ABSTRACT
Aims: Difficulties in diagnosis of phyllodes tumour are well known. Our aim was to observe whether the expressions of satb1, survivin, and ki-67 were helpful in differentiating fibroadenoma and phyllodes tumour. Settings and design: Retrospective. Methods and material: Paraffin embedded tissue samples from 60 female patients with phyllodes tumour and 60 female patients with fibroadenoma were studied. Expression of the gene products was studied and confirmed using immunohisto chemical and western blot analysis. Statistical analysis used: The statistical analysis was performed using Fisher’s exact test to find out the significant changes. Results: Statistically significant difference was observed in Satb1, survivin expression between fibroadenoma and benign phyllodes cases. The difference between the Ki-67 expression in fibroadenoma and benign phyllodes cases was not statistically significant. Conclusions: Our finding of strong stromal expression of satb1 and survivin in phyllodes as compared to fibroadenoma can be helpful for the development of additional diagnostic and prognostic indicators for otherwise difficult cases. Such immunochemical markers can be used to elucidate cellular basis of tumour behaviour. Validation of such immunochemical test in future will reduce diagnostic uncertainty in this rare tumour. In addition to that such parameters may serve as a therapeutic target that could increase effectiveness of chemotherapy or radiation therapy. A study with large number of samples along with clinical and follow-up data is required for confirmation.
KEYWORDS: Phyllodes, Satb1, Survivin, Ki-67, Fibroadenoma, Tumour, Benign Phyllodes, Of Chemotherapy, Radiation Therapy, Immunochemical markers

INTRODUCTION
Phyllodes tumours (PTs) also known as cystosarcoma phyllodes are rare fibroepithelial tumours of breast. They account for an incidence of 0.3–1.0% of all breast neoplasms [1]. Prevalence of this tumour is higher in Latin American white and Asian population [2]. Phyllodes tumours have more stromal cellularity though they contain epithelial elements and connective tissues similar to fibroadenoma [3].

Although phyllodes are usually benign, approximately 20–50% is malignant phyllodes tumours may be classified as benign, borderline, or malignant. Accurate diagnosis of this condition is still a challenging task due to its histological similarity with fibroadenoma. Fine-needle aspiration also usually does not confirm the diagnosis. There are no specific tumour markers that can be used to diagnose PTs. Hence biopsy is considered the definitive diagnostic test. However,
sometimes PTs are wrongly diagnosed as fibroadenoma. Such conditions are very serious as PTs are fast growing tumours which could recur and cause discomfort to the patients as surgical management for both the conditions differs. Thus, a specific tissue protein marker for this condition will aid the surgeons for correct surgical planning. The present study evaluates the potential of Satb1, survivin, and Ki-67 proteins in differentiating PT from fibroadenoma.

Satb1 is a nuclear protein that has been recently reported to be a ‘genome organizer’ which delineates specific epigenetic modifications at target gene loci by directly up-regulating metastasis-associated genes while down-regulating tumour-suppressor genes [4]. It is a necessary contributing factor in the most aggressive forms of breast cancer [5]. It has been observed that breast cancer cells need Satb1 to become metastatic [6]. Thus, Satb1 expression in PT could help early detection of the potentially malignant stromal cells which would eventually be useful in early diagnosis and treatment of PT.

The inhibitor of apoptosis (IAP) is a widely expressed gene family of apoptotic inhibitors [7]. The central mechanisms of IAP apoptotic inhibition appear to be through direct caspases and procaspase inhibition. Survivin, a novel member of IAP family, is a functional protein that suppresses apoptosis and regulates cell division [8], [9]. Survivin is widely expressed in foetal tissues and in human cancers, but generally not in normal adult tissue [8]. It has been shown that survivin expression may be involved in tumour cell resistance to radiation and chemotherapeutic drug [10]. Thus, its expression pattern could be useful in assessing the malignant potential of the PT.

Ki-67 is a cell proliferation marker [11]. It has been demonstrated to be associated with worse outcome in breast cancer [11]. The expression of the human Ki-67 protein has been shown to be associated with cell proliferation [12]–[15]. The fact that the Ki-67 protein is present during all active phases of the cell cycle, but is absent in resting cells, could make it a marker for determining the so-called growth fraction of a given cell population [15]. Researchers also showed the importance of Ki-67 as prognostic factor [16]. Thus, its expression along with the other markers could be useful in deciding treatment strategies in phyllodes tumour.

