Is subtyping of intestinal metaplasia in the upper gastrointestinal tract a worthwhile exercise? An evaluation of current mucin histochemical stains

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ABSTRACT

Intestinal metaplasia is a premalignant condition that occurs in the upper gastrointestinal tract and can be subdivided into three types (I, II and III). Previous studies suggest that type III carries the highest cancer risk. The high iron diamine/alcian blue (HID/AB) technique traditionally has been used to identify this subtype; however, the technique uses reagents that are toxic and potentially carcinogenic. Therefore, in this study we evaluate various alternative histochemical techniques. Our results indicated that the only suitable alternative is Gomori's aldehyde fuchsin/AB technique. The study also revealed that subtyping of intestinal metaplasia is a subjective procedure, open to varying interpretation. Consequently, we suggest that previous work linking cancer risk to metaplasia subtypes should be viewed with some circumspection.

KEY WORD: Barrett esophagus. Mucins. Intestinal metaplasia. Stomach.

Introduction

Intestinal metaplasia (IM) is a form of metaplastic change that can occur in the stomach and the oesophagus (Barrett's oesophagus). It can only be diagnosed by histological examination, and the presence of acidic mucin-containing goblet cells is the hallmark of the condition. Studies suggest that IM of both the stomach and oesophagus may be a risk factor for the development adenocarcinoma at these sites.[1,2] However, IM is present in about 20% of all gastric biopsies[3] and few of these patients will progress to adenocarcinoma of the stomach. Thus, its specificity as a marker is actually too low to be used in surveillance for cancer.

Consequently, in an attempt to improve the specificity of IM, its subtypes have been examined. Conventionally, three main subtypes have been recognised[4] and some studies reveal that there may be a strong link between type III IM and both adenocarcinoma of the oesophagus[2] and intestinal-type gastric adenocarcinoma.[5-7]

The subtypes of IM have been characterised according to their mucin content and morphology (Table I). Discrimination between type I IM and the other two subtypes is easily made using the alcian blue/periodic acid–Schiff (AB/PAS) technique, as the latter two subtypes express acidic mucins in the metaplastic columnar cells, whereas the former type contains no cellular mucin. However, this technique does not allow discrimination between types II and III IM.

As type II IM is characterised by the presence of sialomucins and type III by the presence of sulphomucins within the intervening columnar cells, a histochemical technique called the high iron diamine/alcian blue (HID/AB) technique[8] has been used to distinguish them. The procedure, however, involves the use of reagents that are toxic if inhaled or come into contact with the skin or eyes.[3] Furthermore, the technique is potentially carcinogenic.

The aim of this study is to evaluate alternative, non-toxic histochemical staining procedures to the HID/AB technique that can be used to distinguish the IM subtypes. The histochemical techniques to be evaluated have all been claimed as methods for sulphomucins, and include the combined Gomori's aldehyde fuchsin /alcian blue (GAF/AB) technique,[3] the combined orcein/alcian blue method[9] and the combined alcian blue/ruthenium red technique.[10]

Another suggested technique is that of McFadden *et al.*,[11] which is also claimed to discriminate between sulphomucins and sialomucins. However, this technique involves the use of phenylhydrazine, which is also toxic and potentially carcinogenic.[12,13] Thus, for the purpose of this study, the procedure was considered to be an inappropriate alternative to the HID/AB technique.

Materials and methods

Multiple sets of 4 µm sections were cut and then dried on a hot plate for 20 minutes from five paraffin-processed tissue blocks of normal colon, one of normal ileum and three of normal duodenum. These blocks had been taken in the past from resection margins of surgical cancer specimens and archived in the histopathology department files at the Leeds General Infirmary. Ethical permission was obtained from the local research ethics committee prior to commencement of the study.

Duodenum was chosen as a negative control as it expresses little or no sulphomucin, ileum expresses slightly

Table I. Classification of intestinal metaplasia subtypes as proposed by Jass and Filipe.[4]

		Mucin content	
Subtype	Morphology	Goblet cells	Columnar cells
I	Virtually identical to the appearance of small intestine with fully developed goblet cells and absorptive cells	Sialomucins and or sulphomucins	No mucins (Absorptive cells)
II	Mild architectural distortion with slightly irregular crypts lined by goblet and columnar cells in various stages of maturation. There are few or no absorptive cells.	Sialomucins and or sulphomucins	Sialomucins and neutral mucins
III	As for type II	Sialomucins and or sulphomucins	Sulphomucins

Table 2. Assessment marks for aldehyde fuchsin stains used in the GAF/AB technique, made from basic fuchsin obtained from four different suppliers. Marks were given for the intensity of sulphomucin staining.

