E2F-1: A Proliferative Marker of Breast Neoplasia

Shi Yu Zhang, Shao Chen Liu, Luma F. Al-Saleem, Dana Holloran, James Babb, Xu Guo, and Andres J. P. Klein-Szanto


Abstract

E2F-1 is the best known ultimate transcription factor in the cyclin/cyclin-dependent kinase/retinoblastoma gene pathway and is probably involved in carcinogenesis and tumor progression. Because E2F-1 can be detected in paraffin sections using immunohistochemical techniques, it could be a useful tumor/proliferation marker. We studied the expression of this gene product in 130 breast tissue specimens from 100 patients and compared it with the expression of Mib-1, the widely used prognostic/proliferative marker, to assess E2F-1 as a new marker of neoplastic proliferation. The percentage of E2F-1-positive cells increased from 1.9% in the normal breast (NB) to 6.3% in ductal carcinoma in situ (DCIS) and to 15.3% in invasive ductal carcinomas (IDC). In addition, higher-grade tumors as well as advanced-stage disease correlated with higher expression of E2F-1. A similar trend in Mib-1 expression was observed. There was a positive correlation between the E2F-1 and Mib-1 indices. In an in vitro experiment, we found that a similar difference in the expression of E2F-1 existed between a nontumorigenic breast cell line and two widely used breast carcinoma cell lines. The breast carcinoma cell lines T-47D and MCF-7 had more E2F-1-positive cells than the nontumorigenic cell line MCF-10F by immunohistochemistry and Western blot analysis. Because E2F-1 expression was significantly higher in IDC and DCIS than in NB, this study indicates that deregulation of E2F-1 may be involved in the development of breast IDC. In addition, E2F-1 expression could also be involved in tumor progression because the increased E2F-1 index correlated with the known prognostic predictors of breast cancer, such as histological grade, stage, metastasis status, estrogen receptor/progesterone receptor and Mib-1 expression. Thus, E2F-1 is a promising candidate to become a new prognostic/predictive marker of breast cancer.

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Introduction

Several prognostic indicators have been used in recent years to improve and refine the traditional and powerful morphology-based tumor diagnosis parameters. Among the markers that can be detected by immunohistochemistry, those that are cell proliferation-related have been frequently investigated. A large number of publications on neoplasms of different organs, including the breast, have shown that the expression of proliferation-related antigens, such as PCNA and Ki-67 (Mib-1), shows a good correlation with other significant biomarkers, such as ploidy, and with prognosis. Nevertheless, some contradictory findings and technical pitfalls in the use of PCNA and Ki-67 immunohistochemistry have mitigated the initial interest in these two markers.

With the recent explosion of knowledge in the field of cell cycle, a large number of genes that intervene in its regulation have been identified and characterized. Mammalian cell proliferation is a very complex process controlled by the so-called cell cycle machinery. Multiple components, such as cyclins, Cdkis, RBs, E2Fs, and so forth, are implicated in the transitions from one cell cycle phase to another. For example, D-type cyclins bind and activate Cdk4/Cdk6, which phosphorylate the Rb product pRb, and CdkIs such as p21 and p16 can inhibit the function of Cdkks. As a result of phosphorylation of pRb, E2F is released from pRb-E2F complex. The free E2F is an active transcription factor that promotes the transcription of the genes required for DNA synthesis and drives cells from G1 into S phase.

Several of the gene products of these CCRGs can be detected immunohistochemically and have been successfully used in the study of breast neoplasms. Several investigators have employed promising CCRG products as putative tumor and proliferation markers, cyclin D1 being the most widely studied. Because E2F-1 is the best-known ultimate transcription factor activated in the Cyclin-Cdk-Rb pathway, and antibodies against the gene product are useful in its immunohistochemical detection, it is an ideal candidate to be a new tumor/proliferation marker in pathology studies.

E2F-1 was the first cloned and is the better characterized member of this gene family. In vitro experiments showed that E2F-1 binding activity and mRNA level fluctuate through the cell cycle, peaking at the G1-S phase boundary. Overexpression of E2F-1 can drive quiescent cells into S phase and induce G1 arrested cells into S phase, even in the absence of Cdk activity. Several lines of evidence indicate that E2F-1 is involved in neoplastic development. For example, overexpression of E2F-1 was shown to induce neoplastic transformation in cultured fibroblasts. In addition, amplification of the E2F-1 gene was found in an erythroleukemia cell line.

