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ALCIAN BLUE pH;

THE EFFECT OF STAINING FOR PRIMARY GIT EPITHELIAL TUMOURS IN PATIENTS DIAGNOSED AT MUHIMBILI NATIONAL HOSPITAL, TANZANIA

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ABSTRACT: Background: Alcian blue (AB) is a cationic dye that stains mucins especially acidic mucins into varying shades of blue colour depending on the pH of the dye. GIT comprises of three main portions, fore-, mid- and hind-gut, which develops from different embryological areas. Their epithelial lining presents with varying degree of diversity including their secretions. Studies have shown that there is a tendency of cellular modification (physical and chemical) including secretions during tumour morphogenesis. With recent increase in GIT tumours especially epithelial tumours, Alcian Blue staining of mucins produced by these tumours at different pH more than conventional pH might give valuable information on the property of these tumours. **Setting:** The study was conducted in the Histopathology and Morbid Anatomy unit, department of Laboratory services, Muhimbili National Hospital, Tanzania. **Study design:** This was a hospital based retrospective study, in which archival data and blocks were retrieved. **Objective:** To determine the effect of pH and pattern of Alcian blue staining on primary GIT epithelial tumours tumours. **Materials and Methods:** Information on patients were obtained from cancer registry and patient files. Paraffin blocks were retrieved from archive, sections were cut using rotary microtome at 3μm (SAKURA). Haematoxylin and Eosin (H&E) and AB staining at pH 1, 1.5, 2 and 2.5 was done for each case and control. H&E slides were reviewed for confirmation of the diagnosis primary epithelial tumours and AB stained slides were evaluated for staining reaction and graded. **Results:** Out of 87 GIT primary epithelial tumours which were evaluated, AB staining was positive in 21 (24.1%) cases, the majority of these (11 (52.4%)) were from hindgut. Positive AB staining of GIT epithelial tumours increased as the tumour became more differentiated irrespective of location. Majority of tumours with positive AB staining was observed at pH 2 in GIT epithelial tumours as opposed to the conventional pH of 1 and 2.5 respectively. **Conclusions and recommendation:** The majority of primary GIT epithelial tumours stained positively at pH 2 irrespective of the location. However tumour differentiation influenced AB staining whereby well-differentiated tumours were mostly positively stained. It is recommended that AB at pH 2 should be applied when staining GIT epithelial tumours rather than conventional pH of 1 and 2.5. However the degree of differentiation should be considered since poorly differentiated tumours are likely to give negative results with AB staining.

Key words: Alcian Blue, pH, Staining, GIT Tumours

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INTRODUCTION

Alcian Blue (AB) is large conjugated dye molecule that initially was used for the dying of textile fibers. It is comprised of central copper containing pthalocynine ring linked to Isothiouronium groups via thioester bonds. The Isothiouronium are moderately strong bases and account for the cationic nature of AB, and therefore reaction with anionic groups[1]. The reaction of AB merely depends on the pH of surrounding environment. A variety of different AB dyes have been produced in the past, but for histopathology laboratory, AB 8G (formerly AB 8Gx) is recommended.

Embryologically, GIT develops from three different areas and hence divided into foregut, midgut and hindgut. Foregut includes oesophagus, stomach, first and second part of duodenum and they develop from primitive foregut of yolksac. Midgut which develops from vitello-intestinal duct, includes third and fourth part of duodenum, jejunum, ileum, caecum, appendix and ascending and transverse colons. The hindgut which develops from primitive hindgut, includes

descending and sigmoid colons, rectum and upper two third of anal canal[2]. These areas are lined by different epithelia and produce mucins of varying chemical properties, it is interesting to understand the pattern of staining of these mucins and if these pattern is retained or modified in primary GIT epithelial tumours.

