectal and gastric cancer.[10] [11]

Much of the current attention in the field of Barrett's oesophagus has been focused on its molecular pathology and on possible biological markers, not only to determine the

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Abbreviations: M2-PK, pyruvate kinase type M2; PK, pyruvate kinase
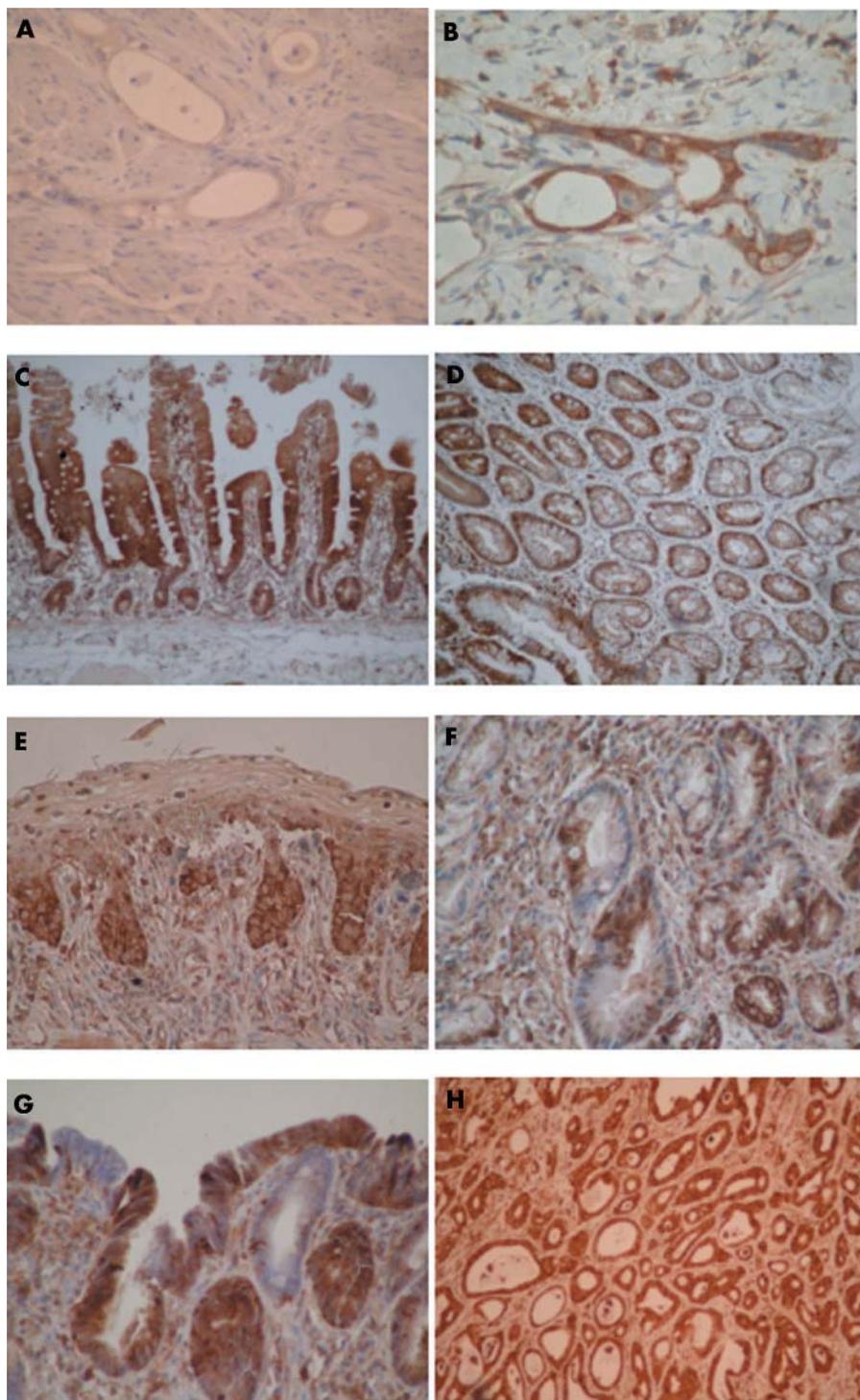
Figure 1 (A) Negative control, no primary antibody in a case of colon cancer. (B) Positive control of colon cancer: all cells of the malignant glands show strong cytoplasmic positivity. (C) Normal small bowel showing strong staining of enterocyte cytoplasm. (D) Normal large bowel showing patchy strong staining of crypt cells. (E) Severe reflux oesophagitis. The lower half of the squamous mucosa shows strong cytoplasmic staining. (F) Barrett's oesophagus. There is patchy strong cytoplasmic staining in groups of cells, whereas others are negative (cf normal small bowel). (G) Barrett's dysplasia. Strong cytoplasmic staining of runs of surface epithelium and glands, whereas some groups are clearly negative. (H) Barrett's adenocarcinoma. Widespread strong cytoplasmic staining of tumour cells.

driving forces behind the maintenance and neoplastic progression of Barrett's oesophagus, but also to enable translational research into treatment. With this in mind, we sought to characterise the expression of the tumour M2-PK marker of proliferation in Barrett's oesophagus, Barrett's dysplasia, and adenocarcinoma.

