Immunohistochemical analysis of biomarkers in patients with adenocarcinoma of the breast: correlation with menopausal status and histological grade

Govindarajah Vinothini1, Paramasivame V. Letchoumy1, Duvuru Prathiba2, Siddavaram Nagini1

Abstract

Introduction: Adenocarcinoma of the breast is the most common cancer worldwide and accounts for the highest morbidity and mortality. The increasing global incidence of breast cancer emphasizes the need to understand the various mechanisms involved in breast tumorigenesis. We investigated the expression of steroid hormone, steroid and growth factor receptors, cytochrome P450 isoforms, oxidative stress markers, and proteins involved in cell survival and proliferation, genomic instability, and apoptosis in breast cancer patients.

Material and methods: Forty-eight breast cancer patients histologically categorized as grade I, II and III, and as pre- and postmenopausal, were chosen for the study. The expression of all the markers in tumour and adjacent tissues was analysed in the same set of patients by immunohistochemical localization.

Results: The expression of oestradiol, oestrogen and progesterone receptor, HER-2/neu, CYP1A1 and 1B1, 4-hydroxynonenal, 8-hydroxy-2'-deoxyguanosine, glutathione S-transferase-P, NFκB, PCNA, p53, and Bcl-2 was significantly increased, whereas the expression of Bax and caspases was significantly lower in breast tumours compared to corresponding uninvolved adjacent tissues. The magnitude of the changes was however more pronounced in premenopausal patients and in grade III tumours.

Conclusions: Our results suggest that analysis of multiple signalling pathways in the same set of patients would be useful in understanding the complex genetic alterations that interplay in the development of breast tumours.

Key words: apoptosis, breast cancer, cell proliferation, oestrogen, oxidative stress.

Introduction

Carcinoma of the breast is the most common cancer in women worldwide, with more than 1,000,000 new cases diagnosed annually [1]. Approximately 75,000 new cases of breast cancer are reported in Indian women every year. Breast cancer is the most common cancer among women in western India and the second most common cancer in South India [2, 3].

The aetiology of breast cancer is predominantly hormonal, with increased lifetime exposure to endogenous or exogenous oestrogens posing a major risk [4, 5]. Metabolism of oestrogens by cytochrome P450s (CYPs), a superfamily of haem-containing monoxygenases, generates genotoxic...
intermediates implicated in breast tumorigenesis [6]. In addition, oestrogen receptor (ER), progesterone receptor (PR) and growth factor receptor HER-2/neu alter hormone responsiveness, increase transcriptional activation and induce oncogenic transformation [7]. Aberrant metabolism of oestrogens is recognized to generate reactive oxygen species (ROS) that can cause DNA damage, cell proliferation, and resistance to apoptosis eventually culminating in mammary carcinogenesis [8, 9].

Studies by Tsuchiya et al. [10] have demonstrated that CYP-mediated channelling of oestrogens into the 2-hydroxy or 4-hydroxy oestradiol pathway is an important determinant of breast cancer risk. Changes in receptor status, cell proliferation and apoptosis have also been documented in breast cancer patients by us and other workers [11-14]. These studies reveal that complex and heterogeneous genetic alterations are involved in the aetiology of breast tumorigenesis. However, this conclusion is based on consolidation of data from literature and suffers from the limitation that the studies were carried out on different sets of breast cancer patients. Analysis of multiple signalling pathways in the same set of patients would be more informative and useful in both diagnosis and prognosis of breast tumours.

The present study was therefore designed to analyse a panel of markers that reflect hormone receptor status, cell proliferation and apoptosis (Bcl-2, Bax, caspases) in the same set of breast cancer patients. Of the 48 breast cancer patients, 24 (50%) were premenopausal and 24 (50%) were postmenopausal. The patients were not using hormones or oral contraceptives and were non-smokers. None of them had concomitant diseases such as diabetes mellitus, liver diseases or rheumatoid arthritis. Informed consent was obtained from all the participants. The Human Ethics Committee, India approved the study. Table I shows the general characteristics of breast cancer patients with TNM classification [15] and histological grading based on Scarff-Bloom-Richardson’s (SBR) classification as modified by Elston and Ellis (Nottingham modification) [16].

