Pan-cytokeratin markers for rapid frozen section immunocytochemistry from head and facial Mohs cases of basal cell carcinoma: a comparison and evaluation to determine the marker of choice

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ABSTRACT

The application of immunocytochemistry in the field of Mohs micrographic surgery (MMS) is well established. This study evaluates the use of pan-cytokeratins (AE1/AE3, MNF116 and AE1/AE3+PCK26) in the assessment of basal cell carcinoma (BCC) on frozen tissue debulk specimens. Fifty-five cases of BCC, all from head and facial sites, were assessed in the study. In addition to staining all cases for the three cytokeratin antibodies under investigation, sections were also stained with haematoxylin and eosin (H&E) to demonstrate tumour architecture and morphology. All sections for immunocytochemistry were stained on a Roche Ventana BenchMark Ultra automated platform employing a rapid frozen section protocol. Results were assessed based on the intensity of staining of keratinocytes (scale: 0–100%), as well as sensitivity of staining determined by the total percentage of keratinocytes stained within the tissue section. AE1/AE3 demonstrated the most consistent staining both in terms of intensity of staining and sensitivity, with a mean of 99.1% and 99.9%, respectively. AE1/AE3+PCK26 average results indicated scores of 70.6% for intensity and 87.2% for sensitivity, with MNF116 scoring 92.9% for intensity but only 57.3% for sensitivity. The data indicate that AE1/AE3 is the best pan-cytokeratin antibody to use in the assessment of BCC in MMS. The use of cytokeratin immunocytochemistry is justified in morphologically complex cases of BCC, or in cases where dense inflammatory infiltrate surrounding any suspicious cells make identification of small numbers of tumour cells difficult to determine with just an H&E stain. The significant rationale is that cytokeratin staining is a valuable adjunct in the study of tumour cell assessment in cases of MMS for BCC. In addition, the use of anti-AE1/AE3 cytokeratin antibodies provides the most consistent staining results for such cases.

KEY WORDS: Carcinoma, basal cell.
Immunohistochemistry.
Keratin.
Micrographic surgery, Mohs.

Introduction

Mohs micrographic surgery (MMS) is a widely employed technique for the removal of cutaneous tumours. The methodology relies on surgical removal and subsequent histological examination of the entire lateral and deep margins. Multiple layers are often taken to ensure complete margin clearance of any given tumour under investigation. The technique has an extremely high cure rate with figures widely published in scientific literature of 95–99%, depending on tumour type and anatomical sites. In addition, it offers the benefits of improved cosmetic result, particularly on facial sites, as preservation of normal uninvolved tissue is fundamental to the procedure.[1,2]

Basal cell carcinoma (BCC) is by far the most commonly encountered tumour for which MMS is performed. Basal cell carcinoma and squamous cell carcinoma (SCC), account for around 90% of all skin cancer (Cancer UK data). Both commonly occur on sun-exposed sites and will predominate in fair-skinned individuals. Interestingly, around 80% of BCCs will occur on the head and neck areas. Most forms of BCC are slow growing and can be treated early with conventional treatments such as radiotherapy or minor surgery. These tumours rarely metastasise; however, if left unchecked they may become more infiltrative and locally invasive, causing tissue damage and destruction within the surrounding tissue. Histologically, BCCs arise from the lower layers of the epidermis (often termed the basal layer), although some may arise from the outer root sheath of the pilosebaceous unit (hair follicle).

In the UK, MMS is mainly performed on facial sites. In more recent developments the use of immunocytochemistry (ICC) as an adjunct to improve the accuracy for the detection of tumour cells within MMS procedures has been gaining popularity. The use of rapid frozen section ICC techniques in MMS are widely discussed in the literature.[1–6] The majority

