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New algorithm to differentiate histochemical types of intestinal metaplasia: G&S2 method

Novo algoritmo para diferenciar tipos histoquímicos de metaplasia intestinal: Método G&S2

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ABSTRACT

Introduction: The classification of intestinal metaplasia by histochemistry methods has been described as the most appropriate. However, current algorithms are not replicable in laboratories due to severe limitations.
Objective: To evaluate a new algorithm to differentiate histochemical types of intestinal metaplasia.
Material and Method: Cross-sectional research in which 512 gastric biopsies with intestinal metaplasia (paraffin blocks) were evaluated by a new algorithm using two types of Alcian Blue dyes during February-March of 2020 in the pathological anatomy service of the Maria Auxiliadora Hospital, Lima, Peru. This evaluation consisted of two steps: visualization of acid mucins in the columnar cells of the gastric mucosa and calculation of the weighted Kappa statistic.
Results: Histochemical types of intestinal metaplasia showed as follows: Type I, 398 (77.7%); Type II, 81 (15.8%) and Type III, 33 (6.5%). The weighted Kappa statistic was 0.79 ($p<0.001$), rated as an important or good concordance.
Conclusion: This new algorithm demonstrated it was useful and capable of identifying and differentiating the histochemical types of intestinal metaplasia, in addition to having statistical reliability.

Key words: gastric mucins; alcian blue; gastric mucosa.

RESUMO

AIntrodução: *A classificação da metaplasia intestinal por métodos histoquímicos tem sido descrita como a mais adequada. No entanto, os algoritmos atuais não são replicáveis ??em laboratórios devido a limitações severas.*
Objetivo: *Avaliar um novo algoritmo para diferenciar tipos histoquímicos de metaplasia intestinal. Material e*
Método: *Pesquisa transversal em que 512 biópsias gástricas com metaplasia intestinal (blocos de parafina) foram avaliadas por um novo algoritmo usando dois tipos de corantes Alcian Blue durante fevereiro-março de 2020 no serviço de anatomia patológica do Hospital Maria Auxiliadora, Lima, Peru. Essa avaliação consistiu em duas etapas: visualização das mucinas ácidas nas células colunares da mucosa gástrica e cálculo da estatística Kappa ponderado.*
Resultados: *Os tipos histoquímicos de metaplasia intestinal mostraram-se a seguir: Tipo I, 398 (77,7%); Tipo II, 81 (15,8%) e Tipo III, 33 (6,5%). A estatística Kappa ponderado foi de 0,79 (p <0,001), classificada como concordância importante ou boa.*
Conclusão: *Este novo algoritmo demonstrou ser útil e capaz de identificar e diferenciar os tipos histoquímicos de metaplasia intestinal, além de possuir confiabilidade estatística.*

Palavras-chave: *mucinas gástricas; azul alciano; mucosa gástrica.*

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INTRODUCTION

Intestinal metaplasia is a gastric pathology which has been described as a risk factor for gastric cancer[1,2]. To improve this prediction and make decisions to treat the specific group of people at the highest risk, intestinal metaplasia is usually classified as type I, II and III, either by morphological or histochemical methods[3,4].

The morphological classification is performed by Hematoxylin-Eosin routine stain and describes cellular characteristics such as the amount of goblet, columnar cells, architectural disorder of the crypts, among others. This method is easy to perform and inexpensive but has been described as imprecise and subjective [5,6].

On the other hand, the best classification due to its greater objectivity and specificity, is through histochemistry and it is based on determining the presence of molecules called acid mucins (sialo and sulfomucins)[7,8]. These acid mucinsare a type of carbohydrates also called glycosaminoglycans acids or acid mucopolysaccharides, which have high molecular weight and are characterized by having complex chains of oligosaccharides bound to the protein by O-glycosidic bonds[9,10].

Throughout history, different histochemical techniques have been performed to identify and differentiate these acid mucins, like High Iron Diamine-Alcian Blue, Gomori's Aldehyde Fuchsin-Alcian Blue and Orcein-Alcian Blue[11-14].

However, the algorithms created from these methods show difficulty in the technique, high cost, toxicity of the reagents and delay in the results, so these features have made them useless for application in most laboratories. A new algorithm derived from histochemistry methods that overcoming these limitations is quite necessary. Therefore, the aim of this research was to evaluate a new algorithm to differentiate histochemical types of intestinal metaplasia.

MATERIAL AND METHOD

This descriptive and cross-sectional research had the following features:

Population and Sample: The population was all gastric biopsies (paraffin blocks) diagnosed with intestinal metaplasia by the Hematoxylin-Eosin routine stain during January-May of 2019 in the pathological anatomy service of the Maria Auxiliadora Hospital, Lima, Peru. The amount was 793 paraffin blocks, and it was decided to choose this entire population as a sample to have the best representativeness. Then, the selection criteria were applied and finally, 512 samples that met these were worked.

