ORIGINAL ARTICLE

Endothelin-1 expression correlates with atypical histological features in mammary phyllodes tumours

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Background and Aims: Endothelin-1 expression is increased in infiltrating duct carcinoma and is associated with larger tumour size, higher histological grade and lymphovascular permeation. This has not been evaluated in phyllodes tumours, which are uncommon fibroepithelial lesions with potential for local recurrences or distant metastasis. While the grading of phyllodes tumours depends on a combination of histological parameters, prediction of their behaviour remains difficult.

Method: A large series of 461 phyllodes tumours (291 benign, 115 borderline malignant and 55 frankly malignant) were evaluated for endothelin-1 expression in both the epithelial cells and stromal cells by immunohistochemistry; results were correlated with the tumour grade.

Results: For benign phyllodes tumours, the epithelial staining of endothelin was negative, weak, moderate and strong in 6%, 26%, 15% and 53% of cases respectively; results were 4%, 18%, 19% and 59% respectively for borderline and 6%, 18%, 6% and 70% respectively for frankly malignant tumours. For the stromal staining, the negative, weak, moderate and strong staining was 32%, 19%, 18% and 31% respectively for benign phyllodes, 24%, 13%, 10% and 53% respectively for borderline and 8%, 16%, 17% and 59% respectively for frankly malignant tumours. There was correlation between epithelial and stromal staining, and the stromal staining correlated with histological features of stromal cellularity, stromal cell nuclear pleomorphism, margin status and stromal overgrowth.

Conclusion: These observations suggest a close relationship between the epithelial and stromal elements in phyllodes tumours; endothelin may play a significant role in the malignant progression of phyllodes tumours.

Endothelins are potent vasoactive peptides that were originally isolated from vascular endothelial cells.¹ To date, the endothelin (ET) group consists of ET1, ET2 and ET3, with ET2 and ET3 differing from ET1 by two and six amino acid residues respectively.^{2–4} ET1 is expressed primarily by endothelial cells, ET2 is found in epithelial cells of the kidney and intestine, and ET3 is found in the brain.⁵ The production of ET has been shown in several types of cancer, including cancer of the lung,⁶ prostate⁷ and ovary.⁸ In breast cancer, expression of ET1 and ET2 is increased in infiltrating duct carcinoma,^{9–11} and the increased expression of ET1 is more commonly associated with larger tumour size, higher histological grade and lymphovascular permeation.¹² ETs have been found to be mitogenic to some cells, particularly fibroblasts, melanocytes, vascular smooth muscle cells and endothelium.^{13–14} The expression of ET1 in ductal carcinoma of the breast appears to be variable,¹⁵ but is lower in ductal carcinoma in situ than in invasive carcinomas.¹²

Mammary phyllodes tumours, together with the much more common fibroadenomas, are fibroepithelial lesions showing proliferation of both epithelial and stromal components. Phyllodes tumours are uncommon, with a reported incidence of 0.3–0.5% of all breast lesions.^{16–20} They usually occur in middle aged females, but are found in younger women, and also in men. Based on a combination of histological criteria, including stromal cellularity, nuclear pleomorphism, mitotic rate, stromal overgrowth and the margin status (whether infiltrative or rounded), phyllodes tumours can be divided into benign, borderline and malignant groups.^{16–21} While all groups of phyllodes tumours show a propensity to recur locally, the borderline and malignant groups may also metastasise to other visceral organs. In some series, only the histological parameters, including the grade, stromal atypia, stromal hypercellularity,

and margin status or stromal overgrowth^{22–25} are shown to be correlated to disease recurrence, whereas other authors also emphasise the importance of complete surgical excision at the time of initial treatment.²⁶ Some other protein products have been assessed, including p53,^{27–28} Ki67,^{29–30} microvessel density and angiogenesis,^{31–32} and c-kit (CD117).^{33–34} None of these have however been consistently shown to correlate with recurrence or metastases prediction. It has been shown previously that for this group of uncommon breast lesions, higher stromal vascularity is associated with a high degree of atypical stromal features as evidenced by increased vascular endothelial growth factor expression of the stromal cells and increased microvessel density.^{31–32} It would be of interest to evaluate the expression profile of ET1 in the group of fibroepithelial lesions, particularly phyllodes tumours, as some of them may behave in a malignant manner. In the literature, only one study has been found; four cases of benign phyllodes tumours and some fibroadenomas were described³⁵; increased ET1 was shown in the former group. In the current study, the expression of ET in the largest series of phyllodes tumours ever reported, encompassing all grades of tumours, was investigated.

