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Diagnostic challenges of motility disorders: Optimal detection of CD117 positive interstitial cells of Cajal

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Abstract

Aims—Several gastrointestinal motility diseases are associated with altered numbers of interstitial cells of Cajal (ICC) and testing for alterations in numbers of ICC has been proposed as one way to improve routine diagnostics in motility diseases. However, the protocols currently used to visualize ICC in formalin fixed, paraffin embedded (FFPE) tissue using antibodies to CD117 have not been optimized for studying motility disorders. Therefore, the aims of this study were to determine the optimal protocol using FFPE tissue, determine normal values for ICC in non-neoplastic human colon, and compare results to those obtained using immunofluorescence (IF).

Methods/Results—Non-neoplastic tissue was collected from patients undergoing resection for colon cancer and fixed for both light (FFPE) and IF testing. Sections were processed for standard immunohistochemistry using different primary antibodies in conjunction with variations in antigen retrieval (EDTA, citrate), antibody dilution, blocking and detection (Mach2, Mach3, Envision+). Best results were obtained with EDTA retrieval, DAKO CD117 antibody and Mach3 detection.

Conclusions—The optimized protocol presented improved CD117 detection in FFPE tissues and showed good concordance with overall localization of CD117-immunoreactive (IR) ICC as detected by IF. As such, this protocol may be more useful than current procedures for diagnostics in motility disorders.

Keywords

c-Kit; immunofluorescence; human; colon; motility disorders

Introduction

Control of gastrointestinal motility requires the coordinated activity of several cell types including nerves, smooth muscle cells and interstitial cells of Cajal (ICC). ICC are of mesenchymal origin and are found throughout the gastrointestinal tract, from the esophagus to the anus¹. ICC are organized in distinct networks² and serve several different functions. ICC generate the electrical slow wave that determines smooth muscle contractile frequency and are therefore known as the pacemakers of the gut^{3, 4}. ICC also set the smooth muscle membrane potential, are mechanosensors and modulate neuronal input to smooth muscle⁵⁻⁸. The requirement for a normal complement of ICC for normal motility has led many investigators to study their distribution and numbers in a variety of gastrointestinal motility disorders. ICC express c-Kit (CD117), a receptor tyrosine kinase, enabling visualization of ICC by a variety of antibodies to CD117. CD117 is also expressed on mast cells in the muscle layers of the gut. Decreased numbers of ICC or abnormal networks of ICC in the gastrointestinal tract have been reported, among other conditions, in achalasia, hypertrophic pyloric stenosis, gastroparesis, intestinal pseudoobstruction, slow transit constipation and Hirschprung's disease⁹⁻¹³. In particular, the association of loss of ICC with slow transit constipation has been reported by several different laboratories and is now well established^{9, 10, 14, 15}. This has led some pathology laboratories to include a stain for CD117 as part of their routine evaluation of specimens resected from patients with slow transit constipation. However, despite the widespread use of CD117 antibodies in this setting, there is no agreement on what constitutes a normal/abnormal number of ICC or what is a normal/disrupted network of ICC. This is primarily because the majority of published studies on the normal distribution of ICC in the gastrointestinal tract have been carried out using fluorescent techniques on non-paraffin embedded tissue without the use of antigen retrieval.

Immunofluorescence (IF), in general, offers higher sensitivity for ICC detection because it utilizes frozen tissue and therefore does not require extensive fixation as is necessary for paraffin embedded tissue samples. IF could potentially be optimized for more standard, pathology fixation procedures such as formalin fixation followed by paraffin embedding (FFPE), but the stain is not permanent and is not compatible with routine diagnostic review by pathologists who predominantly use light microscopy. Hence, if CD117 is to become routine in motility disorder diagnostics, optimization and standardization of ICC detection by light microscopy that can be used on FFPE processed tissue is necessary.