The inclusion criteria were: Gastric biopsies (paraffin blocks) diagnosed with IM by the Hematoxylin-Eosin staining method during January-May of 2019 and registered in the virtual diagnostic storage system, independently of other diagnoses. The exclusion criteria were: Paraffin blocks that do not have residual tissue for new slides, damaged paraffin blocks, paraffin blocks that were not found in the storage cabinet (referred to other hospitals), paraffin blocks exhibiting questionable identification (blurred or damaged frame) and more than one paraffin block by the same person.

The procedures were performed during February-March of 2020. Paraffin blocks stored between January and May of 2019 were chosen to work due to the following reasons:

It is recommended that when molecules are going to be demonstrated in tissues, paraffin blocks with the most recent storage possible be chosen. Investigations have shown that a stored paraffin block can have adequate or useful reactivity for up to two years (48,49). The second reason consisted of own logistical details of the service, which does not allow the removal of very recent stored paraffin blocks because these can be constantly used for procedures after the initial diagnosis.

Procedures: Alcian Blue dye at pH 2.5 value was prepared as follows: 1 g of the Alcian Blue dye (Loba Chemie Pvt. Ltd.) was mixed in 99 mL of distilled water, then 1 mL of glacial acetic acid was added, storing at room temperature. The final pH was evaluated by a potentiometer (Checker-Portable pH Meter, HANNA Instruments), which emitted a 2.5 value, suitable for demonstrating sialomucins and sulfomucins[11,15-17].

Alcian Blue dye at pH 0.5 value was prepared as follows: 1 g of the Alcian Blue dye (Loba Chemie Pvt. Ltd.) was mixed in 99.5 mL of distilled water, then 0.5 mL of hydrochloric acid was added, storing at

room temperature. The final pH was evaluated by a potentiometer (Checker-Portable pH Meter, HANNA Instruments), which emitted a 0.5 value, suitable for demonstrating sulfomucins exclusively[11,15-17].

After choosing the paraffin blocks that met the selection criteria, from each paraffin block, 2 new slides (3 um) were made without any special adherence additive by a conventional rotating microtome and they were processed by the following algorithm.

They were taken to a stove for 20 minutes at 85°C. Subsequently, slides were immersed in xylene (2 changes) for 5 minutes each one. After that, they were immersed in absolute alcohol (2 changes) and 96° alcohol (2 changes) for 5 minutes each one, the hydration was finished by tap water for 1 minute. Then, Alcian blue stain at pH 2.5 and pH 0.5 were applied by drops for 5 minutes and 10 minutes, respectively.

The contrast protocol consisted of washing the slides by tap water for 30 seconds and counterstained with Harris's hematoxylin for 1 minute, after that, they were washed again by tap water for 30 seconds. Then, they were quickly differentiated (5 seconds) by 1% acid alcohol, washed by tap water for 1 minute, immersed in 0.3% ammonia water for 30 seconds and washed by tap water for 1 minute again.

Finally, the slides were dried by a fan for 5-7 minutes and were mounted by synthetic Canada balsam. Note: Positive (duodenum for Alcian Blue at pH 2.5 value and windpipe for Alcian Blue at pH 0.5 value) and negative (Spleen or Liver for both Alcian Blue) tissue controls were added to the slides previously.

New algorithm to differentiate histochemical types of Intestinal Metaplasia

This new algorithm is based on two features:

The use of two types of Alcian Blue dyes (pH 2.5 and pH 0.5). This reagent is worldwide known for detecting acid mucins in different pathologies by the presence of a turquoise color. For the application in intestinal metaplasia, only Alcian Blue at pH 2.5 was previously considered.

The steps that are necessary to follow to define the type of intestinal metaplasia. The first step is to observe the slide stained by the Alcian Blue at pH 2.5, due to it will detect both acid mucins (if they were present) in the columnar cells of the gastric mucosa. However, it will not be possible to differentiate which acid mucin is present yet. After that, the second step is to observe the slide stained by the Alcian Blue at pH 0.5, due to it will detect only sulfomucins in the columnar cells of the gastric mucosa. For these, it is necessary to observe same fields in the two slides (both come from the same paraffin block of the patient, as mentioned in the procedures' section) by a conventional optical microscope.

