Immunoexpression of Ki-67 Labelling Proliferation Index in Phyllodes Tumour of Breast by Polymer-based Detection Method

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Phylloides tumours (PTs) are rare breast neoplasms with a variable clinical course depending on the tumour category**.** The classification of PTs proposed by the World Health Organization (WHO) into benign, borderline and malignant is based on a combination of several histologic features, including Stromal cellularity, nuclear atypia, mitotic activity, stromal overgrowth and tumour margin appearance. However, there are no defined criteria or clear cut-offs for individual histologic parameters. Thus, the diagnosis of PTs based on the integration of morphology remains challenging. Along with the grade, additional study of proliferative markers such as Ki-67 are essential to identify those with potential for aggressive behaviour. This study was undertaken to assess the histopathological characters and correlate Ki-67 expression in different subtypes of PTs. In this study, 30 cases of PTs were studied. Regarding histologic features, routine H&E stains were taken into consideration for diagnosis and classification of tumours. Immunostaining for Ki-67 was also performed by polymer-based detection method. Ki-67 labelling index (LI) was categorized as 0-10, 11-30, 31-50, 51 and above depending on the percentage of positive tumor cells and was correlated with histologic grade and clinical features in each case. Twenty cases (66.7%) of benign phyllodes tumour (BPT), 3 cases (10%) of borderline PT, and 7 cases (23.3%) cases of malignant phylloides tumour (MPT) were examined in this study. Among 20 cases of BPT, 3 cases (15%) were recurrent tumours. Average Ki-67 LI in BPT was 5% (range 1-10%) and borderline PT was 17.5% (range 15-20%). MPT exhibited Ki-67 LI range of 15-35% with average LI of 25%. A significant association was seen between expression of Ki-67 in different grades of PT($p=0.01$). So, Ki-67 LI should be performed in routine histopathology reporting of phylloides for sub-categorisation of phylloides.

Keywords: Phylloides tumours (PTs), Ki-67, Polymer-based detection method, Proliferation Index

INTRODUCTION

Phyllodes tumour is a rare fibroepithelial neoplasm accounting for less than 1% of all breast tumours. It has a leaf-like architecture and infiltrating margins with marked stromal overgrowth and hypercellularity. The behaviour of phyllodes tumours ranges from benign and locally recurrent to malignant and metastatic.[1] The accepted histological classifications into benign, borderline, and malignant subtypes according to features such as tumour margins (pushing or infiltrative), stromal overgrowth of tumour necrosis, cellula ratypia, and number mitoses per high power field[1]. Histological grading has been shown correlate poorly with tumour behaviour though the multiplicity of differing grading systems makes it difficult to be certain whether this effect is in part a result

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of disparities in approach.[1] Because of the difficulty in predicting the clinical outcome, and because even morphologically benign tumours are capable of local recurrence and occasional metastases, ancillary studies in additional to routine morphology for predicting clinical behavior are necessary.[2]

Ki-67 antigen is a cell proliferation-related protein that can be labeled with monoclonal antibody MIB-1. MIB-1 immunostaining can be applied on tissue sections to assess proliferative activity in different types of tumour, including breast carcinoma. The percentage of positive cells, the MIB-1 index, is usually low in benign lesion and increases in malignant tumours.[2] Immuno-histochemistry has an important role in the pathology of breast disease. The polymer-based detection method is highly sensitive and specific because this method utilise a polymer backbone to which multiple antibodies and enzyme molecules are conjugated. In addition, the problem of nonspecific staining can be avoided compared to the Streptavidin-Biotin detection systems due to endogenous biotin.

Inclusion of Ki-67 in routine histopathology reporting of phylloides is mandatory as Ki labelling index proves to be of paramount importance in sub-categorisation of phylloides.[3] Therefore, in this study, the polymer-based detection method was used for detection of the immunoexpression of Ki-67 in phyllodes tumour of breast. It is expected that this study may help to detect the biological behavior and prognosis of phyllodes tumour of breast.