	Supplier of basic fuchsin			
Case	Sigma	BDH	H+W	Polysciences
1	3	2	1	3
2	3	2	1	3
3	3	3	1	3
4	3	2	1	2
5	3	3	1	3
6	3	3	1	3
7	✓	0	✓	0
8	✓	✓	✓	0
9	✓	0	✓	0

0 = false sulphomucin positivity, 1= weak, 2 =moderate, 3 = intense, ✓= no sulphomucin positivity in duodenum. Cases 1-5 = colon, 6 = ileum, 7-9 = duodenum.

more of this acidic mucin, and normal colon is a rich source of sulphomucin, so was used as a positive control.

All sections were initially dewaxed in three changes of xylene and the following techniques were then performed and assessed by the authors.

Gomori's aldehyde fuchsin/alcian blue

Sections were dehydrated in two changes of absolute alcohol and one of 70% alcohol. They were subsequently immersed in aldehyde fuchsin solution for an optimal time of 5 min. The aldehyde fuchsin was prepared by dissolving 1g of basic fuchsin (pararosaniline chloride with a minimum dye content of 88%; Sigma-Aldrich, Poole, Dorset, UK) in 100 mL 60% alcohol, followed by 1 mL concentrated hydrochloric acid and 3 mL paraldehyde (BDH [now VWR International], Leicester, UK). It was allowed to ripen at room temperature for 3 days and then stored at 4°C.

The sections were rinsed in 70% alcohol, followed by water and then counterstained using a solution of 1% alcian blue 8GX (BDH) in 3% acetic acid (pH 2.5) for 3 min. They were subsequently washed in water, dehydrated, cleared and mounted. Other aldehyde fuchsin solutions, prepared using basic fuchsin from different suppliers (Polysciences [Warrington, PA, USA)], BDH and Hopkins and Williams – the latter two VWR International), were also assessed using the same technique.

When using this technique, it has previously been established[3] that sialomucins are alcian blue-positive (blue) whereas sulphomucins are aldehyde fuchsin-positive (purple).

Orcein/alcian blue

Sections were dehydrated in three changes of absolute alcohol, followed by a wash in water. They were subsequently immersed in four different solutions of orcein for 4 h. Each of the four solutions was prepared using orcein obtained from a different commercial supplier – natural orceins from Raymond Lamb (Eastbourne, UK) and BDH, and synthetic orceins from Sigma-Aldrich and BDH.

Each solution was prepared by dissolving 1g of orcein in 100 mL 70% alcohol and then adding 1 mL hydrochloric acid. The sections were then differentiated in 1% acid alcohol for a few seconds, followed by a wash in water and then counterstaining in 1% alcian blue 8GX in 3% acetic acid (pH 2.5) for 1 min. They were then washed in water before dehydration, clearing and mounting.

In addition, the effectiveness of pretreating the sections with a solution of 0.25% potassium permanganate in 0.25% concentrated sulphuric acid for 1 min, then 5% oxalic acid for 2 min followed by water before performing the above technique was also assessed.

It has been stated that this technique stains sialomucins blue (alcian blue-positive), whereas sulphomucins stain red/brown (orcein-positive).[9]

Alcian blue/ruthenium red

Sections were dehydrated in three changes of absolute alcohol before being washed in water and rinsed twice in 0.1 mol/L hydrochloric acid at pH 1.0. They were then treated with 0.5% alcian blue 8GX in 0.1 mol/L hydrochloric acid at pH 1.0 for 30 min, followed by a rinse in 0.1 mol/L hydrochloric acid and two rinses in 3% acetic acid at pH2.5.

The sections were subsequently treated with a solution of 0.5% ruthenium red (Sigma-Aldrich) in 3% acetic acid for various lengths of time, ranging from 5 to 80 min. The sections were then rinsed twice in 3% acetic acid and twice in water, followed by dehydration, clearing and mounting.