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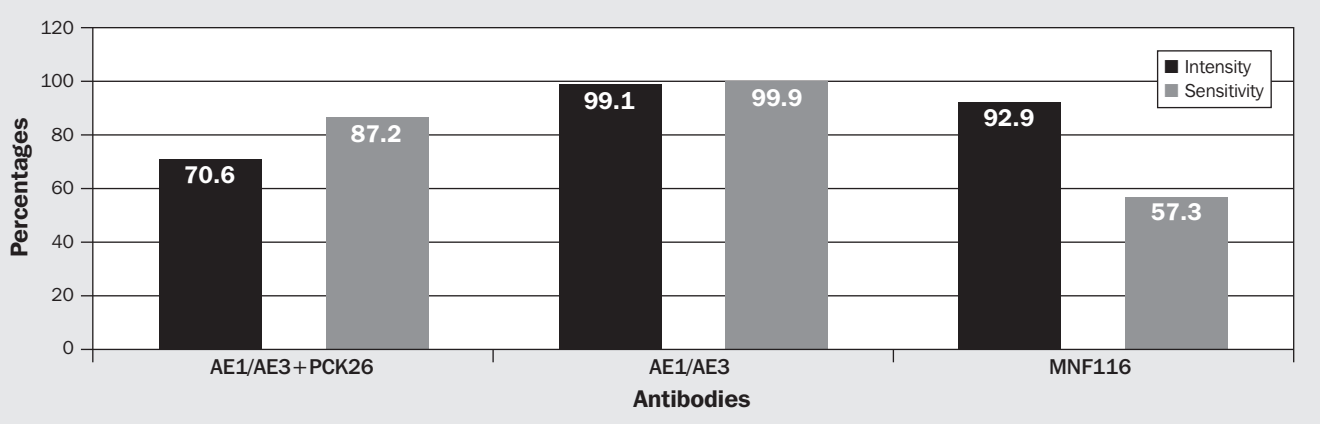

Fig. 1. Histogram of the mean average scores for intensity and sensitivity of staining achieved with all three cytokeratin antibodies across all facial and head anatomical sites.

of studies to date have focused on specific areas of application, most notably cytokeratin studies in cases of BCC and SCC, Melan A studies in cases of lentigo maligna melanoma (LMM) and, to a lesser extent, CD34 staining in cases of dermatofibrosarcoma protuberans (DFSP).[7]

In cases of BCC, the tumour arises from keratinocytes so the most applicable antibodies to employ are those raised against a pan-cytokeratin cocktail of cytokeratins. These are intermediate filament proteins commonly found in nearly all animal cells. They are functionally involved in protection from mechanical trauma, epithelial integrity in general, and possibly communication between surrounding cells.[8]

There appears to be no clear statement on which cytokeratin marker should be used in the case of the studies for BCCs and whether or not any one marker is better than another in terms of sensitivity and intensity of staining of tumour cells. In addition, the clarity of data on differing cytokeratin expression on different anatomical structures of the head and face remain to be fully determined.

In order to investigate these points, the current study evaluates the performance of the pan-cytokeratin antibodies AE1/AE3 (Dakocytomation), MNF116 (Dakocytomation) and AE1/AE3+PCK26 (Roche Diagnostics) on debulk frozen section samples of BCC from 55 cases of scalp and facial tumours.

Material and methods

All 55 cases of BCC used were frozen debulk tissue samples of positively diagnosed tumours from patients undergoing MMS within St. John's Dermatology Laser and Surgery Unit (DSLU).

Following completion of the Mohs procedures, sections were cut (8–10 μm) from the remaining debulk frozen tissue blocks using a Leica CM 1950 cryostat and mounted on Superfrost plus adhesive slides (VWR). Four slides were prepared from each block. One slide was stained with haematoxylin and eosin (H&E), the remaining slides were sent for rapid ICC staining using the primary antibodies AE1/AE3 (Dakocytomation, code M3515), MNF116 (Dakocytomation, code M0821) and AE1/AE3+PCK26 (Roche Diagnostics code, 760-2595).

For H&E staining performed by hand, slides were fixed in ethanol for 1 min and washed in running tap water for 2 min. Slides were then transferred to Harris' haematoxylin for 1 min. Slides were then washed in running tap water for 1 min, differentiated in 0.5% hydrochloric acid in 99% industrial methylated spirit for 30 sec and transferred to running tap water for 2 min. The slides were then 'blued' in 0.4% ammonia alcohol. The slides were then washed in running tap water for 1 min and placed in 0.5% eosin for 1 min, before being washed in tap water and dehydrated in alcohol and cleared through xylene. Sections were mounted in DPX.

