Role of nestin protein in prognosis of breast cancer

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ABSTRACT

Human breast carcinoma is a heterogeneous disease embracing different phenotypes with different biological characteristics. Transcriptional profiling has identified five breast cancer subtypes, of which the “basal epithelial” is the most aggressive. Nestin, an intermediate filament protein, was originally identified as a marker of neuroepithelial stem/progenitor cells in the brain. Recently, it was observed that nestin is expressed in basal/myoepithelial cells of the normal mammary gland and was also later found in cancer stem-like cells of many tumors. Some studies showed that nestin is robustly immunohistochemically expressed in basal epithelial and triple negative breast tumors and that its expression correlates with survival patients. We evaluated the immunohistochemical expression of nestin in breast carcinoma and its correlation with the clinicopathological data. This study investigated sixty cases of invasive duct carcinoma of the breast and five cases of normal breast tissue as non-malignant group. Three paraffin sections were obtained from each block; one is stained by routine Haematoxylin and Eosin and the other 2 sections for nestin immunostaining. In our study nestin is consistently expressed in triple-negative subgroups of breast cancer and correlates with bad clinicopathological and immunohistochemical characteristics.


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1. INTRODUCTION

Breast carcinoma is the most common malignancy in women and is the second leading cause of cancer death in women [1]. Over the last 30 years, deaths from breast cancer have approximately tripled in Japan, which historically has had a low incidence of breast cancer [2]. According to the World Health Organization, more than 1.2 million people will be diagnosed with breast cancer each year worldwide [2]. In Egypt breast carcinoma constitute 33% of all female cancers in Egypt and ranks as number one cancer among Egyptian females [3].

Breast cancer is a heterogeneous disease embracing several different phenotypes with consistently different biological characteristics [4]. Hormonal therapies (tamoxifen, anti-estrogens) and adjuvant chemotherapy (trastuzumab [Herceptin]) have benefited millions of patients with breast cancer [5,6]. Their success, however, is limited to a subset of patients whose tumors express estrogen receptors (ER), progesterone receptors (PR), human epidermal growth factor receptor 2 (HER2/neu) and tumor protein[7]. Therefore, the status of these proteins has prognostic ramifications for breast cancer.

Nestin is an intermediate filament (IF) protein that was originally described in 1990 as a neuronal stem cell/progenitor cell marker during central nervous system (CNS) development [8]. It is also expressed in follicle stem cells and their immediate, differentiated progeny [9]. It has recently received attention as a marker for newly formed endothelial cells. In their study, Teranishi et al. concluded that nestin is an angiogenesis marker for proliferating endothelial cells in colorectal cancer tissue. [10].

Nestin is expressed in dividing cells during the early stages of development in the CNS, peripheral nervous system, myogenic and other tissues. With differentiation, nestin is downregulated and replaced by tissue-specific IF proteins, and therefore, is widely used as a neuronal stem cell marker. Nestin is also...
expressed in immature or progenitor cells in non-neuronal cells in normal tissues[11]. High levels of nestin expression have been detected in oligodendroglial lineage cells, ependymocytes, Sertoli cells, enteric glia, podocytes of renal glomeruli, pancreatic stellate cells, pericytes, and optic nerve [12,13].

In pathological conditions, nestin is expressed in repair processes in the CNS, muscle, liver [14], and infarcted myocardium [15]. Furthermore, increased nestin expression has been reported in various tumor cells, including CNS tumors, pancreatic cancer, gastrointestinal stromal tumors (GISTs), prostate cancer, breast cancer, malignant melanoma, dermatofibrosarcoma protuberances, and thyroid tumors[16,17]. Expression of nestin in several tumors has been reported to be closely correlated with poor prognosis. Recently, nestin has also received attention as a cancer stem cell marker in various tumor tissues and tumor angiogenesis [20]. Detailed analyses of expression patterns of nestin in various tumor tissues and tumor angiogenesis, including gastrointestinal cancer, will be helpful for examining the roles of nestin in mechanisms of tumor growth and invasion and for finding novel therapeutic targets.

