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Review Article

Frozen section tissue staining for non-melanoma skin cancer in Mohs micrographic surgery

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Abstract

Non-melanoma skin cancer has the highest incidence among skin cancers globally. The most common types of non-melanoma skin cancer are basal cell carcinoma and squamous cell carcinoma. Currently, Mohs micrographic surgery is the gold standard in the management of non-melanoma skin cancer because of its higher success rate, lower recurrence rate, and better cosmetic outcome compared to conventional surgery. Hematoxylin and eosin are commonly used for tissue staining of frozen sections in Mohs micrographic surgery. In some cases, other staining such as toluidine blue, hematoxylin and safranin O, periodic acid-Schiff, and oil red O are required to identify the characteristic features of various types of cancer. The use of properly frozen section tissue staining facilitates Mohs micrographic surgeon in determining the boundary of a cancer-free incision.

Keywords: *basal cell carcinoma, frozen section, hematoxylin and eosin, Mohs micrographic surgery, squamous cell carcinoma*

Abstrak

Kanker kulit non-melanoma merupakan kanker dengan insidens terbanyak secara global. Jenis kanker kulit non-melanoma yang paling sering ditemukan adalah karsinoma sel basal dan karsinoma sel skuamosa. Saat ini bedah mikrografik Mohs merupakan terapi baku emas pada tata laksana kanker kulit non-melanoma karena angka keberhasilan yang lebih tinggi. Pewarnaan jaringan frozen section yang umumnya digunakan pada bedah Mohs adalah dengan hematoksilin dan eosin. Pada beberapa kasus diperlukan pewarnaan lainnya seperti toluidine blue, hematoksilin dan safranin O, periodic acid-schiff, dan oil red O untuk mengidentifikasi gambaran karakteristik berbagai jenis kanker. Penggunaan pewarnaan jaringan frozen section yang tepat dapat memudahkan tindakan bedah mikrografik Mohs dalam menentukan batas sayatan bebas kanker.

Kata kunci: Bedah Mohs, hematoksilin dan eosin, karsinoma sel basal, karsinoma sel skuamosa, potong beku

Background

Non-melanoma skin cancer (NMSC) is the type of malignancy with the highest incidence worldwide. The most common types of NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC).[1] The global incidence of NMSC in 2019 was estimated at 6.35 million cases with 57.9% occurring in males.[2] Although the mortality of NMSC is relatively low with a range between three and five deaths per 1000 cases, the predilection of NMSC which is generally on the face can cause significant morbidity.[3] Mohs micrographic surgery (MMS) with a horizontal frozen section is the recommended treatment for NMSC.[4,5] This procedure has many advantages compared to conventional surgery, including higher cure rates, lower recurrence rates, lower risk of scar tissue formation, better cosmetic outcome, and can be performed in an outpatient clinic.[4,6]

Frozen section staining is a technique for staining the tissue which is cut under frozen conditions intra-operatively, allowing rapid evaluation of the specimen. Contrary, the permanent section using paraffin-embedded takes two to three days for processing the specimen.[7] Hematoxylin and eosin (H&E) are commonly used for tissue staining of frozen sections in MMS. In some conditions, H&E staining is difficult to detect tumor cells, for example in poorly differentiated tumors; tumors with single cell spreading pattern; dense infiltration of inflammatory cells around the tumors; tumors invasion of nerve fibers, blood vessels, and fascia; tumor cells within fibrotic tissue; and tumors with pagetoid distribution pattern.[8] Therefore, other histochemical staining methods are needed to facilitate the identification of specimens, including toluidine blue (TB), hematoxylin and safranin O (H&SO), hematoxylin and periodic acid-Schiff (H&PAS), and oil red O (ORO). In addition to conventional histochemical staining, immunohisto-chemical staining is currently being used in MMS.[9]

This review discusses various frozen section tissue staining techniques that are used to diagnose NMSC with the MMS procedure. A more detailed discussion of immunohistochemical staining is not included in this paper. By understanding the various tissue staining options, the Mohs surgeon can determine which stain should be used in a particular case for more precise and easier identification of specimens.