Mucins are high molecular weight glycoproteins that are found dispersed throughout the epithelia of GIT, respiratory and reproductive tract. Mucins are composed of a central protein core with multiple chains of carbohydrates attached. Protein core contain a high content of the amino acid serine and threonine. A defining structure of the mucins is the presence of tandem repeats of specific amino acid sequences within the protein core. From molecular perspective, mucins are categorized into distinct families (Muc 1, Muc 2, Muc 3 etc) based upon difference in the sequence and size of the tandem repeats.

Histochemical reactivity is dependent largely upon the carbohydrate composition of the mucins and not protein core. Histochemically, mucins are classified into neutral mucins and acidic mucins. Acidic mucins contain carbohydrate with carboxylate or sulphonate groups. Both of these groups are ionized at physiologic pH to produce an overall negative charge of these mucins. Carbohydrates of neutral mucins lack acidic groups and thus carry no net charge, they are found primarily on the surface epithelia of stomach, Brunner's glands of duodenum and in prostatic epithelium. The acid mucins are found widely distributed throughout the GIT and respiratory tract.

Expression of mucins is a property of epithelial cell types that exist in relatively harsh environments. Mucin's key characteristic is its ability to form gels; therefore they are a key component in most gel-like secretions, serving functions such as lubrication, cell signaling and forming chemical barriers[3]. In addition, mucins also communicate the information of the external environment to the epithelial cells via cellular signalling through membrane-anchored mucins.

Mucus provides a protective barrier against pathogens and toxins and contributes to the innate defensive system in mucosal immunology. It has been shown that mucins play a role in the processes of tumour progression, invasion and metastasis and also in tumour cell survival and protection against the host immune response[2,4].

Tumours, especially malignant tumours have a tendency to modify cellular properties and their products[5,6,15,16] including physical and chemical properties of native cells from which they develop. Malignant tumours of the gastro-intestinal tract are not as rare as previous studies suggest. Recent studies have indicated increasing incidence[7], where malignant epithelial tumours is leading in its occurrence than other types like sarcomas, carcinoid tumour, Non Hodgkin Lymphoma etc[7,8], it follows therefore that, proper understanding of malignant epithelial tumours of GIT is of paramount importance.

Studies have shown that, there is a tendency of increased production of sialomucins in various pathological conditions, with decreased O-acetylation as a common early feature of malignant and premalignant epithelial disorders, particularly in colon. This has been demonstrated both histochemically and biochemically in colorectal cancer, ulcerative colitis and in colonic adenoma[1,3,5,6].

Staining of mucins especially in epithelial tumours, can add valuable information in the characterization of these tumours. A number of histochemical techniques can be used to stain and characterize these mucins. Commonly used mucin special stains include AB, mucicarmine, colloidal iron and PAS. Mucicarmine, colloidal iron and AB stains acidic mucins because they react with carboxylate or sulphonate group, hence stain mainly mucin of epithelial origin, conversely to PAS, which stain both acidic and neutral mucins apart from other glycoproteins and carbohydrates. For the sake of this study, AB will be used at different pH and evaluate the effect especially for intracellular . AB staining is capable of detecting sulfomucins and sialomucins but not neutral

mucins, which is mainly found on the surface of epithelium. The carboxylated sialomucins do not demonstrate the same magnitude of acidity as the sulfomucins as these are known to stain over a long range of pH[9,10], as a result these groups are not capable of ionization at a pH of 1 or less. The sialomucins therefore are not charged at this pH1. Conversely, sulfomucins are ionized and negatively charged at pH1. It follows that, staining with AB at pH1, is predominantly due to sulfate groups among mucins[9,10,11]. Tissues known to stain positive with AB at pH1 includes cartilage, goblet cells mucins of the large intestines and the mucins of the bronchial serous glands. Acidic epithelial mucins like sialomucins and sulfomucins from large intestines are reactive at pH 2.5. Neutral mucins like those in gastric mucosa and Brunner's glands are not reactive with AB and are mainly found on the surface epithelium, which is beyond the scope of this study. This shows that, mucin produced in different parts of the GIT in normal and abnormal conditions, tends to behave differently[12,13,15,16].