Recently, studies based on Western patients demonstrated that nestin is preferentially expressed in basal/myoepithelial cells of the mammary gland, and that this intermediate filament may be used as a myoepithelial marker [21]. However, the clinical and prognostic implications of nestin as a marker for breast cancer are still unclear.

2. MATERIALS AND METHODS

This study investigated sixty cases of invasive duct carcinoma of the breast and five cases of normal breast tissue as non-malignant group. The cases were taken from Pathology Department, Faculty of Medicine, Menoufia University. The breast biopsies were obtained by core biopsy and radical mastectomy. Clinicopathological data related to the selected cases obtained from the patient's files including patient age, sex and for the malignant cases included the tumor size, lymph node metastasis, hormonal status (ER, PR & HER-2 / neu) and stage of the tumor.

Four microns thick, 3 paraffin sections were obtained from each block; one was stained by routine Haematoxylin and Eosin to evaluate the tumour type, grading and other histopathological characteristics of the tumour. The other 2 sections were cut on poly L lysine coated slides for immunostaining.

2.1. Histopathologic evaluation

Hematoxylin and eosin stained sections were examined microscopically to re-evaluate and verify the following:

2.1.1. Histologic grade

The invasive duct carcinoma cases in this study were graded according to the criteria of Nottingham modification of the Bloom – Richardson system [22]. In this scheme, grading was obtained by adding up the scores for tubular formation, nuclear pleomorphism and mitotic activity; each was given 1, 2 or 3 points as follows:

- Tubule formation: the overall appearance was to be taken into consideration when evaluating the tubule formation and is given as:
  1 point: if tubule formation in > 75% of the tumor;
  2 points: if tubule formation in 10% to 75% of the tumor;
  3 points: if tubule formation in < 10% of the tumor.

- Nuclear pleomorphism: the presence of hairy instead of triangular nuclear projection, absence of nuclear membrane, the presence of chromatin material and basophilia instead of esinophilia in the surrounding cytoplasm [23]. Mitotic count:
  - The criteria for detection of mitoses including absence of nuclear membrane, the presence of hairy instead of triangular nuclear projection, absence of clear zone in the center of the chromatin material and basophilia instead of esinophilia in the surrounding cytoplasm [23].

Mitotic count were estimated at the tumour periphery and in the most mitotic active area, 10 high power fields were evaluated in the same area, however not necessarily to be contiguous.

The points were given according to numbers of mitoses / 10 HPF and the field diameter of the used microscope [24].

The light microscope used in this study was Olympus microscope with field diameter 0.45, so the points for mitosis were given as follow:

- 1 point: if mitosis less than 5 / 10 HPF;
- 2 points: if mitosis between 5-10 / HPF;
- Or 3 points: if mitosis more than 10 / HPF.

The final tumour grade is determined by the summation of points as follows:

- Grade I: 3-5 points;
- Grade II: 6-7 points;
- Grade III: 8-9 points [22].

2.1.2. Vascular invasion

When nests of malignant cells were clearly seen within blood or lymphatic spaces lined by endothelial cells, it was considered positive vascular invasion. The involved vessel must be away from the tumour so it is called peritumoural vascular invasion [25].
2.1.3. Apoptotic count

It was calculated by counting the apoptotic changes in 10 HPF. The apoptotic changes includes cell shrinkage, chromatin condensation, pyknosis, karyolysis, karyorhexis, formation of halo around the perinuclear remnant of the cytoplasm and formation of cytoplasmic blebs and apoptotic bodies [26].

2.1.4. Nottingham prognostic index (NPI)

NPI one of the most important prognostic factors in breast carcinoma and depends mainly on tumour size, tumour grade and lymph node state[22]. The NPI can be calculated as follow:

NPI= Tumour size (cm) X 0.2 + Tumour grade + Stage of lymph node:
   - 1= no lymph node affected;
   - 2 = 1-3 lymph node affected;
   - 3 = > 4 lymph node or more affected.