Table 1. Overall mean average intensity and sensitivity scores for staining across all anatomical sites on the head and face.

Site	Number	AE1/AE3+PCK26		AE1/AE3		MNF116	
	Mohs	Intensity	Sensitivity	Intensity	Sensitivity	Intensity	Sensitivity
Cheek	2	70.0	85.0	100.0	100.0	80.0	45.0
Ear	4	67.5	82.5	95.0	100.0	82.5	57.5
Eye	3	70.0	90.0	100.0	100.0	100.0	60.0
Forehead	7	72.9	91.4	98.6	100.0	94.3	55.7
Lips	3	76.7	83.3	100.0	100.0	100.0	60.0
Scalp	4	70.0	92.5	100.0	100.0	100.0	60.0
Nose	28	70.4	88.2	99.3	99.3	98.6	60.0
Temple	4	67.5	85.0	100.0	100.0	87.5	60.0
All	55	70.6	87.2	99.1	99.9	92.9	57.3

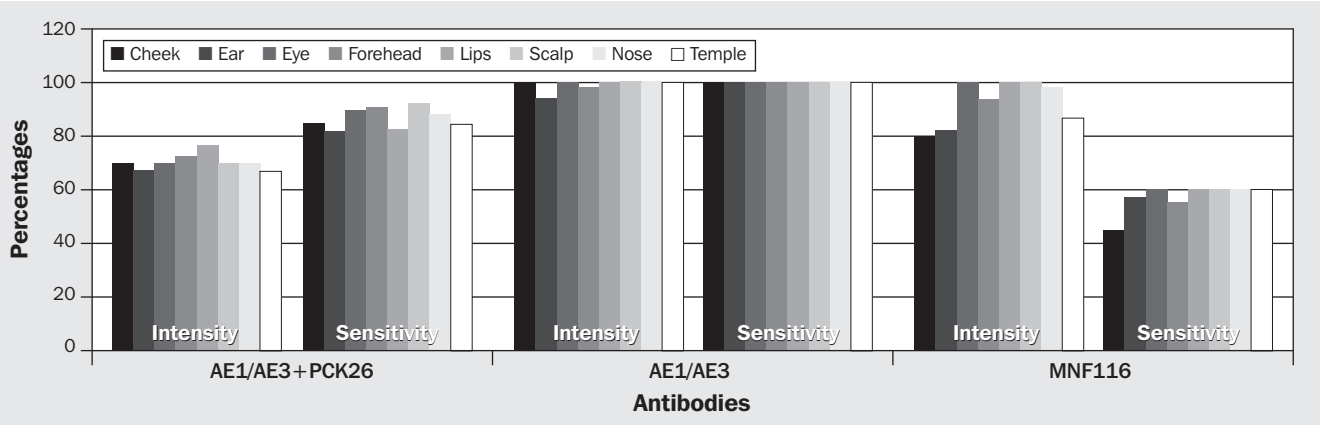

Fig. 2. Histogram of the mean average scores for intensity and sensitivity of staining achieved for each anatomical site with all three cytokeratin antibodies.

For ICC, the slides were fixed in ethanol for 1 min and washed in running tap water for 2 min. Slides were then transferred to the Roche Ventana BenchMark Ultra machine. The primary antibodies used in the study were cytokeratin AE1/AE3 monoclonal mouse anti-human antibody (1 in 100 dilution); ready to use (RTU) primary antibody anti-pan-keratin clones AE1/AE3+PCK26; and cytokeratin MNF116 monoclonal mouse anti-human antibody (1 in 100 dilution). Counterstaining was performed with Roche Ventana Haematoxylin II (Roche Ventana, code 790-2208). The detection kit used was the Optiview DAB IHC detection Kit (Roche Ventana, code 760-700).

