Video Article

Elastic Staining on Paraffin-embedded Slides of pT3N0M0 Gastric Cancer Tissue

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Abstract

The elastic lamina, which is usually located in the sub-mesothelial layer, adjacent to the peritoneum mesothelial cells, is amphiphilic on hematoxylin and eosin (H&E) staining but can be visualized through elastic staining. One of the important benefits of elastic staining is that it makes it easy to determine whether tumor cells have invaded beyond the elastic lamina. This helps in examining the extent of peritoneal surface invasion, thereby distinguishing the pT3 and pT4 stages in gastrointestinal cancer. In this study, we present a protocol to identify elastic fibers in the formalin-fixed, paraffin-embedded pT3N0M0 gastric cancer tissue sections. We prepare 5-µm paraffin sections fixed on slides, and then all the slides are deparaffinated and rehydrated during the staining procedure. Subsequently, all the sections are oxidized by potassium permanganate, bleached by oxalic acid, stained by elastic staining, and counterstained by Van Gieson's. Finally, we examine the effect of staining; elastic lamina is stained blue-black and collagen fibers are stained red, while the cancer cells are stained in varying shades of yellow. We also describe in detail the methods used for determining the positional relationship between cancer cells and elastic lamina. This method is simple, low-cost, and widely applicable in the identification of peritoneal surface invasion in gastrointestinal cancer because of its outstanding selectivity for elastic fibers and authentic results.

Introduction

As per the Tumor, Node, and Metastasis (TNM) cancer staging system, the prognosis of gastric cancer at pathologic stage pT4 is significantly worse than that at pT3 (8th UICC/AJCC)[1]. With gastric cancer, classification of the primary tumor (pT) usually depends on the depth of tumor invasion[2]. Pathologic stage pT3 gastric cancer is defined as cancer invading through the *muscularis propria* into subserosal tissues, whereas pT4 gastric cancer is defined as cancer penetrating to the surface of the visceral peritoneum. The presence of the peritoneal surface invasiveness is the key to distinguish the two stages[2]. However, because the peritoneal mesothelial cell layer is very thin, it could be damaged during the surgery, postoperative treatment, and fixation[3]. Moreover, tumor-related inflammatory changes and fibrosis can damage the normal anatomy of the peritoneum, which makes it very difficult to accurately judge the peritoneal surface invasion using only H&E staining[4]. Current auxiliary diagnosis methods such as cytology and immunohistochemistry are of limited diagnostic value[5,6] because they are false-positive or have a low cost-effectiveness.

The serosal membrane includes the peritoneum, pleura, and pericardium, and is composed of the mesothelium, a basement membrane, and a sub-mesothelial layer[7]. One common histological feature of the serosal membrane is the presence of an elastic lamina in the sub-mesothelial layer, adjacent to the mesothelial cells[8,9]. Elastic lamina has a strong anti-damaging ability and can serve as an alternative prognostic marker if the mesothelial cells are destroyed by severe tumor-related inflammation or fibrosis[3]. Elastic lamina mainly comprises of elastin and microfibril[10], which can be visualized by elastic staining. The principal reason behind the use of the elastic staining is that the elastin forms a hydrogen bond with the phenolic group of resorcinol in the elastin solution, causing elastic fibers to be stained blue-black. After Van Gieson (VG) contrasting staining, collagen fibers can be stained red and muscle fibers and red blood cells can be stained yellow[8,11,12,13].

The eighth edition of the TNM staging of lung cancer defines visceral pleural invasion (T2) as a tumor invasion beyond the elastic lamina or an invasion at the surface of the visceral pleura[2]. Studies on colorectal cancer have shown that the elastic lamina invasion in pT3 colorectal cancer may be the reason for poor prognoses. The 5-year disease-free and overall survival rate of colorectal cancer have also been proven to be like pT4a[13,14,15]. Based on our previous studies on elastic staining, elastic lamina invasion has been found to have a significant negative influence on the prognosis of pT3 gastric cancer and should be treated the same way as pT4a gastric cancer[12]. Hence, this simple and cost-effective method can help eliminate uncertainty in results obtained through H&E staining, and other common limitations associated with other auxiliary diagnosis methods, including cytology and immunohistochemistry, among others. This method can be used efficiently to diagnose peritoneal surface invasions of gastrointestinal cancer, especially in some ambiguous cases. It is convenient since H&E stain and other stains usually make it difficult to identify such invasions. This method also ensures a reliability of results since it is very selective, particularly to elastic fibers. Here, we conduct elastic staining to determine whether tumor cells have invaded the elastic lamina in pT3N0M0 gastric cancer[12].