It is important to note that most commercially available antibodies to CD117 were developed for detection of expression of CD117 in gastrointestinal stromal tumors (GISTs). GISTs form a distinct group of mesenchymal neoplasms with increased expression of CD117 protein visualized as increased numbers of CD117-immunoreactive (IR) cells that are tightly packed together and relatively easy to detect¹⁶. Indeed the majority of GISTs have gain of function mutations in the CD117 gene¹⁷⁻¹⁹. On the contrary, few cases of slow transit constipation have reported mutations in the KIT gene²⁰. Rather the change in CD117 expression that is being measured in slow transit constipation patients is a drop from basal level (normal) expression within the colon^{21, 22}. Immunohistochemical techniques used to stain for strongly expressing CD117 as seen in GISTs might not be sensitive enough to detect normal or reduced expression. Given these limitations with the current protocol, we undertook a study to investigate the optimal protocol for CD117 immunostaining in FFPE non-neoplastic colon tissue, to establish normal ICC counts using this optimal protocol and to compare normal counts using the optimal protocol to those obtained using IF.

Materials and Methods

Tissue

All studies were approved by the Institutional Review Board of the Mayo Clinic College of Medicine. Normal human sigmoid colon was obtained from 7 patients undergoing resection for non-obstructive colon cancer. FFPE tissue was processed by the Histopathology lab at the Mayo Clinic following established clinical guidelines (i.e. fixation in 10% neutral buffered formalin before being embedded in paraffin). Tissues for IF were collected and placed in ice cold F12 medium (Invitrogen, Carlsbad, CA). A piece of tissue 2 cm × 2 cm was dissected from the full thickness of the specimen, pinned out on Sylgard and fixed overnight in cold 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.2. After fixation, the tissue was removed from the substrate, washed four times for fifteen minutes each in cold phosphate buffered saline (0.1 M sodium phosphate, 0.145 M NaCl, pH 7.2) (PBS), then placed overnight in 30% sucrose in PBS to protect the tissue from damage during freezing. The next day the tissue was frozen in OCT embedding compound (Tissuetek) for sectioning. Frozen (paraformaldehyde fixed cryosections) and FFPE sections were both cut at 10 microns for better visualization of ICC processes which are often not in the same plane as the cell body and therefore are better visualized on relatively thick sections. For the DOG1 experiments (see antibody selection below), fresh frozen tissue was fixed in 25 % acetic acid/ 75 % ethanol (v/v) solution for 10 minutes.

Immunostain Optimization for CD117 staining in FFPE

Antibody Selection—Antibodies to be tested were selected based on both a literature search of antibodies previously used with FFPE tissue sections and our own laboratory experience. The DAKO rabbit polyclonal antibody has been used in several published studies^{23, 24} and was approved for in vitro diagnostic use to aid in differential diagnosis of GISTs. Our laboratory has previously published on the use of the MBL rabbit polyclonal antibody for immunofluorescent detection of CD117-IR ICC^{9, 25}. Therefore, these two antibodies were selected for further evaluation. Given that there is considerable variation between lots of the MBL antibody we tested three lots and used the one that best identified ICC. We also tested a mouse monoclonal antibody to DOG1 (discovered on GIST-1) as this has also been reported to specifically identify GISTs^{26, 27}.

Immunostaining Parameters tested—Several variables were tested to find the best CD117 protocol for identifying ICC in colon, including antigen retrieval (EDTA, citrate, and none), primary antibody dilution, blocking and detection kit (Envision+ (DAKO, Carpinteria, CA), Mach 2 (Biocare Medical, Concord, CA), Mach 3 (Biocare)). Each element was tested as follows:

a. Antigen Retrieval: All antigen retrieval was performed using the steamer method. This involved preheating antigen retrieval solution to 90-96°C before adding the slides. The slides were then maintained at that temperature for the optimal time to achieve maximum epitope unmasking. This time may vary from antibody to antibody, so part of the optimization process we undertook involved a titration of the time that the slides were incubated in the steamer. Alternatively a microwave can be used. Although there is no evidence that one method is superior to the other, care must be taken when a microwave is used to maintain uniform heating and avoid excess evaporation of the retrieval buffer^{28, 29}.

EDTA (pH=8.0) retrieval: EDTA solution was heated in a steamer for 20 minutes. After rehydration through xylene and graded alcohols, slides were added and the steamer left on for another 10, 20 or 30 minutes. Slides were then removed and cooled for 5 minutes before being rinsed in water for 5 minutes.