The positive control was a known frozen section of BCC which was not used in the study, but used for the purpose of evaluation of consistency of staining and to determine staining intensity and sensitivity. The negative control was the same positive control tissue, but with the primary antibody replaced with the standard wash buffer.

All cases were assessed and reviewed by two independent assessors (GEO and MS). The H&E staining enabled assessments of the tumour presence and general morphology. The ICC assessments included evaluation of intensity of staining of tumour cells as a percentage (intense staining: 75–100%; moderate intensity staining: 50–74%; weak intensity staining: 0–49%). The evaluation of sensitivity was determined by the percentage of total staining of keratinocytes within the tissue section. This included normal, uninvolved keratinocytes in the epidermal and dermal compartments as well as tumour cells in any given section.

Results

Figures 1 and 2 provide information on staining intensity and sensitivity for each antibody. Figure 1 reflects the mean results for both intensity and sensitivity of staining with all three antibodies employed across all the anatomical sites of the face and head. Figure 2 reflects the mean results for intensity and sensitivity staining with all three antibodies employed in the study for each anatomical site of the face and head.

The results for intensity and sensitivity are summarised in Table 1. It is clearly evident that AE1/AE3 is the most consistent marker, with intensity and sensitivity rates averaging 99.1% and 99.9 %, respectively. This indicates that AE1/AE3 consistently stained all the keratinocytes within the tumours studied and also the normal, uninvolved keratinocytes within the surrounding tissue with high staining intensity. Results for MNF116 indicate that this antibody produced on average lower rates of intensity and sensitivity (92.9% and 57.3%, respectively). Finally, the results for AE1 AE3+PCK26 produced the intensity and sensitivity scores of 70.6% and 87.2%, respectively.

Of note is the drop in average sensitivity scores for MNF116 compared to the other two antibodies. This was due to the fact that normal epidermal keratinocytes above the basal layer were far more variably stained or unstained with MNF 116. The intensity of staining, however, was consistently high and indicated that all the tumour cells were in fact staining in the majority of cases. (Figs. 3a–c and Figs. 4a–c)

Table 2. Product specification details of the antibodies AE1/AE3 and AE1/AE3+PCK26 and MNF116.

Antibody	AE1/AE3		AE1/AE3+PCK26			MNF116
Clone	**AE1**	**AE3**	**AE1**	**AE3**	**PCK26**	**MNF116**
Product supplier	Dako	Dako	Ventana	Ventana	Ventana	Dako
Molecular weights (kDa)	56.5, 54, 50, 48, 40	65, 67, 64, 59, 58, 56, 54, 52	56.5, 50, 48, 40 (Also 64, 51 Nordiqc.com)	65-67, 64, 59, 56, 52	58, 56, 52	58-40
Family type: Acidic=I Neutral-basic=II	Acidic=I	Neutral-basic=II	Acidic=I	Neutral-basic=II	Neutral-basic=II	Acidic=I Neutral-basic=II