The abbreviations used are: PCNA, proliferating cell nuclear antigen; Cdk, cyclin-dependent kinase; RB, retinoblastoma gene; pRB, retinoblastoma protein; NB, normal breast; DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ER, estrogen receptor; PR, progesterone receptor; CdkI, Cdk inhibitor; CCRG, cell cycle regulating gene.
line (18), 4% of gastric carcinomas, and 25% of colorectal carcinomas (19). Furthermore, increased expression of E2F-1 mRNA was observed in 40% of gastric carcinomas and in 60% of colorectal adenocarcinomas (19). A recent study showed that loss of E2F-1 reduced the frequency of pituitary and thyroid tumors in Rb(+/−) mice (20). Recently, an immunohistochemistry study has demonstrated that E2F-1 is overexpressed in some non-Hodgkin’s lymphomas (21). In contrast, E2F-1 gene transfection induced apoptosis in some cell lines (22, 23) and E2F-1 knockout mice developed a variety of tumors (24). A recent study of E2F-1 in the bladder cancer showed that patients with lower E2F-1 protein expression had an adverse outcome (25). These findings suggest that E2F-1 could function as either an oncogene or a tumor suppressor gene, depending on tissue type and experimental conditions.

Abnormalities of the cyclin/Cdk/Rb pathway are very common in breast carcinomas. Among the most frequent changes, we should point out amplification and overexpression of cyclin D1, overexpression of cyclin E, and decrease or absent expression of Rb, p16, and p27 (for reviews, see Refs. 26–27). It was calculated that one or more of these alterations could be seen in up to 90% of breast carcinomas (26). To our knowledge, there is no information in the literature regarding the expression of E2F-1 in primary breast carcinomas. Thus, with the availability of a specific anti-E2F-1 monoclonal antibody, we studied the expression of this CCRG product in 130 specimens, including NB, DCIS, and IDC of different grades and stages and compared with the expression of Mib-1, the known prognostic/proliferative marker, to investigate the feasibility of its use as a tumor/proliferation marker in these neoplasms.

Materials and Methods

One hundred formalin-fixed, paraffin-embedded IDCs from 1995 through 1997 were retrieved from the Department of Pathology, Fox Chase Cancer Center. The patients ranged in age from 30 through 85 years with a mean age of 58.5. In addition, 15 specimens of NB tissue and 15 DCIS found in other blocks of the same patients were included in this study. The diagnosis, histological grade, and stage were classified according to the American Joint Committee on Cancer (AJCC) system (28). The status of ER and PR of all IDCs were available from the Department of Pathology, Fox Chase Cancer Center. The main clinicopathological information of the cases is presented in Table 1. Follow-up time of these patients ranged from 20 to 36 months from diagnosis. Cell lines used in this study were the mammary epithelial cell line MCF-10F and breast carcinoma cell lines T-47D and MCF-7. The cell lines were the mammary epithelial cell line MCF-10F and breast carcinoma cell lines T-47D and MCF-7. The cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

Immunohistochemistry. Five-μm sections were cut from representative blocks from each case. The sections were deparaffinized in xylene and rehydrated with graded ethanol. Antigen retrieval was performed in 1 mM EDTA (E2F-1) or 0.1 M sodium citrate (Mib-1) for two sets of 5 minutes each, followed by 3% H2O2 and normal horse serum treatment for 30 min at room temperature, respectively. Then the sections were incubated with antihuman E2F-1 monoclonal antibody sc-251 (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Mib-1 monoclonal antibody (1:100 dilution, Immunotech, Westbrook, ME) at room temperature for 1 h. The sc-251 antibody is mouse monoclonal IgG2a raised against an epitope of E2F-1 between amino acids 342 and 386. It is specific for this transcription factor as described and characterized by Hein et al. (29). Mib-1 is a mouse monoclonal IgG1 that specifically recognizes the Ki-67 nuclear protein. It was raised against a recombinant human peptide corresponding to a 1002-1026 Ki-67 cDNA fragment (30). Reactions were visualized with a biotinylated antimouse ABC kit (Vestastain Vector Laboratories Inc., Burlingame, CA) and chromogen 3,3’-diaminobenzidine and were counterstained with hematoxylin. E2F-1 transgenic mouse tumors (a kind gift from Dr. D. Johnson, The University of Texas M. D. Anderson Cancer Center, Smithville, TX) were used as positive control for E2F-1 staining. Normal mouse IgG sc-2025 (Santa Cruz Biotechnology, Inc.) replaced the primary antibody as negative control.

