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Immunohistochemical expression of heparan sulfate correlates with stromal cell proliferation in breast phyllodes tumors

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Phyllodes tumors are fibroepithelial neoplasms typified by stromal proliferation. We have previously shown the role of pathologic parameters and the prognostic significance of p53 and CD117 protein expression in these tumors. In this study, we evaluated the expression of heparan sulfate, which has been implicated in many biological processes such as cell adhesion, embryogenesis, and tumorigenesis (including malignant transformation of mammary cells) in 232 breast phyllodes tumors. We used a monoclonal antibody, 10E4, to examine the localization of heparan sulfate in phyllodes tumors by immunohistochemistry. The immunoreactivity of both epithelial and stromal components was examined and analyzed with pathological parameters and other immunohistochemical markers, including p53, MIB1, bcl2, and CD117. Stromal 10E4 expression was significantly associated with tumor grade, stromal p53, and MIB1 expression in proliferating cells, suggesting that heparan sulfate may participate in malignant tumor growth.
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Heparan sulfate is an important biomolecule that is essential in maintaining cell–cell and cell–extracellular matrix adhesion, mediating receptor-ligand binding and regulating the activities of growth and motility factors.[1–3] Its primary structure is characterized by repeats of disaccharide units of a uronic acid and a derivative of glucosamine. The biosynthetic process of heparan sulfate is highly complicated whereby it can undergo modifications such as sulfation, epimerization, and acetylation to generate a great structural diversity of heparan sulfate chains.[4,5] These chains are covalently attached to core proteins to form heparan sulfate proteoglycans. There is accumulating evidence highlighting the influence of heparan sulfate in modulating many physiological processes and diseases such as cancer. Studies have indicated that this molecule may be altered structurally during malignant transformation of colon cancer cells and indirectly promote growth factor signalling, leading to tumor cell proliferation.[6] Differential heparan sulfation patterns in breast cancer cells have been demonstrated by various groups.[7–10] Safaiyan *et al*[11] have shown that there is selective reduction of 6-O sulfation in heparan sulfate from transformed breast epithelial cells. Several reports have also shown that syndecan 1 is an important heparan sulfate proteoglycan in cell signalling and tumor cell progression in breast cancer.[11–15]

Phyllodes tumors of the breast, originally termed cystosarcoma phyllodes, are uncommon fibroepithelial neoplasms,[16] typified by hypercellular stroma and elongated ducts with irregular leaf-like patterns due to stromal proliferation.[17] Its numbers are much fewer than breast carcinomas, with a proportion of only 1.5% compared to breast carcinomas. Phyllodes tumors have a higher frequency in Asian women.[18,19] Classification into three categories of benign, borderline and malignant is based on a

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spectrum of histological features such as stromal hypercellularity, mitotic rate, and nature of microscopic margins. However, there are still difficulties in accurate categorization of phyllodes tumors and predicting their clinical outcome.[18]

Several biological molecules such as p53, MIB1, and CD34 have been demonstrated to be possible prognostic indicators in phyllodes tumors.[16,20–22] As heparan sulfate has been shown to be differentially expressed in many forms of cancers including breast cancers, we aimed to investigate its relationship with malignant progression in phyllodes tumors by immunohistochemistry using the 10E4 monoclonal antibody. The epitope of 10E4 has been shown to consist of mixed N-sulfated and N-acetylated heparan sulfate.[23] Apart from correlating the immunohistochemical results with histological parameters, we also compared its expression with MIB1, p53, bcl2, and CD117 expression. To the best of our knowledge, this is the first study evaluating the role of heparan sulfate in breast phyllodes tumors.

Materials and methods

Clinical Materials

Previously constructed tissue microarray blocks of archival breast phyllodes tumor specimens from the Department of Pathology, Singapore General Hospital were used for this study. [19] Clinicopathological information collected included patient age, tumor size, macroscopic features of hemorrhage, myxoid or necrotic changes, cystic degeneration; microscopic alterations of mitotic activity, stromal hypercellularity and overgrowth, nature of borders and stromal cytologic atypia. Immunohistochemical data on the proliferating index (MIB1), p53, bcl2, and CD117 were also available.[24]

Immunohistochemistry

10E4 monoclonal antibody was purchased from Seikagaku Corporation (Tokyo, Japan). Briefly, paraffin-embedded tissue microarray sections (4 μm thickness) were deparaffinized, rehydrated, and endogenous peroxidase activity was quenched with 3% H_2O_2 for 15 min. Antigen retrieval was performed by incubation with 0.1 mg/ml testicular hyaluronidase for 2 h at room temperature prior to overnight incubation at 4°C with 10E4 antibody (1:150 dilution). After washing with Tris-buffered saline, biotinylated secondary antibody was added and incubated for 1 h at room temperature. Visualization was achieved by the avidin–biotin-complex technique (ABC kit, Vector Laboratories) using diaminobenzidine as the substrate, followed by counterstaining with hematoxylin.