The tissues were fixed in 10% formalin, embedded in paraffin and mounted on polylysine-coated glass slides. One section from each specimen was stained with haematoxylin and eosin. The remaining sections were used for immunohistochemical staining.

### Immunohistochemistry

The tissue sections were deparaffinised by heat at 60°C for 10 min, followed by three washes in xylene. After gradual hydration through graded alcohol, the slides were incubated in citrate buffer (pH 6.0) for two cycles for 5 min in a microwave oven for antigen retrieval. The sections were allowed to cool for 20 min and then rinsed with Tris-buffered saline (TBS). The sections were treated for 15 min with 3% H_2O_2 in distilled water to inhibit endogenous peroxidase activity. Non-specific antibody binding was reduced by incubating the sections with universal power block (BioGenex, San Ramon, CA, USA) for 10 min. The sections were then incubated with mouse monoclonal antibodies for ER, PR, HER-2/neu, PCNA (Dako, Carprinteria, CA, USA), CYP1A1 (provided by Dr. John J. Stegeman, ...
Woods Hole Oceanographic Institute, USA), CYP1B1 (Santa Cruz Biotechnology, CA, USA), 4HNE (provided by Dr. Koji Uchida, Nagoya University, Japan), 8-OHdG (JaICA, Japan), p53 (Neo Markers, USA), Bcl-2 (BioGenex, USA), and rabbit polyclonal antibodies for GST-P (BioGenex, USA), NFκβ (Neo Markers, USA), Bax, caspases -3, -8 and -9 (Santa Cruz Biotechnology, CA, USA) at room temperature for two hours.

The antibodies for ER, PR, HER-2/neu, PCNA, GST-P, p53 and Bcl-2 were used in the prediluted form. The following antibody dilutions were used for the other markers: 1:300 for CYP1B1, 1:200 for CYP1A1, 4-HNE, 8-OHdG, NFκβ, and caspases -3, -8 and -9; and 1:100 for Bax. The slides were washed with TBS and then incubated with anti-mouse and anti-rabbit biotin-labelled secondary antibody followed by streptavidin-biotin-peroxidase (both Dako) for 30 min each at room temperature. The immunoprecipitate was visualized by treating with 3,3’-diaminobenzidine (Dako) and counterstaining with haematoxylin. For negative controls, the primary antibody was replaced with TBS. Positive controls were also processed simultaneously.

**Statistical analysis**

Immunohistochemical staining was scored according to the number of positively stained cells per 100 counted cells. The data for immunohistochemical analyses are expressed as mean ± SD. Statistical comparisons were performed by paired sample t-test. The values were considered statistically significant if the P value was less than 0.05.

**Results**

The expression of oestradiol, ER, PR and HER-2/neu in breast tumour tissues of pre- and postmenopausal patients are illustrated in Tables II and III and representative photomicrographs of immunostaining are shown in Figure 1. While immunostaining of ER and PR showed nuclear localization, HER2-neu was localized in the membrane. The expression of oestradiol, ER, PR and HER-2/neu was significantly increased by 94, 80, 66, and 70% respectively in premenopausal breast tumours and by 64, 79, 38, and 55% respectively in postmenopausal breast tumours when compared to adjacent normal tissues. The extent of increase was greater in premenopausal breast tumours compared to postmenopausal patients. The expression of oestradiol and ER was significantly higher in grade III patients compared to grade I and II. Wide variations in the expression pattern of PR and HER-2/neu within grading were seen in both pre- and postmenopausal patients. In premenopausal patients, PR and HER-2/neu expression was negative in grade III and II respectively, whereas in postmenopausal patients, PR was negative in grade II, and HER-2/neu was negative in grade I.

