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Which one to choose for anti-cytokeratin immunohistochemistry in small gastric biopsies: Bleached periodic acid schiff- Alcian blue (PAB) or May Grunwald Giemsa (MGG) sections?

Yasemen ADALI[1,*], Hatice BEŞEREN[2]

[1]Department of Pathology, Faculty of Medicine, İzmir University of Economics, İzmir, Türkiye
[2]Department of Pathology, Kafkas Health Research and Application Hospital, Kafkas University, Kars, Türkiye

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Abstract

Endoscopic biopsies are commonly used for diagnosis in gastrointestinal tractus diseases. The dimensions of the biopsy samples taken during endoscopic examination may sometimes not allow additional examinations or the tissues may become smaller during routine technical procedures. In gastric endoscopic biopsies, hematoxylin & eosin (H&E) staining, periodic acid schiff-Alcian blue (PAB) histochemistry for intestinal metaplasia, and giemsa histochemistry for Helicobacter pylori are used routinely in many in laboratories. In cases where tumor is suspected, immunohistochemical staining is performed in addition to these stainings. The most commonly performed staining is anti-cytokeratin. For various reasons, additional staining applied to materials with reduced tissue size may not yield results. In this case, the patient may need to undergo an endoscopic examination again for definitive diagnosis. To avoid or at least minimize this situation, this study is planned to evaluate the diagnostic usability of restaining with anti-cytokeratin on bleached PAB and MGG sections that were performed for routine examination.

Keywords: Anti-cytokeratin, bleaching, restaining, periodic acid schiff- Alcian blue, May Grunwald Giemsa

1. Introduction

Hematoxylin & eosin (H&E) has been used as the main staining in pathology laboratories since Wissowzky published the first study in the 1870s in which hematoxylin and eosin were used together (1). However, as scientific developments have increased, various histotechnical methods have emerged and support H&E in diagnosis. In earlier times histochemistry, later immunohistochemistry, and nowadays molecular methods serve as beneficial tools for a definitive diagnosis and enable subtyping to guide treatment.

There are many detection methods for the nature and/or content of the tissue in histochemistry applications. These methods, also called histochemical stains, are still frequently used in pathology laboratories (2). Immunohistochemical (IHC) applications also function as important building blocks of many pathology laboratories. Immunohistochemistry, whose basic logic is based on the formation of antigen-antibody complexes, is applied to sections on positively charged slides due to the chemicals used. There are many pre-staining, inter-staining and post-staining factors to obtain appropriate stained sections (3).

The fixation solution, the quality and dimensions of the tissues can be counted among the factors affecting the pre-staining. Occasionally, there are cases where there is no tissue left for IHC staining after H&E sections are taken from small tissues. For this reason, in some routinely applied histochemical examinations, slides are prepared before removing the block and trimming after the H&E section and kept unstained until they are needed. Gastric endoscopic biopsies are one of the best examples of this situation. In many pathology laboratories, 1 H&E section for routine histopathological evaluation in gastric endoscopic biopsies, 1 Giemsa stained section for helicobacter pylori evaluation and 1 Alcian blue (AB) or periodic acid shiff-Alcian blue (PAB) stained section for intestinal metaplasia evaluation are prepared. The small tissue in these biopsy materials, which require IHC from time to time, especially in neoplastic conditions, create technical problems. The tissue in the new sections taken may not be sufficient for evaluation. In such cases, previously stained slides are bleached and IHC staining is performed in these bleached slides.

Since the main histopathological evaluation stain is H&E, bleaching the histochemical stained slides seems to be a more reasonable choice to make comparisons. However, the compatibility of post-bleached slides with directly stained slides should be evaluated in terms of IHC standardization and

*Correspondence: yasemenadali@hotmail.com

quality. For this reason, in this study, it was aimed to compare the IHC staining of the gastric endoscopic biopsies proven to be neoplastic, in slides stained after the histochemically stained slides bleached, and the direct IHC staining of the new slides.