Mohs Micrographic Surgery

The MMS technique consists of several stages performed on the same day. Mohs surgeons also act as pathologists who are examining tissue specimens, thereby increasing the accuracy of clinicopathological correlations. Therefore, the Mohs surgeon's knowledge of dermatopathology and frozen section interpretation is very important in determining the success of this procedure.[6]

Excision Technique in Mohs Micrographic Surgery

The procedure is started with identification and marking at a distance of 2 to 3 mm beyond the edge of the lesion (Fig. 1.A). The area of surgery is cleaned with anti-septic and then injected with local infiltrative anesthesia (1% lidocaine and epinephrine 1:100,000).[6,10] Clinically visible tumors are excised with a curette or scalpel (debulking) (Fig. 1.B). Sharp excision with a scalpel is preferred over curettage if the tumor is large, deep, and the lesion is to be sent for standard pathological examination.[11-13] The first layer is then excised with a scalpel at an angle of 30^{o} from the skin surface to facilitate flattening of the specimen edges (Fig. 1.C). Once the edges of the lesion have been freed, deeper tissue can be excised using a scalpel or bent scissors. Next, the specimen is cut into smaller pieces to facilitate the histopathological examination process (Figure 1.D). Hemostasis is carried out either with vessel ligation or electrocoagulation. The wound is temporarily closed using wet gauze during tissue processing for 20 minutes to 1 hour.[6,10–12] Specimens are then marked with colored ink on each side and schematic drawings are made on paper that represent the various colors in the specimens (Figure 1.E and 1.F). Consistency of marking the specimen and drawing an appropriate schematic figure is recommended to improve accuracy and avoid misorientation.[6]

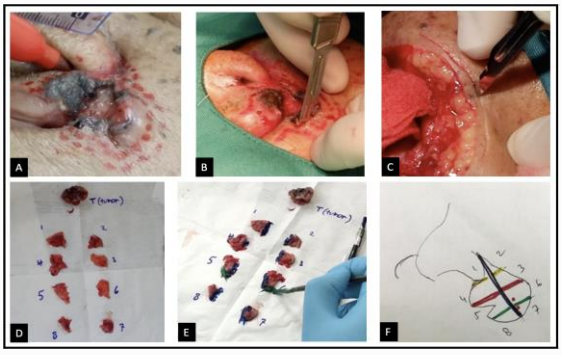

Figure 1. Mohs micrographic surgery technique. (A) Clinically visible tumor is marked 2-3 mm from the edge of the lesion. (B) Debulking of the clinically visible tumor using a scalpel. (C) Excision of normal-looking skin (2-3 mm from the edge of the lesion) is performed using a scalpel with a bevel angle of 30°. (D) Incision of the specimen into smaller pieces using a scalpel and numbering for orientation purposes. (E) Specimens are marked with various colors of ink at the edges of the tissue. (F) Schematic drawing according to the anatomical position and orientation of the specimen on paper.
Source: documentation of Adhimukti T. Sampurna, M.D.

Frozen Section and Tissue Processing

The tissue is placed on a frozen iron plate (chuck) and a freezing medium is added (Figure 2.A). Freezing of MMS specimens is a very important step to obtaining a good quality frozen section, allowing more accurate histopathological examination. The freezing process of the specimen must be carried out as quickly as possible to avoid the formation of ice crystals on the tissue. The frozen tissue is cut horizontally using a microtome (Fig. 2.B) and then attached to a glass slide for later staining with H&E or other stains by a laboratory technician (Fig. 2.C).[6,11,12,14] Mohs surgeon examines the stained specimen using a microscope. If tumor cells are still detected, the location will be marked on the schematic figure that has been drawn previously. Then the excision procedure is repeated according to the guidelines on the schematic figure until a tumor cell-free edge of the tissue was obtained.[6]