Citrate (pH=6.0) retrieval: Citrate buffer was heated in the steamer for 20 minutes. After rehydration through xylene and graded alcohols, slides were then added and the steamer left on for another 10 or 20 minutes. Slides were then cooled for 5, 10 or 20 minutes before being rinsed in water.

No retrieval: After rehydration through xylene and graded alcohols, samples were not subjected to any antigen retrieval.

b. Primary Antibody Dilution: The DAKO antibody dilution was tested from 1:50 to 1:1600 (stock concentration: 10 μ g/ μ l; range 200 μ g/ml-6.25 μ g/ml). The MBL antibody dilution was tested from 1:100 to 1:1000 (stock concentration: 1 μ g/ μ l; range 10 μ g/ml-1 μ g/ml). All dilutions were done in TBS.

c. Detection kits: Given the high level of endogenous biotin in colon tissue, polymer based kits were chosen for testing. Envision+ (DAKO) and Mach 2 (Biocare) are two-step approaches while the Mach 3 (Biocare) is a three-step approach. All secondary antibodies come ready to use so the only parameter varied in this step was the incubation time: DAKO (30 min to 1 hour), Mach 2 (30 min to 1 hour), Mach 3 (10 min to 1 hour). Final development was done with diaminobenzidine (DAB) for all kits and counterstaining was with hematoxylin.

Detection of CD117-IR ICC using IF

Frozen sections were warmed to room temperature then washed three times in PBS and incubated with 10% normal donkey serum (Jackson ImmunoResearch, West Grove PA), 0.3% Triton X-100 (Pierce, Rockford IL) in PBS. ICC were labeled by incubation with a primary antibody raised in rabbit against human c-Kit (MBL, Nagoya Japan, 5 μ g/mL) diluted in 5% normal donkey serum, 0.3% Triton X-100 in PBS. Bound primary antibody was detected after washing with PBS using Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, 1.4 μ g/mL) in 2.5% normal donkey serum, 0.3% Triton X-100 in PBS. The sections were then washed 5 times and mounted in SlowFade™ with DAPI to allow the number of ICC bodies and processes in each section to be counted. All classes of ICC (myenteric, intramuscular and submucosal) were visualized using this method. To compare DOG1-IR with CD117-IR, fresh frozen tissue was fixed in 25 % acetic acid/ 75 % ethanol (v/v) solution for 10 minutes. After that, the tissue was blocked for 2 hours at room temperature using 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and incubated overnight at 4 °C with affinity-purified polyclonal antibody for c-Kit (1:500, IBL Co. LTD, Gunma, Japan) and monoclonal DOG-1 (1:250, Applied Genomics Inc, Burlingame, CA, USA). The next day after washing, the tissue was incubated for 1 hour at room temperature with a Cy3 conjugated anti-rabbit secondary antibody (1:800, Chemicon International, Temecula, CA, USA) and a Cy5 conjugated anti-mouse secondary antibody (1:200, Chemicon International, Temecula, CA, USA). Labeled cells were visualized using an Olympus BX51WI fluorescence microscope (Olympus America Inc, Center Valley, PA, USA).

Determining CD117-IR ICC numbers

Each IF case (n=7) was stained in quadruplicate and each light microscopy case (n=7) stained in duplicate. The circular muscle, longitudinal muscle and myenteric plexus regions of each slide were evaluated individually. A total of 30 fields per region per slide were counted at a final magnification of 400X, referred to as a high powered field (hpf) in the text. Both ICC bodies and processes were included in the counts. Total counts were averaged for final comparison. The myenteric plexus region did not take up the entire field of view. For the myenteric ICC counts, only ICC within the myenteric region were counted.

Results

Immunostain Optimization

Antigen retrieval—Eliminating the pretreatment resulted in little or no staining of mast cells (Figure 1), the internal positive control for CD117 and no visible ICC. Therefore this approach was not continued. A comparison between EDTA and citrate indicated increased sensitivity with the EDTA pretreatment (Figure 2). There was a slight increase in non-specific background with EDTA retrieval, but this was easily discernible from true staining. Optimal pretreatment time was 30 minutes with a 5 minute cool down. Pretreatment times less than 30 minutes resulted in inconsistent staining.