It has been proposed[10] that when using this technique the sialomucins are ruthenium red-positive, whereas sulphomucins stain blue (due to the low pH of the alcian blue).

Subtyping sections displaying intestinal metaplasia

Archived formalin-fixed, paraffin-embedded tissue blocks were selected from 20 intestinal-type gastric cancers and 20 Barrett-associated adenocarcinoma resection specimens that demonstrated IM in the non-neoplastic mucosa. The 40 cases

Table 3. Assessment marks for the orcein/AB technique using orcein obtained from four different suppliers. Marks were given for the intensity of sulphomucin staining.

Case	Synthetic orcein supplier				Natural orcein supplier			
	Sigma		BDH		Raymond Lamb		BDH	
	N	P	N	P	N	P	N	P
1	1	2	1	2	2	2	1	2
2	1	2	1	2	2	2	2	2
3	1	2	1	2	2	3	2	2
4	1	2	1	2	1	2	2	2
5	1	2	1	2	2	2	2	2
6	1	2	1	2	1	2	1	3
7	✓	✓	✓	✓	✓	0	✓	0
8	✓	✓	✓	✓	✓	0	✓	0
9	✓	✓	✓	✓	✓	✓	✓	✓

0 = false sulphomucin positivity, 1 = weak, 2 = moderate, 3 = intense, ✓ = no sulphomucin positivity in duodenum. Cases 1–5 = colon, 6 = ileum, 7–9 = duodenum. P = potassium permanganate pretreatment, N = no pretreatment.

were labelled 10 to 49. IM was identified in the archived haematoxylin and eosin-stained sections by the presence of goblet cells. Multiple sets of 4-μm sections were then cut from the blocks, dried on a hot plate for 20 min and subsequently stained with the GAF/AB technique, using Sigma basic fuchsin to prepare the aldehyde fuchsin solution, as previously described.

The percentage of columnar epithelium showing IM was then subjectively estimated by each observer. Where estimations differed when viewing a particular slide the mean of the two percentages was taken. Areas showing dysplasia or neoplasia were not included in the estimations. The metaplastic epithelium was then subtyped according to the mucin content found in the columnar cells between the metaplastic goblet cells (i.e., the intervening columnar cells). The percentage of each subtype was then estimated in each section.

Results

Gomori's aldehyde fuchsin/alcian blue

All sections showed blue, purple or blue/purple staining in the goblet cells. Elastic fibres stained purple and the background was clean. The staining intensity of alcian blue (blue) in the goblet cells remained constant; however, the staining intensity, pattern and quality of staining of the aldehyde fuchsin (purple) in these cells varied depending on the aldehyde fuchsin solution used.

Orcein/alcian blue

The sections showed blue, red/brown or a mixture of blue/red/brown staining in the goblet cells. Elastic fibres and nuclei were red/brown. In addition, red/brown background staining was present, and was particularly prominent in the sections stained with the synthetic orceins.

