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Developing a Double-label Immunohistochemistry Panel for Diagnosis of Skin Cancers Using Commercial Kits

Skin cancer is common cancer diagnosed in Australia. Since the high incidence rates, more efforts have been put in to look for a more reliable tool to diagnose the tumour. Immunohistochemistry technique, especially double-labelled immunohistochemistry, has become a useful and expected tool in differentiating different types of skin cancers because it provides more details in demonstrating proliferated activities of tumour cells, compared with routine histopathology test. Some dual stain markers have been studied and reported. Based on the analysis, it is believed that developing double-labelled IHC panels could significantly help to make more accurate analysing of the varying tumours, although most applications of double-labelled immunohistochemistry are still in the research fields. Moreover, a new technology, next-generation sequencing, in the molecular area, has been mentioned. The applications of next-generation sequencing in diagnosing of two different skin cancers has been briefly discussed.

Keywords : Skin cancer, Immunohistochemistry (IHC), Biomarkers, Double stain, Dual stain

Skin cancer is one of the commonly diagnosed cancers in Australia. Based on where the cancer cells develop, skin cancer is categorised into melanoma and non-melanoma skin cancer (NMSC). NMSC encompasses basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) which are common NMSCs, and rare NMSCs. The incidence rates of skin cancer increase each

year. The Australian Institute of Health and Welfare (AIHW) has estimated that there will be more than 15, 200 new cases of melanoma and 800 new cases of NMSC diagnosed in 2019. The rising incidence of skin cancer has been linked to chronic exposure to ultraviolet radiation.(1, 2)

As the high occurrence of skin cancer, it is necessary to look for a reliable diagnostic tool to confirm the disease. There are two steps for diagnosing skin cancer. The first step usually is that the doctor combines the medical history of the patient with the physical exam on the skin to determine whether the lesion is skin cancer suspected. If the tumour is suspected, further testing should be done to confirm the diagnosis. The second step is that the suspicious area of skin will be removed for skin biopsy in the histopathology laboratory. Performing skin biopsy is a definitive examination to diagnose the suspect lesion and find out the stage or extent of skin cancer. The unique laboratory tests, such as immunohistochemistry (IHC), fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH) will be performed if the routine Haematoxylin and Eosin (H&E) stain cannot confirm the diagnosis. (3-5)

Immunohistochemistry uses the method of immune reaction to detect and localise specific structure which cannot observe in routine histopathology test. Since Coons et al. first came up with the basic principles of direct IHC method in 1941 and the principles of indirect IHC method of amplification were conceptualised in 1979, IHC technique has gradually become a pivotal tool in histopathology for classification and diagnosis of neoplastic diseases.

(6, 7) In skin cancers, the aim of using IHC is a taxonomy of the tumours or cell of origin. Currently, the use of IHC technique in the diagnosis of <https://assignbuster.com/double-label-immunohistochemistry-panel-for-diagnosis-of-skin-cancers-using-commercial-kits/>

neoplastic skin disease has significantly and rapidly developed with the evolution of commercial biomarkers. Newly detected antibodies are becoming more and more complete, although the practices of the new markers in the clinic still need to be validated. (7-9)

Double-label IHC identifies two different antigens simultaneously within the same section. In this technique, the proper stain can only attain when the appropriate markers and enzymatic system are chosen. (10) This article will focus on the method of double stain IHC, existing dual IHC stain markers for diagnosing skin cancers and discussing a new technology which may be an essential tool in the diagnosis of skin cancers in the future.

Introduction to Immunohistochemistry

Immunohistochemistry is a diagnostic method to localise the antigen in the tissue sections by using specific antibodies and visualise the immune reaction by markers. Theoretically, IHC can demonstrate any protein (antigen) which can be retained in testing tissue sections or cells. (7, 11) The technique involves knowledge in Immunology, Histology, and Chemistry.

In the immune response, antibodies are the critical component to recognise a specific antigen expression site (epitope) and then bind to the region, particularly with high affinity. The representation of antibody structure shows “ Y” shape, which is composed of four polypeptide chains, containing two light chains and two heavy chains. The situation of the antigen-antibody binding site, also known as an epitope, is at the tip of the Y-shaped arms. In the traditional IHC method, the epitope remains typically masked. During fixation, tissue processing, and antigen retrieval, the tertiary structure of the <https://assignbuster.com/double-label-immunohistochemistry-panel-for-diagnosis-of-skin-cancers-using-commercial-kits/>

target protein can be changed notably. The chemical modification, for instance, temperature, oxidation and cross-linking fixatives, can lead to alternation in the structure of the protein. Therefore, it is significant to test different fixative methods and antigen retrieval solutions to see whether the antigen-antibody reaction can still be identified after tissue processing. (11)

