

Comparison between pH 2.5 Alcian blue stain and Hale's dialyzed iron technique for demonstrating acid mucin in colorectal carcinoma

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This study compared the value of pH 2.5 Alcian blue and Hale's dialyzed iron methods for demonstrating acid mucin in colorectal carcinoma. A total of 40 colorectal biopsies from known positive cases were examined. Sections of the biopsies were stained using pH 2.5 Alcian blue method and Hale's dialyzed iron technique, and classified as 'good' or 'excellent' according to staining intensity and efficiency, using a semi-quantitative scoring system. The pH 2.5 Alcian blue method was found to provide a higher frequency of 'excellent' scores compared to Hale's dialyzed iron technique, 32 (80%) versus 21 (52.5%) respectively ($P = 0.018$). Both methods provide at least a minimal level of satisfactory staining results and seem to demonstrate the same amounts of acid mucins; however, if high quality Alcian blue dyes are available, pH 2.5 Alcian blue method is especially recommended to stain colorectal carcinoma acid mucins because it provides a stronger staining intensity and coloration compared to Hale's dialyzed iron technique.

Keywords: Acid mucin, Alcian blue, Colorectal carcinoma, Hale's dialyzed iron

Introduction

Colorectal cancer is the third most common cancer throughout the world, and accounts for approximately one million new cases each year (9.4% of the world total).¹

In the Sudan, large bowel cancer has increased in frequency, mainly in the northern parts of the Sudan; the disease affects the population at a younger age than has been generally reported in the literature.² Mucins are a family of high molecular weight, highly glycosylated glycoproteins and have a role in tumor metastasis, progression, and invasion, and also in survival and protection of tumor cells against the immune response.³⁻⁸ Mucins secreted by carcinoma of large bowel may be intracellular, in gland-like lumina or in large extracellular pools, usually as a mixture of neutral mucins, sialomucins, and sulfomucins. Sialomucins generally predominate in mucoid carcinomas.⁹

Many of the commercial powders of Alcian blue available in the Sudan (with some few exceptions) do not work properly. We therefore evaluated the use of Hale's dialyzed iron method in comparison to Alcian blue method.

Materials and Methods

Study design

This study compared pH 2.5 Alcian blue and Hale's dialyzed iron techniques. Forty human colon biopsies were selected for this study; all were formalin-fixed, paraffin wax embedded blocks and had previously been diagnosed as colorectal adenocarcinoma.

Fixation

Each biopsy was fixed in 10% formalin for 24 hours.

Tissue processing

The biopsies were transferred to an automatic processing machine, subsequently dehydrated in 70 and 90% alcohol for 1 hour each, and then placed in four changes of absolute alcohol for 1 hour each. Specimens then were cleared in two changes of xylene for 2 hours each and impregnated in two changes of paraffin wax at 60°C for 2 hours each.

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The specimens were embedded with paraffin wax, left to solidify at room temperature, and then cooled in the refrigerator.

Microtomy

A total of 80 labeled and albumenized slides were prepared. Using a rotary microtome, 3 μ m sections were cut, two sections from each block.

Staining

All sections were de-waxed with xylene, and hydrated in descending alcohols from absolute through 90 and 70% to distilled water, for 2 minutes in each solution.

One of the two sections from each block was stained by one of two different mucin staining methods described by Bancroft and Stevens, with one modification of using a light eosin counter stain.^{10,11} Aqueous eosin solution (0.5%; Fine-chem Limited, Mumbai, India) was used in both methods.

pH 2.5 Alcian blue technique for acid mucin

A pH 2.5 solution of Alcian blue was used for comprehensive staining of acid mucin. To obtain this pH, 1 g of Alcian blue (Reactifs RAL, Paris, France) was dissolved in 3% acetic acid. The sections were:

1. treated with Alcian blue for 5 minutes;
2. rinsed in tap water;
3. counterstained with 0.5% eosin for 2 minutes;
4. rinsed in tap water;
5. dehydrated in ascending concentrations of alcohol starting from 70%, 90%, and absolute alcohol for 30 seconds in each, cleared in xylene, and mounted using DPX mounting medium (Scharlauchemies, Barcelona, Spain).

