Stromal nitric oxide synthase (NOS) expression correlates with the grade of mammary phyllodes tumour


Background: Nitric oxide synthase (NOS), particularly endothelial and inducible forms (e/i-NOS), are expressed in various cancers, including breast cancer. In mammary fibroepithelial lesions, NOS expression in stromal cells has been reported to be lower in fibroadenomas than in phyllodes tumours. Aims: To investigate NOS expression in phyllodes tumours of varying degrees of malignancy. Methods: One hundred and sixty seven mammary phyllodes tumours (97 benign, 47 borderline malignant, and 23 frankly malignant) were evaluated for e-NOS and i-NOS expression by immunohistochemistry. Correlations with previously reported expression of stromal vascular growth factor (VEGF) and microvessel density were also performed. Results: Stromal expression of e-NOS was absent, weak, moderate, and strong in 43%, 31%, 13%, and 13% of benign tumours; 17%, 26%, 13%, and 44% of borderline malignant tumours; and 17%, 35%, 13%, and 35% of frankly malignant tumours, respectively. Stromal expression of i-NOS was 77%, 18%, 4%, and 1% in benign tumours; 42%, 28%, 19%, and 11% in borderline malignant tumours; and 43%, 13%, 26%, and 18% in frankly malignant tumours, respectively. Stromal expression of both i-NOS and e-NOS was significantly different between the benign and malignant (borderline and frank) groups of phyllodes tumours (p<0.0001). Furthermore, the expression of i-NOS correlated with stromal VEGF expression and microvessel density. The expression of NOS in the epithelial cells was strong, and showed no differences between the different groups of tumours. Conclusions: Higher stromal expression of NOS in phyllodes tumours is associated with malignancy, suggesting a possible role in malignant progression, particularly metastasising potential.

Nitric oxide (NO) is a free radical gas synthesised by a family of isoenzymes called NO synthases (NOS). NO acts as an intracellular secondary messenger in various pathways, including those involved in vasodilation, neuronal transmission, and cytotoxicity of inflammatory cells. The NOS isoenzymes are classified into two groups, namely: (1) the calcium dependent, constitutively expressed constitutive NOS enzymes, which include the endothelial NOS (e-NOS) and the neuronal NOS forms; and (2) calcium independent inducible NOS (i-NOS), which is involved mainly in tumour induced immunosuppression and macrophage mediated cytotoxicity. The role of NO in tumour biology is still poorly understood, with many authors reporting that NO has both protumour and antitumour effects. Its protumour effects include promotion of DNA strand breakages, impairment of the function of the tumour suppressor p53, promotion of neovascularisation or enhancement of blood flow, and the development of metastases. NO can also have antitumour effects, and this may or may not be related to the observed effect of inducing apoptosis in tumour cells.

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NOS activity has been demonstrated in a variety of human malignancies, including ovarian cancer, gastric cancer, colon cancer, brain tumours, head and neck squamous cell carcinoma, lung tumours, prostate carcinoma, urinary bladder transitional cell carcinoma, oesophageal adenocarcinoma, and Kaposi’s sarcoma. In breast cancer, NO expression and its correlation with various parameters including stage, grade, metastasis status, and precursor lesions has been the subject of much investigation, but with conflicting results. Most studies have concentrated on i-NOS, with smaller numbers reporting on the relation between breast lesions and e-NOS. Many authors have found e-NOS expression to be present in breast carcinoma, and its expression has been variably reported as reduced or increased compared with normal breast epithelium. It has also been reported that e-NOS expression is variably related to tumour grade, ranging from no correlation to an inverse correlation with tumour grade (higher expression in lower grade cancers). Other authors have only detected e-NOS in metaplastic apocrine cells of the breast and not in carcinomas. The presence of e-NOS positive vessels in the peritumorous tissue but not within the tumour has also been observed.

Expression of i-NOS in breast cancer has been more extensively studied. Most authors have found increased expression of i-NOS in breast cancers, compared with weak or absent expression seen in benign breast epithelium; others have found decreased expression compared with benign breast epithelium. Among those studies showing increased i-NOS expression in breast cancers, some found a correlation between increased expression and tumour grade (higher grade, higher expression), whereas others demonstrated an inverse relation (higher grade, lower expression). In addition, i-NOS expression was thought to correlate with metastasis development in some studies.

Abbreviations: e/i-NOS, endothelial/inducible nitric oxide synthase; NO, nitric oxide; VEGF, vascular endothelial growth factor.
Very few studies have documented the expression of NOS in fibroepithelial lesions of the breast. In one report, negative i-NOS expression was noted in four cases of fibroadenoma. Another report noted the presence of nitrate and nitrite but negative NOS activity in cell culture of a single case of phyllodes tumour of unknown malignant potential. The largest study of mammary fibroepithelial lesions reported in the literature so far found that i-NOS expression was higher in epithelial cells and lower in stromal cells of fibroadenomas compared with phyllodes tumours.