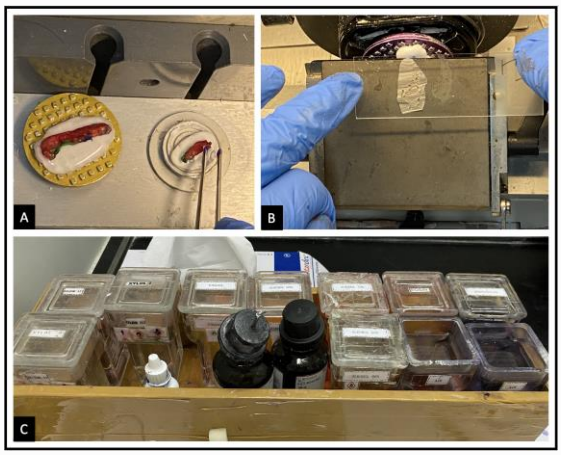

Figure 2. Frozen section tissue processing. (A) The specimens are flattened on a frozen iron plate (chuck) and added to the freezing medium. (B) Specimens are cut using a microtome and attached to a glass slide. (C) Specimens are stained using hematoxylin and eosin.
Source: Department of Anatomical Pathology, Faculty of Medicine Universitas Indonesia.

Examination of Frozen Section Tissue

Most of the frozen section examinations in skin malignancies are needed to evaluate the BCC margin with an accuracy rate of up to 90 – 95%.[15] Frozen section is also widely used in surgery in other locations, for example in breast, neurology, gynecology, pulmonary, genitourinary, gastrointestinal, ear, nose, and throat, and soft tissue tumors. Frozen section examination is a quick process, allowing for examining the specimens intraoperatively so that diagnostics and decisions to explore further tumors can be made in real-time. In contrast, the permanent section with a paraffin-embedded block takes two to three days for obtaining a specimen ready for examination.[16] Frozen section examination is recommended for lesions with unclear clinical boundaries, infiltrative growth patterns, chronic large lesions, recurrence lesions, or in areas requiring more skin preservation. In cases of nodular type BCC with clear clinical boundaries, excision without a frozen section has an effectiveness of up to 90%, therefore, routine frozen section examination in these cases has not much benefit. In cases of malignant melanoma, frozen section examination is not recommended because the depth of invasion and grade of atypical cells are difficult to determine accurately. Examination with a paraffin-embedded permanent section is more suitable for cases of malignant melanoma to increase diagnostic value.[15]

Although frozen section has its advantages in terms of rapid intraoperative diagnostic, there are several drawbacks of this technique, including difficulty in distinguishing between hair follicles and BCC; an inflammatory cell that can be mistaken for a nest of tumor cells; a dense inflammatory cell that can obscure the appearance of tumor cells below; and atypical melanocytes which could be mistaken for dysplastic keratinocytes in actinic keratoses. In these circumstances, a permanent section examination is recommended for a more accurate diagnosis.[15]

Hematoxylin and Eosin Staining

Hematoxylin and eosin are the most common staining for tissue histopathological examination. Hematoxylin is a natural dye derived from the bark of the logwood tree. Hematoxylin is a positively charged nucleophilic base that binds to the negatively charged material of the nucleus (DNA and RNA) by producing a blue-black color. Eosin is an acid that is used after hematoxylin (counterstaining) and aims to stain positively charged protein components in the cytoplasm, connective tissue, and erythrocytes by producing variations of pink, orange, and red colors. The combination of these two types of stains applied sequentially will produce a contrast between the cell nucleus and cytoplasmic components.[17,18]

There are various H&E staining methods used in MMS, however, the staining process generally consists of seven steps, including fixation, hydration, hematoxylin staining, blue staining, eosin staining (counterstaining), dehydration, and cleaning. Fixation is the most important step in determining the staining quality. The most often used material for fixation is ethanol (70%-

90%) because it works quickly and produces clear details of the cell nucleus. Other materials that can be used are methanol and acetone. After fixation, hydration is performed with distilled water and continued with hematoxylin staining. Acidic substances, such as alcohol, are used to remove residual dyes. Then the specimen is added with a blue dye, such as Scott's tap water, ammonia, or lithium carbonate before staining with eosin. The next step is dehydration using 95%-100% alcohol. The last process is cleaning the specimen with xylene for clearing the tissue.[14,18]