Table IV and Figure 2 illustrate the expression of CYP1A1, CYP1B1, 4HNE and 8-OHdG in breast tumour tissues of pre- and postmenopausal patients with respect to histological grading. The expression of all the markers was significantly increased in both pre- and postmenopausal breast tumours when compared to adjacent uninvolved tissues. The extent of increase was however greater in premenopausal breast tumours than postmenopausal breast tumours as well as in grade III patients compared to grade I and II patients. Furthermore, the increase in CYP1B1 expression was greater than CYP1AI expression both in premenopausal and postmenopausal patients. While CYP1A1 and 4-HNE showed cytoplasmic localization, 8-OHdG was nuclear and CYP1B1 showed both cytoplasmic and nuclear staining (Figure 3).

The results of immunohistochemical analysis and representative photomicrographs of PCNA, GST-P, NFκβ and p53 immunostaining in tumour and adjacent uninvolved tissues of pre- and postmenopausal breast cancer are shown in Table V, and Figures 4 and 5. While immunostaining of PCNA and p53 showed nuclear localization, NFκβ was localized in the cytosol and GST-P showed both nuclear and cytoplasmic staining. The expression of PCNA, GST-P, NFκβ and p53 was

**Table II.** The expression of oestradiol, ER, PR and HER-2/neu in breast cancer patients with respect to menopausal status (mean ± SD)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Premenopausal patients</th>
<th>% Increase</th>
<th>Postmenopausal patients</th>
<th>% Increase</th>
</tr>
</thead>
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<td></td>
<td>(n=24)</td>
<td>tumour tissue</td>
<td>adjacent tissue</td>
<td></td>
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<tr>
<td>Estradiol</td>
<td>65.72±5.6 *</td>
<td>33.88±3.6</td>
<td>94</td>
<td>60.93±5.4 **</td>
</tr>
<tr>
<td>ER</td>
<td>71.90±7.4 **</td>
<td>39.99±3.5</td>
<td>80</td>
<td>61.61±5.5 **</td>
</tr>
<tr>
<td>PR</td>
<td>43.95±3.8 *</td>
<td>26.50±1.9</td>
<td>66</td>
<td>33.02±3.8 *</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>61.68±5.9 *</td>
<td>36.21±4.2</td>
<td>70</td>
<td>59.64±5.5 **</td>
</tr>
</tbody>
</table>

*Significantly increased when compared to adjacent normal tissues, P<0.05 (paired sample t-test)  
**Significantly increased when compared to adjacent normal tissues, P<0.01 (paired sample t-test)
significantly increased in both pre- and postmenopausal breast tumours when compared to adjacent normal tissues. Although protein expression was significantly increased in breast tumours of different histological grading, the expression of all the markers in grade III was significantly higher than in grade I. No significant changes in protein expression were seen in grade II compared to grade I and III.

Table VI and Figures 7 and 8 show the expression of Bcl-2, Bax, Bcl-2/Bax ratio, and caspases-8, -9 and -3 in tumour and adjacent uninvolved tissues of pre- and postmenopausal breast cancer patients. While Bcl-2 expression was significantly increased in pre- and postmenopausal breast tumours, the expression of Bax, and caspases-8, -9 and -3 was significantly decreased compared to adjacent normal tissues. Bcl-2 expression was significantly

<table>
<thead>
<tr>
<th>Marker</th>
<th>Premenopausal patients (n=24)</th>
<th>Postmenopausal patients (n=24)</th>
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<tr>
<td></td>
<td>grade I (n=8)</td>
<td>grade II (n=8)</td>
</tr>
<tr>
<td></td>
<td>tumour tissue</td>
<td>adjacent tissue</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>65.72±6.8*</td>
<td>33.88±3.1</td>
</tr>
<tr>
<td>ER</td>
<td>65.97±5.7*</td>
<td>36.64±3.8</td>
</tr>
<tr>
<td>PR</td>
<td>56.31±6.1*</td>
<td>39.12±3.7</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>61.68±5.9**</td>
<td>36.21±4.2</td>
</tr>
</tbody>
</table>

*Significantly increased when compared to adjacent normal tissues, P<0.05 (paired sample t-test)
**Significantly increased when compared to adjacent normal tissues, P<0.01 (paired sample t-test)
♥ As compared with grade I and II, P<0.05 (paired sample t-test)