Interpretation of NPI:
   - Good prognosis < 3.4;
   - Moderate prognosis 3.41- 5.4;
   - Poor prognosis > 5.4.

2.2. Nestin immunostaining

The primary antibody is monoclonal, goat anti nestin anti-body 1ml concentrated (Novocastra, UK). The detection kit is avidin-biotin peroxidase complex followed by DAB kit (3,3-diaminobenzidine color development) (Neomarkers’ cat. # TP-012-HDX).

(A) Preliminary steps:

One liter of phosphate buffer saline was prepared with the following:
7.75 gram sodium chloride (NaCl);
1.5 gram (K2 Hpo4) Dipotassium hydrogen orthophosphate;
0.2 gram (KH2 po4) potassium dihydrogen orthophosphate;
This was then mixed with 1000 CC distilled water, and the pH was adjusted to 7.6 by pH meter using 0.1 M NaOH and 1.0 M HCl. The solution is stable for one week at 3-8 °C.

Citrate buffer solution for antigen retrieval was prepared by adding 2.1 gram citrate to 1000 CC distilled water and adjusting pH to 6.0.

(B) Immunostaining steps:

1. Paraffin sections were cut by microtome at 4-micron thickness and mounted on the glass slides.

2. The sections were deparaffinized in xylene 2 times, 30 minutes each.

3. Deparaffinized sections were rehydrated in distilled water through 100%, 95%, and 70% ethanol, 5, minutes each.

4. To block endogenous peroxidase the sections were incubated in 3% hydrogen peroxide in absolute methanol for 10 minutes.

5. Then, sections were rinsed in phosphate – buffered saline (PBS) 2 changes, 5 minutes each.

6. For antigen retrieval: Heat induced epitope retrieval (HIER) procedure was used.
   - The deparaffinized sections were washed in distilled water three times 2 minutes each.
   - The slides were put in a slide rack emerged in Pyrex container, which contain 5000 ml of citrate buffer (pH 6.0).
   - The Pyrex rack was put on a hot plate and the solution was kept boiling for 10 minutes, then removed and allowed to cool 20 minutes in the room temperature.
   - Then slides washed in distilled water several times.

7. The sections were washed in phosphate-buffered saline (PBS), 2 changes 5 minutes each.

8. Ultra-V block was put on the slides for minutes.

9. Monoclonal mouse nestin (Novocastra immunohistopathology catalogue, 2002) was then put on the sections. The slides were then incubated horizontally in humidity chamber overnight at room temperature.

10. Excess reagent was thrown off and slides were rinsed in 2 changes of (PBS), 5 minute each rinse.

11. After blotting of excess buffer, 1-2 drops of biotinylated secondary anti-immunoglobulin were applied for 20 minutes and incubated horizontally in a humidity chamber at room temperature. Sections were then rinsed with (PBS) 2 changes, 5 minutes each.

12. After blotting of excess buffer, 1-2 drops of pre – formed streptavidin biotin complex were applied for 20 minutes and incubated horizontally in a humidity chamber at room temperature. Sections were then rinsed as before.

13. While the slides were in the (PBS), the chromogen substrate was prepared as prescribed in the supplied data sheet. The chromogen used was diaminobenzidine tetrahydrochloride (DAB), 1-2 drops for 10-20 minutes until the desirable brown color was obtained. Then the slides were washed in distilled water.

14. Counter-staining was done using Mayer's hematoxylin for 3-5 minutes according to intensity for nuclear and cytoplasmic staining.

15. Then sections were washed in tap water and dehydrated in 70%, 95% and 100% ethanol.

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Slides were cleared in xylene, 2 changes, 5 minutes each.