This method is used for identifying elastic fibers in tissues on formalin-fixed, paraffin-embedded sections, and may be used for frozen sections as well. Here, we choose paraffin-embedded sections for the best maintenance of cellular and tissue morphology. All the steps in this protocol take place at room temperature. A commercial staining kit which includes a potassium permanganate solution, an oxalic acid solution, an elastin solution, and a Van Gieson's solution is used in the presented study.

Protocol

Between 1994 and 2005, patient samples were selected by two experienced gastrointestinal pathologists in the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, from patients who suffered gastrectomy of stomach neoplasms, with a postoperative pathological diagnosis of gastric cancer at the pT3N0M0 stage (according to the seventh TNM staging). This study was approved by the ethics review committee.

1. Sectioning

1. Place the paraffin block into a fixed holder of the sledge microtome, which can move backward and forward across the blade. Adjust the optimal angle between the block and the microtome knife.
   NOTE: The optimal angle not only depends on the blade's geometry but also on the cutting speed and technique.
2. Trim the paraffin block as necessary and cut it into 5-µm-thick cross sections.
3. Place the paraffin 5-µm sections in a water bath at about 40 - 45 °C and mount it, subsequently, from the water onto the glass slide, one section per slide.
4. Press the mounted section carefully against a paper towel to remove any residual water and potential air bubbles.
5. Allow sections to air-dry for 30 min and then bake them in an oven at 45 - 50 °C for 2 h.

2. Deparaffinization and Rehydration of All Slides

1. Immerse the slides in xylene in a Coplin jar for 10 min.
2. Take the slides out from the first xylene jar and immerse them in xylene in another jar for 10 min.
3. Now, immerse the slides in 100% ethanol in a Coplin jar for 5 min.
4. Immerse the slides in 100% ethanol in another Coplin jar, also for 5 min.
5. Immerse the slides in 95% ethanol in a Coplin jar for 2 min.
6. Immerse the slides in 70% ethanol in a Coplin jar for 2 min.
   NOTE: Ensure that the slides are completely immersed in the solutions while performing the above steps.
7. Rinse the slides briefly with distilled water in a new jar.

3. Staining

1. Before proceeding for staining, wipe off any excess solutions around the tissue on the slide with a tissue paper.
2. Place the slides inroom temperature and room humidity conditions to avoid drying of the tissue on the slide throughout the entire staining process.
3. Oxidize the slides with a few drops of potassium permanganate for 5 min. Ensure that the volume of potassium permanganate is enough to cover the tissue section on each slide.
4. Rinse the oxidized slides with distilled water in a Coplin jar with 2 - 3 changes. Observe the blue color of the tissue.
5. Now, bleach these slides by adding a few drops of oxalic acid for 5 min. Ensure that the volume of oxalic acid is enough to completely cover the tissue section on each slide.
6. Rinse the slides by immersing them in the Coplin jar filled with distilled water. Perform the rinse 2x - 3x.
7. Now, wash these slides briefly in 95% alcohol, and then immerse the slides into an elastin solution (5 g/L) for 8 - 24 h.
   NOTE: Elastin solution is volatile, and its staining time is relatively long, so it is best to immerse the slides with elastic staining in a sealed container, such as a transparent glass bottle with a sealing cap. The capacity of the container and the volume of the elastin solution depends on the number of immersed slides, as long as it enables the complete immersion of all the slides.
8. Immerse these slides directly in 95% ethanol for 1 - 2 min to differentiate each tissue section well.
   NOTE: Differentiation refers to the change in the charge of the tissue section, which removes the excess staining absorbed by the tissue and makes the colors clearer. After elastin solution staining, samples should not be washed with distilled water to avoid any difficulty in differentiation. Check microscopically, as necessary, for blue-black elastic fibers staining and gray background. It is better to slightly underdifferentiate the tissue since the subsequent Van Gieson's counterstain can also somewhat extract the elastic stain.
9. Fully rinse these slides with distilled water after differentiation.
10. Counterstain the slides in a Van Gieson's solution for 1 min.
    NOTE: The volume of the Van Gieson's solution needs to be enough to be able to immerse the tissue section on each slide completely.
11. Rapidly drop 95% ethanol onto these slices for a few seconds to differentiate each tissue section well.
    NOTE: Do not rinse the slides with any water after the Van Gieson's counterstaining, as that could weaken or even elute the color of the Van Gieson's counterstaining.

4. Dehydration and Mounting

1. Immerse the slides in 95% alcohol for 5 min to let them dehydrate quickly.
2. Continue to dehydrate these slices by 2 changes of 100% alcohol, each time immersing the slides for 5 min.
3. Immerse the slides into 2 changes of xylene, each time for 3 min.