**MATERIALS AND METHODS**

**Tissue Specimens**

The present study included tissues from 60 untreated, histologically confirmed, and graded female patients with PT, their ages ranging from 30 to 60 years (median age, 45 years).

Histopathologically the tumours were categorized as benign, borderline, and malignant phyllodes (20 cases each) using Bloom Richardson’s scoring and grading.

Patients with fibroadenoma a benign breast lesions consisting of 60 female patients, their ages ranging from 18 to 52 years (median age, 35 yrs), were included in the study.

Paraffin-embedded samples between the year 2002 and 2012 were randomly selected for the study. All samples were histopathologically proven and were fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical analysis.

The work was approved by our institutional ethic committee. The clinical data of each patient were maintained inclusive of age and sex.

**Inclusion/Exclusion Criteria**

All biopsies were collected from patients in a specified age group who did not receive any therapy before surgery. Cases other than phyllodes and fibroadenoma were not included.

**Immunohistochemical Analysis**

Tissue sections were cut at 5 µm and were mounted on poly-l-lysine-coated glass slides (sigma, St. Louis, MO, USA). Antigen retrieval was then performed by
heating slides immersed in citrate buffer, pH 6, in a microwave oven [17]. Primary rabbit polyclonal antibodies obtained from ABACAM against Satb1 (Sc-715), survivin (Sc-834), and Ki-67 (Sc-834) were used at a dilution of 1:100 in 1% bovine serum albumin (BSA). Biotinylated antirabbit IgG (NA 934V) obtained from GE Healthcare UK Lt. (Buckinghamshire, UK) was used as a secondary antibody, and the bound antibody was detected using streptavidin-conjugated horse radish peroxidase with 3, 3’-diaminobenzidine as a substrate and Harris’ hematoxylin as a counterstain. Known positive tissue sample of invasive ductal carcinoma was used as positive control and was stained with each batch of slides. Negative control was done by staining the positive control specimen with secondary antibody alone. The correlation between the expression level and the histological grade was analysed using the Fisher exact test.

The immunohistochemical analysis of Satb1, survivin, and Ki-67 expression in fibroadenoma and various grades of phyllode tumours was carried out on the basis of the percentage of cells showing staining.

The level of expression was scored as follows:
0 = negative with less than 5% of cells staining.
1+ = weak staining between 6% and 25% of cells staining.
2+ = moderate staining between 26% and 50% of cells staining.
3+ = medium strong staining between 51% and 75% of cells staining.
4+ = strong staining more than 75% of cells staining.

**Western Blot Analysis**

The presence of expression of various markers was confirmed using a western blot technique carried out as described [18]. Proteins were extracted from paraffin-embedded tissue sections. Follin Lowry estimation was carried out to estimate concentration of proteins [19], [20]. Electrophoresis of known amount of protein sample was performed using 5% and 10% SDS polyacrylamide gel. Separated proteins were transferred to nitrocellulose membranes, and incubated with primary antibodies for 2 h. Then, after washing with tris saline buffer with Tween 20 it was treated with secondary antibodies linked to horse radish peroxidase. Then, Pierce chemiluminescence mixture was added. Maintaining proper dark conditions blots were exposed to the X-ray film in dark room as quickly as possible. Films were then developed using Kodak developing solution.

**Statistical Analysis**

Statistical analysis was performed using Graphpad Instat 3 statistical software. Fisher exact test was used to find out the significant difference in expression of sat b1, survivin, and Ki67 in fibroadenoma and various grades of phyllodes cases.

**RESULTS**

In phyllode tumour Sat b1 expression was observed in epithelial as well as in stromal cells. It was mainly observed to be localized in the nucleus. Overall about 32 of 60 (53%) phyllode cases showed Sat b1 positivity (Figure 1B) in epithelial as well as in stromal cells. Nine out of 20 cases (45%) of benign phyllode and borderline phyllode each were found to exhibit Satb1 expression in epithelial cells from weak to moderate, whereas 14 out of 20 (70%) cases of malignant phyllode cases showed strong positivity of satb1 in epithelial cells.

