Evaluation of haematoxylin subtypes for the optimal microscopic interpretation of cutaneous malignancies

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Introduction

Non-melanoma keratinocyte carcinomas are on the rise with around 156,000 cases diagnosed annually in the United Kingdom [1]. Basal cell carcinoma (BCC) is the most predominant form that is encountered [3]. In recent years Mohs micrographic surgery has gained popularity for the treatment of BCC due to the rapid result turnaround, improved surgical results and preservation of healthy tissue [2]. The Mohs technique employs mainly H&E-stained frozen sections for surgical margin assessment of cutaneous excisions, utilising microscopic evaluation of the complete, circumferential, peripheral and deep margins. This study aimed to determine which mordant based haematoxylin (Ehrlich's, Cole's, Mayer's, Gill's I, Gill's II, Gill's III, Weigert's, Harris' or Carazzi's) produced the optimal morphological clarity of staining for the identification of cellular morphology of cutaneous BCC.

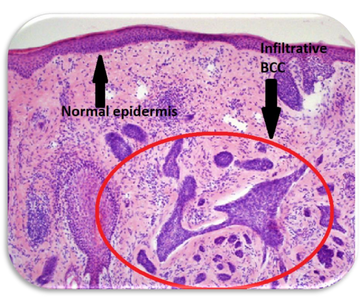

Figure 1- H&E stained skin section showing normal epidermis and infiltrative basal cell carcinoma tumour (highlighted in red).

Method- Sample cohort

In total, 100 patient cases were selected, who were undergoing Mohs micrographic surgery (MMS) at Guy's Cancer Centre in London for BCC tumour removal. The tissues used were patient consented anonymised remaining samples, that was no longer required for diagnostic purposes.

Method- optimisation and staining

To determine the optimal haematoxylin subtype, all staining was performed on the linistat linistainer (Thermo Scientific) to allow for increased standardisation and reproducibility. All samples were sectioned on the Leica CM1950 cryostat at 15um thickness and picked up on super frost plus poly-L-lysine coated slides.

Initially, all nine haematoxylin subtypes (Ehrlich, Coles, Mayer's, Gill's I, Gill's II, Gill's III, Weigert's, Harris and Carazzi's) were individually optimised on the linistat linear stainer within the parameters available on this platform, by increasing or decreasing immersion times in haematoxylin and acid alcohol. The concentration, time and volume of the remaining reagent constituents [A] [B] remained identical for each staining protocol. As part of the staining process, each haematoxylin was filtered before us. The only variation to this process was Weigert's haematoxylin which was produced before each run by mixing equal quantities of solution A and B. The optimisation procedure was performed on positive BCC debulk specimens from anonymised patient samples where prior consent had been obtained. The test slides were then stained according to the protocols in table 1.

Name of Haematoxylin	Time in Haematoxylin (Seconds)	Wash in running water (Seconds)	Time in Acid alcohol (Seconds)	Wash in running water (Seconds)	Time in Scott's Tap water (Seconds)	Wash in running water (Seconds)	Time in Eosin (Seconds)	Wash in Water (Seconds)	IDS 99% (Seconds)
Carazzi's	20	10	10	10	10	10	10	10	20
Cole's Ehrlich's Gill's I Mayer's	50	10	10	10	10	10	10	10	20
Gill's II	40	10	10	10	10	10	10	10	20
Gill's III Weigert's	10	10	20	10	10	10	10	10	20
Harris	30	10	10	10	10	10	10	10	20

Table 1: Optimised H&E protocols times on the linistat linistainer for each haematoxylin subtype.

Method-assessment criteria

Upon completion of the staining of all 100 cases with each haematoxylin subtype, the slides were independently evaluated by two assessors. The scoring criteria were based on a modified UKNEQAS CPT Mohs scheme assessment criteria [4]. Each assessor allocated scores between 1 to 5 based on the scoring criteria [4]. The assessment focused mainly on the quality of the haematoxylin staining highlighted in Table 2. The results assigned by each observer for the specificity and sensitivity of each slide were then combined to generate an overall score for each slide out of 10. These results were then added together and divided by 100 to calculate the mean and then a sensitivity and specificity score was generated as a percentage for each dye. These sensitivity and specificity scores were critically evaluated to determine if a particular haematoxylin preparation provided a better pathological assessment of BCC tumours.