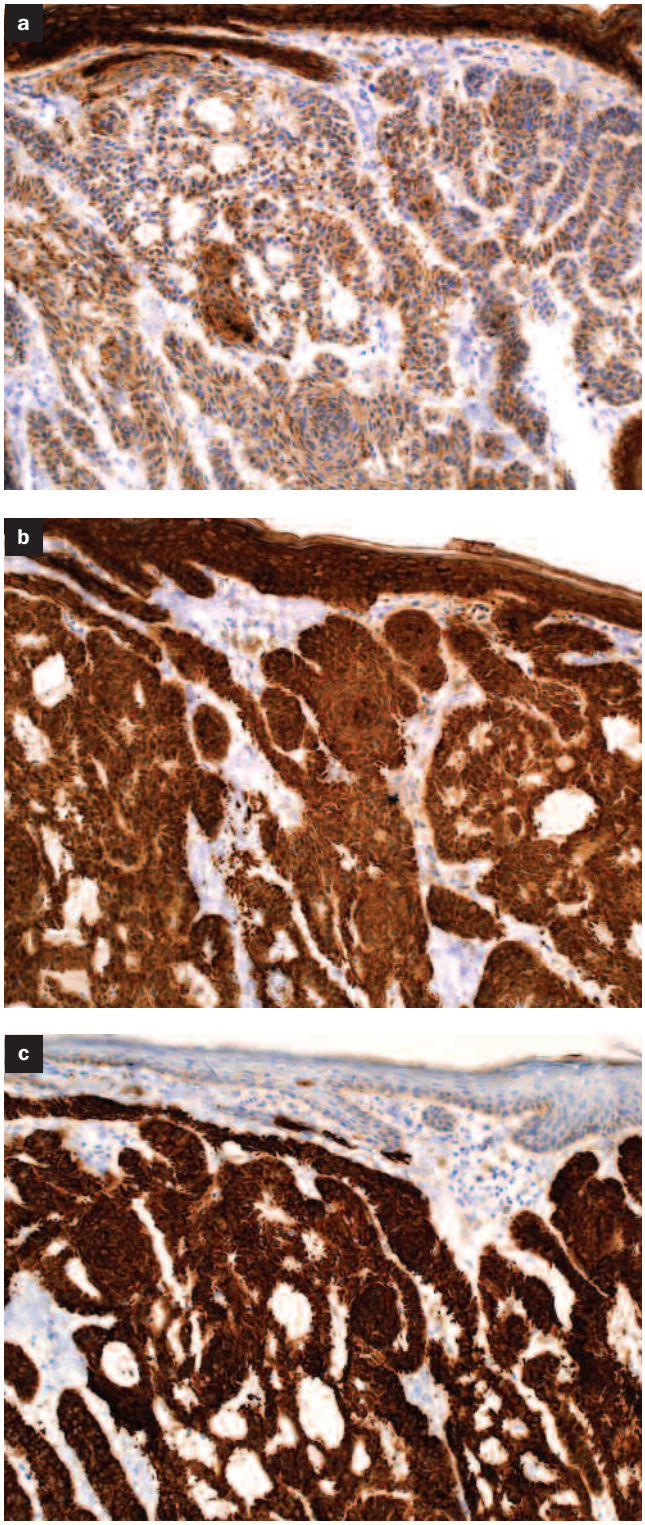

Fig. 3. a) Anti-AE1/AE3+PCK26 labelling of a nodular BCC, demonstrating uniform staining of the epidermal compartment and patchy and less intense staining of dermal BCC tumour cells (original magnification x20); **b)** Anti- AE1/AE3 labelling of the same case with the same field view, demonstrating uniform staining of all keratinocytes in both the epidermal and dermal compartments and with good intensity of staining (original magnification x20); **c)** Anti-MNF116 labelling of the same case with the same field view, demonstrating basal keratinocyte labelling within the epidermal compartment with strong, intense staining of the tumour cells within the dermal compartment (original magnification x20).

Table 2 provides information on the cytokeratin molecular weights for each antibody employed in the study. This reflects the differences between each antibody in terms of its recognised labelling profile.

Discussion

This study clearly demonstrates that AE1/AE3 is the cytokeratin antibody of choice for the assessment of BCC in MMS frozen tissue. It provides the advantage of high intensity of staining with accompanying high levels of sensitivity for keratinocyte demonstration, irrespective of anatomical site on the head or face.

Table 2 highlights the molecular weights of the cytokeratins contained within each of the three antibodies used in this study. Clearly, the cocktail of molecular weight cytokeratins in AE1/AE3 is more broadly distributed than those in MNF116. This would help to explain the uniformity of staining of all the keratinocytes in the tissue sections seen with AE1/AE3, irrespective of location in the epidermal or dermal (pilosebaceous gland) skin compartments.

MNF116 seemed to be strongly expressed on basal keratinocytes, pilosebaceous keratinocytes and tumour cells in general. However, staining for suprabasal keratinocytes within the epidermal compartment was often missing or weak (Figs. 3a–c), which contributed to the lower recording for sensitivity with MNF116 in this study. This would seem to indicate that the higher molecular weight cytokeratins are more prevalent in the suprabasal keratinocytes and therefore detected more precisely with AE1/AE3 than with MNF116.