4. Add one drop of resinous mounting medium to the slide and place the coverslip on the top.

5. Microscopic Observation of the Slides

1. Find the serosal direction and observe the results of elastic staining: elastic fibers (lamina) are stained blue-black, collagen fibers are stained red, and muscle fibers are stained yellow.
2. Look for the cancer cells in the whole slide: cancer cells are stained yellow[12].
3. Determine the positional relationship between the cancer cells and elastic lamina.
   NOTE: There will be two situations here. As long as there is one tumor cell that has invaded beyond the elastic lamina in the whole slide, then this case could be considered as elastic lamina invasion. On the other hand, only when there is no tumor cell that has invaded beyond the elastic lamina in the whole slide, this case could be considered as elastic lamina non-invasion.

Representative Results

A successful elastic staining clearly reveals elastic lamina and cancer cells in pT3 gastric cancer. **Figure 1** - low-power field, microscopically checked after elastin solution staining, prior to Van Gieson's counterstain as per the protocol mentioned - shows the elastic lamina is close to the serosal mesothelial cells and is stained blue-black in the form of filaments, and there is still a certain distance between the tumor cells and the elastic lamina. **Figure 2** is a typical elastic staining with Van Gieson's counterstain, where elastic lamina is stained blue-black, collagen fibers are stained red, and muscle fibers and cancer cells are stained yellow. Elastic lamina invasion can be considered as cancer cells penetrating the elastic lamina (**Figure 2B**), whereas elastic lamina non-invasion can be considered as cancer cells that are close to the elastic lamina, but not invading (**Figure 2A**). In case the elastic staining reveals a repetitive structure, which suggests there is more than one layer of elastic lamina, if the tumor invasion does not invade beyond the outermost elastic lamina (indicated by arrows), the sample should be diagnosed as elastic lamina non-invasion (**Figure 2C**).

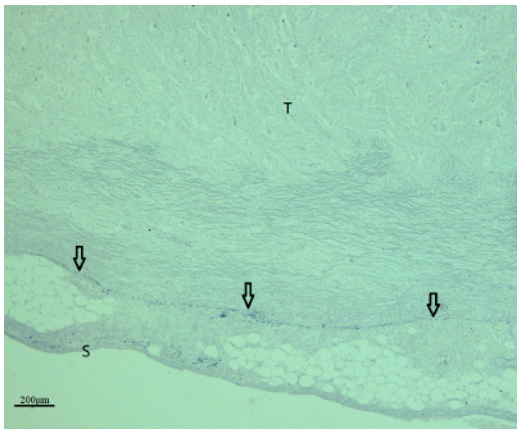

Figure 1: The microscopic image of elastic staining before Van Gieson's counterstaining. The elastic lamina is marked by arrows, "T" represents tumor cells, "S" represents the direction of the serosal membrane. The elastic lamina can be clearly visualized by elastic staining. The arrows indicate elastic lamina. The scale bar is 200 μm. Please click here to view a larger version of this figure.

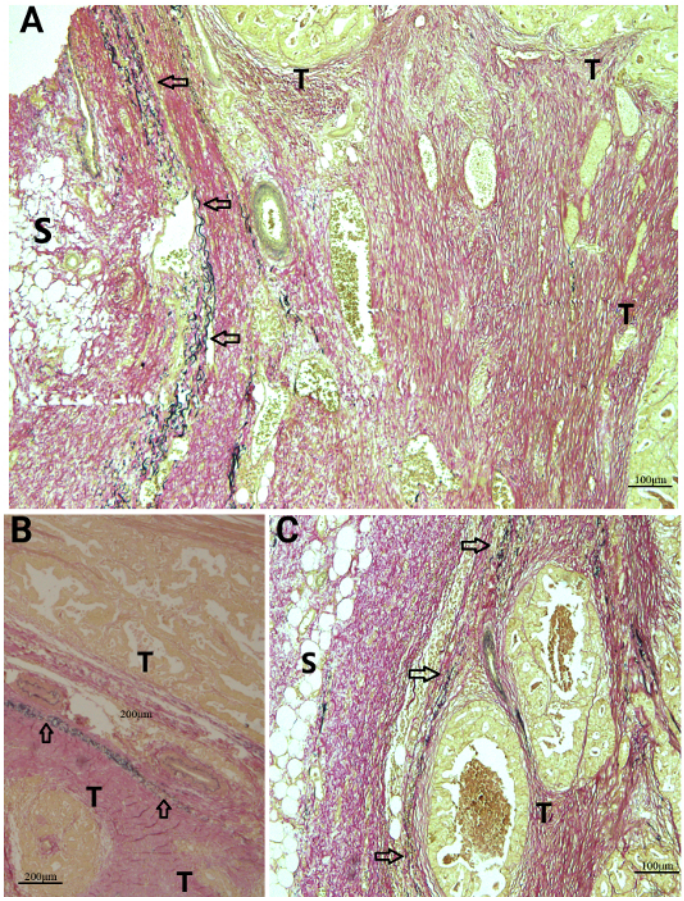

Figure 2: The representative result of elastic staining with Van Gieson's counterstain. The elastic lamina is marked by arrows, "T" represents tumor cells, "S" represents the direction of the serosal membrane. (**A**) Tumor cells grow near the elastic lamina without invading beyond it. (**B**) Tumor cells invade beyond the elastic lamina. (**C**) Tumor cells exhibit invasion in the vicinity of elastic lamina but do not penetrate beyond the outermost elastic lamina. Please click here to view a larger version of this figure.