A frozen section tissue with good quality H&E staining yields the same detail as H&E staining of a permanent section with formalin fixation and paraffin-embedded.[18] To date, there is no standard protocol for H&E staining to detect BCC in Mohs surgery. There is a comparative study evaluating nine types of hematoxylin staining (Appendix 1) (Ehrlich, Coles, Mayer's, Gill's I, Gill's II, Gill's III, Weigert's, Harris, or Carazzi's) based on a mordant to produce the most optimal frozen section tissue morphology staining in BCC. This study found that Carazzi's type of hematoxylin produced the clearest and sharpest pattern of cell nucleus staining. The nucleolus, chromatin, and mitotic processes are also seen more clearly compared to other types of hematoxylin staining.[19] Another comparative study evaluated the conventional H&E staining protocol with the shortened one with a difference in staining time of 125 seconds. This study revealed that the image quality of shortened staining protocol was inferior compared to the conventional staining evidenced by the presence of air bubbles, faded eosin painting, and decreased color contrast when the specimen is stored for a long time.[20]

In most cases of BCC, nuclear staining with H&E is the key to diagnosis. The presence of a basophilic nucleus, hyperchromatic, apoptotic bodies, mitosis, pleomorphism, peripheral palisade pattern, retraction artifacts, and extracellular keratinization support the diagnosis of BCC.[21] In the case of SCC, histopathological examination with H&E showed necrosis, pyknotic cells, keratin pearls, mast cells in the stroma surrounding the tumor, and perineural infiltrates and invasion.[21,22] Findings of perineural invasion on frozen section have similar accuracy as permanent section examination, and findings of perineural inflammation on frozen section indicate perineural invasion so that further histopathological examination is required.[22] However, Mohs surgeons have to be careful in interpreting the findings of perineural invasion, because several normal structures can be seen as perineural invasion in frozen section SSC preparations, for example, the arrector pili muscles, eccrine ducts, blood vessels, and perineural fibrosis. If definitive results cannot be concluded from morphological findings or additional frozen section examinations, immunohistochemical staining is considered.[23]

Toluidine Blue Staining

Toluidine blue (TB) is the second most common tissue staining method used in the frozen section of MMS. This stain is used by 13 to 17% of Mohs surgeons, in particular, to identify BCC.[24] Toluidine blue is a cationic thiazine that has been widely used for biological staining since 1856. It is soluble in water and alcohol and exhibits basophilic staining in specimens containing anionic radicals. Toluidine blue staining is favorable for detecting alterations in DNA chromatin-protein complexes, nucleolus locations, proteoglycan extracellular matrix complexes, and glycosamino-glycans. It stains mast cells metachromatically, resulting in purple color for their granules.[25]

The TB staining process followed several steps, including immersing the specimen in absolute alcohol for 30 seconds and then rinsing with running water for 60 seconds. Next, the specimen is immersed in 0.5% TB solution for 60 seconds and then rinsed with running water for 60 seconds. After the remaining dye is rinsed off, the specimen is immersed again in alcohol for 90 seconds and the last process is cleansing for 90 seconds. The total time for TB staining is seven minutes, two minutes faster than the H&E staining.[26] The staining process can be shortened to less than 2.5 minutes by the addition of an alkalinizing agent, sodium borate.[27]

Currently, TB staining is increasingly being used in the frozen section of MMS because of its simplicity to H&E staining. The main advantage of TB staining is it can identify stromal reactions around tumor nests. The finding of mucopolysaccharides with TB staining is a characteristic feature of BCC. Mucopolysaccharides are stained magenta or pink, obviously visible around the BCC tumor nests, while tumor cells are stained dark blue. This finding is especially important in BCC with few tumor cells and infiltrates. Although it can also be found around the papillary dermis of hair follicles, the finding of mucopolysaccharides in MMS provides a clue to the surgeon to search for residual tumors more thoroughly.[21,27,28] Other features that can be found in BCC with TB staining are inflammatory cells consisting of T lymphocytes,

mast cells, and fibroblasts. Mast cells around the tumor nest are larger with abundant and larger granules than the normal mast cells.[26]

The term “the setting sun sign” is to describe the loss of tumor cell nests and the magenta zone in the serial frozen section of BCC which resembles the setting sun at dusk. In some cases of BCC, no nests of tumor cells were found, but only the reaction of the surrounding stroma, which appeared as a magenta zone. By identifying “the setting sun sign”, Mohs' surgeons will be more confident they have operated on the right area.[26] Toluidine blue is also beneficial to distinguish between BCC and folliculocentric basaloid proliferation which is difficult to evaluate by H&E staining. Folliculocentric basaloid proliferation is a benign lesion characterized by vertical and axial folliculocentric proliferation with a well-defined hyaline basement membrane and a normal appearance of the surrounding stroma. This benign lesion is commonly found on the frozen section of MMS in the perinasal area. With TB staining, no magenta zone was found in this case as in BCC.[29]