2. Materials and Methods

48 cases diagnosed with adenocarcinoma in endoscopic gastric biopsies performed in a tertiary healthcare institution between 2014 and 2017 were included in the study. Slides that were in the pathology archive of the cases and stained histochemically with PAB (Facepath) and May Grunwald Giemsa (MGG) (Biognost) according to the steps in table 1 were bleached using the technique in table 1. Anticytokeratin staining (Anti-cytokeratin Cocktail, Biogenex, USA) was performed manually on these bleached slides and new slides obtained from paraffin blocks of the cases, immunohistochemically as given in Table 2. Anticytokeratin stained slides obtained by direct staining of the new section and anticytokeratin stained slides obtained after histochemical staining bleaching were evaluated for staining intensities semi quantitatively (0: no staining, 1: mild staining, 2: moderate staining, 3: strong staining), staining frequencies (0: no staining, 1: 1-33% staining, 2: 34-66% staining, 3: 67-100% staining) and H-scores. The H-score was evaluated both numerically and categorized as 0: no staining, 1-3: mild staining, 4-6: moderate staining, 7-9: strong staining. Statistical analyzes were performed with SPSS package program 15.0 (Released 2006. SPSS for Windows, Version 15.0. Chicago, SPSS Inc.). In the analyzes performed at the 95% confidence interval, the conformity of the data to the normal distribution was evaluated with the Shapiro-Wilk test, and the Spearman test was used in the correlation analysis, and $p<0.05$ values were considered statistically significant.

Table 1. May Grunwald Giemsa (MGG) and Periodic acid schiff-Alcian blue (PAB) staining protocols and histochemical staining bleaching protocol

Order of process	MGG staining	PAB staining	Bleaching
1	4 µm thickness slides were kept in 70 ˚C oven for 15 minutes.	4 µm thickness slides were kept in 70 ˚C oven for 15 minutes.	The slides were kept for 3-4 hours in a 60-degree oven in xylol inside the chalet to open the coverslips.
2	Kept in xylol for 5 minutes.	Kept in Alcian Blue-Ph 2.5 solution for 30 minutes.	The opened coverslips were taken
3	Kept in 100% alcohol for 2 minutes.	Washing was done in tap water for 2 minutes.	It was kept in xylol for 1-2 hours until the entellan on the tissue was completely dissolved.
4	Kept in 95% alcohol for 1 minute.	Kept in Periodic Acid solution for 10 minutes.	Absolute alcohol 10 times dipping
5	Kept in 90% alcohol for 1 minute.	Washing was done in distilled water.	95% alcohol 10 times dipping
6	Washing was done in distilled water for 1 minute.	Kept in Schiff's solution for 30 minutes.	Tap water 10 times dipping
7	Kept in May Grunwald stain for 5 minutes.	Washing was done in tap water for 5 minutes.	1% acid alcohol (with 70% alcohol) was left for 5 minutes-1 hour
8	Washing was done in tap water until the color ran clear.	Passed through distilled water.	The acid was removed under running water for 10 minutes.
9	Kept in 10% Giemsa stain for 20 minutes.	Kept in Harris Hematoxylin solution for 3 minutes.	Passed through alcohol series.
10	Washing was done in tap water until the color ran clear.	Washing was done in tap water for 2 minutes.	Clearing was done with xylene.
11	After the slide was passed through alcohol series and cleared with xylene, it is closed with mount.	After the slide was passed through alcohol series and cleared with xylene, it is closed with mount.	The slide is closed with mount.

Table 2. Anti-cytokeratin immunohistochemical staining protocol

Order of process	Anti-cytokeratin immunohistochemical staining protocol
1	Sections of the tissues were taken on 4 micron thick poly-lysine slides. Sections were kept in an oven at 80 degrees for 1 hour.
2	Deparaffinization was done.
3	Antigen retriever was performed with 1/10 Citrate buffer solution using a pressure whistle.
4	Tissues were taken into distilled water and kept for 2 minutes.
5	The tissue was scratched with Pappen. 3% hydrogen peroxide was dripped and left for 10 minutes.
6	They were incubated in phosphate buffered solution (PBS) for 2 minutes.
7	Kept in Wblock for 5 minutes. Washing was not done.
8	Antibody was dripped and waited according to the time written in the datasheet.
9	Washing was done with PBS for 4 minutes and biotin was incubated for 10 minutes.
10	Washing was done with PBS for 4 minutes and streptavidin was incubated for 10 minutes.
11	Washing was done with PBS for 4 minutes and chromogen was incubated for 10 minutes.
12	Slide was washed in distilled water for 5 minutes and kept in Mayer hematoxylin for 5 minutes.
13	Slide was washed in tap water for 5 minutes, passed through alcohol series, cleared with xylene, and then closed with a mount.