To determine the E2F-1 and Mib-1 expression, the average stained region as near as possible to the central portion of the section was selected. More than 500 cells were counted at ×400 with light microscopy. Only a nuclear stain was counted as positive. E2F-1 and Mib-1 expression index was calculated as the number of positive cells divided by the sum of positive and negative cells, respectively.

Twenty thousand cells from each cell line were seeded in chamber slides (Nunc Inc. Naperville, IL) with the medium and cells were allowed to grow to subconfluency. The cells were fixed in cold 70% ethanol at 4°C for 15 min. Then immunostaining was performed on the cells with the E2F-1 antibody as described above.

Western Blot Analysis of Cell Lines. Western analysis was performed as described previously (31). In brief, cell lines MCF-10F, T-47D, and MCF-7 were harvested with trypsin when they were subconfluent and were washed twice with cold PBS. The cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM Na2HPO4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 10 μg/ml trypsin inhibitor] for 30 min on ice. The cell lysates were clarified by centrifugation at 13,000 rpm (13,793 × g) for 5 min. The protein concentration of the supernatants was determined spectrophotometrically at 750 nm (Beckman DU-7 spectrophotometer, Beckman, Irvine, CA) with the Bio-Rad DC assay kit (Bio-Rad Labs, Hercules, CA) as suggested by the manufacturer. Eighty μg of total protein from each sample were subjected to 8% Tris-glycine gel electrophoresis, as suggested by the manufacturer (Novex, San Diego, CA) and were transferred onto a nitrocellulose membrane (Amersham, Arlington Heights, IL). Then the membrane was immunoblotted with the E2F-1 antibody mentioned above. Antihuman actin monoclonal antibody (sc-8432, 1:200 dilution, Santa Cruz Biotechnology) was used as the loading control. Immunodetection was performed with an ECL kit (Amersham Corp., Arlington Heights, IL). The E2F-1 bands in the exposed films were normalized with the actin bands and were evaluated densitometrically (NIH Image 1.61 software, NIH, Bethesda, MD).

Statistical Analysis. Differences in immunohistochemical expression across patient groups, defined by age (≤50 versus >50 years), histological grade, clinical stage, and lymph node, ER, or PR status, were assessed using the Kruskal-Wallis test. The Pearson product-moment and Spearman rank correlation coefficients were used to evaluate the association between the E2F-1 and MIB-1 indices. The NB, DCIs, and IDC tissue blocks were compared using a matched-pairs Wilcoxon signed-ranks test or a Mann-Whitney test, depending, respectively, on whether or not the tissue blocks being compared were obtained from the same patient. Logistic regression was implemented to determine whether MIB-1 and/or E2F-1 expression was a significant predictor of histological grade, clinical stage, lymph node metastasis, or ER and PR status. The association between
MIB-1 and E2F-1 expression and the time to recurrence, metastasis, or disease-related death was evaluated using both the log-rank and the generalized Wilcoxon test.

Results

Immunohistochemistry and Western Analysis of the Breast Cell Lines. In the first stage of our analysis of E2F-1 expression in breast epithelial cells, we studied three cell lines of different biological characteristics. The MCF-10 cell line, an immortalized but nontumorigenic cell line, exhibited relatively low expression of E2F-1. This was seen both when the percentage of positive cells (E2F-1 index) was counted (8.3%) as well as when the intensity of nuclear immunoreaction was evaluated. As can be seen in Fig. 1, the tumorigenic carcinoma-derived cell line T-47D (Fig. 1B) exhibits intense immunostaining in a larger number of cells than the MCF-10F cell line.
The former cell line also exhibited a much higher E2F-1 index of 35.7%. MCF-7, another tumorigenic cell line derived from breast cancer, exhibited similar staining characteristics (data not shown), and its E2F-1 index was 34.8%. Western blot analysis confirmed that the MCF-10 cells expressed less E2F-1 protein than the breast cancer-derived cell lines (Fig. 2).