MATERIALS AND METHODS

Histological preparations and evaluation

All phyllodes tumours of the breast were identified from the computer based records of the four participating institutions (Prince of Wales Hospital, The Chinese University of Hong Kong; Singapore General Hospital; Royal Prince Alfred Hospital, Sydney; and National University Hospital, Singapore) (4–15 years' collections). The paraffin blocks of

Abbreviations: ET, endothelin; ETR, endothelin receptor; VEGF, vascular endothelial growth factor

Table 1 Patient demographics

	Benign (n = 291)	Borderline (n = 115)	Malignant (n = 55)	Total (n = 461)*
Age (y), mean (range)	39.0 (14–68)	41.7 (15–77)	37.5 (19–70)	41.3 (14–77)
Tumour size, mean cm in diameter (range)	4.3 (0.8–22)	6.3 (1–22)	6.9 (1.5–22)	5.0 (0.8–23)
Histopathological data	% of cases (n)	% of cases (n)	% of cases (n)	% of cases (n)
Tumour margin				
Round	83 (241)	57 (66)	18 (10)	71 (317)
Irregular	17 (50)	43 (49)	82 (45)	29 (144)
Nuclear pleomorphism				
None	90.7 (264)	42 (48)	4 (2)	70 (314)
Mild to moderate	9 (26)	54 (62)	45 (25)	23 (113)
Severe	0.3 (1)	4 (5)	51 (28)	7 (34)
Stromal cellularity				
Low	77 (223)	26 (30)	9 (5)	58 (258)
Moderate	23 (68)	60 (69)	36 (20)	33 (157)
High	0 (0)	14 (16)	55 (30)	9 (46)
Stromal overgrowth				
None	92.4 (269)	63 (73)	16 (9)	76 (351)
Slight	6.9 (20)	27 (31)	22 (12)	14 (63)
Prominent	0.7 (2)	10 (11)	62 (34)	10 (47)
Mitotic counts, mean (range)	1.5 (0–4)	5.4 (1–22)	15.3 (0–80)	4.0 (0–80)

*The total number of patients recruited was 647. There were 167 and 480 breast tumour specimens in individual slide format and tissue microarray format respectively. A total of 294 specimens of tissue microarray samples and all 167 specimens in individual slide format (overall total 461) showed sufficient materials for assessment of ET1 immunostaining.

the tissue specimens were retrieved; 4 µm slides were prepared routinely and stained with H&E for histopathology. All the slides were reviewed for the following histological parameters: stromal cellularity; nuclear pleomorphism; stromal overgrowth; mitotic rate; and margin of the tumour, whether infiltrative or rounded. The first two parameters were graded as low/mild, moderate or severe; stromal overgrowth was graded as mild, moderate (scanty epithelial elements within a low power field ($\times 40$, Nikon Labophot, field area 1.9 mm²) or severe (absence of epithelial elements within a low power field)); and the mitotic count was given as the number of mitotic figures per 10 high power fields ($\times 400$, Nikon Labophot, field area 0.19 mm²). A diagnosis of benign phyllodes tumour was made when the tumour fulfilled most of the following criteria: low to focally moderate stromal cellularity, absent stromal overgrowth, and mild to focally moderate stromal nuclear pleomorphism, a rounded margin and a mitotic count of 4 or less per 10 high power fields. Frankly malignant phyllodes tumour was diagnosed when the tumour fulfilled most of the following criteria: moderate to high degree of stromal nuclear pleomorphism, mitotic count of 5 or more per 10 high power fields, stromal overgrowth, and an infiltrative margin. Borderline malignant phyllodes tumour was diagnosed when the histological features were intermediate between these two categories. Clinical and follow up data were collected, including the patient's age and ethnicity, size of the lesion, and whether or not there were recurrences or metastases.