MATERIAL AND METHODS
Tissue samples
One hundred and ninety cases were selected from the histopathology archives as follows: 17 cases of reflux oesophagitis, 37 cases of Barrett's intestinal metaplasia, 21 cases of high grade dysplasia in Barrett's mucosa, and 38 cases of adenocarcinoma arising in Barrett's oesophagus. An additional 74 cases of oesophageal adenocarcinoma with well documented survival data were selected from the oesophageal laboratory of Birmingham Heartlands Hospital. The local research ethics committees approved our study.

Immunohistochemistry
Formalin fixed, paraffin wax embedded, 3 µm thick tissue sections were subjected to a heat mediated antigen retrieval

method using a microwave oven. Briefly, dewaxed, hydrated sections were simmered in hot citrate buffer (pH 6.0) at 95℃ for 60 minutes. Sections were removed and placed in cold water, ensuring that at no time were they allowed to dry out. Endogenous peroxidase was blocked with 1% hydrogen peroxidase in methanol for 10 minutes. Sections were washed well in running water and incubated with the primary anti-M2-PK antibody (monoclonal mouse antihuman antibody directed against dimeric M2-PK (tumour M2-PK): clone DF4; ScheBo Biotech UK Ltd, Basingstoke, UK), diluted 1/10 in 0.01M phosphate buffered saline, for 60 minutes at room temperature. Sections were washed with 0.01M phosphate buffered saline and the primary antibody was detected using the Strepavidin ABC duet kit A0492 (Dako, Ely, Cambridgeshire, UK). Staining was visualised with diaminobenzidine and a light Mayers haemalum counterstain was applied.

Positive tissue controls stained in parallel with the test slides included sections of normal colon, of small bowel, and of three cases of adenocarcinoma of the colon. Negative controls were test oesophageal, normal colon and small bowel, and colonic adenocarcinoma sections stained in parallel but omitting incubation with the primary antibody.

Histological assessment

Staining was assessed with regard to intensity on a semiquantitative scale of 0, +, and ++ (no staining, weak staining, and strong staining, respectively). The proportion of cells stained was also assessed semiquantitatively as follows: 0, up to 30%, 30–60%, and 60–100%. A note of the location of the staining within the cells and of any staining patterns was also made.

RESULTS
Controls
Negative controls were uniformly negative (fig 1A). The positive tissue controls all showed staining. The three cases of adenocarcinoma of the colon were strongly positive (++) in the cytoplasm of almost 100% of the tumour cells (fig 1B). Normal small bowel showed strong staining of the cytoplasm of the enterocytes (fig 1C), but normal large bowel showed only patchy staining of crypt cells (fig 1D).

Test groups (table 1)
All 17 cases of reflux oesophagitis showed positive (++) staining in the cytoplasm of all cells in the lower third of the squamous epithelium. Occasional accentuation of the basal layer was noted, and occasional nuclear staining was seen. In more severe cases of oesophagitis, the lower half or even lower two thirds of the squamous mucosa showed strong cytoplasmic staining (fig 1E).

In three of the 37 cases of Barrett's metaplasia 100% of the cells stained, 16 cases showed more than 60% positivity, 13 cases showed 30–60% positivity, and five cases showed less than 30% positivity. In these samples, the intestinal mucosa goblet cells were negative and it was both the intestinal absorptive cells and the mucous secreting cells that were positive. The variation in staining might result from the variable proportions of these two cells types. Some nuclear staining was occasionally noted (fig 1F).

All 21 cases of high grade dysplasia showed positive (++) staining. In 19 of these cases more than 60% of cells were positive, and two cases showed 30–60% positivity. Where dysplastic cells were seen along the surface of the mucosa, staining was the most pronounced in these cells. Some cases showed continuous runs of positively stained cells interspersed with occasional groups of negatively stained cells (fig 1G).

All 112 cases of adenocarcinoma were positive (++), with almost 100% of the cells staining (fig 1H). Nuclear staining for tumour M2-PK was seen in some sections of adenocarcinoma and in some inflammatory and endothelial cells.

Two independent assessors graded the staining and their counts were compared. The correlation coefficient ($R = 0.85$; $p < 0.01$) indicated that interindividual reproducibility was good.