MATERIALS AND METHODS

Haematoxylin and eosin staining

Tissue sections was dewaxed in xylene I, II and III for 2 minutes each. Xylene from the sections was removed in absolute alcohol for 2 minutes. The sections were rehydrated in 90% alcohol, 70% alcohol and tap water for 2 minutes each. Nucleus were stained with Harris' alum haematoxylin for 15 minutes. Excess stain was washed with water. The nuclear stain was differentiated with 1% acid-alcohol to remove unwanted cytoplasmic stain. The nuclear stains changed to blue color under running alkaline tap water for about 10 minutes. The nuclear staining was checked by ordinary light microscope. Counter stain was used by eosin for 1-2 minutes. Excess eosin stain was washed with water and checked again by ordinary light microscope for differentiation of nuclear and cytoplasmic staining. The sections were dehydrated in 70%, 90% and absolute alcohol for 2 minutes each, cleaned in xylene I, II, III for 2 minutes each and then the sections were mounted with DPX. Nuclei were seen as blue to blue black, cytoplasm as shades of pink and RBCs as bright orange to red.

Three-Tiered Grading System for phyllodes tumours based on 2012 World Health Organization Classification

Histologic features	Benign	Borderline	Malignant
Stromal cellularity	Mild	Moderate	Marked
Stromal atypia	Mild	Moderate	Marked
Mitosis (per-10 HPF)	<5	5-9	>10
Stromal overgrowth	Absent	Absent or focal	Present
Tumour margin	Well-defined	Well-defined or focal infiltrative	Infiltrative

Polymer-based immunohistochemical staining procedure

Staining procedure

The tissue sections were put on salinized slides and labelled. Positive and negative controls were included in every batch of immunohistochemical staining. The tissue sections were deparaffinized in xylene I, xylene II and xylene III for 5 minutes each and were rehydrated in absolute alcohol, 90% alcohol and 70% alcohol for 2 minutes each. Then, they were washed with distilled water and were placed in the slide carrier rack and placed in citrate buffer (pH 6.0) making sure that slides will be completely sunk in the buffer. They were left in

microwave oven at high temperature for 10 minutes for antigen retrieval. The procedure was repeated another two times with new fresh citrate buffer.

Before changing slides from one buffer to next one, the new buffer was pre-warmed for two minutes each time. Then, the tissue section slides were cooled at room temperature for at least 20 minutes and washed well in distilled water. For blocking endogenous peroxidase activity, endogenous peroxidase was neutralized using peroxidase block for 5 minutes. The tissue sections were washed for 5 minutes in phosphate buffer saline (PBS) for two times. For blocking non-specific staining, tissue sections were incubated with Protein Block for 5 minutes and were washed for 5 minutes in PBS for two times. The tissue sections were covered with primary antibody (Ki-67) for 30 minutes.

After that, the slides were washed for 5 minutes in PBS for two times. Then, they were incubated with secondary antibody for 30 minutes and washed for 5 minutes in PBS for two times. They were incubated with Novolink™ Polymer for 30 minutes and washed for 5 minutes in PBS for two times with gentle rocking. Then, they were applied freshly prepared 3-3' diaminobenzidine (DAB) solution (chromogen) for 5 minutes and the slides were washed with distilled water, and were counterstained with haematoxylin for a few seconds. After that, these were rinsed in distilled water, and were dehydrated, cleared and mounted with DPX and coverslip. Sections from known case of invasive duct carcinoma of breast (no special type) were used as positive control in every batch and also the omission of the application of primary antibody to the section for negative control.

Interpretation of result

Positive staining for Ki-67 expression was regarded as brown nuclear staining in tumour cells.

Ki-67 immunoexpression: Ki-67 labelling index (Ki-67 LI) was scored on a scale from 0 to 3 according to Papantoniou *et al*, 2004, as follows: 0=negative (0-10% positive cells), 1=positive (11-30% positive cells), 2=positive (31-50% positive cells), 3=positive (>51% positive cells).

Nuclei of tumour cells showing diffuse brown nuclear staining were taken as positive regardless of the staining intensity. The percentage of positive cells was determined by counting 1000 cells in different areas of each section.[4] The Ki-67 positive cells were counted by using a Miller ocular. This is an eyepiece giving a square field, in the corner of which is a smaller ruled square (5 mm^2), one-ninth the area of the total square. The Ki-67 positive cells were examined in the larger square and the total number of cells and multiplied by nine were counted in the small square.

Ethical consideration

This study was approved by the Institutional Review Board of University of Medical Technology, Yangon.