In the Prince of Wales Hospital, Royal Prince Alfred Hospital, and National University Hospital, additional 4 µm slides were cut for ET1 immunostaining (167 cases). In Singapore General Hospital, tissue microarray blocks were constructed using the histological blocks (480 cases). The arrays were constructed with a 2 mm punch on a Beecher arrayer. The array layout in grid format was designed using a Microsoft Excel spreadsheet. H&E-stained sections were reviewed and the area of interest marked on the slide. Using a marker pen, the corresponding region was circled on the archival "donor" paraffin block. The samples were then arrayed on to a "recipient" blank block. The 2 mm punch was selected as it allowed easy manipulation during punching, with the larger individual sample size offering more lesional tissue for assessment. Difficulties

encountered in the construction of tissue microarrays were overcome as described previously.³⁶

ET1 immunohistochemistry

Immunostaining was performed using anti-ET1 antibody (Affinity BioReagent, Golden, USA; clone TR.ET.48.5, dilution 1:25). All the slides were examined by two pathologists; cytoplasmic staining intensity was scored as 0, 1, 2, and 3, indicating absent, weak, moderate and strong staining respectively. The percentage of cells showing such staining was also recorded. A staining score was defined as the product of the staining intensity and the percentage of positive stained cells; thus the score could range from 0 (no positively stained cells) to 300 (strong staining in all (100%) cells). Epithelial cells and stromal cells were assessed separately. Staining was considered negative if the score was 0, weak if the score was 1–20, moderate if the score was 21–50, and strong if the score was 51–300.

Figure 1 Photomicrograph showing a phyllodes tumour of borderline malignancy (H&E, $\times 100$).

Statistical analysis

One-way ANOVA was used to assess the relationships between clinicopathological parameters, including pathological types of tumours, tumour margin, nuclear pleomorphism, stromal cellularity, stromal overgrowth, and recurrence, and ET1 staining scores. Bonferroni correction was used for adjustment of p-values for determination of significance among sample groups in multiple comparisons. Correlations between mitotic count rate and staining scores and between epithelial and stromal ET1 staining scores were estimated by Spearson's *r* correlation analysis. The Fisher exact test was used to determine the association between pathological type of tumour and tumour recurrence.

RESULTS

A total of 647 cases of phyllodes tumours have been collected in the series, including 167 cases with tissue sections on individual slides and 480 cases in tissue microarray. Of the latter, 186 arrays were cut through or could not be assessed, giving an overall number of 461 examined cases in this study. Of these 461 cases, 291 cases (63%) were diagnosed as benign, 115 (25%) as borderline malignant and 55 (12%) as frankly malignant. The majority (66%, 303 cases) of the patients were Chinese, followed by Malays (13.1%, 63 cases). Caucasians and Indians each accounted for 7.0% (30 cases) of our patient cohort.

Clinicopathological characterisation

Table 1 summarises the histological characteristics of the three groups of phyllodes tumours in this study. The tumour size in the borderline (6.3 (0.5) cm in diameter) and frankly malignant tumours (6.5 (0.6) cm) was larger than that of benign tumours (4.3 (0.2) cm, $p < 0.001$). Of all the cases, 33 were recurrences (15 benign, 12 borderline and 6 malignant phyllodes). These recurrent tumours showed a higher frequency of being malignant (borderline and frankly) than benign ($p = 0.038$, Fisher exact test).

Endothelin-1 immunostaining

The epithelial cells in all three groups showed cytoplasmic staining for ET1. For the benign group, 6%, 26%, 15% and 53% respectively showed negative, weak, moderate and strong staining (no epithelial element was found in 4 cases); for the

Figure 3 Photomicrograph showing a benign phyllodes tumour (H&E, $\times 100$).

borderline group, the staining was 4%, 18%, 19% and 59% respectively (no epithelial element was found in 3 cases); and for the frankly malignant group, the staining was 6%, 18%, 6% and 70% respectively (no epithelial element was found in 15 cases).

The stromal cell staining for the benign group was 32%, 19%, 18% and 31% respectively for negative, weak, moderate and strong staining. For the borderline malignant group, the staining was 24%, 13%, 10% and 53% respectively for negative, weak, moderate and strong staining. For the frankly malignant group, the staining was 8%, 16%, 17% and 59% respectively for negative, weak, moderate and strong staining (figs 1–4).