Discussion

This method proposed here provides an accurate approach for identifying the subserosal elastic lamina. The method can be used to evaluate whether tumor cells have invaded the elastic lamina in pT3N0M0 gastric cancer[12]. Elastic lamina is reddish in H&E-stained sections, which could not be easily distinguished from collagen fibers, and other existing auxiliary diagnosis methods, such as cytology and immunohistochemistry, also exerted limited diagnostic value. The finding of tumor cells in the peritoneal irrigating fluid does not necessarily indicate the presence of a serosal invasion of the primary tumor, which may be due to the lymph node metastasis or other distant metastasis[5]. As the mesothelial cells are likely to be destroyed by fibrous inflammation, an immunohistochemistry diagnosis may not feasible, and the workload is relatively large[6]. However, elastic staining as a classic, special staining method can make the elastic lamina clearly visible[11,12,13]. After standard operating, according to this protocol, the elastic lamina can be stained blue-black, collagen fibers can be stained red, and muscle fibers and red blood cells can be stained yellow, which makes it easier for pathologists to assess the distribution of tumor cells and elastic lamina. Elastic staining may also have some limitations in the diagnostic criteria. For example, in some cases, the structure of elastic lamina is not clear due to obvious tumor-related fibrous inflammation. Different observers may have differences in the understanding and practical application of the diagnostic criteria in one case, although some pathologists still believe that if the tumor tissue invades beyond one side of the remaining elastic lamina, it can be diagnosed as an elastic lamina invasion[17]. Nonetheless, with the continued popularization of the application of elastic staining and the standardization of diagnosis consensus of pathologists, this limitation will be weakened in the future.

Critical steps in this protocol include proper control of the differentiating process and standard tissue specimen selection. After elastin solution staining, slides should not be washed by distilled water, but differentiated slides need to be placed directly in 95% ethanol for 1 - 2 minutes to avoid the difficulty in differentiation. After Van Gieson's counterstaining, contact of the slides with water should be avoided; instead, the differentiated slices rapidly need to be washed in 95% ethanol for a few seconds. Otherwise, the color of the Van Gieson's counterstain will be weakened or even eluted. As for the tissue specimen selection, the most important part is the area where the peritoneum changes direction, especially the acute angle area, rather than the area covered by flat mesothelial tissue[4]. All the cases in this study were drawn according to this method, and the depth of the tissue specimen selecting was as deep as possible in order to assess the relationship between the tumor and the serosa. In addition, all the steps in this protocol take place at room temperature. Since elastin solution is easy to volatilize and the staining time is relatively long, it is best to immerse the slides in elastic staining solution in a sealed container. As for the microscopic observation, the result of elastic staining is accurate and reliable. However, in some special cases, some reasonable diagnostic criteria need to be taken into account, as proposed by Grin *et al.*[16]. In cases where tumor-related fibrous inflammation results in an obvious instability of the elastic lamina structure, when the tumor tissue invades beyond the residual elastic lamina on one side, it can be diagnosed as elastic lamina invasion. In the case of mucinous

adenocarcinoma, only if the cellular mucous component invades beyond the elastic lamina can it be diagnosed as elastic lamina invasion. If elastic staining shows a repetitive elastic lamina structure, the sample can be diagnosed as elastic lamina invasion only when the tumor invasion is beyond the outermost elastic lamina. If the above condition occurs in one or more sections, the case can be diagnosed as elastic lamina invasion[16].

This protocol demonstrates a straightforward technique for visually identifying the elastic lamina in gastric cancer. This technique is widely adaptable and may be expanded to judge the serosal invasion for other prognostic predictions, such as esophageal or ovarian cancer[17]. It also can be utilized to identify blood and lymphatic vessel invasion in colorectal cancer as well as in other cancers, especially in some difficult cases, for the reason that elastic lamina also exists in vessel and lymphatic endothelium. Also, elastic staining is cheap and can be easily adopted by pathologists at different sites[18]. Furthermore, this technique is not limited to tumor tissues, but can also be used for diagnoses of dermatologic and vascular diseases, such as discoid lupus erythematosus, alopecia, and carotid artery lesion[19,20]. With the development of the elastic staining technology and accumulating studies of tumors with large sample sizes, we expect that elastic staining can be accepted by the AJCC staging system in judging the serosal invasion of gastric cancer and colorectal cancer.

Disclosures

The authors have nothing to disclose.

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