3. Results

The IHC staining profiles of the cases after PAP and MGG bleaching and the direct IHC staining profiles are given in Fig. 1. In Fig. 2 and 3; H&E sections of tumor tissues, direct IHC staining, IHC staining after PAB and MGG bleaching of two cases are demonstrated. In direct immunohistochemical staining, mild frequency staining was not observed. The mean frequency of direct immunohistochemical staining was calculated as 2.58±0.50, mean intensity was 2.58±0.65, and mean H-score was 6.92±2.62. IHC staining after PAP bleaching revealed mean frequency 1.50±0.65, mean intensity 2.08±0.87, mean H-score 3.58±2.72. The mean IHC staining frequency after MGG bleaching was 1.23+0.43, mean intensity was 1.75±0.84, and mean H-score was 2.23±1.55.

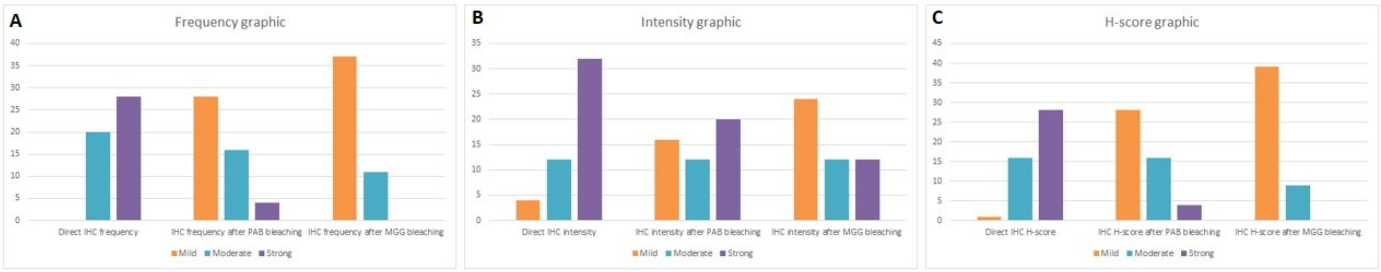

Fig. 1. A: The frequency of staining scores of direct IHC, IHC after PAB bleaching, IHC after MGG bleaching; B: The intensity of staining scores of direct IHC, IHC after PAB bleaching, IHC after MGG bleaching; C: Categorized H-scores of staining scores of direct IHC, IHC after PAB bleaching, IHC after MGG bleaching