Antibody selection—Comparisons between the CD117 antibody from MBL and DAKO showed that regardless of retrieval or detection, the MBL antibody consistently demonstrated more non-specific background than the DAKO antibody on FFPE tissue. Multiple blocking steps including 2% bovine serum, Sniper (Biocare) and dual endogeneous block (DAKO) were used, but no significant decrease in background was observed. Based on these observations, we concluded that the DAKO antibody provided a better specific to non-specific staining ratio.

Detection Kits—Envision+ and Mach 2 are similar two step approaches. The primary difference is that different polymers are used. The staining using Envision+ and Mach 2 was comparable. In contrast, the Mach 3, which is a three step detection system, markedly improved sensitivity. Figure 3 shows two sections stained using the Envision + or the Mach 3 and the DAKO CD117 antibody (1:250; stock concentration 10µg/ul; final concentration: 40µg/ml). Given the increased staining seen with the Mach 3, we decided to complete the optimization using this kit. We titrated the CD117 antibody (1:200 to 1:1600; 50-6.25µg/ml) after EDTA retrieval. Using Mach 3 for final detection, we determined that 1:700 (14.3µg/ml) was the optimal dilution to obtain strong signal with the maximum specificity to background ratio (Figure 4). The optimal incubation time for the secondary and tertiary antibodies using Mach 3 was 25 minutes.

Determination of ICC counts in FFPE stained sections of normal human colon using light microscopy

To determine normal values for the human colon circular muscle, longitudinal smooth muscle and myenteric plexus region, CD117-IR ICC were counted in each of these 3 regions. The mean number of CD117-IR ICC for the circular muscle was 6.2 ± 0.8 cells/hpf, for longitudinal muscle 3.3 ± 0.5 cells/hpf and for the myenteric plexus 4.3 ± 0.7 cells/hpf.

Comparison of ICC counts in FFPE sections of normal human colon between immunofluorescence and light microscopy

Immunohistochemistry on FFPE detected fewer CD117-IR ICC cells in all three areas analyzed (Figure 5). Immunohistochemistry on FFPE detected 42% of ICC detected by IF in the circular muscle layer (6.2 ± 0.8 versus 14.8 ± 1.8 ICC/hpf), 38% in the longitudinal muscle (3.3 ± 0.5 versus 8.7 ± 1.1 ICC/hpf) and 42% (4.3 ± 0.7 versus 10.2 ± 1.0 ICC/hpf) in the myenteric plexus region. The relative number of ICC was consistent between both techniques, i.e. the circular muscle had the most CD117-IR ICC and the longitudinal had the least (Figure 5). Therefore, although the absolute number of ICC visualized with the two techniques varied, there was good concordance between the two approaches.

An alternative approach to visualizing ICC

In view of the recent publications that suggest that an antibody to DOG1 is of use to identify GISTs^{26, 27} and small intestinal ICC²⁶ we also tested DOG1 on human sigmoid tissue. As

can be seen in Figure 6, ICC in the human colon identified by an antibody to Kit were also visualized by the DOG1 antibody suggesting it may also be of future use to identify and quantify ICC in motility disorders.

Discussion

Immunohistochemical techniques have proven to be useful in the diagnosis of many disease processes, often leading to novel clinical diagnostic tests. But immunohistochemistry also has its challenges, primarily achieving maximum sensitivity without sacrificing specificity. Because of this, it is important to consider the clinical application of the immunostain and undertake optimization to ensure this balance is met for that specific use²⁹. The CD117 protocol presented here is an excellent example of how different clinical applications can require different technical approaches. CD117 is primarily used for diagnostic purposes in GISTs which typically strongly express the CD117 protein.^{30, 31} For this application current procedures appear to be relatively adequate. In contrast to the strong expression of CD117 in GISTs, the diagnosis of motility disorders is associated with decreased, not increased, numbers of CD117-IR ICC¹⁵. Therefore, using a protocol designed to detect strong expression of CD117 may be inadequate for the diagnosis of gastrointestinal motility disorders.