Sensitivity factors	Specificity Factors
-Haematoxylin intensity too strong (defined as excessive staining which obscure nuclear visualisation including chromatin and nucleoli detail). -Haematoxylin Intensity too weak (defined as reduced staining with pale nuclear visualisation including chromatin and nucleoli detail). -Haematoxylin colour not purple/blue -Clarity of chromatin detail -Crisp and clear demonstration of nucleoli.	-Haematoxylin background staining -Uneven staining -Stain deposit present -Non-specific staining of cells/tissue. -Poor haematoxylin to eosin balance.

Table 2: Factors that assessed the sensitivity and specificity of all haematoxylin subtypes.

Results

All 900 slides stained as expected with each haematoxylin dye subtype demonstrating nuclear staining at different degrees of intensity (Figures 3-11). Staining was limited to the maximum capacity that was possible on the linistainer of 2 minutes 30 seconds. The specificity and sensitivity results for each haematoxylin subtype based on the criteria that were set out in Table 2 are shown in Table 3. Figure 2 shows the sensitivity and specificity scores of all the haematoxylin subtype dyes graphically.

Haematoxylin Subtype	Mordant	Specificity(%)	Sensitivity (%)
Carazzi's	Potassium Alum	99.2%	85.0%
Gill's III	Aluminium Sulphate	98.4%	80.4%
Ehrlich's	Potassium Alum	98.2%	81.6%
Harris	Potassium Alum	85.0%	80.2%
Weigert's	Ferric chloride	80.0%	83.4%
Gill's II	Aluminium Sulphate	62.2%	79.2%
Mayer's	Ammonium or Potassium Alum	60.6%	61.6%
Gill's I	Aluminium Sulphate	50.4%	59.8%
Cole's	Potassium Alum	40.0%	40.8%

Table 3: Breakdown of specificity and specificity result for each haematoxylin subtype.

The scores generated for specificity identified Carazzi's haematoxylin as best performing (99.2%) followed by Gill's III (98.4%), Ehrlich's (98.2%) and Harris' (85.0%).

The sensitivity score again identified Carazzi's as producing the best result (85.0%) followed by Weigert's (83.4%), Ehrlich's (81.6%) and Gill's III (80.4%).

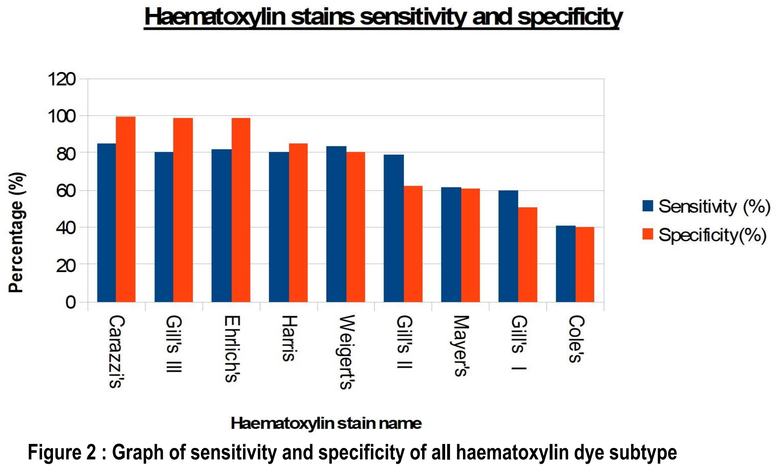

Figure 2 : Graph of sensitivity and specificity of all haematoxylin dye subtype

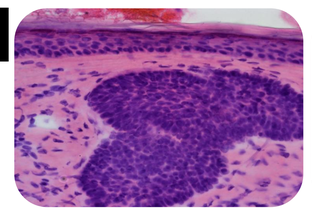

Figure 3. H&E stained section of a case with tumour foci of infiltrative BCC stained with Carazzi's haematoxylin (X40)