It is known that, mucins produced in the GIT present with varying degree of properties; this is true because these cells develops from different part from embryological point of view. An increase in the GIT tumour prevalence has been noted in past few years most especially, epithelial tumours which are associated with production of mucin. These tumours have a tendency to modify cellular properties and their products[5,6] including physical and chemical properties of native cells from which they develop. It is important to characterize these tumour properly including mucins they secretes.

There is a need to characterize mucins produced by GIT epithelial tumours to understand whether AB staining pattern at convectional pH of 1 and 2.5 is retained or is changed and at which pH these mucins stain positively. This will help to understand and properly characterize these tumours into fore-, mid- and hind-gut in the GIT using simple and cost effective technique, for low resource country like Tanzania. Understanding specific pH at which particular tumours stain necessitated introduction of other pH ranges rather than using the convectional pH of 1 and 2.5 only. Immunohistochemistry or combination of techniques for mucins was not considered because of cost and time of the study. Staining GIT primary epithelial tumours with varying pH of AB dye, can give valuable information in the characterization of these tumours

MATERIALS AND METHODS

The objective of the study was to determine the effect of pH and pattern of Alcian blue staining on primary GIT epithelial tumours and characterize them in relation to location (fore-, mid, and hind-gut).

The study was a hospital based retrospective study, in which archival data and blocks were retrieved which was conducted in Histopathology and Morbid Anatomy unit, department of Laboratory services, Muhimbili National Hospital, Tanzania from 2010 to 2012.

The information on patients were obtained from cancer registry and patient files. The blocks from 88 patients diagnosed to have GIT primary epithelial tumour were taken as cases and 40 blocks with non tumourous conditions like inflammation were regarded as "normal". Appendix following appendectomy of non tumourous patient was taken as external control for AB at pH 1 and transverse colon without a tumour as external control for pH 2.5. Thereafter blocks were retrieved from archive, sections were cut with rotary microtome at 3□m (SAKURA) and Haematoxylin and Eosin (H&E) and AB staining at pH 1, 1.5, 2 and 2.5 was done for each case and control. H&E slides were reviewed by a pathologist for confirmation of the diagnosis primary epithelial tumours and AB stained slides were evaluated for staining reaction and graded.

Data Collection and Analysis

Data collection was done in designed structured in tables. Microscopy to determine AB staining of glandular cell mucins was done independently by a histotechnologist and pathologist, where pathologist grading was regarded as gold standard. AB staining was grouped into positive

and negative, where positive cases were graded as mild (+) for pale blue staining, moderate (++) for blue staining and strong (+++) for deep blue staining while negative cases were those without intracellular epithelial AB staining. The intense staining was taken as the best pH at which the case stained.

Ethical issues

Permission to access blocks and patient files, use of machines and other materials was sought from Head of Histopathology Unit. Also ethical clearance was sought from Muhimbili University of Health and Allied Sciences (MUHAS) ethical clearance committee.

pH meter measurement.

pH solution was measured with a pH meter Consort C830 a mult parameter analyzer (manufactured by Consort Electrophoresis power supplies, Tumhout, Belgium) which measures pH in two decimal places. Before AB solution pH measurements, the pH electrode previously stored in KCL solution was then rinsed in a beaker containing distilled water and thereafter, the electrode was immersed in a container that contained raw AB solution and the pH was recorded

Microtomy

Sections of 3μm were cut from paraffin blocks cooled on ice blocks, using rotary microtome (SAKURA Model SRM 200 CW), where four (4) sections were cut per block, then sections floated on water bath at 45^{o}C, mounted on standard frosted glass slides each labeled with ID number and respective pH to be stained. Slides were allowed to drain before being put on hot plate at 60oC for 15 min.