Figure 1. Photomicrographs of immunohistochemical localization of oestradiol, ER, PR and HER-2/neu in premenopausal and postmenopausal human breast tumour tissues (×10)
Evaluation of biomarkers in breast cancer patients

Table IV. The expression of CYP1A1, CYP1B1, 4HNE and 8-OHdG in breast cancer patients with respect to menopausal status (mean ± SD)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Premenopausal patients</th>
<th>Postmenopausal patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tumour tissue</td>
<td>adjacent tissue</td>
</tr>
<tr>
<td>CYPIA1</td>
<td>61.6±5.4**</td>
<td>36.2±3.9</td>
</tr>
<tr>
<td>CYPIB1</td>
<td>75.89±7.7**</td>
<td>39.48±4.1</td>
</tr>
<tr>
<td>4HNE</td>
<td>80.42±7.9</td>
<td>44.84±4.7</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>65.23±6.2</td>
<td>38.42±3.1</td>
</tr>
</tbody>
</table>

*Significantly increased when compared to adjacent normal tissues, P<0.05 (paired sample t-test)
**Significantly increased when compared to adjacent normal tissues, P<0.01 (paired sample t-test)

Figure 2. Expression of CYPIA1, CYPIB1, 4HNE and 8-OHdG in breast cancer patients with respect to menopausal status and histological grading (mean ± SD)

* As compared with corresponding adjacent tissues P<0.05 (paired sample t-test)
** As compared with corresponding adjacent tissues P<0.01 (paired sample t-test)
♣ As compared with grade I and II P<0.01 (paired sample t-test)

higher in grade III tumours compared to grade I and II, whereas the expression of Bax and caspases was significantly higher in grade I tumours compared to grade II and III. Representative photomicrographs showing cytoplasmic staining of the apoptotic markers are shown in Figures 7 and 8.

Discussion

The breast tumours analyzed in the present study were characterized by increased expression of oestriol, hormone and growth factor receptors and CYPI enzymes associated with enhanced expression of markers of oxidative stress, cell survival and cell proliferation, genomic instability and apoptosis.

Increased lifetime exposure to oestrogen has long been linked to the development and progression of breast cancer. Oestrogens mediate their biological effects via interaction with ER, and the oestrogen-ER complex is recognized to influence the expression of genes regulating cell proliferation, differentiation and...
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apoptosis [17, 18]. In addition to ER, PR and HER-2/neu have also been implicated in breast tumorigenesis. The expression of these receptors during breast cancer development is reported to be interrelated [7, 19, 20]. Several studies and a report from this laboratory have documented overexpression of ER, PR and HER-2/neu in breast carcinomas [13, 21, 22]. To our knowledge this is the first study to evaluate the expression of all these receptors in breast tumours with respect to menopausal status and histological grading. Although all the breast cancer patients showed increased ER expression, an inverse correlation was seen between PR and HER-2/neu expression irrespective of menopausal status and histological grading. While HER-2/neu positive tumours showed lower or negative PR expression, PR positive tumours were negative for HER-2/neu expression. Altered expression of steroid hormone receptors and growth factor receptor seen in the present study is consistent with similar reports in the literature [23, 24].

Both endogenous and exogenous oestrogens undergo metabolic activation in the mammary gland by the CYP family of enzymes. The E2-ER complex binds to the oestrogen response element in the CYP gene promoter with increased metabolism of E2. While CYP1A1 catalyses 2-hydroxylation of E2, CYP1B1 catalyses 4-hydroxylation [10]. The greater increase in CYP1B1 relative to CYP1A1 in breast cancer patients observed in the present study suggests increased channelling into the 4-hydroxy pathway. In a study involving 393 patients with primary breast cancer, Haas et al. [25] showed an association between strong expression of CYP1B1 with poor tumour differentiation and unfavourable prognosis.