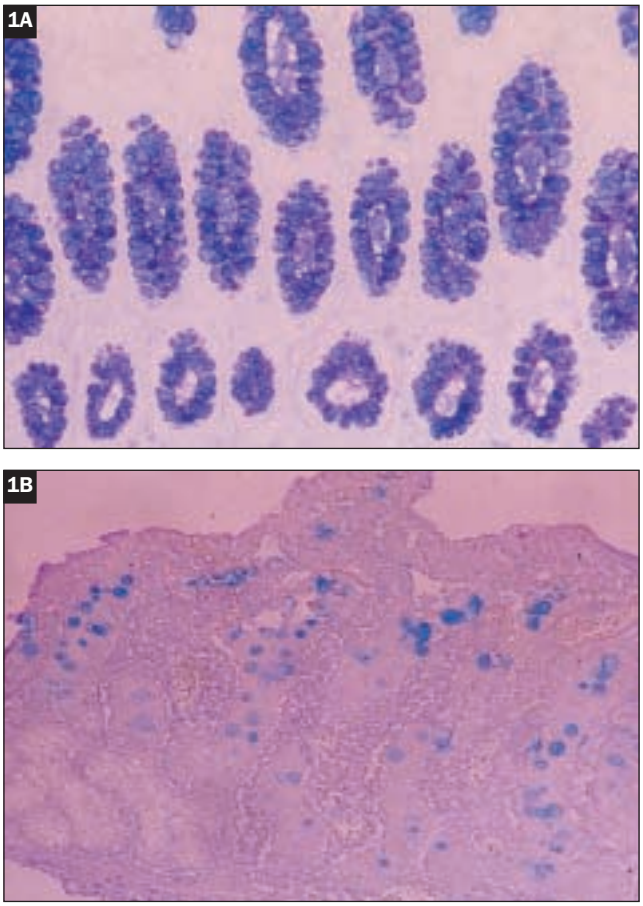

Fig. 1. Sigma GAF/AB-stained sections: (A) Colon – mixture of sulphomucins (purple) and sialomucins (blue) within goblet cells; (B) Duodenum – sialomucin positive (correct staining pattern)

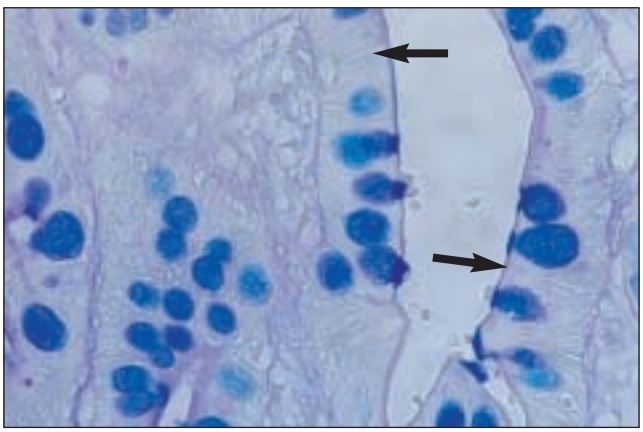

Fig. 2. Type I intestinal metaplasia demonstrated using the GAF/AB technique (original magnification x40). Goblet cells secrete either sulphomucin (purple) or sialomucin (blue); however, no mucin is found in the columnar cells that intervene the goblet cells (arrows). These are absorptive cells with a well-marked glycocalyx.

As with the GAF/AB technique, staining intensity of the alcian blue-positive mucin in the goblet cells remained constant. However, staining intensity, pattern and quality of staining of the goblet cells altered depending on which orcein stain was used. Furthermore, the pretreatment step caused considerable alterations in staining pattern, particularly in the goblet cells.

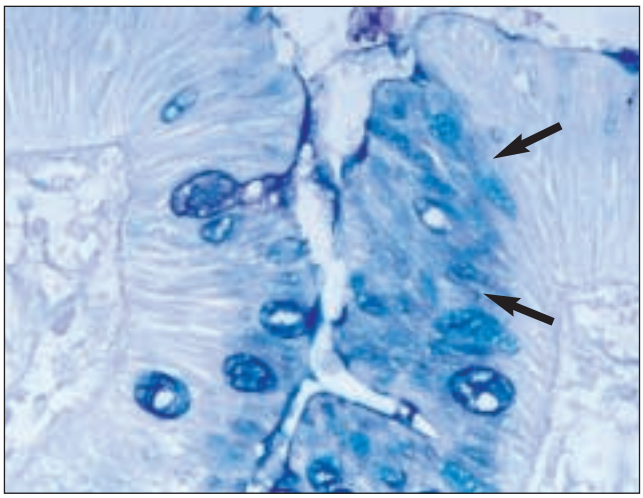

Fig. 3. Type II intestinal metaplasia demonstrated using the GAF/AB technique (original magnification x40). Goblet cells secrete either sulphomucin or sialomucin; however, the intervening columnar cells only secrete the latter so are only alcian blue-positive (arrows).

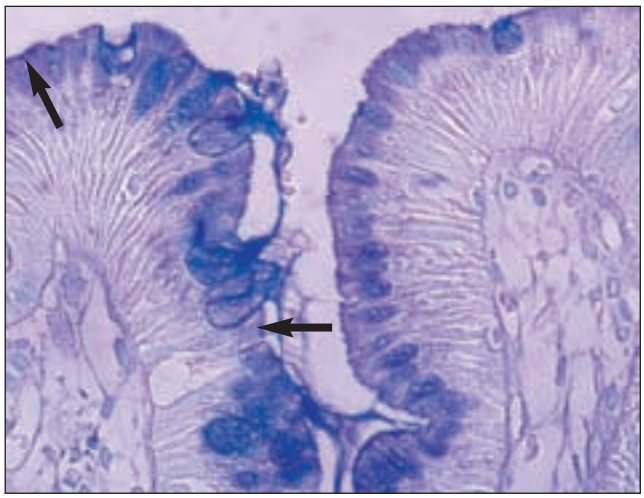

Fig. 4. Type IIIb intestinal metaplasia demonstrated using the GAF/AB technique (original magnification x40). Goblet cells secrete either sulphomucin or sialomucin. The intervening columnar cells are strongly sulphomucin-positive (purple) (arrows).