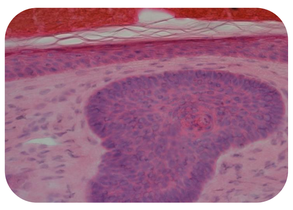

Figure 4. H&E stained section of a case with tumour foci of infiltrative BCC stained with Cole's haematoxylin (X40)

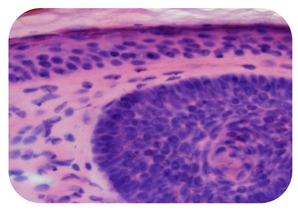

Figure 5. H&E stained section of a case with tumour foci of infiltrative BCC stained with Ehrlich's haematoxylin (X40)

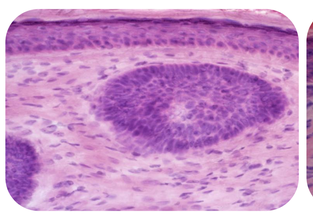

Figure 6. H&E stained section of a case with tumour foci of infiltrative BCC stained with Gill's I haematoxylin (X40)

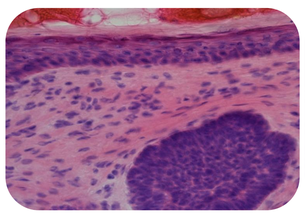

Figure 7. H&E stained section of a case with tumour foci of infiltrative BCC stained with Gill's II haematoxylin (X40)

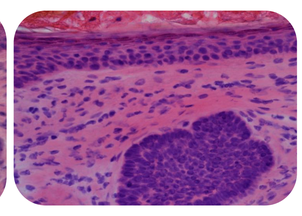

Figure 8. H&E stained section of a case with tumour foci of infiltrative BCC stained with Gill's III haematoxylin (X40)

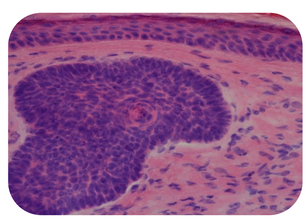

Figure 9. H&E stained section of a case with tumour foci of infiltrative BCC stained with Harris haematoxylin (X40)

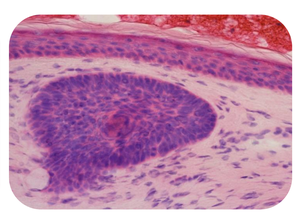

Figure 10. H&E stained section of a case with tumour foci of infiltrative BCC stained with Mayer's haematoxylin (X40)

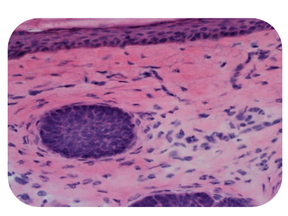

Figure 11. H&E stained section of a case with tumour foci of infiltrative BCC stained with Weigert's haematoxylin (X40)

Discussion and Conclusion

The diagnosis and classification of most neoplastic disorders rely on the information gathered from the evaluation of H&E stained sections, with the interpretation of haematoxylin stained nuclear detail playing a key role in determining morphological characteristics. This study identified Carazzi's haematoxylin as the most optimal staining dye for the identification of BCC tumours for use as part of the Mohs procedure.

The use of Carazzi's haematoxylin as part of any frozen section procedure, including Mohs, has not been widely assessed. However, this study has highlighted the vastly improved and clear visualisation of nuclear and chromatin detail of Carazzi's haematoxylin when used as part of the H&E staining process. This was reflected in the higher sensitivity and specificity scores that Carazzi's obtained overall in this study. Nationally in the UK, there is no standardised staining protocol for use in the MMS procedure. This study helps towards quantifiably determining an optimal H&E staining protocol that can be used as part of this procedure.

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