Data published on cytokeratin expression in suprabasal cells in wound healing or the regenerative epidermis suggests that that there is a temporary abnormal staining pattern, meaning that expression of cytokeratin in epidermal keratinocytes changes during the process of wound healing.[9] Basal cell carcinomas can create ulceration in some cases, resulting in repair and regeneration.

Suprabasal keratinocyte staining offers good internal control for monitoring staining performance and is a clear advantage. However, BCCs arise from the basal layer or pilosebaceous units in general and so the suprabasal keratinocytes are not directly involved with BCC tumour genesis. Interestingly, the results achieved for AE1/AE3+PCK26 demonstrate that there is no benefit over AE1/AE3 alone. In fact, AE1/AE3 used on its own gave superior results not only in terms of intensity of staining but also, to a lesser degree, in terms of sensitivity (Figs 4a–c). This may be due to the fact that the antibody used was in prediluted form and both the AE1/AE3 and MNF116 antibodies were diluted from the concentrated forms of the antibody. There could also be changes to the composition of the cocktail as a result of the addition of PCK26, which may have altered its sensitivity.

Expression of keratins in human tumours, particularly carcinomas, is well documented in the literature and examples include CK5, -7, -8/18, -19 and -20, some of which are particularly useful when evaluating metastatic tumour deposits. Highlighting tumour cells by ICC staining provides the Mohs surgeon with the ability to pinpoint residual tumour cells at the surgical margins. This process should be undertaken during the final round of sections taken on the