Mammary phyllodes tumour is an uncommon stromal epithelial neoplasm that usually occurs in middle aged women. Determining its malignant potential is difficult, but based on a combination of histological criteria, phyllodes tumours can be divided into benign, borderline malignant, and frankly malignant groups. Although all groups of phyllodes tumours show a propensity to recur locally, the borderline and frankly malignant groups may also metastasise to other visceral organs.

In our study, we investigated the expression of NOS activity, including both endogenous (e-NOS) and inducible (i-NOS) forms, in a large series of mammary phyllodes tumours and assessed its correlation with tumour grade. We also compared the expression of i-NOS with the previous assessment of vascular endothelial growth factor (VEGF) expression and microvessel density in the same group of lesions.

MATERIALS AND METHODS

The histological files of four participating institutions in three different countries were searched for phyllodes tumours of the breast. The number of years searched ranged from four to 15. The paraffin wax blocks were retrieved and 4 μm sections were prepared routinely and stained with haematoxylin and eosin. All the slides from the various institutions were collected in one centre, where all the immunohistochemical staining was performed. The haematoxylin and eosin slides were reviewed by each author and the diagnosis was compared with the original one. Discrepant cases (<5%) were reassessed together with the submitting pathologist and a consensus diagnosis was made. The following histological parameters were assessed: 1, stromal cellularity; 2, nuclear pleomorphism; 3, stromal overgrowth; 4, mitotic rate; and 5, margin of the tumour (whether infiltrative or rounded). Parameters 1 and 2 were graded as low/mild, moderate, or severe; stromal overgrowth was graded as present or absent; and the mitotic count was expressed as the number of mitotic figures for each 10 high power fields (×400 magnification; Nikon Labophot, field area 0.19 mm²). As described previously, a diagnosis of benign phyllodes tumour was made when there was low cellularity, no stromal overgrowth, mild pleomorphism, a rounded margin, and a mitotic count of ≤2/10 high power fields. Malignant phyllodes tumour was diagnosed when the mitotic count was ≥5/10 high power fields, together with stromal overgrowth and an infiltrative margin (fig 1). Phyllodes tumour of borderline malignancy was diagnosed when the criteria for malignant phyllodes tumour were not totally fulfilled.

For the assessment of e-NOS and i-NOS expression, a representative slide from each case was stained using an antibody against e-NOS and i-NOS (both from Transduction Laboratories, Lexington, Kentucky, USA; 1:200 dilution) with the avidin biotin method and microwave antigen retrieval. Cytoplasmic staining of the epithelial and stromal cells was assessed for intensity (graded as 0 if there was no staining, low, intermediate, or high if the staining was weak, moderate, or strong, respectively; fig 2). For e-NOS, staining of the endothelium was taken as strong positive. The overall percentage of stained cells was also assessed. The overall staining pattern was considered negative if there was no staining, weak if there was only weak staining, moderate if there was moderate to strong staining in less than 50% of the cells, and strong if there was moderate to strong staining in 50% or more of the cells. The slides from each case were assessed by three of the authors from the same centre, and a consensus was reached regarding the staining intensity and percentage of cells stained.

The phyllodes tumours in our series had previously been assessed for VEGF expression by immunohistochemistry and for microvessel density by the hot spot method (applying to CD31 positive structures in the stroma), the results of which have been published. Because increased vascularity has been cited as one of the purported mechanisms for the actions of i-NOS, VEGF expression and microvessel density of the stroma were also assessed for a correlation with i-NOS expression.

For statistical analysis, the χ² test was used to compare patients’ ages, tumour size, and e-NOS and i-NOS expression in the epithelial cells and stromal cells between the benign, borderline malignant, and frankly malignant groups of
phyllodes tumours. ANOVA was used to assess the correlation between i-NOS, VEGF expression, and microvessel density of the stroma. Multinominal logistic regression was used to evaluate whether i-NOS, VEGF expression, or microvessel density was a predictor of malignancy of phyllodes tumours. Significance was established at p < 0.05.

RESULTS

In total, 167 mammary phyllodes tumours were retrieved: 97 benign, 47 borderline malignant, and 23 frankly malignant tumours. These tumours were derived from 163 patients, with two tumours (one original and one recurrent) from each of two patients, and the original tumour and two recurrences from one patient. In the remaining 160 tumours, there were 148 tumours that did not recur, three original tumours that later recurred, eight first recurrences, and one second recurrence.