Sections were examined using an Olympus BX 51 microscope (Olympus, Tokyo, Japan) and photographed with an Olympus DP50 CCD camera. Two images were selected randomly of each specimen and analyzed using Image J v1.33 software (NIH, USA) with the color deconvolution plugin to measure staining intensities of the areas of interest.[25] The mean heparan sulfate staining intensities of the stromal and epithelial compartments of phyllodes tumors were measured separately in arbitrary units and later converted into an immunoreactivity score where 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. For MIB1, p53, bcl2, and CD117, any staining of nuclei (MIB1, p53) or cytoplasm (bcl2, CD117) were considered positive reactivity. Table 1 shows details of these antibodies.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 4 for Windows (GraphPad Software, San Diego, CA, USA). Immunoreactivity scores for heparan sulfate staining were correlated with clinicopathological parameters and immunohistochemical staining results of MIB1, p53, bcl2, and CD117, using Chi-square test or Fisher's exact test. The Mann–Whitney *U*-test was used to compare means between variables. Differences were considered to be statistically significant when *P*-values were <0.05.

Results

We have documented the clinicopathological data of the largest phyllodes tumor series in literature to

Table 1 Details of antibodies, dilutions and antigen retrieval methods

Antibody	*Catalogue No.*	*Dilution*	*Pretreatment method*	*Clone*
MIB1	Dako M7240	1:300	Microwave (using National MW oven NN-S650 WF) in Milestone T/T Mega, at 98°C for12 min in Ventana CC1 Solution, Code No. 950–124	SP6
p53	Dako M7001	1:70	Microwave (using National MW oven NN-S650 WF) in Milestone T/T Mega, at 98°C for12 min in Ventana CC1 Solution, Code No. 950–124	D07
Bcl2	Dako M0887	1:10	Pressure cook in microwave oven, Milestone T/T Mega, at 98°C for 10 min in DakoCytomation Target Retrieval Solution, High pH , Code No. S3307	124
CD117	Dako A4502	1:200	Microwave (using National MW oven NN-S650 WF) in Milestone T/T Mega, at 98°C for12 min in Ventana CC1 Solution, Code No. 950–124	Ra

MW, microwave.

date of 335 women with breast phyllodes tumors.[19] Owing to loss of some sections of individual cores during cutting of the tissue microarray blocks, immunohistochemical analysis using 10E4 was performed on 232 microarrays out of the original 335 cases. Among these, 163 were diagnosed as benign, 36 as borderline, and 33 as malignant.

As shown in Figure 1, the 10E4 epitope was present in higher amounts in the basement membranes and perithelial regions of phyllodes tumors, with 11.2% of cases demonstrating accentuated decoration of the basement membrane and stroma immediately around epithelial elements of these tumors. Stromal and epithelial expression of 10E4 was detected in 35.3 and 46.6% of phyllodes tumors, the latter in the cytoplasm of epithelial cells. The findings are summarized in Table 2. There was no significant association between the intensity of 10E4 epithelial staining and tumor grade ($P=0.400$). However, there was a significant difference in 10E4 expression in the stromal component of phyllodes tumors among the tumor grades ($P=0.0188$, Figures 1 and 2; negative control, Figure 3).

The expression of 10E4 epitope in the stromal compartment of phyllodes tumors was compared with other histological features, as depicted in Table 3. We also compared 10E4 expression with immunohistochemical detection of MIB1, p53, bcl2 and CD117. Interestingly, statistically significant correlations were found between 10E4 stromal positivity with p53 and CD117 stromal staining. We had previously reported the latter two parameters