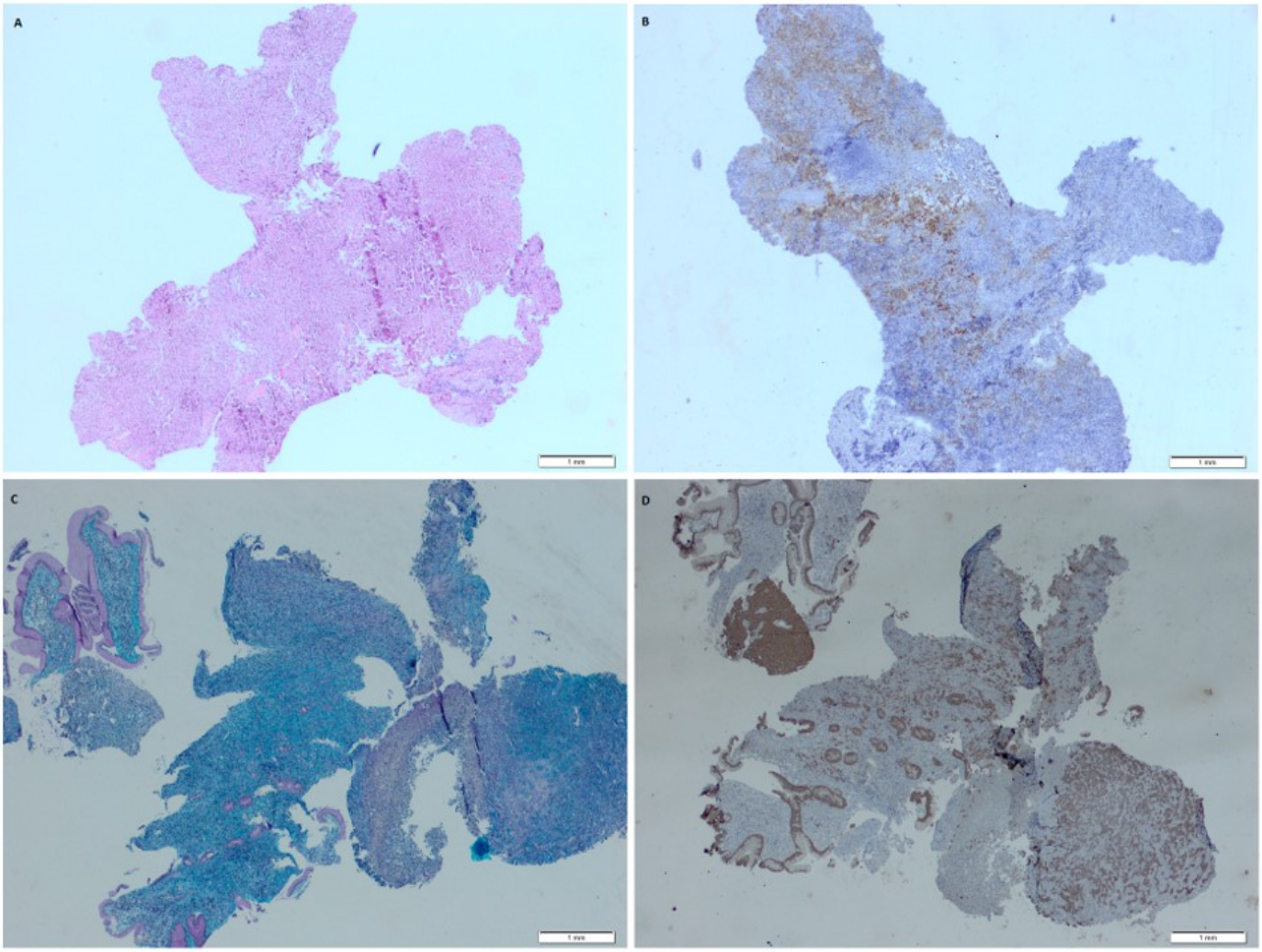

Fig. 2. A: H&E stained slide of endoscopic biopsy material diagnosed as adenocarcinoma, 40x; B: Direct anti-cytokeratin stained slide endoscopic biopsy material of figure 2A, 40x; C: PAB stained slide of endoscopic biopsy material of figure 2A, 40x; D: Anti-cytokeratin stained slide of figure 2C after PAB bleaching, 40x