16. Mounting the cover – slip by Canada balsam was done.

Interpretation of the immunostaining results

Nestin immunostaining interpretation

2.3. Nestin expression evaluation

Nestin expression was classified semiquantitatively according to the following criteria:

0 if <1% of neoplastic cells discretely expressed nestin in their cytoplasm;

1+ if more than 1% and less than 10% of morphologically unequivocal neoplastic cells discretely expressed nestin in their cytoplasm; and

2+ if 10% or more of morphologically unequivocal neoplastic cells discretely expressed nestin in their cytoplasm.

Samples scored as 1+ or 2+ were considered positive [127].

2.3.1. Reassessment of ER , PR & HER-2 / neu

Nuclear staining for ER and PR was graded as follows:

1 if <10% of the cells were stained;

2 if 10–50% of the cells were stained;

3 if >50% of the cells were stained.

Grades 2 and 3 were considered positive, whereas absence of staining and grade 1 staining were considered negative. Similar standards were used for staining intensity in HER-2 / neu; only grade 3 (high intensity) was considered positive.

2.3.2. Statistical analysis

Data were collected and statistically analyzed by using Statistical Package for Social Sciences (SPSS) version 11software program (2003). Two types of statistics were done:

A) Descriptive statistics: e.g. mean (X), standard deviation (SD) and percentage (%).

B) Analytic statistics:

- Student’s t-test was used to compare between two groups containing quantitative variables as age.
- Chi-square test ($\chi^2$) was used to study the association between two qualitative values.
- Multivariate F-test (ANOVA) was used to compare between more than two groups containing quantitative variables. Differences were considered statistically significant with $p \leq 0.05$[28].

3. RESULTS

Patient characteristic: Sixty cases of invasive ductal carcinomas and five cases of normal breast tissue as a control group were included in this study. The mean age of 65 patients was 51.04 years (range, 27–80 years). There were 46 patients with lymph node metastasis. In this study 36 cases had grade 2, 23 had grade 3 and only one case with grade 1.

Nestin expression: Positive expression of nestin, was found in 14 cases of invasive duct carcinoma. The all non malignant control group showed positive nestin expression in myoepithelial cells, while the epithelial cells showed little or no staining.

Correlations between nestin expression and clinicopathological features: After analysis, the mean age of patients was similar among, (ER+, ER-ve), (PR+, PR-ve), (HER2/ neu+, HER2/ neu-ve) cases (52.57, 49.48, 48.88, 50.49, 51.29, and 49.91 years, respectively; $P > 0.05$). The mean age of nestin positive cases was younger than that of nestin negative cases and this difference was statistically significant (40.8±10.5 an55±9.66). Moreover the nestin expression was associated with higher grade, larger size, vascular invasion, bad NPI and positive surgical margin.

Fig. 1. A case of invasive duct carcinoma grade I (HX&Ex 200).
Fig. 2. A case of invasive duct carcinoma grade II (HX&Ex 200).

Fig. 3. A case of invasive duct carcinoma grade III (HX&Ex 400).

Fig. 4. A case of in-situ duct carcinoma (HX&Ex 200).
Fig. 5. (A) Nestin expressed in myoepithelial cells of normal breast tissue; (B & C) Nestin located in the cytoplasm in triple-negative breast cancers.

Regarding the tumour type the nestin expression was higher in triple negative type, as 70% of triple negative breast carcinoma showed positive nestin expression, while only about 20% of Her2 +ve type and about 8% of luminal type showed positive nestin expression.

We also evaluated the relationship between nestin expression and clinicopathological features in triple-negative breast cancers.

Interestingly, significantly increased nestin expression rates were observed in patients with lymph node metastasis compared with those without node metastasis (25.00% vs 76.92%; P = 0.032). A similar phenomenon was observed in IDC compared with DCIS (16.67% vs 73.33%; P = 0.046). However, no similar phenomenon was observed in the patients with other types of breast cancers.
**Table 1.** Relationship between nestin expression in tumour cells and different clinicopathologic features.