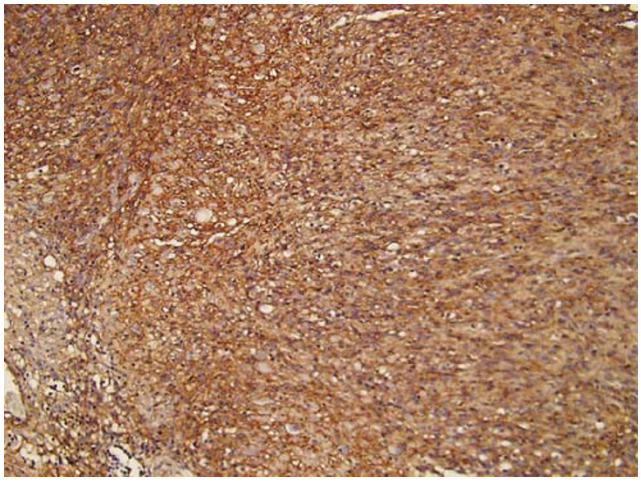

Figure 2 Diffuse intense stromal staining for heparan sulfate 10E4 in a malignant phyllodes tumor with stromal overgrowth.

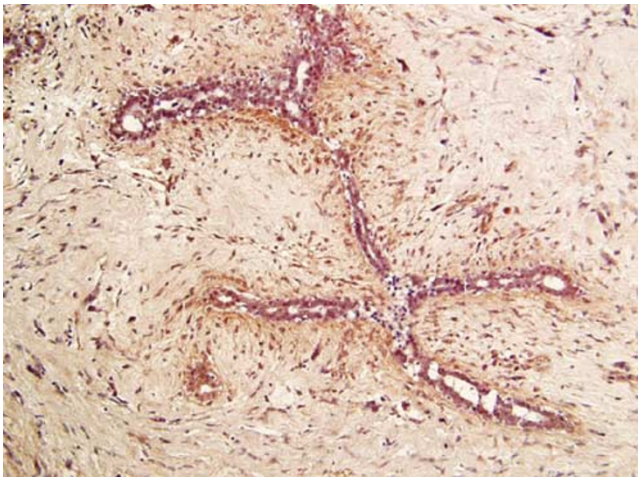

Figure 1 Perithelial accentuation of heparan sulfate 10E4 immunohistochemical staining in a benign phyllodes tumor.

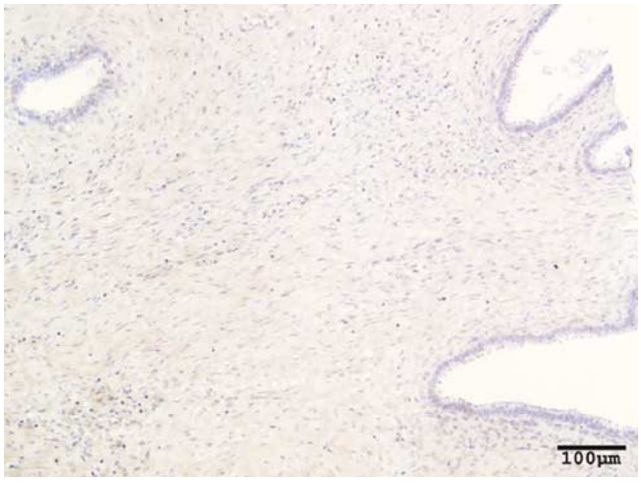

Figure 3 Negative control section accomplished by omitting the primary antibody.

Table 2 Immunohistochemical expression of heparan sulfate 10E4 in benign, borderline and malignant phyllodes tumors

	10E4 expression in stromal compartment				*10E4 expression in epithelial compartment*			
	Total number	*IRS≤0*	*IRS>0*	P-*value*	*Total number*	*IRS≤0*	*IRS>0*	P-*value*
Benign	163	114	49		159	89	70	
Borderline	36	21	15		34	17	17	
Malignant	33	15	18	0.0188[a]	28	12	16	0.4001
Total	232	150	82		221	118	103	

IRS: immunoreactivity score.
[a]Statistically significant result.