Therefore, unstained columnar cells in both histological slides mean that no acid mucin is present (Intestinal metaplasia - type I). Turquoise columnar cells in the slide with Alcian Blue at pH 2.5, but unstained in the slide with Alcian Blue at pH 0.5 means that there are sialomucins predominantly (Intestinal metaplasia - type II). Turquoise columnar cells in both histological slides means that there are sulfomucins predominantly (Intestinal metaplasia - type III).

During visualization of the slides, more than one type of IM was found, so the highest grade was considered.

Statistical Analysis: To determine the agreement, two experts in the area were invited to participate in this research, one with more than 25 years of experience and the other with 4 years of experience. This difference between the experts was chosen because it was desired to create a real application situation, in which the laboratories will always have experts with diverse capacities and experience.

The weighted Kappa statistic was calculated by STATA 16.0. This type of kappa statistic was chosen because "types of intestinal metaplasia" was a polytomic variable. Statistical significance was considered by a p value less than 0.05 and the Fleiss weight scheme was used because this is the best approximation to the intraclass correlation coefficient.

Finally, to determine the prevalence of the types of intestinal metaplasia, the values issued by the most experienced expert were those considered.

Ethical Aspects: This research was approved by the Ethics Committee of the Maria Auxiliadora Hospital.

RESULTS

Prevalence of the types of intestinal metaplasia: As a main result, this new algorithm could identify the acid mucinsin all the samples. They were classified according to the turquoise color in columnar cellsof the gastric mucosa (goblet cells are also shown only to demonstrate the differences between the cells).

For type I, no color was observed in the cytoplasm of the columnar cells in both histological slides (example is given in Figure 1).

For type II, there was observed a turquoise cytoplasm of the columnar cells in the slide with Alcian Blue at pH 2.5, but unstained in the slide with Alcian Blue at pH 0.5 (example is given in Figure 2).

For type III, there was observed a turquoise cytoplasm of the columnar cells in both histological slides (example is given in Figure 3).

Types of intestinal metaplasia were as follows: Type I, 398 (77.7%); Type II, 81 (15.8%) and Type III, 33 (6.5%).

Kappa statistic: The contingency table and the analysis of the results generated by the two experts are shown in Table 1.

The weighted Kappa statistic calculated was 0.79, rated as an important or good concordance. The null hypothesis that agreement between the experts' results is due to chance, was rejected ($p<0.001$).

DISCUSSION

This research is one of the first to be carried out in Latin America, since only two investigations have been published to date on the typification of intestinal metaplasia by histochemistry[18,19]. Furthermore, this study is the first to work with only the Alcian blue dye andpresents the largest sample size, which would make the statistical precision of the results more adequate.

The prevalence of the types of intestinal metaplasia is similar to the results of other studies carried out in the western region[20,21], higher than those carried out in Europe[22,23] and less compared to the eastern region[24-26]. The first difference could be because Europeans are less frequently exposure to risk factors, such as *Helicobacter pylori* infection. The second difference could be due to own features of Asian population, for instance, genetic factors, gastronomy, and lifestyles.

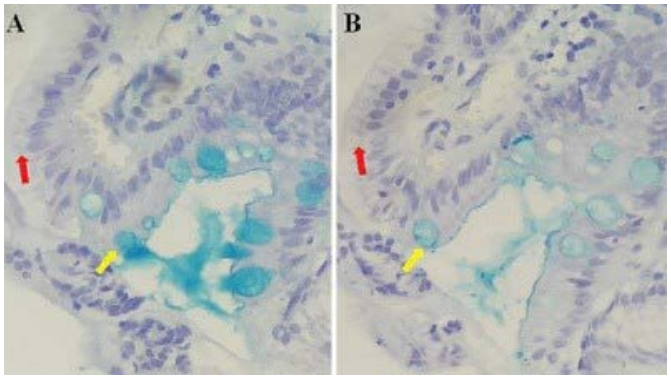

FIGURE 1 – *Presence of sialo or sulfomucins in the Goblet cells with Alcian Blue at pH 2.5 but Columnar cells not (A). Confirmation of sulfomucins in the Goblet cells with Alcian Blue at pH 0.5 but Columnar cells not (B). Interpretation of the algorithm: Intestinal Metaplasia-Type I. Red arrow: Columnar Cell. Yellow arrow: Goblet cell. Objective: 40X.*