Alcian blue/ruthenium red

Only alcian blue positivity (blue) was seen in the goblet cells. No ruthenium red positivity (apparent red staining) was observed in any of the sections, regardless of incubation time. Therefore, the method was not evaluated further.

Assessment of GAF/AB and orcein/AB techniques

As the different basic fuchsin and orcein dyes used in these two techniques gave different staining results, the assessors awarded each stained section an assessment mark. Marks were given for the intensity of sulphomucin staining within goblet cells as follows (Tables 2 and 3):

0 False sulphomucin positivity was observed in the sections of duodenum
1 Weak
2 Moderate
3 Intense
✓ Sulphomucin positivity was absent in sections of duodenum (correct staining pattern)

Optimum method

On the basis of these assessments we decided that the GAF/AB technique using Sigma-Aldrich basic fuchsin to prepare the aldehyde fuchsin solution was the optimum method (Fig. 1). Therefore, this technique was used to subtype the cancer-associated IM in the surgical specimens.

Subtyping sections displaying intestinal metaplasia

A mixture of normal, dysplastic, neoplastic and metaplastic (both intestinal and gastric metaplasia in the oesophagus) epithelium were observed in each of the 40 haematoxylin and eosin-stained sections. It should be noted that cases 48 and 49 were eliminated from the study as these sections displayed dysplastic metaplasia rather than true IM.

The GAF/AB-stained sections showed aldehyde fuchsin and/or alcian blue positivity within some of the neoplastic areas; however, no staining was observed in the areas of dysplasia. The areas of IM were very apparent due to an intense blue, purple or blue-purple staining of the goblet cells. In most sections, the intervening columnar cells showed a mixture of blue, purple or blue–purple staining, indicating that a 'mosaic' pattern of subtypes was present.

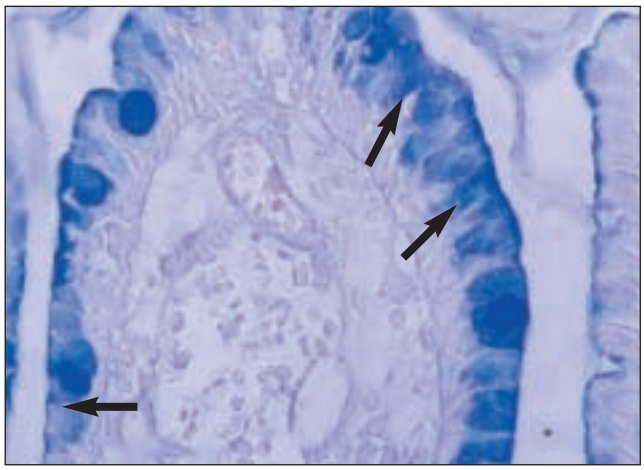

Fig. 5. Type IIIa intestinal metaplasia demonstrated using the GAF/AB technique (original magnification x40). Goblet cells secrete either sulphomucin or sialomucin. Intervening columnar cells secrete predominantly sialomucin (blue) but a trace of sulphomucin positivity (purple) is observed at high power (arrows).

In attempting to subtype the metaplastic areas according to the classification of Jass and Filipe[4] (as described in Table I), type I was generally readily identified (Fig. 2). However, we found that type II (pure blue staining of the intervening columnar cells [Fig. 3]) was almost non-existent and there seemed to be many intervening columnar cells that contained purple-stained sulphomucin. However, there was substantial variation in the amount of sulphomucin contained in each aldehyde fuchsin-positive cell; some showed dense uniform purple staining (Fig. 4), while others were either predominantly blue or colourless when viewed at low power (x16) but showed faint granular aldehyde fuchsin positivity when viewed at high power (x40) (Fig. 5).