DISCUSSION
Progression to adenocarcinoma from Barrett's oesophagus is thought to be associated with the intestinal metaplastic type of Barrett's oesophagus, which contains goblet cells. At a molecular level, expression of the oncogene products p53, p16, and adenomatous polyposis coli, among others, has been shown to be associated with malignant progression, although these markers are not accurate predictors of patient survival.[12] In addition, some high risk individuals with a strong family history have E-cadherin mutations[4] and interleukin 1β polymorphisms,[13] which could provide an objective basis for screening. More recently, the importance of cyclooxygenase 2[14] and tumour necrosis factor α in the process of neoplastic progression has been recognised, with the potential to inhibit their actions pharmacologically.

There is still a need to find markers that could help in stratifying patients with Barrett's oesophagus into high and low risk groups for targeted surveillance. Recently published data on tumour M2-PK concentrations in EDTA plasma samples from patients with oesophageal cancer showed that this marker was raised at least as often as the carbohydrate antigen 72-4 and substantially more frequently than carbohydrate antigen 19-9 or carcinoembryonic antigen.[15] Tumour M2-PK was the best of these four markers at discriminating between patients with localised oesophageal cancer and a non-malignant control group. A smaller study published earlier[16] suggests that tumour M2-PK may be a valuable marker for the detection of gastrointestinal cancers in general. Therefore, we undertook a simple immunohistochemical study to look at the distribution of the tumour metabolic marker tumour M2-PK in Barrett's oesophagus, Barrett's dysplasia, and adenocarcinoma.

Table 1 Tumour pyruvate kinase type M2 (M2-PK) staining in reflux oesophagitis, Barrett's metaplasia, Barrett's dysplasia, oesophageal adenocarcinoma, and colorectal adenocarcinoma

| Tissue type (number of cases) | Staining intensity (cytoplasmic) | | | Proportion of cells positive % | | | | |
	0	+	++	0	<30	30–60	>60	100
Reflux oesophagitis (17)	0	0	17	Lower 1/3–1/2 of mucosa				
Barrett's metaplasia (37)	0	0	37	0	5	13	16	3
Barrett's dysplasia (21)	0	0	21	0	0	2	19	0
Adenocarcinoma (112)	0	0	112	0	0	0	0	112
Control tumours (3)	0	0	3	0	0	0	0	3

Take home messages

- Dimeric pyruvate kinase type M2 (tumour M2-PK) was expressed in all cases of Barretts' oesophagus, and increased cytoplasmic expression was seen with progression along the metaplasia–dysplasia–adenocarcinoma sequence
- All cases of adenocarcinoma showed 100% staining so that tumour M2-PK would not be useful as a prognostic marker
- Tumour M2-PK may be important as a marker of transformed and highly proliferating clones during progression along the metaplasia–dysplasia–adenocarcinoma sequence

Oesophagitic squamous epithelium expressed tumour M2-PK, with an increased depth corresponding to more severe oesophagitic change (fig 1E). All cases of Barrett's metaplasia expressed tumour M2-PK in their cytoplasm. Staining was variable, ranging from less than 30% cells positive to 100% positive, with both the intestinal absorptive cells and mucous secreting cells being positive and goblet cells negative. In the dysplastic cases, there was also variability in the proportion of stained cells, but a greater proportion of cells was stained overall (fig 1G). The variation in staining could be the result of the variable proportions of cells types. Alternatively, we must consider the issue of clonality: in Barrett's mucosa there are probably multiple clones of metaplastic cells, which are selected out along the metaplasia–dysplasia–adenocarcinoma sequence. In metaplasia and dysplasia, it is possible that the expression of tumour M2-PK reflects clones of cells within the epithelium with a metabolic switch leading to abnormally high anaerobic metabolism and enhanced neoplastic characteristics, such as increased proliferation and invasive potential. In all the cases of adenocarcinoma in Barrett's oesophagus, almost 100% of the tumour cells stained strongly (fig 1H). The nuclear staining seen in some of our adenocarcinoma sections has been noted by other investigators,[17] although currently a physiological explanation is lacking.

''There is still a need to find markers that could help in stratifying patients with Barrett's oesophagus into high and low risk groups for targeted surveillance''

The expression of Ki67 and the accumulation of p53, as measured by immunocytochemisty, have been reported to be only very weak markers of cancer risk in Barrett's oesophagus.[18] Therefore, we thought that there would be an association between the coexpression of these molecules and M2-PK, but that this association data would not provide definitive evidence that M2-PK is useful clinically. We found that the expression of tumour M2-PK increased as the Barrett's metaplasia–dysplasia–adenocarcinoma sequence progressed. Because all cases of adenocarcinoma were almost homogeneously stained, we recognise the limitations of tumour M2-PK as a prognostic marker for patient survival. However, tumour M2-PK is a common marker of neoplasia (vide infra) and, although not specific for Barrett's adenocarcinoma, it may help to draw the pathologist's attention to specific areas of tissue. Tumour M2-PK does indicate the extent of the ''stress response'' during mucosal inflammation in Barrett's metaplasia. As such, these data provide additional information of the cell types at risk of oxidative stress, in addition to the early and sustained tissue damage and resultant clonal evolution of adaptive cells.

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