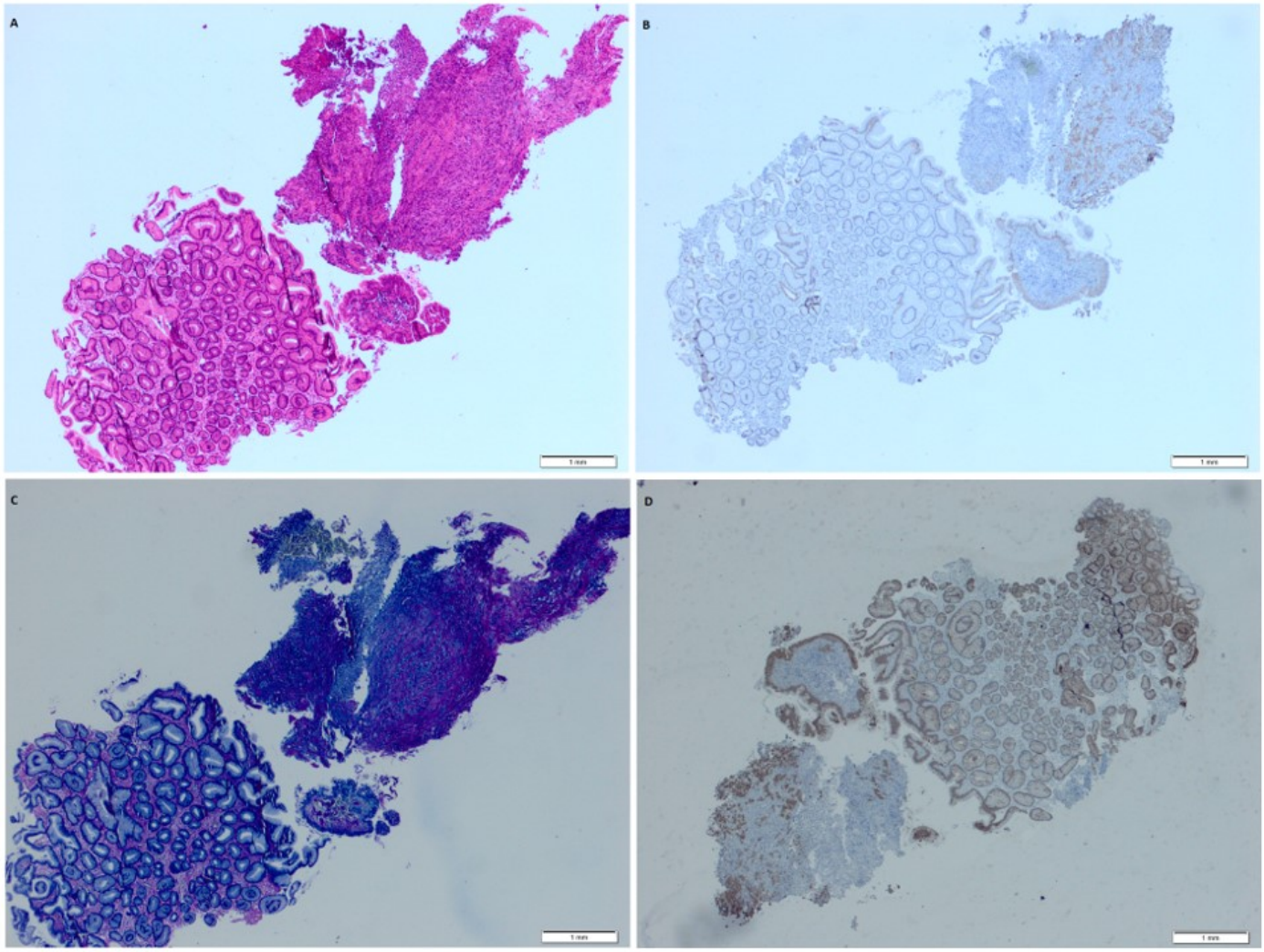

Fig. 3. A: H&E stained slide of endoscopic biopsy material diagnosed as adenocarcinoma, 40x; B: Direct anti-cytokeratin stained slide endoscopic biopsy material of figure 3A, 40x; C: MGG stained slide of endoscopic biopsy material of figure 3A, 40x; D: Anti-cytokeratin stained slide of figure 3C after MGG bleaching, 40x

Spearman's test was used in correlation tests, since it was determined that the data were not normally distributed in Shapiro-Wilk test -even though data were not far from the normality-. While direct IHC staining and the staining frequency after PAB bleaching were correlated in the analyzes (p= 0.006, correlation coefficient 0.391), the same correlation was not found with the staining frequency after MGG bleaching (p= 0.777, correlation coefficient -0.420). Additionally, while the intensity of direct IHC staining and the intensity of staining after PAB bleaching were correlated (p= 0.000, correlation coefficient 0.515), the same correlation was not found with the staining intensity after MGG bleaching (p= 0.255, correlation coefficient 0.168). Furthermore, the numerical evaluation and categorical evaluation of the H-score are similar, and the numerical (p= 0.004, correlation coefficient 0.411) and categorized (p= 0.004, correlation coefficient 0.407) analysis of direct IHC H-scores and IHC after PAB bleaching show a statistical correlation. No correlation was found between the numerical H-score of direct IHC and IHC after MGG bleaching (p= 0.404, correlation coefficient 0.123) as well as categorized H-score (p= 0.953, correlation coefficient 0.009)