ALCIAN BLUE (AB) Staining procedure

The AB staining at varying pH was made using the following combination of reagents

Reagents

AB pH 2.5

- Alcian blue 8GX…….. 5.0g
- 3% Acetic acid solution…. 100ml

AB pH 2.0

- Alcian blue 8GX ……. 5.0g
- 3% Acetic acid …….. 100ml

AB pH 1.5

- Alcian blue 8GX …….. 5.0g
- 0.1N Hydrochloric acid ……. 100ml

AB pH 1

- Alcian blue 8GX …….. 5.0g
- 0.1N Hydrochloric acid ……. 100ml

Weighing scale (ADAM) was used to measure alcian blue powder and pH was measured by pH meter (CONSORT C830) and adjusted using glacial acetic acid, potassium hydroxide and hydrochloric acid as required.

Sections were dewaxed and brought down to distilled water. Sections were dipped in AB stain of appropriate pH for 30minutes following mordanting in 3% acetic acid for pH 2.5 and 2 or 0.1N Hydrochloric acid for pH 1 and 1.5, then washed in water, counterstained with Neutral red for 5 minutes, washed in water, dehydrated, cleared and mounted using automated mounting machine.

Slides were examined using light microscope, where first the controls slides were examined to assess the technique and staining results and then the staining of tumour cases and normal epithelium was examined for quality and intensity of staining, whereby the intensity of positive results was graded into + for mild, ++ for moderate and +++ for strong AB staining.

RESULTS

The study comprised of 88 epithelial malignant tumour cases from GIT, diagnosed based on H/E staining. There were no cases of benign tumour in this study. Out of 88 GIT tumour cases 75 (85.2%) were adenocarcinoma and 13 cases (14.8%) were other malignant epithelial tumours especially squamous cell carcinoma. Histomorphological diagnoses showed that 35 (39.8%) of these tumour were well differentiated in which 26 were adenocarcinomas, 8 squamous cell carcinomas and 1 carcinoid tumour. Twenty eight (28), (31.8%) were moderately differentiated which consisted of 26 adenocarcinoma and 2 squamous

cell carcinoma and 25 (28.4%) were poorly differentiated in which 23 were adenocarcinoma and 2 squamous cell carcinoma.

Forty two (42) cases were from foregut of which 31 were adenocarcinoma and 11 other epithelial tumours particularly squamous cell carcinoma, 15 cases from midgut out of which 13 were adenocarcinoma and 2 squamous cell carcinoma and 31 cases found in hindgut were all adenocarcinomas.

Fifty seven (57), (64.8%) cases were male patients and 31 (35.2%) female patients. The age ranged from 1day to 93years, with large group of patients aged 30 to 80 years Of the 38 GIT "normal" cases without tumour were regarded as controls and stained with AB at varying pH; 11 were from foregut, 14 from midgut and 13 from hindgut. Of 38 GIT controls studied, 25 (65.8%) stained positively, and out of these 22(88.0%) stained intensely at pH 2 and 3(12.0%) stained intensely at pH 2.5. There was no staining on foregut "normal" tissues (Table I)

Of 87 primary GIT epithelial tumour cases studied only 21 (24.1%) were positively stained with AB, of these 11(52.4%) were from hindgut, 4(19.0%) from midgut and 6(28.6%) from foregut especially from epithelial tumours of the secretory part. Majority (18(85.7%)) of GIT epithelial tumour cases stained with AB intensely at pH 2 (Table II).

Regarding the degree of differentiation of tumours, 13 (62.0%) which stained positively with AB were well differentiated and 4 (19.0%) were moderate and poor differentiated. There was an increase in the number of cases stained with AB as differentiation of tumours decreased from well to poor (Table III).