ET1 specifically stains the cytoplasm of epithelial and stromal cells of phyllodes tumours of the breast. The epithelial ET1 score as the product of staining intensity and percentages of positive stained cells statistically correlated with the stromal scores (Spearson's *r* correlation coefficient = 0.572, $p < 0.001$). Both epithelial and stromal scores showed correlation with stromal mitotic count in tumours with Spearson's *r* correlation coefficient as 0.15 ($p = 0.002$) and 0.32 ($p < 0.001$) respectively.

Figure 2 Photomicrograph of the same tumour as in fig 1, showing positive stromal (score 3) and epithelial (score 3) staining for endothelin-1 (H&E, $\times 100$).

Figure 4 Photomicrograph of the same tumour as in fig 3, showing positive stromal (score 1) and epithelial (score 2) staining for endothelin-1 (H&E, $\times 100$).

Figure 5 Endothelin-1 (ET1) staining scores versus tumour diagnosis (A), tumour margin (B), nuclear pleomorphism (C), stromal cellularity (D) and stromal overgrowth (E).

Stromal ET1 scores increased from 48.2 (3.5) (mean (SEM)) in benign tumours to 74.6 (7.1) in borderline cases and further increased to 105.4 (12.4) in frankly malignant cases (overall $p < 0.001$, fig 5A). For the epithelial ET1 scores, there was a statistically significant increase in malignant tumours (121.5 (16.2), $p = 0.016$) as compared to those in benign tumours (81.9 (4.5), fig 5A). Tumours with irregular margin showed relatively higher stromal ET1 staining scores (79.6 (7.0)) than those with round margin (53.7 (3.6), $p < 0.001$, fig 5B). However, the epithelial scores of tumours with round and irregular borders were comparable. The stromal scores also showed significant difference between tumours with different degrees of nuclear pleomorphism, increasing from 50.0 (3.5) in tumours with no pleomorphism, to 79.7 (7.3) in tumours with mild pleomorphism, and to 111.2 (14.2) in tumours with severe pleomorphism (overall $p < 0.001$, fig 5C). When the scores with stromal cellularity and overgrowth were examined, the stromal ET1 staining scores showed a significant increase with high cellularity and degree of overgrowth. The mean stromal score of

tumours with low cellularity was 50.1 (4.0); it increased to 65.9 (5.6) in tumours with moderate cellularity, and further increased to 110.7 (13.4) in tumours with high cellularity (overall $p < 0.001$, fig 5D). However, there was no statistically significant difference in epithelial ET1 staining scores among tumours with different degrees of stromal cellularity. The tumours with no stromal overgrowth showed weak ET1 staining, with 52.3 (3.4) as the score compared to those with slight overgrowth (67.4 (9.6)) and to those with prominent overgrowth (120.2 (12.6), overall $p < 0.001$). Interestingly, the epithelial scores also showed a statistically significant increase from 81.8 (4.2) in tumours without stromal overgrowth to 144.8 (17.8) in tumours with prominent overgrowth ($p < 0.001$, fig 5E).

In summary, there was an increase in both epithelial and stromal vascular endothelial growth factor (VEGF) expression, with the increased stromal expression correlated with all atypical histological features, and the increased epithelial expression correlated with only the overall diagnosis and

stromal overgrowth. There was no significant difference in either epithelial or stromal VEGF expression between the recurrences and the primary tumours. ET1, endothelin-1.