To explore this possibility, we undertook an optimization strategy, investigating antibody selection, antigen retrieval and detection method, to determine the optimal immunohistochemical technique for use in human colon. We tested these parameters using normal colon specimens to establish normal values that can be used for future studies. Our results indicate that techniques optimized for clinical use in GISTs do not demonstrate adequate sensitivity to be useful for routine use in the study of the pathology of motility disorders. Furthermore, our optimized protocol significantly improved the ability to detect CD117-IR ICC. We also established normal values for networks of CD117-IR ICC in normal human colon using this optimized immunohistochemical technique.

The need to optimize immunohistochemical techniques is not surprising^{32, 33}. Indeed, a recent review article by Goldstein et al outlined the recommendations of an ad-hoc consensus conference that attempted to address the tremendous variability in immunohistochemical staining that exists within the field. They put forth multiple factors to consider throughout the immunohistochemistry process from pre-analytic fixation and processing to post-analytic assessment³⁴. The current study focused on the actual analytic portion of the process and addressed the majority of components Goldstein et al outlined for consideration at this stage. Interestingly, one of the antibodies that they cited as a specific concern, CD117, has become a target of increasing interest with regard to optimization in multiple settings using FFPE tissue³⁵⁻⁴⁰. Goldstein et al focused on CD117 immunohistochemistry in GISTs in particular, noting that neoplasms have varying degrees of expression. As a result, if the test optimization set was done with tumors that have the greatest positivity, the immunoreactive threshold set for the assay may miss weakly positive tumors³⁴, let alone staining tissue with normal or reduced levels of expression of CD117. We also recognized the potential for biological variability even among normal tissues and therefore tested multiple normal colon samples to ensure the final protocol would encompass the full range of basal expression levels.

Although this is the first report to investigate optimizing CD117 detection in normal colon, others have previously investigated CD117 optimization in GISTs. For instance, Saito et al compared staining sensitivity of three mouse monoclonals, a rabbit monoclonal and a DAKO rabbit polyclonal, the same one used in the current study³⁹. They combined this with the Envision+ detection system (DAKO) and tested known CD117-expressing and -non-expressing tumors. Their data indicated that the DAKO antibody positivity was consistently higher than that seen with the mouse monoclonals. The rabbit monoclonal had similar results

as the DAKO antibody overall, but was less sensitive in cases that were known to be only weakly positive³⁹. These data are in agreement with our finding that the DAKO antibody is more sensitive for detection of CD117-IR cells in FFPE.

Unlike Saito et al, we chose not investigate any monoclonal antibodies, either rabbit or mouse, for this initial study. The reason for this is that the principle behind tissue fixation, such as with formalin, is that proteins are cross linked to preserve the tissue in a “lifelike” state for pathologic review⁴¹. Unfortunately, this process frequently also masks the antigenic targets of antibodies. Because of this, immunoreactivity for many antigens is lost, requiring antigen retrieval (unmasking) to restore this reactivity. The process may not restore all epitopes or, in some instances, may damage epitopes. Therefore, monoclonal antibodies which are raised against a single epitope may be less likely to regain antigenicity even after treatment. A polyclonal antiserum, which may recognize several epitopes, is more likely to have at least a portion of the target epitopes revealed²⁹. Therefore, taking this inherent challenge into consideration, we concluded that polyclonal antibodies had the greater likelihood of success in this setting.

Whether an antibody is a polyclonal or a monoclonal may be less relevant than the epitope against which the antibody is made. For example, a monoclonal raised against the full length c-kit protein may be more effective than a polyclonal raised against shorter epitopes. This is an interesting possibility, given that the antibodies used in both the current study and the study by Saito et al were raised against shorter epitopes primarily located in the C-terminus. However, a recent study by Went et al²³ investigated seven different c-kit antibodies and tested for sensitivity and specificity using a panel of GIST tumors. They included monoclonal and polyclonal antibodies to the C-terminus, N-terminus and full length protein. Their investigations still found that the DAKO (A4502) antibody was the most sensitive and specific, detecting 100% of the GIST tumors. No other antibody reached that level of sensitivity, with the monoclonal antibody to the full length protein detecting only 13%. Given the limitations of these antibodies in detecting CD117 in a situation of over-expression, we did not attempt to optimize these again in our experiments which entailed only basal level CD117 expression. After completion of this study, we did briefly test three other antibodies (Santa Cruz c-19 [Santa Cruz, CA] and Invitrogen ZMD.519 [Carlsbad, CA]) and one monoclonal (Vector [Burlingame, CA], clone T595, monoclonal), but the DAKO A4502 still gave the best results (data not shown). It is interesting that the protocol that Went et al used (citrate retrieval and 1:300 dilution) was not adequate to detect ICC in normal colon in our hands. This again highlights the necessity of optimizing antibodies for each particular diagnostic situation.