Table 3 Clinicopathologic features of phyllodes tumors correlated with 10E4 stromal immunostaining

	Negative 10E4 stromal staining	*Positive 10E4 stromal staining*	P-*value*
Age (mean, in years)	39.53	44.35	[a]0.0261
Tumor size (mean, in mm)	46.81	62.43	0.0698
Grade			
Benign	114	49	
Borderline	21	15	[a]0.0188
Malignant	15	18	
Gross margins			
Well circumscribed	128	64	
Poorly circumscribed	5	8	0.1193
Not available	16	9	
Cystic degeneration			
Absent	131	66	
Present	17	14	0.4088
Not available	1	1	
Gross necrosis			
Absent	145	76	
Present	3	5	0.2405
Not available	1	1	
Gross hemorrhage			
Absent	139	64	
Present	9	17	[a]0.0013
Not available	0	1	
Stromal hypercelluarity			
Mild	82	33	
Moderate	55	38	0.2006
Marked	12	10	
Stromal overgrowth			
Absent	132	63	
Present	17	18	[a]0.0351
Microscopic borders			
Circumscribed	106	45	
Focally permeative	39	27	
Widely permeative	4	9	[a]0.0088
Stromal atypia			
Mild	106	54	
Moderate	30	21	0.5901
Marked	13	6	
Stromal metaplasia			
Absent	145	76	
Present	5	4	0.7227
Mitotic activity/10hpf (mean)	4.24	5.561	0.5601
Myxoid change			
Absent	16	12	
Present	133	69	0.4016
PASH			
Absent	36	19	
Present	113	62	1
Microscopic hemorrhage			
Absent	29	15	
Present	120	64	1
Infarction or necrosis			
No infarction or necrosis	119	56	
Infarction	27	21	0.2766
Tumour necrosis	2	3	
Both infarction and tumour necrosis	1	1	
Epithelial hyperplasia			
Absent	44	21	
Mild	49	26	0.6456
Moderate	44	21	
Marked	12	10	
No epithelium present	2	0	
Epithelial metaplasia			
Absent	146	77	
Present	4	3	0.6966
p53 stromal staining			
Absent	105	45	
Present	45	37	[a]0.0308
bcl2 epithelial staining			
Absent	11	10	
Present	139	72	0.237
bcl2 stromal staining			
Absent	10	6	
Present	140	76	1
CD117 epithelial staining			
Absent	58	41	
Present	91	41	0.1265
CD117 stromal staining			
Absent	146	71	
Present	4	11	[a]0.0034
MIB1epithelial staining			
Absent	77	43	
Present	73	39	0.8915
MIB1 stromal staining			
Absent	103	42	
Present	47	40	[a]0.0107

hpf, high power fields; PASH, pseudoangiomatous stromal hyperplasia.
[a]Statistically significant result.

as possible prognostic biomarkers of breast phyllodes tumors.[24]

10E4 stromal immunostaining in each grade of phyllodes tumor was further analyzed with histological parameters. There was no association of 10E4 stromal immunopositivity with other histological parameters in the groups of benign and borderline phyllodes tumors. However, as shown in Table 4, the expression of 10E4 in the stromal compartment of malignant tumors was associated with intensity of p53 stromal immunohistochemical expression ($P = 0.0329$) and inversely with the intensity of bcl-2 epithelial immunohistochemical expression ($P = 0.0342$).

Table 4 Immunohistochemical expression of p53 and bcl2 compared against stromal 10E4 expression in malignant phyllodes tumors

	10E4 expression in stromal component			
	Total number	*IRS≤0*	*IRS>0*	P-*value*
p53 stromal expression				
IRS≤1	19	12	7	
IRS>1	14	3	11	0.0329[a]
Total	33	15	18	
bcl2 epithelial expression				
IRS≤0	11	2	9	
IRS>0	22	13	9	0.0342[a]
Total	33	15	18	

IRS, immunoreactivity score.
IRS for p53 stromal and bcl2 epithelial expression is based on the intensity of immunohistochemical staining of the stromal cell nuclei and the epithelial cell cytoplasm respectively, quantified from 0 to 3.
[a]Statistically significant result.

Discussion

In the present study, we have shown that heparan sulfate recognized by the 10E4 antibody is found in the basement membranes and perithelial regions of phyllodes tumors, with expression being stronger in the perithelial stromal region. 10E4 antibody is a widely-used antibody for the detection and localization of heparan sulfate in tissues.[26–28] Leteux *et al*[29] reported that 10E4 recognized a heparan sulfate tetrasaccharide in which an N-unsubstituted GlcN residue was required for the binding to 10E4. More recently, it was shown that a mixed N-sulfated/N-acetylated epitope was necessary for 10E4 binding.[23] Unfortunately, to date, the exact structure of the heparan sulfate epitope recognized by the 10E4 antibody is still not fully characterized.