Consequently, a new subtyping classification was proposed (Table 4). The appearance of each subtype (IIIa and IIIb) was the same in the sections displaying gastric intestinal metaplasia and those with Barrett's oesophagus.

Table 4. New classification for the identification of intestinal metaplasia subtypes using the GAF/AB technique.

IM type	Cell	Mucin content	Colour
I	Goblet	Sialomucin and/or sulphomucin	Blue and purple
	Columnar	No mucin	None
II	Columnar	Sialomucin	Blue
IIIa	Columnar	Some sulphomucin	Blue or clear with trace of purple
IIIb	Columnar	All sulphomucin	Purple

Accordingly, the percentages of each 'new type' of IM were estimated in the metaplastic areas of the test sections and the figures are shown in Tables 5 and 6.

Discussion

In 1997 Shah *et al*.[3] evaluated the GAF/AB technique for the demonstration of IM subtypes and suggested that it was suitable for their routine identification. In that study, the use of Sigma-Aldrich basic fuchsin for the preparation of aldehyde fuchsin was recommended, and we agree with that. From the results of the present study, the other aldehyde fuchsin solutions either gave weak, inadequate staining or false staining patterns (i.e., sulphomucin positivity was observed in goblet cells of the duodenum). The reason for this is unclear.

Disappointing results were also obtained when using the orcein/alcian blue and alcian blue/ruthenium red staining procedures. The former gave either weak staining (also accompanied by background staining in many cases) or false staining patterns, particularly in the sections that had undergone the potassium permanganate pretreatment. In our hands, this latter histochemical procedure failed to give any ruthenium red positivity. Perhaps increasing the concentration or altering the pH of the ruthenium red solution could be examined in a future evaluation.

All the test sections displaying IM were stained with the GAF/AB technique, using Sigma-Aldrich basic fuchsin for the preparation of the aldehyde fuchsin solution because of its superior results in the first part of the study.

Subtyping of the metaplastic regions was attempted initially using the classification suggested by Jass and Filipe.[4] However, as already discussed, we felt that a new classification should be proposed (Table 4). When analysing previous work on this subject it is difficult to determine whether or not specimens were categorised according to the predominant type or, in the case of type III IM, merely on its presence. Thus, the frequencies expressed are open to question.

For instance, in a small group of gastric cancers (n=14), Baracchini *et al*.[14] found Type III in eight (57 %) cases. However, this was always in combination with another type of IM – type I in six cases, type II in one, and all three types in a further case. There is no indication as to the predominant type present.

To our knowledge, no previous study has assessed the relative proportions of the IM subtypes in cancer cases. With this proviso, comparison of subtyping results (Table 7) shows

Table 5. Estimated proportion of non-neoplastic/dysplastic columnar epithelium exhibiting IM and the extent of the newly defined intestinal metaplasia subtypes found in cases of gastric cancer-associated intestinal metaplasia. The frequency of the IM subtype as the predominant type present is also shown.

Case	Columnar epithelium showing IM (%)	Subtypes in metaplastic area (%)				
		I	II	IIIa	IIIb	IIIa+IIIb
10	1	100	0	0	0	0
11	85	5	10	10	75	85
12	10	20	0	80	0	80
13	90	80	5	15	0	15
14	30	90	0	5	5	10
15	15	95	0	5	0	5
16	75	40	0	15	45	60
17	100	40	0	60	0	60
18	90	10	5	70	15	85
19	40	90	0	10	0	10
20	10	40	0	20	40	60
21	80	30	5	40	25	65
22	15	60	0	35	5	40
23	30	80	0	10	10	20
24	50	70	5	25	0	25
25	50	90	0	10	0	10
26	75	95	0	5	0	5
27	10	80	0	20	0	20
28	60	50	0	20	30	50
29	50	40	5	35	20	55
Mean	48.3	60.25	1.75	24.50	13.50	38.0
SD	32.17	30.59	2.94	22.59	20.40	29.10
Range	1-100	5-100	0-10	0-80	0-75	0-85
Frequency (predomin-ant type)	-	55	0	20	5	45

that the frequency of type III (our IIIa and IIIb) IM associated with gastric cancer (45%) is higher than those of Filipe *et al*.[15] and Silva and Filipe.[16] However, comparing the frequency of types I and II, there is no correlation between any of the results.