<table>
<thead>
<tr>
<th></th>
<th>Nestin expression</th>
<th>Test of Significance</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>Negative (n=46)</td>
<td>Positive (n=14)</td>
<td></td>
</tr>
<tr>
<td><strong>Age (mean ± SD)</strong></td>
<td>55±9.66</td>
<td>40.8±10.5</td>
<td>U= 2.643</td>
</tr>
<tr>
<td><strong>Size (mean ± SD)</strong></td>
<td>3±0.00</td>
<td>6±2.82</td>
<td>U=2.686</td>
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<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Grade 1 = 1</td>
<td>1 (2.1%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Grade 2 =36</td>
<td>33 (71.7%)</td>
<td>3 (21.4%)</td>
<td>$X^2=12.45$</td>
</tr>
<tr>
<td>Grade 3 =23</td>
<td>12 (26.2%)</td>
<td>11 (78.6%)</td>
<td></td>
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<tr>
<td><strong>Apoptotic index</strong></td>
<td>13.2±8.035</td>
<td>11.2±7.82</td>
<td>U = 0.813</td>
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<tr>
<td>(mean ± SD)</td>
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<tr>
<td><strong>Mitotic index</strong></td>
<td>5.59±4.14</td>
<td>7.64±3.89</td>
<td>$X^2=1.215$</td>
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<tr>
<td>(mean ± SD)</td>
<td></td>
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<tr>
<td><strong>Lymph node status</strong></td>
<td></td>
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<tr>
<td>Negative (14)</td>
<td>13 (28.3%)</td>
<td>1 (7.1%)</td>
<td>$X^2=2.67$</td>
</tr>
<tr>
<td>Positive (46)</td>
<td>33 (71.7%)</td>
<td>13 (94.9%)</td>
<td></td>
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<tr>
<td><strong>NPI</strong></td>
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<tr>
<td>Good NPI</td>
<td>5 (10.8%)</td>
<td>0 (0%)</td>
<td></td>
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<tr>
<td>Moderate NPI</td>
<td>24 (52.1%)</td>
<td>1 (7.1%)</td>
<td>$X^2=13.45$</td>
</tr>
<tr>
<td>Bad NPI</td>
<td>17 (37.1%)</td>
<td>13 (94.9%)</td>
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<tr>
<td><strong>Vascular invasion</strong></td>
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<tr>
<td>Present (n= 23)</td>
<td>15 (32.6%)</td>
<td>8 (57.1%)</td>
<td>$X^2=7.94$</td>
</tr>
<tr>
<td>Absent (n= 37)</td>
<td>31 (67.4 %)</td>
<td>6 (42.9%)</td>
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<tr>
<td><strong>Necrosis</strong></td>
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<tr>
<td>Present (n= 32)</td>
<td>24 (52.1%)</td>
<td>8 (57.1%)</td>
<td>$X^2=0.106$</td>
</tr>
<tr>
<td>Absent (n= 28)</td>
<td>22 (47.9%)</td>
<td>6 (42.9%)</td>
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<tr>
<td><strong>Paget disease</strong></td>
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<tr>
<td>Present (n= 17)</td>
<td>12 (26%)</td>
<td>5 (35.7%)</td>
<td>$X^2=0.49$</td>
</tr>
<tr>
<td>Absent (n= 43)</td>
<td>34 (74%)</td>
<td>9 (64.3%)</td>
<td></td>
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<tr>
<td><strong>Surgical margin</strong></td>
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<tr>
<td>Positive (n= 5)</td>
<td>1 (2.1%)</td>
<td>10 (71.5%)</td>
<td>$X^2=14.26$</td>
</tr>
<tr>
<td>Negative (n= 55)</td>
<td>45 (97.9%)</td>
<td>4 (28.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>Types</strong></td>
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<tr>
<td>Her2 + ve (n= 24)</td>
<td>19 (79.2%)</td>
<td>5 (20.8%)</td>
<td>$X^2=15.2$</td>
</tr>
<tr>
<td>Triple –ve (n= 10)</td>
<td>3 (30%)</td>
<td>7 (70%)</td>
<td></td>
</tr>
<tr>
<td>Luminal (n= 26)</td>
<td>24 (92.3%)</td>
<td>2 (7.7%)</td>
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</table>