The 4-hydroxymetabolite of E2 is reported to bind strongly to ER, increasing transcriptional activation and oncogenic transformation. In addition, the

Table V. The expression of PCNA, GST-P, NFκβ and p53 in breast cancer patients with respect to menopausal status (mean ± SD)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Premenopausal patients (n=24)</th>
<th>% increase</th>
<th>Postmenopausal patients (n=24)</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tumour tissue</td>
<td>adjacent tissue</td>
<td></td>
<td>tumour tissue</td>
</tr>
<tr>
<td>PCNA</td>
<td>74.33±6.7’’</td>
<td>39.84±5.6</td>
<td>87</td>
<td>54.26±5.3’’</td>
</tr>
<tr>
<td>GST-P</td>
<td>80.06±8.5’’</td>
<td>42.05±3.8</td>
<td>90</td>
<td>63.78±6.7’’</td>
</tr>
<tr>
<td>NFκβ</td>
<td>64.94±6.0’’</td>
<td>39.96±4.1</td>
<td>63</td>
<td>60.93±6.3’’</td>
</tr>
<tr>
<td>P53</td>
<td>72.67±7.5’’</td>
<td>40.84±3.6</td>
<td>78</td>
<td>60.11±5.7’’</td>
</tr>
</tbody>
</table>

*Significantly increased when compared to adjacent normal tissues, P<0.05 (paired sample t-test)  **Significantly increased when compared to adjacent normal tissues, P<0.01 (paired sample t-test)
Evaluation of biomarkers in breast cancer patients

**Figure 4.** The expression of PCNA, GST-P, NFκβ and p53 in breast cancer patients with respect to menopausal status and histological grading (mean ± SD)

* As compared with corresponding adjacent tissues P<0.05 (paired sample t-test)

** As compared with corresponding adjacent tissues P<0.01 (paired sample t-test)

*** As compared with corresponding adjacent tissues P<0.001 (paired sample t-test)

♠ As compared with grade I P<0.01 (paired sample t-test)

**Figure 5.** Photomicrographs of immunohistochemical localization of PCNA, GST-P, NFκβ and p53 in premenopausal and postmenopausal human breast tissues (×10)
Table VI. The expression of Bcl-2, Bax, Bcl-2/Bax ratio, caspases-8, -9 and -3 in breast cancer patients with respect to menopausal status (mean ± SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Premenopausal patients (n=24)</th>
<th>% increase* /decrease*</th>
<th>Postmenopausal patients (n=24)</th>
<th>% increase* /decrease*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tumour tissue</td>
<td>adjacent tissue</td>
<td></td>
<td>tumour tissue</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>84.36±7.3**</td>
<td>52.43±5.6</td>
<td>61♣</td>
<td>76.03±7.3*</td>
</tr>
<tr>
<td>Bax</td>
<td>58.35±6.8*</td>
<td>73.88±6.9</td>
<td>21♠</td>
<td>66.6±6.9**</td>
</tr>
<tr>
<td>Bcl-2/Bax</td>
<td>1.45</td>
<td>0.71</td>
<td>104♣</td>
<td>1.15</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>41.76±4.3**</td>
<td>53.15±6.2</td>
<td>21♠</td>
<td>46.43±5.1***</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>50.74±4.9*</td>
<td>59.08±5.1</td>
<td>14♣</td>
<td>52.41±5.8*</td>
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<tr>
<td>Caspase-3</td>
<td>45.89±4.8*</td>
<td>58.15±5.2</td>
<td>21♠</td>
<td>48.73±4.3**</td>
</tr>
</tbody>
</table>

*Significantly increased when compared to adjacent normal tissues, P<0.01 (paired sample t-test) **Significantly increased when compared to adjacent normal tissues, P<0.001 (paired sample t-test) ♣ Significantly decreased when compared to adjacent normal tissues, P<0.05 (paired sample t-test) ♠ Significantly decreased when compared to adjacent normal tissues, P<0.01 (paired sample t-test) ♦ Significantly decreased when compared to grade I and II, P<0.01 (paired sample t-test)