It is important to note that our data indicated that, even when optimized, immunohistochemistry using FFPE was less sensitive than IF using frozen tissue. This is not surprising given that without the fixation process required for FFPE, the epitopes in frozen sections remain readily available for antibody binding. Drawing conclusions from this comparison is further complicated by our inability to optimize the MBL antibody in FFPE as it would have been ideal to have used the same antibody for both procedures. Despite multiple attempts using the MBL antibody, we were unable to achieve the desired balance of sensitivity and specificity sought in routine pathological diagnostics. However, our comparison of the two techniques does suggest that while immunohistochemistry using FFPE cannot be used to determine absolute numbers of ICC it can be used to determine relative numbers as the immunohistochemistry results showed good concordance with overall localization of CD117-IR ICC as detected by IF. That is, no particular network of ICC was better or less well visualized by immunohistochemistry using FFPE, making it likely that a decrease from normal in a particular population of colonic ICC would be detected by immunohistochemistry using FFPE as long as this optimized technique is used.

The results obtained with the use of the DOG1 antibody are promising and suggest that DOG1 can be used to identify and quantify human colonic ICC. However, these results need to be replicated and expanded to other regions of the human gastrointestinal tract before any recommendations can be made on the use of this antibody in the routine identification of ICC.

In conclusion, the current FFPE immunohistochemistry protocol for detection of CD117-IR cells developed for GISTs is not sensitive enough to detect normal expression of CD117 in ICC. The optimization presented here allows for improved CD117-IR ICC detection in nonneoplastic human tissue. Utilization of standardized immunohistochemical approaches such as the one described here is crucial to more fully elucidate the pathology of motility disorders and improve diagnostics in this patient group.

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Figure 1.

Antigen retrieval. Dako CD117 antibody (1:200; 50 µg/ml) and Mach 3 detection with (panel A) and without (panel B) antigen retrieval. Panel B shows only weak mast cell staining (arrow) compared to panel A suggesting antigen retrieval is required for formalin fixed paraffin embedded tissue to detect CD117.

Figure 2. Comparison between EDTA and citrate retrieval. Dako antibody (1:250; 40 µg/ml) with Envision + detection using EDTA retrieval (panel A) or citrate retrieval (panel B). EDTA retrieval resulted in improved intensity of staining and better visualization of the ICC processes compared to citrate retrieval.

Figure 3.

Comparison between Mach 3 and Envision + detection. The DAKO antibody (1:250; 40µg/ml) with EDTA retrieval was used for both detection systems. Mach 3 (panel A) detected more ICC and resulted in better visualization of ICC processes than Envision +. The CD117 antibody was able to be diluted further with Mach 3.

Figure 4. DAKO antibody at 1:100 (100 μ g/ml; panel A) and 1:700 (14.3 μ g/ml; panel B) dilution with EDTA retrieval and Mach 3 detection. Sections were blocked with dual endogenous block and Sniper before incubation with the primary antibody. The 1:700 dilution markedly reduced the background while preserving strong ICC staining

Figure 5. Number of CD117-IR ICC as detected by immunofluorescence on frozen tissue (black bars) and by immunohistochemistry on formalin fixed paraffin embedded human colonic tissue (white bars). In all regions (circular, longitudinal, MPR=myenteric plexus region) immunofluorescence detected significantly more ICC than immunohistochemistry on formalin fixed paraffin embedded tissue

Figure 6. Double labeling of human colonic circular muscle with CD117 and DOG1 antibodies. The left panel shows CD117-IR ICC as detected by immunofluorescence and the right panel the same section double labeled using the antibody to DOG1. DOG1 selectively identified the Kit positive ICC.