In fibroadenoma, only 9 out of 60 (15%) patients showed sat b1 expression in epithelial cells. None of the fibroadenoma cases expressed sat b1 in stromal cells (Figure 1A). The degree of sat b1 expression was distinctly found to increase with increasing grade of the phyllode tumours (Figure 2A) in epithelial cells as well as in stromal cells. We observed statistically significant increase in stromal satb1 expression (p=0.0006, Fisher’s exact test) and epithelial satb1 expression (p=0.0111, Fisher’s exact test) in benign phyllodes in comparison with fibroadenoma.
Survivin expression was found to be localized in nucleus as well as cytoplasm. Overall about 51 out of 60 (85%) phyllode cases showed epithelial as well as stromal positivity to survivin whereas 31 out of 60 (52%) fibroadenoma showed survivin positivity basically in epithelial cells. Positivity in stromal cells of fibroadenoma was almost negligible (Figure 2B).

Grade-wise analysis of phyllode cases indicated 17 out of 20(85%) of benign phyllodes, 18 out of 20 (90%) of borderline phyllode, and 16 out of 20 (80%) of malignant phyllodes expressed strong survivin staining in epithelial cells and 11 out of 20(55%) of benign phyllodes, 11 out of 20 (55%) of borderline phyllode, and 16 out of 20 (80%) of malignant phyllode expressed strong survivin staining in stromal cells, whereas fibroadenoma showed weak to moderate expression of survivin in epithelial cells but stromal staining was almost negligible (7%).

Thus, we observed statistically significant increase in stromal survivin expression ($p<0.0001$, Fisher’s exact test) and epithelial survivin expression ($p=0.0090$, Fisher’s exact test) in benign phyllodes in comparison with fibroadenoma, whereas in malignant phyllodes tumour there was statistically significant increase ($p=0.0001$) in survivin expression was observed in stromal cells as compared to fibroadenoma.

Immunohistochemical analysis was done for Ki67. Its expression was found to be more localized in nucleus.

About 12 out of 60 (20%) phyllodes tumour cases showed Ki67 expression as compared to 13 out of 60 (22%) in fibroadenoma. Thus, the difference in the expression of Ki67 in fibroadenoma and phyllodes is not significant statistically. Also in phyllodes cases, the increase in expression of Ki67 is not very significant with increase in the grade of tumour. As far as expression of Ki67 in stromal and epithelial cells of phyllodes cases concern there is no significant difference observed with respect to intensity and percentage of positive cells (Figure 2C).

To confirm the presence of proteins the western blot analysis was carried out. Figure 1H represents the protein bands at molecular weight 85K for sat b1, 16K for survivin using 10% polyacrylamide gel and two bands at 395K and 345K for Ki67 using 5% polyacrylamide gel.

**DISCUSSION**

In the present study, we studied the expression of Sat b1, survivin, and Ki67 in benign breast lesion, i.e., fibroadenoma and phyllodes tumours.

It has been documented that alteration in gene expression plays very important role in cancer progression and metastasis [21]. Chromatin structure is important in expression of various genes during progression and metastasis of cancers [22]. Sat b1 is a protein that has been shown to attach to characterized matrix attachment region of chromatin and thus modify gene expression [23]. Sat b1 has now demonstrated to play contributing factor in most aggressive form of breast cancer [22].

In the present study, significant increase in expression as well as intensity of Sat b1 was observed in all grades of phyllodes tumours as compared to fibroadenoma. Strong positivity was observed in epithelial as well as stromal cells. The percentage positivity was also shown to be increased from benign to borderline to malignant cases of phyllodes Figure 1C.

The mammary gland is a complex tissue, as the epithelium is embedded in a mixture of stromal cells that regulates its proliferation, differentiation, and survival. All stages of mammary gland development depend on epithelial stromal interactions. Thus, stromal participation plays important role in pushing epithelium to progress to malignancy.

The strong expression of sat b1 in epithelial and stromal cells of phyllodes tumour in our finding may suggest that there could be possible relation between its expression and promotion of disease proliferation.