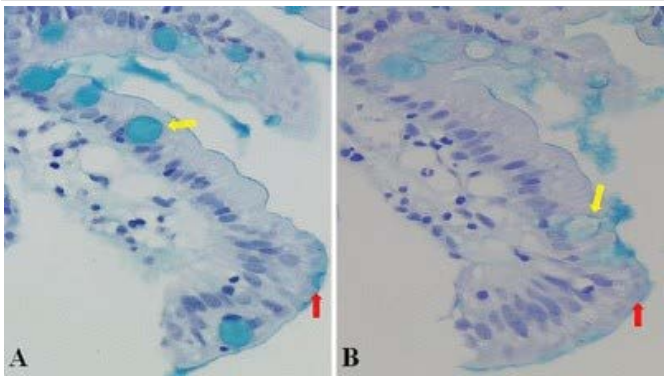

FIGURE 2 – *Presence of sialo or sulfomucins in the Goblet and Columnar cells with Alcian Blue at pH 2.5 (A). Confirmation of sulfomucins in the Goblet cells with Alcian Blue at pH 0.5 but Columnar cells not (B). Interpretation of the algorithm: Intestinal Metaplasia- Type II. Red arrow: Columnar Cell. Yellow arrow: Goblet cell. Objective: 40X.*

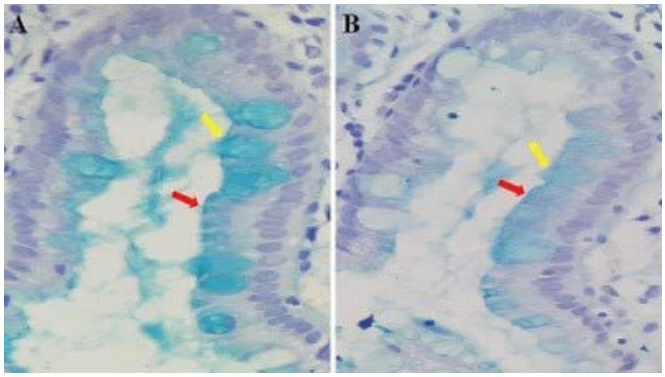

FIGURE 3 – *Presence of sialo/sulfomucins in the Goblet and Columnar cells with Alcian Blue at pH 2.5 (A). Confirmation of sulfomucins in the Goblet and Columnar cells with Alcian Blue at pH 0.5 (B). Interpretation of the algorithm: Intestinal Metaplasia-Type III. Red arrow: Columnar Cell. Yellow arrow: Goblet cell. Objective: 40X.*

TABLE 1 – Contingency table and concordance of the results generated by the two experts.

Observer 1	Observer 2				
	IM I	IM II	IM III		TOTAL
IMI[a]	384	14	0		398
IMII[b]	33	38	10		81
IMIII[c]	0	12	21		33
TOTAL	417	64	31		512
Agreement	Expected agreement	Kappa	Standard error	Z	p
96.63%	83.95%	0.79	0.04	17.94	<0.001

[a]Intestinal metaplasia - Type I. [b] Intestinal metaplasia - Type II. [c]Intestinal metaplasia - Type III

Different methods have been described for the demonstration of acid mucins. In the case of both types of mucins (sialo and sulfomucins), the Alcian Blue dye at pH 2.5 is the most widely used due to its selectivity[14,27]. For greater specificity with sulfomucins, it has been described that the High Iron Diamine method is the reference reagent.

However, studies have revelated by chromatography analysis, that also the best reagents are the Alcian Blue at pH 0.5-1.0 and Azure A at pH 0.4-1.5[11,28,29]. Hence, these three would be the bestor reference reagents for detecting sulfomucins.

This new histochemical algorithm uses reagents with very less toxicity, in contrast to the High Iron Diamine and Gomori's aldehyde-fuchsinhistochemical solutions. In addition, the reagents are very accessible, with an immediate application (not maturation), room temperature storage and the staining protocol with the main solution is the fastest one (5 minutes for the Alcian Blue at pH 2.5 and 10 minutes for the Alcian Blue at pH 0.5), in comparing with High Iron Diamine: 24-48 hours; Gomori's Aldehyde Fuchsin: 20-30 minutes; Orcein: 4-6 hours.

The weighted Kappa statistic was rated as an important or good concordance, and it could be due to the fact that visualization of the slides only implies focusing on a single color and cell compared to the other methods described. To improve this kappa statistic, the authors recommend trying other nuclear dyes, such as neutral red (because this could help to recognize the columnar cell faster), and to recategorize the types of intestinal metaplasia into only two categories: unchanged (type I) and with changes in columnar cells (type II and type III). The latter because the type II is an intermediate phenotype and sooner or later it will evolve towards type III.

The process of learning to recognize the type of intestinal metaplasia requires, though short, a training period and anatomopathologists may not be very used to reading slides in which histochemistry methods were applied. These are limitations.

To conclude, the evaluation of this new algorithm using the Alcian Blue dye demonstrates that it was useful and capable of identifying and differentiating the histochemical types of intestinal metaplasia, in addition to having statistical reliability.

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Conflicts of Interests: The authors declare they have no competing interests.

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