	Staining intensity (pH)													
	1			1.5			2			2.5				
Location	+	++	+++	+	++	+++	+	++	+++	+	++	+++	-ve	+ve
Foregut	-	-	-	-	-	-	-	-	-	-	-	-	11	0
Midgut	-	-	-	-	-	-	-	3	8	1	-	1	1	13(92.9%)
Hindgut	-	-	-	-	-	-	-	3	8	-	-	1	1	12(92.3%)
Subtotal	-	-	-	-	-	-	-	6	16	1	-	2	13	
Grand Total	0			0			22			3			13	25(65.8%)

Table-I. Staining intensity of "normal" GIT epithelium in relation to pH
"Normal": - controls

	Staining intensity (pH)													
	1			1.5			2			2.5				
Location/ Tumour	+	++	+++	+	++	+++	+	++	+++	+	++	+++	-ve	+ve
Foregut	-	-	-	-	-	-	2	3	-	1	-	-	35	6(14.6%)
Midgut	-	-	-	-	-	-	1	3	-	-	-	-	11	4(26.7%)
Hindgut	-	-	-	-	-	-	4	3	2	2	-	-	20	11(26.8%)
Subtotal	-	-	-	-	-	-	7	9	2	3	-	-	66	21(24.1%)
Grand Total	0			0			18			3			66	21

Table-II. Intensity of AB staining of primary GIT tumours according to location at varying pH
** Carcinoid tumour not included*

	Differentiation							
Location	PD		MD		WD		Total	
	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
Foregut	9	1(10.0%)	13	1(7.1%)	13	4(22.2%)	35	5
Midgut	4	1(20.0%)	4	1(20.0%)	3	2(40.0%)	11	4
Hindgut	3	2(40.0%)	7	2(22.2%)	10	7(41.2%)	20	11
Total	16	4(19.0%)	24	4(19.0%)	27	13(62.0%)	66	21

Table-III. AB staining of GIT primary tumours in relation to Location and Differentiation
**Carcinoid tumour not included.*

Key:
WD: Well differentiated MD: Moderately differentiated PD: Poorly differentiated
+: Mild AB staining ++: Moderate AB staining +++: Strong AB staining
+ve: Positive results with AB staining -ve: Negative results with AB staining

DISCUSSION

Alcian Blue (AB) staining of controls ("normal") of GIT epithelium was consistent with the expected normal GIT staining pattern, however the staining intensity showed that, majority of "normal" epithelium (88 %), stained at pH 2, this finding can be explained by the facts that "normal" epithelium in this study was taken from non tumourous conditions like inflammatory diseases. This might have affected the outcome of staining.

AB staining of GIT primary epithelial tumours were positive only for 21 (24.1%) cases, of these 11 (52.4%) were from hindgut, 4(19.0%) from midgut and 6(28.6%) from foregut. This shows that there were more AB staining of GIT tumour cases observed in hindgut than other sites, although this might have been attributed to the number of tumour cases studied in each location. Also the predominance acidic mucin produced in hindgut as compared to midgut which has a mixture of both neutral and acidic mucin and also predominance of neutral mucin produced from foregut may be a characteristic nature of the respective location which is retained in tumours arising from these sites.

The staining pattern for the majority of GIT primary epithelial tumours were observed to stain intensely at pH 2, this might have been attributed to the fact that most of tumours were preceded by inflammation which could have changed the physiochemical nature of mucin secreted. These findings is supported by other studies[5], who found in their study that inflammation and other pre malignant conditions increased production of sialomucins with decreased O-acetylation therefore changing physiochemical nature of mucin produced.

Regarding the degree of differentiation, majority of well differentiated GIT primary epithelial tumours 13 (62.0%) positively stained with AB while 4(26.7%) and 6(14.6%) were found in midgut and foregut respectively. This might be explained by the fact that, well differentiated tumours retain a significant number of their normal genes and therefore express the same physiochemical and genetic characteristics during tumorigenesis.

Despite more positive results with well differentiated tumours compared to poor and moderate differentiated tumours, a significant number of cases were stained negatively with AB. This might be explained by the facts that, during tumorigenesis the switch off mechanism for genes controlling mucin production occurred very early, hence physiochemically the tumour cells failed to secrete mucin. Furthermore; molecular changes of genes that control protein core of mucin may have played a big role toward this finding as supported by previous studies[1,15,16].