Survivin a novel member of IAP family is a bifunctional protein that suppresses apoptosis and regulates cell division [24]. It was shown to be associated with more
Figure 1: (A) Fibroadenoma showing no Satb1 expression. (B) Benign phyllodes lesion showing strong nuclear and cytoplasmic stromal and epithelial Satb1 expression. (C) Malignant phyllodes lesion showing strong Satb1 expression. (D) Benign phyllodes showing survivin expression. Epithelial discharge strongly stained. (E) Benign phyllodes lesion showing strong stromal survivin expression. (F) Malignant phyllodes showing strong stromal survivin expression. (G) Benign phyllodes lesion showing strong stromal Ki-67 expression. (H) Protein expression by western blot.
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Unlike fibroadenoma phyllodes tumours can recur and progress to malignancy. It has been demonstrated that potential of phyllodes tumours to recur and progress is mainly because of their stromal characteristics [26]. Sawhney et al. describe [27] the presence of mitotic figures within preductal stroma as compared to stroma away from epithelium in benign phyllodes. In the present study, we obtained only 7% of fibroadenoma showed stromal expression of survivin as compared to 55% stromal expression in benign phyllodes tumours which is statistically significant ($p<0.0001$ Fisher extract test).

Survivin expression in epithelial cells also significantly increased in benign phyllodes (85%) as compared to fibroadenoma (52%) ($p=0.0090$ Fisher extract test). In our finding, we observed that there is strong correlation seen between histological grade and survivin expression. Epithelial and stromal expression of survivin increased from benign to borderline to malignant phyllodes (Figure 2B). In fibroadenoma stromal cells are seen but they are hardly proliferated, whereas in phyllodes stromal proliferation occurs predominantly. These results strongly support the findings which demonstrate that survivin expression indicates the poor prognosis [28]. Ribeiro Silva et al. [29] also reported that survivin antiapoptotic stimuli predominated in sarcomatous cells of PT. Thus, we can say that survivin can be a potential marker for differentiating phyllodes from fibroadenoma in diagnostically difficult cases.

Ki67 is said to be a proliferative marker [30] and fraction of Ki67 positive tumour cells is often correlated with the clinical course of cancer [12]. We attempted to study its usefulness in differentiating fibroadenoma and phyllodes tumours.

In our investigation we found no significant difference in fibroadenoma and benign phyllodes as well as in borderline and malignant phyllodes tumour (Figure 2C). However, Jara Lzaro et al. [31] reported increased Ki67 positivity in phyllodes tumours. Kleer et al. [32] also had analysed a group of benign and malignant phyllodes tumour to determine its usefulness as diagnostic and prognostic parameter. According to their findings Ki67 expression may assist in distinguishing benign and malignant phyllodes tumours in diagnostically difficult cases. We, however, could not get any statistical significance between Ki67 expression in epithelial as well as stromal cells of fibroadenoma and phyllodes. The gradewise change in expression of Ki67 in epithelial and stromal cells of phyllodes tumour was also not noticed (Figure 2C). Thus, we observed that Ki67 could not reveal any diagnostic information in differentiating between fibroadenoma and phyllodes tumours as well as between different grades of phyllodes tumours.

Figure 2: (A) Stomal and epithelial expression of Satb1 in fibroadenoma and phyllodes lesions. (B) Stomal and epithelial expression of survivin in fibroadenoma and phyllodes lesions. (C) Stomal and epithelial expression of Ki-67 in fibroadenoma and phyllodes lesions.
The role of pathologist in preoperative diagnosis of phyllodes tumour is critical for appropriate planning of surgery. However, reliable differentiation of phyllodes from fibroadenoma remains difficult task. Extensive research has been conducted over several years for understanding the genetic changes that occur in normal cells to proceed them towards transformed state \cite{33}, \cite{34}. There must be complex interactions involved for development and progression of cancer cells. Epithelial tissue consisting of specialized cells carries out various activities which in turn depend on immediate signals from stroma. Involvement of stromal cells in development and progression of cancer cells is always an area of interest and lot of research is taking place in this context \cite{35}, \cite{36}.

Our finding of strong stromal expression of sat b1 and survivin in phyllodes as compared to fibroadenoma may suggest the stromal role in progression of cancer. Such immunochemical markers can be used to elucidate cellular basis of tumour behaviour. These markers can be helpful for the development of additional diagnostic and prognostic indicators for otherwise difficult cases. Validation of such immunochemical test in future will reduce diagnostic uncertainty in this rare tumour. In addition to that such parameters may serve as a therapeutic target that could increase effectiveness of chemotherapy or radiation therapy. A study with large number of samples along with clinical and follow-up data is required for confirmation.

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