4. Discussion

Endoscopy and histopathological examinations, which are the most important methods in diagnosing stomach diseases, are frequently performed today. The dimensions of the biopsy samples taken during endoscopic examination may sometimes not allow additional examinations or the tissues may become smaller during routine technical procedures. Therefore, the presented study evaluated the diagnostic usability of restaining with anti-cytokeratin on bleached PAB and MGG sections that were performed for routine examination.

Although the continued progress of molecular techniques has led to a breakthrough in the pathological examination of neoplasms, the immunohistochemical staining technique, which has evolved with many different steps since Marrack's production of reagents for Typhus and cholera microorganisms (4), is now used quite frequently in many pathology laboratories in addition to routine sections. In the past, immunohistochemistry was referred to as the "Brown revolution" in pathology laboratories, which has many uses

such as the differentiation of benign and malignant lesions, subgrouping of neoplasms or determination of the nature of undifferentiated tumors, finding the primary focus in metastatic lesions, providing therapeutic and prognostic data (5-8).

In immunohistochemical examinations, many pre-, inter- and post-staining factors starting from taking the biopsy affect the staining result (3, 9). Small biopsy materials are vulnerable to many negative factors in terms of staining results, especially shedding and tissue loss. In this case, bleaching the existing sections and staining them again immunohistochemically comes to the fore. In one of the limited studies in the literature on the subject, H&E sections of 105 materials in prostate tru-cut materials were bleached and re-stained. It has been reported that a definitive diagnosis was obtained in 58% of the bleached and restained sections (10). Another study in which H&E is bleached and restained was performed on rat liver and kidney sections. In this study, it was reported that an image similar to the H&E imaage could be obtained in restaining with iNOS (11). In a relatively newer study, it is stated that archived H&E sections can be re-used for multiplex protein biomarker analysis (12). Moreover, novel methods are defined for decolorization of the H&E and masson-trichrome which can be re-used for additional tests including immunohistochemistry (13). However, a study on the semi-quantitative evaluation of immunohistochemical examination performed after histochemical bleaching method in small biopsy materials such as gastric tumors does not draw attention in the literature.

In the present study, the staining frequency, intensity and H-scores of direct IHC staining and IHC staining after PAB and MGG bleaching were evaluated. As a result, it was determined that IHC staining after PAB bleaching showed a positive correlation with direct IHC staining in terms of frequency, intensity and H score, but it was noted that the same correlation was not observed with IHC staining after MGG bleaching. The bleaching of MGG was harder than PAB and PAB showed relatively better bleaching than MGG. Therefore, the lack of correlation detected in MGG bleached sections can be associated with this situation.

Since H&E is the most frequently used stain routinely, it is obvious that H&E sections will be preferred over PAB or MGG stain as an archive or consultation material due to revealing cytoplasmic and nuclear details more clearly and in terms of eye familiarity for pathologists. In the light of the data, we have obtained as a result of current study, it is suggested that IHC staining in PAB bleached sections can be used when IHC examination is required in small endoscopic biopsy materials in order to protect H&E sections as an archive or consultation material. The use of IHC staining after MGG bleaching that does not correlate with direct IHC staining, therefore it is not recommended.

Ethical statement

This study was approved ethically, with the decision number 80576354-050-99/148 and dated 27.09.2017 by Kafkas University, Faculty of Medicine, Ethics Committee.

Conflict of interest

The authors report there are no competing interests to declare.

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None to declare.

Authors' contributions

Concept: Y.A., Design: Y.A., H.B., Data Collection or Processing: Y.A., H.B., Analysis or Interpretation: Y.A., H.B., Literature Search: Y.A., H.B., Writing: Y.A.

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