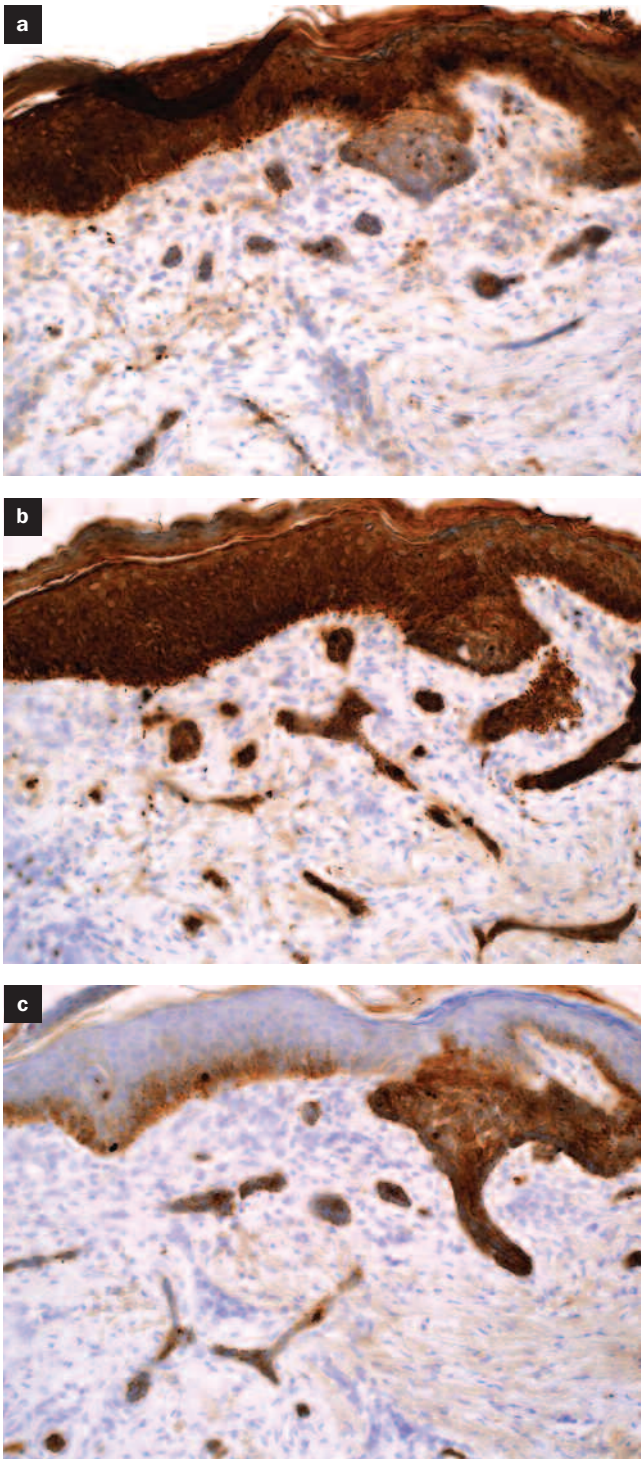

Fig. 4. a) Anti-AE1/AE3+PCK26 labelling of a superficial BCC tumour nest, demonstrating good epidermal keratinocyte labelling with good intensity, but weaker labelling with reduced intensity of tumour deposits budding down from the basal layer and entering the dermal compartment (original magnification x20); **b)** Anti-AE1/AE3 labelling of the same case with the same field view, demonstrating uniform staining of all keratinocytes in both the epidermal and dermal compartments, with good intensity of staining (original magnification x20); **c)** Anti-MNF116 labelling of the same case with the same field view, demonstrating basal keratinocyte labelling within the epidermal compartment, with strong and intense staining of the tumour deposits budding down from the basal layer and entering the dermal compartment (original magnification x20).