The well-known MIB1 antibody recognizes Ki-67, a nuclear nonhistone protein that is strictly linked to cell proliferation.[30,31] The expression of Ki-67 in active phases of the cell cycle (G_1, S, G_2 and M) and its absence in the G_0 resting phase makes it a common and useful marker for assessing the degree of cell proliferation.[32,33] Previously, studies have shown a high degree of correlation of stromal Ki-67 expression with phyllodes tumor grade.[34,35] In our present study, a significant correlation between stromal 10E4 expression and stromal MIB1 expression was observed. Thus, we postulate that heparan sulfate chains detected by the 10E4 antibody may have a role in controlling the proliferation of stromal cells in phyllodes tumors. Heparan sulfate is an accessory molecule essential for stabilizing the binding of growth factors to their receptors. For example, it regulates the activation of fibroblast growth factor receptors by fibroblast growth factors to elicit a series of downstream events, such as cell proliferation.[36–38] Several reports have suggested that alterations in the expression or biosynthesis of heparan sulfate are responsible for the differential affinities of fibroblast growth factor 1 and fibroblast growth factor 2 with their receptors, thus promoting breast cancer cell progression, metastasis and angiogenesis.[39–41] Hence, it is possible that heparan sulfate may manipulate the proliferative acitivity of the stroma in phyllodes tumors through the fibroblast growth factor/fibroblast growth factor receptor signalling pathways.

p53 is a tumor suppressor that has a main role as a gatekeeper in preventing cells with damaged DNA from proceeding further in the cell cycle. Defective p53 expression can result in neoplastic transformation in many different cell types, including breast epithelial cells.[42–44] Thus far, it has been reported that p53 stromal expression increases from benign to malignant grades in phyllodes tumors and its expression has been related to various histological parameters, such as mitotic count and stromal overgrowth.[20,21,45] Our findings show a statistically significant relationship between 10E4 stromal staining and p53 expression in the stroma, suggesting the possibility that 10E4-specific heparan sulfate species may play an indirect role in phyllodes tumor pathogenesis and increased p53 expression. Furthermore, more intense p53 stromal staining is associated with 10E4 stromal positivity in malignant tumors. Shpitz *et al* has reported that the expression of p53 tends to be higher in phyllodes tumors of higher malignant potential.[46] Thus, it is possible that heparan sulfate may cooperate with p53 in regulating cell cycle progression in phyllodes stromal cells. Also, the epithelial expression of bcl2, a well-known antiapoptotic protein, is decreased in malignant phyllodes tumors with little or no 10E4 staining. Several studies have reported an inverse relationship between p53 and bcl2. Krajewski *et al*[47,48] concluded that the regulation of bcl2 is associated with alterations in p53. The correlation of 10E4 epitope with p53 and bcl2 suggests that these molecules may be involved in malignant transformation of cells in phyllodes tumors.

We also found a close association of 10E4 stromal staining with CD117 stromal staining. CD117 is a membrane bound tyrosine kinase receptor that is well known for controlling many cell signalling processes through the Ras/MAP kinase pathway and JAK/STA signalling. It is a well-known marker for gastrointestinal stromal tumors.[49] Some phyllodes tumors may be thought to bear similarity to gastrointestinal stromal tumors in that the latter also contain a spindle cell component of neoplastic CD34 positive cells.[50] In concert with recent reports, we had previously suggested CD117 as a possible prognostic biomarker for phyllodes tumors and that it may be involved in the early development of these tumors.[24,50,51] In this study, we hypothesize that heparan sulfate may be involved in ligand activation of CD117 and lead to cellular proliferation.

In summary, we have demonstrated that higher stromal expression of heparan sulfate is present in

higher grades of phyllodes tumors. The correlation of heparan sulfate immunopositivity with p53, bcl2 and CD117 provides strong evidence that they may work together to control cell cycle progression in these tumors. In the majority of the associations discussed above, expression of various immunohistochemical markers was confined within the stromal region, supporting the notion that neoplastic activity occurs mostly in the stromal rather than epithelial component in phyllodes tumors.[20,52] Categorization of such tumors into their respective grades can be potentially aided by using a combination of 10E4 antibody together with antibodies against other well-known biomarkers MIB1 and p53. The relevance of heparan sulfate expression in breast phyllodes tumors in routine surgical pathology practice may lie in not only more accurate assignment of tumor grade, but in the prediction of biologic behavior. Confirmation of heparan sulfate expression in phyllodes tumor stroma can lead to further understanding of the pathogenesis of this tumor and impact on possible therapeutic modulations.

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