All the patients were female, and included 98 Chinese, 29 whites, 11 Malaysians, seven Indians, and seven other Asians; in 11 patients the ethnicity was not known. The overall age range was 17–77 years, with a mean of 42. Eighty one tumours were on the left side, and 85 were on the right. In one tumour, the side was not known. The tumour size range was 0.8–22 cm, with a mean of 4.8. There was a progressive increase of the patient’s age and tumour size from benign to borderline malignant to frankly malignant phyllodes tumours (p < 0.05).

Table 1 shows the overall expression of i-NOS and e-NOS in the entire series, and tables 2 and 3 show the expression of i-NOS and e-NOS among the different categories of phyllodes tumour.

In general, staining for both i-NOS and e-NOS was positive for the epithelial component of all grades of phyllodes tumour, but stromal expression of both forms of NOS increased with increasing grade.

There was no significant difference in the epithelial expression of i-NOS or e-NOS in the entire study population of phyllodes tumours or between tumours of differing degrees of malignancy.

For the stromal expression of both i-NOS and e-NOS, the differences between the benign and borderline malignant groups and between the benign and frankly malignant groups were significant (p < 0.05). On further analysis, when the tumours were grouped into two categories—namely, benign and malignant (which includes the borderline and frankly malignant subgroups)—the difference between these two groups of tumours became highly significant (p < 0.0001). There was no significant difference between these groups for the epithelial component.

In the entire series, there were 13 recurrent phyllodes tumours: five benign, six borderline malignant, and two frankly malignant tumours. The epithelial and stromal expression of i-NOS and e-NOS showed no correlation with tumour grade.

Previously, we found that the expression of VEGF in the stromal cells of phyllodes tumours was about 30%,42 and the level of expression correlated with the degree of malignancy. Higher levels of VEGF expression were seen in higher grade phyllodes tumours (p < 0.001). A strong correlation between microvessel density and tumour grade was also demonstrated previously (p < 0.0001).42 There was also a strong correlation between stromal VEGF expression and stromal microvessel density (p < 0.0001).42 The expression of stromal i-NOS correlated with stromal VEGF expression and with stromal microvessel density (p < 0.001). However, using multinominal logistic regression, stromal i-NOS and microvessel density (p < 0.001), but not stromal VEGF expression, were predictors of malignancy in phyllodes tumours.

DISCUSSION

Reports on the activity of NOS in breast cancer have been conflicting, with different or completely opposing findings being reported. Most authors reported the presence of NOS activity in carcinoma.1 5 25 26 30–33 Among these, some reported higher NOS activity in malignant than in benign breast tissue,5 10–13 encompassing both a positive correlation between NOS activity and tumour grade10 13 31 and a negative correlation,1 25 31 whereas others reported the reverse,1 28 and in benign apocrine metaplasia but not in carcinoma.28 These paradoxical results may be explained by the fact that at low

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### Table 1

Expression of i-NOS and e-NOS in the epithelial and stromal components of phyllodes tumours

<table>
<thead>
<tr>
<th>NOS isoform expression</th>
<th>Epithelium</th>
<th>Stroma</th>
</tr>
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<tbody>
<tr>
<td>i-NOS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>12 (7%)</td>
<td>105 (63%)</td>
</tr>
<tr>
<td>Weak</td>
<td>58 (35%)</td>
<td>33 (20%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>14 (8%)</td>
<td>19 (11%)</td>
</tr>
<tr>
<td>Strong</td>
<td>83 (50%)</td>
<td>10 (6%)</td>
</tr>
<tr>
<td>Total</td>
<td>167 (100%)</td>
<td>167 (100%)</td>
</tr>
<tr>
<td>e-NOS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>4 (2%)</td>
<td>53 (32%)</td>
</tr>
<tr>
<td>Weak</td>
<td>45 (28%)</td>
<td>50 (30%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>0 (0%)</td>
<td>22 (13%)</td>
</tr>
<tr>
<td>Strong</td>
<td>118 (70%)</td>
<td>42 (25%)</td>
</tr>
<tr>
<td>Total</td>
<td>167 (100%)</td>
<td>167 (100%)</td>
</tr>
</tbody>
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i-NOS, inducible nitric oxide synthase.

e-NOS, endothelial inducible nitric oxide synthase.
Increased vascularity as one of the possible effector mechanisms. Further evaluation of those cases with recurrences or metastases, in particular NOS/VEGF expression and stromal microvessel density, may provide new insights into the pathogenesis and facilitate the prognostication of phyllodes tumours.

**Take home messages**

- Nitric oxide synthase (NOS) is expressed in both the epithelial and stromal cells of phyllodes tumours
- There was a positive correlation between NOS expression in stromal cells and tumour grade
- There was a strong correlation between inducible NOS and both VEGF and microvessel density
- Higher stromal expression of NOS in phyllodes tumours was associated with malignancy, suggesting a possible role in malignant progression, particularly metastasising potential

**REFERENCES**