DISCUSSION

ET1 is an important member of the so called endothelin axis, which includes the three endothelins (ET1, ET2, ET3), two receptors (ET-receptor A, and ET-receptor B), and two endothelin converting enzymes (ECE-1, ECE-2).⁵ While this axis is active in many cancers including breast cancer, it should be noted that there is a relative low level of synthesis of endothelins by inflammatory cells such as macrophages and monocytes.^{37, 38} Within the endothelin axis, ET1 preferentially binds to ETR-A compared to other ETs (ET2, ET3), as well as ETR-B. The binding of ET to the receptors will lead to activation of phospholipase C and MAPK pathways, an increase in intracellular calcium and the induction of immediate early genes.^{39, 40} Furthermore, endothelin may also lead to expression of vascular endothelial growth factor.⁴¹

In breast cancer, there is increased expression of ET1, ET2, ETR-A and ETR-B.^{9, 11} This expression is higher than in benign tumours (fibroadenomas) or pre-invasive ductal carcinoma in situ.¹⁰ Furthermore, this increased expression is also found to be associated with invasive ductal carcinomas with larger size, higher histological grade and the presence of lymphovascular permeation.¹² While the underlying mechanism of increased ET secretion in breast ductal carcinoma has not been fully elucidated, there is evidence that it is related to the degree of hypoxia within the tumour microenvironment.⁹ In addition, there is further evidence that ETs are implicated in the invasiveness of breast cancer, as the expression of ET and ETR was highest at the invasive edges of breast carcinomas,⁴² and a relation between ETR expression and tumour recurrence and metastasis has been shown.^{12, 43}

The expression of ET in other non-carcinomatous breast tumours has not been studied extensively. In the literature, there were only two studies evaluating this phenomenon in fibroadenoma and phyllodes tumours, and in the latter, only four cases were assessed. In one series of six fibroadenomas, the lesions showed moderate and weak staining for ET1 in 1 (17%) and 5 (83%) cases respectively, but there was no differentiation whether the staining was in the epithelial cells or in the stromal cells.¹¹ In another study, ET1 expression was found to be higher in the four cases of benign phyllodes tumours compared to the 14 fibroadenomas.³⁵ In our current study, we have shown that epithelial expression of ET1 in phyllodes tumours is common, with variable staining in more than 90% of all the cases. In addition, there is a significant difference between the various histological grades of phyllodes tumour, with the expression of ET1 progressively increasing with the grade of the tumour.

The phenomenon of stromal expression of ET1 in mammary fibroepithelial lesions (phyllodes tumours or fibroadenomas) has not been previously reported in any substantive series in the literature. The most important finding of this series is the variable expression of ET1 in the stromal cells of phyllodes tumours; the expression shows a progressive increase, in parallel with the grade of the tumour. This observation is in partial agreement with the previous study,³⁵ in which the stromal cells of the four benign phyllodes tumours were negative for ET1. Thus, higher stromal ET1 expression is associated with higher histological grade of the phyllodes tumour. This has not been previously shown.

When one looks in greater detail, it appears that while the stromal VEGF expression correlated with all atypical histological parameters of the stromal cells, the epithelial VEGF expression did show a parallel increase, particularly with

Take-home messages

- Endothelin-1 (ET1) expression in stromal cells and epithelial cells of phyllodes tumours correlates with the grade of the tumour.
- There is also correlation between the stromal ET1 expression and atypical histological features including nuclear pleomorphism, tumour margin pattern, stromal cellularity and stromal overgrowth.
- Correlation of epithelial and stromal ET1 expression may suggest epithelial participation in the stromal proliferation of phyllodes tumours.
- Similar ET1 expression patterns in primary and recurrent phyllodes tumours suggests a limited role of ET1 in tumour recurrence.

stromal overgrowth, stromal cellularity and stromal nuclear pleomorphism, even though only the first feature attained statistical significance. Thus we may infer that there is intimate interaction between the epithelium and the stroma; as ET1 can act in a paracrine fashion, this suggests that the ET axis may be involved in the pathogenesis of this group of fibroepithelial lesions, indicating that the almost always benign epithelial component may be an active participant of the pathogenesis, instead of being an innocent bystander. The association of epithelial VEGF expression with stromal overgrowth and cellularity may further suggest that somehow the epithelial cells stimulate the proliferation of even the malignant progression of the stromal cells.

The implication of this finding is not yet clear. Whether this is related to the metastasising or recurrence potential, or to the observation of increased angiogenesis in the stroma of high histological grade phyllodes tumours^{31, 32} remains to be elucidated. We were however unable to show any difference in the ET1 staining in those tumours and recurrences. One can thus extrapolate that the ET axis is probably not a direct determinant of tumour recurrence, warranting exploration of other potential markers.

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