Basic Protocol of Double-Labelled Immunohistochemistry

Double-label IHC, based on the IHC method, applies two immunoenzymatic systems or one immunoenzymatic system with different substrates to achieve two colours in one section. (10) The process usually uses the formalin-fixed and paraffin-embedded tissues for analysing. Adequate fixation is essential in preserving tissue. Commonly, it is recommended that the tissue should be fixed in 10% neutral buffered formalin (NBF) for a minimum of 8 hours. Prolonged fixation may damage the cellular structure and lead to diminishing antibody binding capability. Furthermore, some antigens can only be preserved by rapid freezing which is another conventional technique. Either paraffin-embedded or fresh-frozen tissue preparation technique is working well in small soft tissues. (7, 11)

IHC starts from antigen retrieval. Antigen retrieval is aimed to unmask the antigenic sites since the methylene bridge cross-links generated during the formalin-fixed process which hide antigens. To undo the antigen-masking effect, Heat Induced Epitope Retrieval (HIER) by using microwave or pressure cooker or Proteolytic Induced Epitope Retrieval (PIER) by using Proteinase, Trypsin and Pepsin can be used. A combination of two methods can also be utilised. (7, 11)

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The second step is blocking the endogenous enzyme. The purpose of this step is to inactive enzymatic activity in tissue samples, which avoids staining the background of tissues. The first blocking reagent is usually Hydrogen Peroxidase (HRP) (7, 11-14)

The third step is performing the first primary antibodies, which are to target the interested proteins. Antibodies can be either monoclonal or polyclonal. These two types of antibodies have different affinities and specificities, which are the two main factors contributing to antibody-antigen interaction. There are two kinds of antibodies, and the differences are compared in Table 1. Immunohistochemistry contains two methods: direct and indirect methods. In the direct method, the antibody is designed to identify epitope on the target of interest, and the label is directly conjugated to the primary antibody. In the indirect method, the design of the primary antibody is intended to bind to the target of interest, and the antibody is unconjugated. The label is conjugated to the secondary antibody, and the secondary antibody then binds to the primary antibody. Hence, for the indirect IHC, the fourth step is performing the first secondary antibodies to amplify the reaction. (7, 11-14)

Table 1. Comparison between monoclonal antibodies and polyclonal antibodies. (7, 11-14)

	Monoclonal Antibodies	Polyclonal Antibodies
Reaction with epitope on antigen	only one epitope on antigen	Directly react with many epitopes on antigen
Affinity	Lower	Higher
Sensitive	Less	High
Specificity	High	Low
Cross-reactivity	No	More

The final step for the first part of the IHC stain is visualisation. The colours generated by using chromogen. The uses of different chromogens are based on the blocking enzyme in the second step. The most common used chromogens are shown in Table 2. (7, 11-14)

Table 2. Widely used chromogens and the colour production after reacting with the enzyme. (7, 11-13)

Common Chromogen	Substrate	Colour
Diaminobenzidine (DAB)	HRP	Brown
Fast Red	HRP	Red
Aminoethyl Carbazole (AEC)	Alkaline Phosphate (AP)	Red

The second part of the IHC stain will start from blocking the section in AP. The second primary antibody will then be applied. Following the application of the second secondary antibody, finally, the tissue section will be stained in AEC or permanent red. (13)

Double-Labelled IHC in Diagnosis of Skin Cancers

With the significant development of immunohistochemistry in the last two decades, single-stain IHC has gradually become a routine diagnostic tool in skin cancers. (8, 9, 15) However, nowadays, the uses of double-stain IHC in skin cancers are still only in academic researches and private laboratories. As Nielsen et al. in 2011, Kristian et al. in 2012, Arnaud et al. in 2015 and Rafael et al. in 2017 have done more studies, it is confirmed that the dual-stain IHC is a more reliable diagnostic tool to provide more

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specific interpretation of proliferated tumour cells compared with the routine H&E examination. (16-20)

Some combinations of biomarkers for diagnosing skin cancers have been designed and evaluated the diagnostic utilities in the last decade. With more studies have been completed, some dependable combinations of antibodies have been provided.