RESULTS

Age distribution among study population

During the one year study period, total phylloides tumours (PT) studied were 30 cases and age range was 17-79 years with mean age of 46.37 years for all PTs. Average age of BPT patients were younger than the borderline and malignant PT patients (Table 1).

Table 1. Age distribution of phyllodes tumours

Phyllodes tumours	Mean age of the study (years)
All phyllodes tumours	46.37
Benign phyllodes tumour	45.10
Borderline phyllodes tumour	47.00
Malignant phyllodes tumour	49.71

Samples processed by routine H &E method

Among 30 cases, 20 cases (66.7%) of benign, 3 cases (10%) of borderline, and 7 cases (23.3%) of malignant phylloides tumor were noted. Three out of 20 cases (15%) of benign phyllodes tumours were recurrent tumours.

Ki-67 proliferation labelling index observed by polymer-based detection method

Average Ki-67 labelling index (LI) in benign PTs was 5% (range 1-10%) but 3 cases of recurrent PT tumours reveal average Ki-67 LI of 9% (range 8-10%). Average Ki-67 LI of borderline PTs was 17.5% (range 15-20%) and malignant PTs exhibited Ki-67 LI range of 15-35% with average LI of 25%, respectively (Fig. 1).

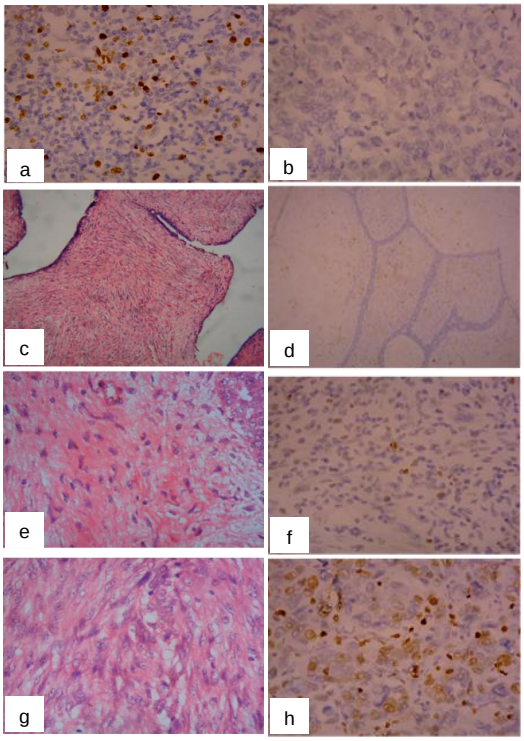

Fig. 1. (a) Ca Breast Ki-67 Positive Control IHC 40X, (b) Ca Breast Ki-67 Negative Control IHC 40X, (c) Benign phyllodes tumour H&E 10X, (d) Benign phyllodes tumour IHC 10X Ki-67 LI-0 (2%), (e) Borderline phyllodes tumour H&E 40X, (f) Borderline phyllodes tumour IHC 40X Ki-67 LI-1 (15%), (g) Malignant phyllodes tumour H&E 40X, (h) Malignant phyllodes tumour IHC 40X Ki-67 LI-2 (35%)

Table 2. Ki-67 labelling index on different types of phyllodes tumour

Ki-67 index		H & E			t-test
		Benign phyllodes tumour	Boderline phyllodes tumour	Malignant phyllodes tumour	
	n	n	n	n	
Negative	20	20	0	0	
1+	9	0	3	6	0.011
2+	1	0	0	1	

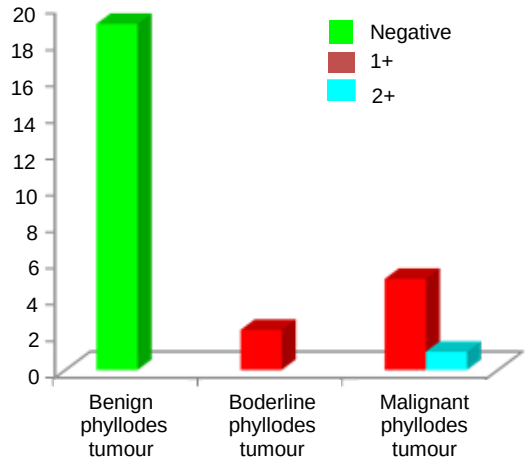

Fig. 2. Ki-67 labelling index on different types of phyllodes tumour

Statistical analysis

Descriptive statistics was done and mean, standard deviation and t test were conducted. P value less than 0.05 was considered to be significant. Ki-67 LI was 0 (negative) in all benign PTs. All Borderline PTs showed Ki-67 LI 1 (positive) and all malignant PTs revealed Ki-67 LI 1 to 2 (positive). Although benign PTs showed negative Ki-67 LI, recurrent PTs revealed higher average Ki-67 LI of 9% (range 8-10%). So, higher Ki-67 LI is associated with the higher grade of PTs and it was statistically significant (p= 0.011). (Table 2 & Fig. 2).