Research has shown that additional TB staining increases the diagnostic accuracy of BCC in physicians undergoing training in MMS.[28] Toluidine blue staining is specifically used in BCC, whereas H&E is used more commonly in SCC and melanoma in situ.[30]

In SCC specimens, TB produces more detail and sharper staining of tumor cell nuclei compared to H&E staining.[21,31] Nuclear detail is an essential clue in diagnosing SCC in the frozen section. Pleomorphism and nuclear hyperchromatism are characteristics related to the degree of tumor differentiation (more prominent in poorly differentiated tumors). Toluidine blue can also identify individual cell keratinization and necrosis with similar quality as H&E staining. However, pyknotic cells are clearer and easy to observe with H&E (eosinophilic) compared to TB (greenish blue).[21] Toluidine blue staining can detect perineural SCC invasion even in conditions where tumor cells are not clearly visible. This is known as the “perineural corona sign” which describes a pink to magenta coloration due to the deposition of mucopolysaccharides around the nerve fibers.[32] The finding of perineural invasion in SCC is classified as high risk because it is associated with frequent recurrence and metastases even after the MMS has been performed.[33,34] However, a comparative study reports that there is no statistically significant difference between H&E and TB staining for Mohs surgeons to detect SCC.[35]

Toluidine blue staining is also useful for detecting spindle cell-type SCC in frozen sections.[31] Spindle cell-type SCC is a rare malignancy with a predilection for sun or radiation exposed areas, particularly in elderly Caucasian males. This type of SCC is commonly seen in patients with a history of radiation, post-burn scarring, and organ transplant recipients.[36] The tumor is highly aggressive with rapid growth, local recurrence, and metastatic potential.[37] Histologically, the tumor is composed of clustered spindle-cell with various histopathological findings of SCC. Tumors may be found in the dermis, with or without connection to the overlying epidermis. At the periphery, tumor cells are usually scattered individually without forming a tumor nest. This makes it difficult to identify tumor cells by H&E staining on frozen sections. Toluidine blue can identify stromal reactions by producing a pink to magenta color due to the deposition of mucopolysaccharides around tumor cells, although it is not as clear as the stromal reaction in BCC. Toluidine blue staining also enhances nuclear detail, enabling the detection of squamous cells with abundant eosinophilic cytoplasm and vesicular nuclei.[31]

Toluidine blue staining is beneficial in cases of dermatofibrosarcoma protuberans (DFSP). The histopathological picture of DFSP is a monomorphic spindle cell proliferation with a storiform pattern which is usually surrounded by subcutaneous fat to form a honeycomb appearance.[38] In the tumor margin area, which is usually difficult to visualize tumor cells, TB produces better color contrast between tumor cells against their background when compared to H&E staining.[39]

Toluidine blue staining has also been reported to be useful in cases of microcystic adnexal carcinoma. This tumor is a rare malignancy with a predilection on the head, neck, and mid-face. These tumors commonly occur in the Caucasian population. The clinical manifestations of microcystic adnexal carcinoma are papules, nodules, plaques, or cysts that grow slowly with local invasion and have the potential to infiltrate nerve fibers, muscle, and subcutaneous fat. The most effective treatment is MMS because the recurrence rate is relatively low (0-12%) compared to conventional surgical excision (40-60%). Stromal reactions occur in the form of mucopolysaccharide and hyaluronic acid deposition.