Figure 6. Expression of Bcl-2, Bax, caspases -8, -9 and -3 in breast cancer patients with respect to menopausal status and histological grading (mean ±SD) 
* As compared with corresponding adjacent tissues P<0.05 (paired sample t-test) **As compared with corresponding adjacent tissues P<0.01 (paired sample t-test) ♣ As compared with grade I and II P<0.01 (paired sample t-test)
4-hydroxy metabolite is believed to undergo redox cycling to generate ROS that can cause damage to cellular macromolecules, especially DNA [10]. ROS is also known to induce mutations in proto-oncogenes and tumour suppressor genes, eventually culminating in malignant transformation [26]. Enhanced expression of 4-HNE and 8-OHdG, markers of ROS-induced lipid peroxidation and DNA damage respectively, associated with increased expression of mutant p53 in breast cancer patients in the present study suggest ROS involvement. Several studies including our own have shown increased lipid peroxidation as well as high levels of oxidative base lesions in tumour tissues of breast cancer patients compared to normal adjacent tissues. The results of the present study are in line with these findings [27-29].

Mutational inactivation of the p53 gene, a key step in neoplastic transformation, has been extensively documented in breast tumours. Increased expression of mutant p53 may facilitate cell proliferation, leading to genomic instability and resistance to apoptosis [30]. Overexpression of PCNA, GST-P and NFκB in breast cancer patients in the present study is indicative of increased cell proliferation and is consistent with similar findings by us and other workers [12, 13, 31, 32]. Besides cell proliferation, PCNA, GST-P, NFκB and mutant p53 are involved in apoptosis inhibition. Interaction of PCNA with Gadd45 and its homologue MyD118 was shown to impede the functions of these proteins as negative regulators of growth control, implicating the antiapoptotic role of PCNA [33]. GST-P, NFκB and mutant p53 have been reported to inhibit apoptosis by upregulating expression of antiapoptotic Bcl-2 and downregulating Bax expression [30, 31, 34, 35].

The Bcl-2/Bax ratio is a critical determinant of the overall propensity of a cell to undergo apoptosis. In addition, apoptosis is also regulated by caspases. Activated caspases cleave a broad range of cellular targets and execute the cell death programme [36]. The increase in Bcl-2/Bax ratio with downregulation of caspases signifies apoptosis evasion in breast cancer patients in the present study.

Analysis of biomarkers associated with hormone and receptor status, CYP enzymes, oxidative stress, cell proliferation and apoptosis has revealed that breast tumour tissues have a proliferative and apoptosis resistant phenotype compared to the uninvolved adjacent tissues. These changes were more pronounced in premenopausal breast cancer patients compared to postmenopausal patients. This may be because premenopausal breast tumours tend to be generally more aggressive than breast tumours that develop in women after menopause. This aspect is especially significant in countries like India where the incidence of breast tumours is more common in premenopausal women [37].

The alterations in oestradiol expression and receptor status were more pronounced in grade III tumours compared to grade I and II, indicating that oestrogen exposure may stimulate clonal proliferation of ER positive cells. In contrast to reports by Hou et al. [38], we found a positive association of HER-2/neu with ER and an inverse correlation with PR status, suggesting that PR- tumours although ER+ are more likely to express HER-2/neu. While expression of cell survival and cell proliferation markers NFκB, GST-P and PCNA showed increased expression with histological grading, a shift of balance from the proapoptotic phenotype in low grade tumours to the anti-apoptotic phenotype in high grade tumours was seen, as reflected by the Bcl-2/Bax ratio and caspase expression. Apoptosis evasion, a hallmark capability of cancer, increases cell
survival, facilitates metastasis, promotes resistance to immune-based destruction and confers resistance to chemotherapy [39, 40]. Increased expression of proapoptotic proteins in low grade tumours may be indicative of better response to therapy.

Thus the results of the present study demonstrate that multiple signalling pathways are aberrant in breast carcinogenesis, including hormone and receptor status, ROS generation, cell survival and proliferation, and apoptosis. Further studies on the expression of steroid hormone metabolizing enzymes and their isoforms, NFκB and ROS signalling, and the crosstalk between different apoptotic pathways, are required to unravel the intricate molecular mechanisms involved in the development of breast cancer.

References


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