DISCUSSION

The term cystosarcoma phyllodes was first introduced by Muller in 1838. It is derived from the Greek word sarcoma, meaning flesh appearance, and phyllon, meaning leaflike. This term may be misleading because the majority of phyllodes tumours (PTs) are benign. Benign phyllodes tumour (BPTs) comprises 60% to 75% of all PTs. The local recurrence rate has been reported to be about 20%. These tumours are characterized by mildly increased stromal cellularity and mild nuclear atypia. Mitoses are rare, usually fewer than 5 per 10 high-power fields (HPF).[5] It is in accordance with this study in which among 30 cases of PTs, 20 cases (66.7%) were benign PT.

The mitosis cut-off for the diagnosis of borderline PT has been clearly defined as 5 to 9/10 HPF in the WHO classification of 2012.[5] In this study, 3 out of 30 cases (10%) were borderline PT and this finding is nearly the same as that of Zhang & Kleer, in which the percentage of borderline PT ranges from 12% to 26%. According to Zhang & Kleer, local recurrence rate of PT has been reported to be 14% to 25%. This study also found out that 3 out of 20 cases (15%) of benign phyllodes tumours were recurrent tumours.

Reports suggest that approximately 10% to 15% of PTs are malignant. Malignant PT is characterized by marked stromal cellularity and nuclear pleomorphism, stromal overgrowth, and more than 10 mitoses per 10 HPF.[5] In this study, 7 cases (23.3%) of malignant phylloides tumour were noted so that the higher prevalence of malignant tumours was noted.

According to Chan *et al* , 11 out of 13(85%) malignant tumours but only 8 out of 50(16%) benign tumours showed Ki-67 antigen increased >10% ($p<0.005$). In this study, average Ki-67 expression in benign PT was 5% (range 1-10%), Ki-67 labelling index 0 and borderline PT was 17.5% (range 15-20%), Ki-67 labelling index 1, respectively. Malignant PT exhibited Ki-67 range of 15-35% revealing Ki-67 labelling index 1 to 2. In 2004, Chan *et al*, found that 3 out of 50 benign PT progressed from benign to malignant tumours and all the first and recurrent tumours in these 3 patients showed Ki-67 >10%. In this study, range of Ki-67 positive cells in recurrent tumours are from 8-10% and average Ki-67 positive cells is 9% and Ki-67 labelling index is 0.

According to Chan *et al* and Soong & Yoon, in tumors with benign morphology but having a Ki-67 antigen >10%, it is necessary to treat the patient and follow up properly to avoid recurrence and malignant transformation. In this study, although Ki-67 LI is 0, percentage of Ki-67 positive cells was higher in recurrent PT than the benign PT and the minimum is 8% and maximum is 10%.

According to Soong & Yoon,[6] Ki-67 can play an important role in predicting the prognosis and for possibly employing the additional therapy besides the role of the conventional prognostic factors in the treatment of PT patients. Tariq, Haroon and Kayani,[7] stated that there is a significant association between Ki-67 LI and recurrence of PT. In 2016, Vani *et al*[8] also reported that a significant association was seen between expression of Ki-67 in different grades of PTs. This study also found out that higher Ki-67 LI is associated with the higher grade of PTs and it was statistically significant ($p=0.011$).

Conclusion

Most published studies have a limited number of samples, in particular a smaller number of borderline and malignant tumors. Also, in this study, only 30 cases of PTs can be studied within one year. This study found out the importance of Ki-67 LI for sub-categorisation of phyllodes tumours and recommended that Ki-67 LI should be performed in routine histopathology reporting of phyllodes. Further studies including a large series of well-characterized phyllodes tumours with follow-up data are needed.

Competing interests

The authors declare that they have no competing interests.

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