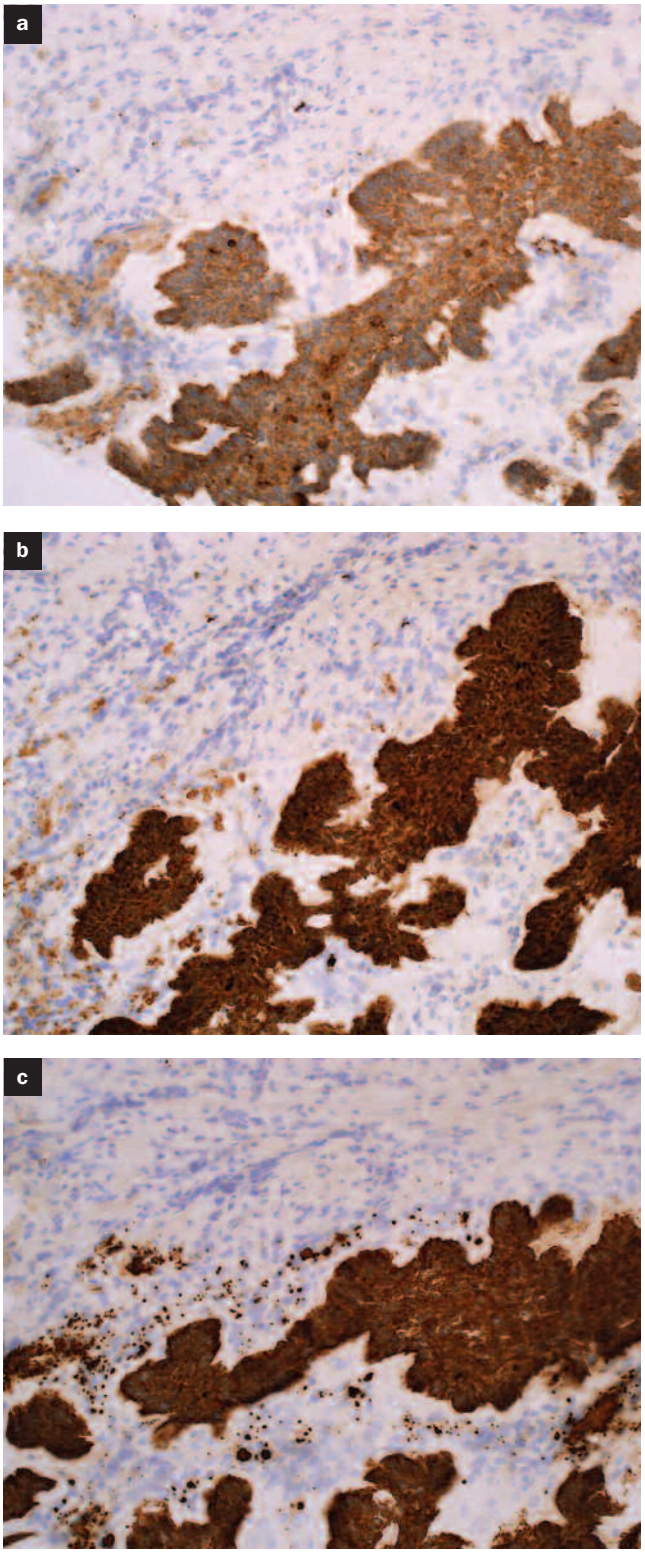

Fig. 5. a) Anti-AE1/AE3+PCK26 labelling of a dermal nest of BCC with surrounding staining of amorphous amyloid K deposits (light brown) (original magnification x10); **b)** Anti-AE1/AE3 labelling of the same case with the same field of view, demonstrating, staining of a dermal nest of BCC with surrounding staining of amorphous amyloid K deposits (light brown) (original magnification x10); **c)** Anti-MNF116 labelling of the same case with the same field of view, demonstrating staining of a dermal nest of BCC with surrounding staining of amorphous amyloid K deposits (light brown) (original magnification x10).

final layer of tissue removed and ideally deemed clear with routine H&E staining. It acts as a confirmation of the H&E findings. This can be critical in some instances (e.g., when residual tumour cells are of an unusual histological appearance or exhibit a particularly aggressive or infiltrative growth pattern).

Similarly, cytokeratin staining can be useful in cases where prolonged surgical procedures have resulted in a busy or dense inflammatory infiltrate masking any suspicious cells, making identification of small numbers of tumour cells difficult to determine by H&E staining alone.

Of interest in this study was the detection of amyloid K deposits in association with the BCCs studied. This was seen in 10 of the 55 cases studied in this series. Amyloid K is derived from the epidermal keratinocytes comprising the BCC tumour, stained by the cytokeratin antibodies. The formation of amyloid K is believed to be due either to abnormal keratin maturation or degeneration of the basaloid cells involved in tumour genesis. There is also evidence to suggest that any insult to the skin leading to degenerative cell change may stimulate the synthesis of amyloid K deposits.[10,11] Subsequently, the amyloid K contains cytokeratin components. In an H&E-stained preparation, the protein appears as amorphous, pale pink deposits surrounding and lying between BCC tumour cell nests. Staining with pan-cytokeratin antibodies demonstrates a pale staining with the chromogen employed. In this study, 3-3 diaminobenzidine (DAB) was employed which produced a paler but clearly detectable pale brown staining of the amyloid K compared to the intense staining of the BCC tumour cells (Figs. 5a–c).

Finally, the use of cytokeratin antibodies to aid the final determination of tumour clearance in any given case of MMS is significant. In a recent study by Smeets,[2] in which 49 patients with 51 BCCs were assessed using MNF116, one out of 143 stained slides showed MNF116-positive staining where the H&E was negative. This represented a failure rate of 0.7% of the slides assessed but this single slide represented a failure rate of nearly 2% of the treated patients. The significant rationale is that cytokeratin staining is a valuable adjunct in the study of tumour cell assessment in cases of MMS for BCC.

Findings from this study would suggest that it is important to ensure that the choice of cytokeratin antibody used is appropriate. The detection of small clusters of residual tumour deposits will depend on an antibody that displays high sensitivity and intensity of staining. In this regard, anti-AE1/AE3 is the cytokeratin antibody of choice. Also noteworthy is the delicate balance between the cost of, and labour involved with, performing such additional tests. The Mohs surgeon requires a fast turnaround time with an end product that is quality assured and guaranteed. Automated platforms will ensure the latter requirement, but, currently, they are not as time efficient and are more costly than manual and labour-intensive methods. Continual developments in automated technology will provide platforms that will improve on turnaround times and enable an acceptable level of proficiency to be reached, while keeping costs per slide rates for ICC tests to an acceptable minimum. □

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