The acid mucins were stained blue, and other tissues components were stained pink-red.^{10,11}

Hale's dialyzed iron technique for acid mucin

The sections were:

1. treated with equal parts of dialyzed iron and acetic acid for 10 minutes;
2. rinsed in several changes of distilled water;
3. treated with Perls' reagent (equal parts of 2% aqueous potassium ferrocyanide and 2% hydrochloric acid) for 10 minutes;
4. rinsed in tap water;
5. counterstained with 0.5% eosin for 2 minutes;
6. rinsed in tap water, dehydrated in ascending concentrations of alcohol starting from 70%, 90%, and absolute alcohol for 30 seconds in each, cleared in xylene, and mounted using DPX mounted medium.

The acid mucin stained blue; other tissue components stained pink-red.^{10,11}

Scoring system

To compare the quality of Hale's dialyzed iron and pH 2.5 Alcian blue methods, the stained sections were given scores from 0 to 9, according to the efficiency of the acid mucin staining, including the staining

intensity and amount of mucins demonstrated. Scores were assigned as follows: 7–9, excellent staining results, in which acid mucin was clearly visible and intensely stained; 4–6, good staining results, in which acid mucin was visible, but stained less intensely; 0–3, poor staining, in which acid mucin was barely stained, making it hard to visualize.

All results were documented in a master sheet, and then entered into a computer for analysis using the GraphPad software (GraphPad QuickCalcs). A chi-square test was used to analyze the variance between the two groups. A *P* value of <0.05 was considered significant.

Results

Of the 40 sections, excellent staining results were found in 32 (80%) of sections stained with pH 2.5 Alcian blue, compared to 21 (52.5%) of sections stained with Hale's dialyzed iron method. These findings indicate that pH 2.5 Alcian blue method provides better staining results, as compared to Hale's dialyzed iron method. This was found to be statistically significant (*P*=0.018), as shown in Table 1.

Table 1 Staining quality for the two acid mucin staining methods

| Staining quality | pH 2.5 Alcian blue | | Hale's dialyzed | |
|------------------|--------------------|-----|-----------------|------|
| | <i>n</i> | % | <i>n</i> | % |
| Excellent (7–9) | 32 | 80 | 21 | 52.5 |
| Good (4–6) | 8 | 20 | 19 | 47.5 |
| Total | 40 | 100 | 40 | 100 |

Discussion

In this study, pH 2.5 Alcian blue stained sections were given a score of 'excellent' more frequently than those stained using Hale's dialyzed iron method. The main advantage of pH 2.5 Alcian blue over Hale's dialyzed iron was the intensity of the stain, which was very strong with pH 2.5 Alcian blue, compared to Hale's method. Both methods demonstrated approximately the same amount of acid mucin in each case. Although Hale's dialyzed iron method provided results that ranged from good to excellent in all examined sections, it has the disadvantage of being a more complicated technique than pH 2.5 Alcian blue, and may also confuse mucin with hemosiderin, due to non-specific staining.

The present findings agree with those reported by Bancroft and Stevens,¹⁰ who confirmed that Alcian blue is the most common and favorite technique for staining acid mucin, due to its specificity, strong coloration, permanence of results, and insolubility of

the stained product. We noted that several sections from the same specimens stained intensely by pH 2.5 Alcian blue method and less intensely by the Hale's technique. We propose that the variation in the staining quality between the pH 2.5 Alcian blue and Hale's dialyzed iron methods may be due to the diverse affinities of acid mucins produced by tumor cells in colorectal carcinoma. These mucin types include variable production of sialomucin and sulphomucin in tumor cells, as reported by Hadi and co-workers.⁹

Conclusion

In conclusion, pH 2.5 Alcian blue remains the preferred stain for acid mucins due to its specificity and strong coloration compared to Hale's dialyzed iron method. However, in the absence of high-quality Alcian blue dyes, Hale's dialyzed iron method can be used to provide acceptable results for staining acid mucins, if false-positive results due to hemosiderin are excluded.

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