CONCLUSION AND RECOMMENDATION

The majority of primary GIT epithelial tumours stained positively at pH 2 irrespective of the location. However the tumour differentiation influenced AB staining, whereby well differentiated

tumours were mostly positively stained.

From our study, we recommend that AB at pH 2 should be applied when staining GIT epithelial tumours rather than convectional pH of 1 and 2.5. However the degree of differentiation should be considered since poorly differentiated tumours are likely to give negative results with AB staining.

REFERENCES

1. Bancroft J D, Gamble M. **Theory and Practice of Histological Techniques.** Churchill Livingstone Elsevier. Sixth edition (2008).

2. Morson BL, Dawson IMP, Day DW, Jass JR, Price AB, Williams GT. Morson & Dawson's Gastrointestinal Pathology. Blackwell Scientific Publications. Third edition.

3. Ali U, Nagi AH, Naseem N, Ullah E. **Mucin Histochemistry in Tumours of Colon, Ovaries and Lung.** Journal of Cytology and Histology 2012. 3: 163. doi:10.4172/2157- 7099.1000163.

4. Komatsu M, Yee L, Carraway KL. **Over expression of sialomucin complex, a rat homologue of MUC4, inhibits tumor killing by lymphokine-activated killer cells.** Cancer Research 1999 May; 59(9):2229-36.

5. Milton J D, Eccleston D, Parker N, Raouf A, Cubbin C, Hoffman J, Hart C A, Rhodes J M. **Distribution of 0-acetylated sialomucin in the normal and diseased gastrointestinal tract shown by a new monoclonal antibody.** Journal of Clinical Pathology 1993; 46:323-329.

6. Habib NA, Smadja C, Dawson P, Wood CB. **Histochemical changes of the Intestinal mucins in benign and malignant lesions of the colon and rectum.** Gastroenterology and Clinical Biology 1985; 9:491-494.

7. Abdulkareem FB, Faduyile FA, Daramola AO, Rotimi O, Banjo AA, Elesha SO, Anunobi CC, Akinde OR, Abudu EK. **Malignant Gastrointestinal tumours in South Western Nigeria; a Histopathologic analysis of 713 cases.** West Africa Journal of Medicine 2009; 28(3)173-176.

8. Galkin VN, Maistrenko NA. **Non-epithelial tumours of Gastrointestinal Tract: Diagnosis and Surgical treatment.** Khirurgia (Mosk)2003, (1)22-26.

9. Jones R and Reid L. **The Effect of pH on Alcian Blue staining of epithelial acid glycoproteins. I. Sialomucins and Sulphomucins (Singly or in simple combinations).** Histochemical journal 1973; 5(1) 9-18.

10. Jones R and Reid L. **The Effect of pH on Alcian Blue staining of epithelial acid glycoproteins. II. Human bronchial submucosal gland.** Histochemical journal 1973; 5(1) 19-27.

11. Niv Y. **MUC1 and colorectal cancer pathophysiology considerations.** World Journal of Gastroenterology April 2008. 14 (14): 2139–41.

12. McNeer RR, Price-Schiavi S, Komatsu M, Fregien N, Carraway CAC and Carraway K L. **Sialomucin Complex in Tumours and Tissues.** Frontiers in Bioscience 2, d449-459, September 15, 1997.

13. Corfield AP, Carroll D, Myerscough N, Probert CS. **Mucins in the gastrointestinal tract in health and disease.** Frontiers in Bioscience; a Journal and Virtual library, 2001 Oct 1;6:D1321-57.

14. Carson FL. **Histotechnology,** A self Instructional Text. ASCP.

15. Wabinga HR. **Mucin secretion by gastric carcinoma cells; PAS-AB stain study.** East Africa Medical Journal. 1996 May; 73 (5suppl):S19-20.

16. Ionila M et al. **Mucinous adenocarcinoma of the colon; A histochemical study.** Romania Journal of Morphological embryology. 2011:52(3): 783-90.