I. Double-Staining IHC Markers for Diagnosis of Melanoma

- Melan-A/Ki-67

The experimental results from Nielsen *et al.*, Puja *et al.* and Leigh *et al.* exhibited that all the test samples staining in Melan-A and Ki-67 double staining helped to diagnose melanoma. Melan-A focused on the histogenesis of melanosomes. Ki-67 highlight other proliferation of cells, for example, histocytes, keratinocyte and lymphocytes at the same time. Therefore, this dual-stain combination is useful in demonstrating the proliferation of melanocytes in the deeper lesions. (9, 17, 21-23)

- H3 (PHH3)/ MART-1

Kristian et al. in 2012 indicated that H3 (PHH3)/ MART-1 is an independent marker for the staging of melanoma by evaluation of mitotic rates, and when combined the H3 (PHH3)/ MART-1 double-label IHC test with routine H&E test, the diagnosis is more reliable. (18) In this group of biomarkers, H3, the sensitive and specific marker for detecting mitotic activities, has been multiplexed with MART-1, the sensitive marker for proliferating melanocytes. (18, 24-26)

After the study by Kristian et al., further studies have been performed by Michael et al., Patricia et al. and Thomas et al., and more evidence confirmed the value of the dual stain marker in diagnosing melanoma. Thomas et al. also demonstrated that people do not need to pay attention to the cells, which only staining with H3 during the mitotic count since these cells are mitotically active cells but melanocytes. This conclusion can avoid reporting false-positive results and improve diagnostic rates. (24-26)

II. Double-Staining IHC Markers for Non-Melanoma Diagnosis

- S-100/ p63

Noah *et al.* built a novel dual stain IHC protocol- S-100/ p63 and succeeded in using this dual IHC stain to increase the detection of SCC. In this protocol, S-100 was used for labelling nerve and p63 was for labelling nucleus of tumour cells. The experiment showed that the double label IHC improved the diagnostic rate of SCC in 2.17 times comparing with using routine H&E stain only which was 13 in 57 versus 6 in 57 (95% confidence interval 0.89-5.30, $p = 0.08$). (27)

- INMS1/CK20

In 2017, Patrick et al. reported that INMS1 could be conjugated with CK20 in double stain IHC to provide a better evaluation and diagnosis of Merkel cell carcinoma (MCC). MCC is an uncommon and aggressive cutaneous neuroendocrine (NE) tumour, and the disease is usually confirmed by dual stain IHC. INMS1, a marker, is highly expressed to neuroendocrine neoplasm. (28, 29) The study by Patrick et al. was also concluded that INSM1 is

localised to the nucleus, and the stain of the nuclei was strong and well-
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differentiated in the tumour (14 in 15 cases, 93.3% sensitivity). CK20 stains cytoplasm and it often assist in distinguishing between MCC and other NE neoplasms in MCC. The result: INMS1-/ CK20- indicated that the lesion is non-MCC and with NE characteristics. (29)

Advantages and Disadvantages of Double-Labelled IHC

Naturally, every method has its benefits and drawbacks. The summary of the pros and cons for double-labelled IHC are listed in Table 3. (12, 14, 30)

Table 3. Advantages and disadvantages of double stain IHC comparing with the single stain IHC. (12, 14, 30)

Advantages	Disadvantages
<ul style="list-style-type: none"> • Saving tissue samples • Saving time, due to staining two proteins at the same time • Saving 	<ul style="list-style-type: none"> • Need optimisation for all the new combinations of biomarkers • There is only a small number of antigens that can be stained in

- one tissue section
- reagents , cheaper
- Avoid the problem s coming from staining series slides
- Can only perform the primary antibody originated from the specific species
- Difficult in achieving dual staining

New Advances other than Double-Labelled IHC Applied to Diagnose Skin Cancers

Next-generation sequencing (NGS) technology is currently being used in studying melanoma and NMSC cases with the mutant gene in research purposes, which may be a useful method to diagnose skin cancers. NGS has become a diagnostic tool and helped to improve the sensitivity in detecting various DNA mutations. Ihle et al. evaluated NGS technology on melanoma cases, and the technology showed 100% specificity and 97.5% sensitivity. They also discussed the value of NGS for using in molecular diagnosis of melanoma, which may contribute to the diagnosis of melanoma in the future.

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The study which was done by Francesco demonstrated similar results. In the diagnosis of melanoma, NGS exhibited 100% specificity and 98% sensitivity. (31-33) Furthermore, Jason et al. indicated that NGS could help the clinic to identify the origin of SCC. With more further studies being done, NGS may play an essential role in diagnosing NMSC diseases. (34)

It is unavoidable that NGS will take the place of all molecular diagnostic method that we have used in the recent day, although NGS has disadvantages, such as requiring more tumour materials, having longer turn-around time and requiring special equipment. Since NGS technology has highly specific and sensitive in the detection of gene mutations. (33)

Conclusion

Double-labelled IHC is now used in research institutions for the diagnosis of different skin cancers. Some dual stain IHC markers have confirmed to be highly reliable biomarkers in the diagnosis of melanoma. Although the downsides of double stain IHC are inevitable, with more efforts devoting in the field, it is believed that the double-labelled IHC will be a powerful technique in interpreting the proliferated activity of tumour cells. In the end, more and more new technologies have been studied to aid in determining the unknown cases. It is no doubt that NGS will be a practical diagnostic tool for skin cancer in the future.

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