Nonetheless, it is interesting that the amount of type I IM observed in the present study is rather high. It appears that the increased amount of this subtype is at the expense of type II. It is possible that some intervening cells were understained for sialomucin and thus false-negative cells were catagorised as type I. The alcian blue incubation time is not stated in the other studies. However, other morphological features like the presence of a glycocalyx on absorptive cells come into the identification of type I IM.

Previous subtyping of IM in Barrett's oesophagus has also produced variable results. For instance, one study[2] found the frequency of type III IM to be 60%, while another[17] found it to be as high as 93.8%. We found type III (IIIa and IIIb) in 89% of cases, and as the predominant subtype in 78%.

Our study has revealed inherent difficulties in the

Table 6. Estimated proportion of non-neoplastic/dysplastic columnar epithelium exhibiting IM and the extent of the newly defined intestinal metaplasia subtypes found in cases of Barrett-associated adenocarcinoma. The frequency of the IM subtype as the predominant type is also shown.

Case	Columnar epithelium showing IM (%)	Subtypes in metaplastic area (%)				
		I	II	IIIa	IIIb	IIIa+IIIb
30	100	0	5	95	0	95
31	100	5	5	5	85	90
32	100	10	75	10	5	15
33	100	5	0	60	35	95
34	100	0	0	95	5	100
35	100	0	10	60	30	90
36	100	60	0	20	20	40
37	100	0	0	40	60	100
38	100	5	0	10	85	95
39	100	25	0	75	0	75
40	95	20	0	80	0	80
41	100	5	10	25	60	85
42	100	15	30	40	15	55
43	5	100	0	0	0	0
44	100	5	15	70	10	80
45	100	0	0	0	100	100
46	100	20	0	10	70	80
47	50	100	0	0	0	0
Mean	91.67	20.83	8.33	38.61	32.22	70.80
SD	24.61	32.23	18.39	34.21	34.95	34.18
Range	5-100	0-100	5-75	0-95	0-100	0-100
Frequency (predomin-ant type)	-	17	6	39	33	78

identification of IM subtypes because there are many variables and inconsistencies involved.

Some sulphomucin-positive cells contain trace amounts only identifiable at high power – can these really be identified as the same subtype as strong sulphomucin expressors? We propose a new classification to take account of this.

There were occasional difficulties in distinguishing 'true' goblet cells from vacuolated sialomucin-positive intervening columnar cells. Are the latter 'immature' goblet cells in otherwise type I metaplasia or indicative of type II IM?

Some areas of IM that were morphologically type I (i.e., goblet cells and absorptive cells) were sometimes seen to express sialomucins or sulphomucins in the intervening 'absorptive-type' cells, indicating categorisation as type II or III. Is it correct to disregard the morphology of the cells and only place importance on its histochemistry, which may be vague anyway for the above reasons?

With these problems in mind, it appears that the subtypes of IM cannot be placed into neat categories, as there is overlap in both distribution and development. It is evident that there are both 'mosaics' of different subtypes and

Table 7. Frequency of predominant IM subtypes (%) in gastric cancer-associated IM: comparative data from other studies.

	n	Type I	Type II	Type III
Filipe *et al* [15]	24	29	36	35
Silva and Filipe[16]	72	43	21	36
Present study	20	60	2	38

transitional forms that are impossible to categorise. The existence of mosaic patterns was emphasised previously by Baracchini *et al*., who found all combinations of IM subtypes present in 41% of 217 cases of gastric IM.[14] They concluded that this provided additional evidence of impaired maturation and aberrant differentiation along various pathways that incorporated both small- and large-intestinal characteristics.

In conclusion, the results of this study showed that the GAF/AB technique, using Sigma-Aldrich basic fuchsin, is a suitable alternative to the HID/AB staining procedure for the discrimination of sulphomucins and sialomucins. As previously suggested, type III IM is recognised by the presence of sulphomucins in intervening columnar cells, and could be used to help identify this subtype.

However, this study also revealed that not all areas of IM can be classified in the clearly defined way that some authors have implied. Indeed, there are many variables and discrepancies involved in identifying the different subtypes. Therefore, we conclude that IM subtyping using histochemical analysis is a highly subjective procedure, open to varying interpretation. Consequently, previous work that has defined cancer risk based on the identification of metaplasia subtypes by mucin histochemistry should be viewed with some circumspection. □

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