These stromal changes were more easily identified with TB staining, resulting in a pink to magenta coloration around the tumor nest. In tumors that infiltrate the nerves, it is also easy to find a magenta pattern within and around the nerve fibers.[42]

Hematoxylin and Safranin O Staining

Safranin O is generally used in the field of plant histology to stain the nucleus of plant cells. Staining with hematoxylin and safranin O (H&SO) is considered less expensive than H&E. This substance stains the keratin into dark red, while other structures such as muscle cells, hair follicles, sebaceous glands, and mast cell granules into pink to red. A comparative study evaluating H&E with H&SO for diagnosing SCC and BCC showed better or equivalent quality for H&SO staining. The color contrast of the SCC nucleus and keratin cells was better on H&SO staining compared to H&E. In the case of Bowen's disease, H&SO increases the contrast between cells and surrounding tissue, allowing identification of the typical pattern of Bowen's disease. For morphea-form BCC, H&SO increased the intensity and contrast of the cell nucleus, increasing diagnostic accuracy.[43] However, another researcher assumed that the results reported in that previous comparative study had a drawback due to the poor quality of H&E staining, as evidenced by generally darker stains, blue shadows, and pale pink. This was possibly due to the poor H&E staining protocol.[18]

Hematoxylin and Periodic Acid-Schiff

Hematoxylin and periodic acid-Schiff (H&PAS) stain glycogen and mucoproteins containing neutral mucopolysaccharides into red and the cell nucleus into the blue. This staining is generally used in cases of extramammary Paget's disease because it can distinguish intraepidermal adenocarcinoma from other cells such as Langerhans and epidermal cells. This staining is also used to identify eccrine/apocrine tumors and BCC with eccrine differentiation, which generally contain an abundance of glycogen.[44]

Oil Red O Staining

Oil red O (ORO) staining produces a red color in triglycerides and intracytoplasmic lipids. It is often used for cases of sebaceous carcinoma. This staining can distinguish between periorbital sebaceous carcinoma and the Meibomian gland. The presence of a “pagetoid” pattern, which is a clear lipid-filled cell in the epidermis, is a characteristic of sebaceous carcinoma. Basal cell carcinoma with sebaceous differentiation also showed a positive intracytoplasmic lipid appearance with ORO staining.[44]

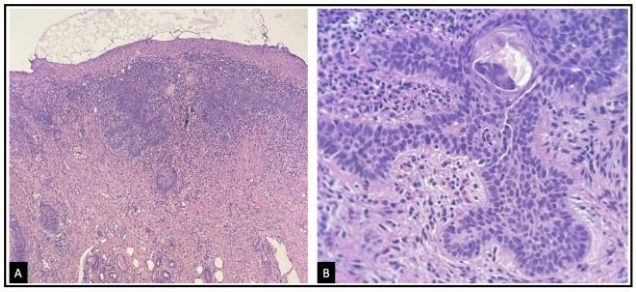

Figure 3. Histopathology of basal cell carcinoma with hematoxylin and eosin staining. A. 40x, B. 400x magnification.

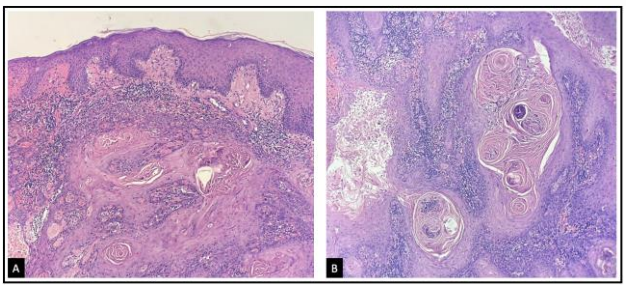

Figure 4. Histopathology of squamous cell carcinoma with hematoxylin and eosin staining. A. 40x, B. 400x magnification

Conclusion

Mohs micrographic surgery is the gold standard in the management of NMSC. The staining of frozen section tissue that is commonly used in MMS is H&E, however other stains are needed to improve diagnostic accuracy in some cases. Toluidine blue is useful for detecting mucopolysaccharides, which is a characteristic finding in BCC. Good quality H&SO has an accuracy equivalent to H&E staining for diagnosing SCC and BCC. Hematoxylin and periodic acid-Schiff staining are beneficial in cases of extramammary Paget's disease, while ORO staining is specific for detecting sebaceous carcinoma. A comprehensive understanding of the various tissue staining options allows the Mohs surgeon to determine which stain should be used in a particular case, therefore identification and diagnoses of various NMSC could be easier and more precise.

Conflict of Interests

The authors have no financial conflicts of interest.

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