* Significant ** Highly significant

**4. DISCUSSION**

Breast cancer is a clinically heterogeneous disease. Histological type, grade, tumor size, lymph-node involvement, and ER and HER2 receptor status all influence the prognosis and probability of response to systemic therapies, but do not fully capture the varied clinical course of breast cancer[29]. Endocrine therapy and trastuzumab adjuvant treatment have benefited patients with ER+ cancers and HER2-overexpressing cases[7]. Therefore, the status of these proteins has prognostic ramifications in breast cancer. Consequently, much effort is focused on understanding the clinical significance of known markers, finding relationships between them and discovering new ones – all aimed at optimal utilization of the available therapies and development of novel therapies based on improved cancer models.
Nestin is an intermediate filament protein has the shortest head domain (N-terminus) and the longest tail domain (C-terminus) of all the intermediate filament proteins[30]. Nestin is expressed by many types of cells during development, although its expression is usually transient and does not persist into adulthood[31]. Nestin is expressed in dividing cells during the early stages of development in the central nervous system (CNS), peripheral nervous system (PNS), myogenic, and other tissues[32]. Nestin is utilized as a marker of proliferating and migrating cells; however, very little is known about its functions or regulation.

A study based on Western patients suggested that nestin is preferentially expressed in basal-like breast carcinomas, predominantly expressed in triple-negative breast cancers[21]. Parry et al., reported that, although nestin expression was associated with basal-like and triple-negative phenotypes, high proliferation rates and p53 nuclear expression – all markers of poor prognosis – it was not associated with metastasis-free survival or breast cancer-specific survival [33].

In our study, the nestin expressed in 14 cases of invasive duct carcinoma. The all non-malignant control group showed positive nestin expression in myoepithelial cells, while the epithelial cells showed little or no staining. Nestin associated with higher grade, larger size, vascular invasion, bad NPI and positive surgical margin.

In addition nestin was predominantly expressed in triple-negative cancers, corroborating previous findings. Interestingly we observed that nestin expression was significantly correlated with lymph node metastasis in the triple-negative cancers alone. Moreover, nestin expression was associated with poor prognoses in the lymph node-positive group in our study – supporting the concept that nestin-positive cancer cells may have higher lymphatic metastasis capability. There are some differences between our results and previous reported results. Parry et al. reported that nestin was not found to be associated with metastasis-free survival or breast cancer-specific survival in their study[33]. There may be some reasons causing these differences, such as different subgroups and ethnic differences. It demonstrated that nestin might play an important function in breast carcinoma development and metastasis. However, the specific role of nestin in breast cancer is unclear.

In summary, our findings show that nestin is consistently expressed in triple-negative subgroups of breast cancer and correlates with bad clinicopathological and immunohistochemical characteristics. It demonstrated that nestin might be a new potential marker for breast cancer. However, the underlying mechanisms of nestin’s involvement are still unclear, so more and larger studies are warranted to investigate the prognostic impact of nestin expression in of breast carcinomas.

5. CONCLUSION

From our study, we can conclude that nestin is expressed in triple-negative subgroups of breast cancer and correlates with bad clinicopathological and immunohistochemical characteristics. It demonstrated that nestin might be a new potential marker for breast cancer. However, the underlying mechanisms of nestin’s involvement are still unclear, so more and larger studies are warranted to investigate the prognostic impact of nestin expression in breast carcinomas.

AUTHOR CONTRIBUTIONS

MIS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. SSE-G managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript. Patients consent where obtained where. The study was done on archived paraffin section blocks.

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947-956.