Immunohistochemistry and Quantitative Analysis of Breast Tissues. One hundred thirty samples from 100 patients were analyzed for immunohistochemical expression of E2F-1 and Mib-1. These samples included 100 IDCs, 15 DCIS, and 15 histologically NB tissue blocks. All of the samples of IDC, DCIS, and NB showed positive staining for E2F-1 and Mib-1. The E2F-1 immunoreaction was localized to the nuclei, with a few cases exhibiting light cytoplasmic staining. The majority of positive nuclei in the three different types of tissues had intense or moderately intense nuclear staining. The percentage of positively stained cells (E2F-1 index) varied both within and between the different tissue types. Nevertheless, there was a gradual increase in E2F-1 index from normal tissue to DCIS and to IDC. Overall, the mean E2F-1 index was 1.9% for NB, 6.3% for DCIS, and 15.4% for IDC (Table 1; Fig. 1, C–F). Expression of E2F-1 in IDC was significantly higher than in NB (P < 0.0002). In addition, the expression of E2F-1 in IDC was significantly higher than in DCIS (P < 0.001; Table 1). A similar significant increase in staining pattern was observed using the Mib-1 antibody: the mean Mib-1 index was 1.9% for NB, 8.2% for DCIS, and 21.1% for IDC (Table 1; Fig. 1, G and H). In general, the Mib-1 index was slightly higher than the E2F-1 index. In particular, among IDC samples, Mib-1 expression was higher than E2F-1 expression in 79 out of 100 tissue samples and had a significantly higher median (16.6%) than E2F-1 (median, 13.0%; P < 0.0001). As evidenced in Fig. 3, there was a strong, statistically significant (P < 0.001), positive correlation between the E2F-1 and Mib-1 indices in the IDC tissue blocks (the Pearson product-moment and Spearman rank correlation coefficients were 0.78 and 0.82, respectively).

Table 2 summarizes the comparisons and statistical data. IDC patients 50 years old or younger had higher average E2F-1 and Mib-1 expression than did patients older than 50. IDCs with higher histological grade (Fig. 1, F and H) had significantly higher E2F-1 and Mib-1 expression than lower-grade carcinomas (Fig. 1, E and G). Similarly, higher clinical stages had increased expression of both markers when compared with low stage (Table 2). IDCs with lymph node metastases had significantly higher immunohistochemical expression than IDCs without lymph node metastases whereas expression was significantly lower among IDC patients with positive ER or PR status than among patients with negative status (Table 2).

Logistic regression was implemented to determine whether Mib-1 and/or E2F-1 expression was a significant predictor of histological grade, clinical stage, lymph node metastasis, or ER and PR status. The results showed that E2F-1 was a statistically significant predictor of each and every factor, whereas Mib-1 was not a significant predictor of either lymph node metastasis or PR status. Overall, Mib-1 was found to be the most efficient single predictor of histological grade and ER status, and E2F-1 was the best predictor of each of the three remaining factors.

Using both the log-rank and the generalized Wilcoxon tests, E2F-1 and Mib-1 were each observed to be significantly associated (P < 0.03) with the time to recurrence, metastasis, or death.
Table 2: Immunohistochemical staining of E2F-1 and Mib-1 in IDCs

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>E2F-1</th>
<th>Mib-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50</td>
<td>0.0116</td>
<td>0.015</td>
</tr>
<tr>
<td>&gt;50</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>19.4 ± 10.7</td>
<td>25.8 ± 16.3</td>
</tr>
<tr>
<td>II</td>
<td>13.4 ± 8.3</td>
<td>19.0 ± 15.8</td>
</tr>
<tr>
<td>III</td>
<td>19.7 ± 9.1</td>
<td>29.8 ± 17.3</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11.9 ± 7.6</td>
<td>17.0 ± 14.7</td>
</tr>
<tr>
<td>II</td>
<td>16.8 ± 10.0</td>
<td>22.8 ± 17.3</td>
</tr>
<tr>
<td>III</td>
<td>18.6 ± 10.4</td>
<td>25.5 ± 16.9</td>
</tr>
<tr>
<td>IV</td>
<td>20.0 ± 13.3</td>
<td>26.5 ± 12.7</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>13.4 ± 8.9</td>
<td>18.9 ± 17.0</td>
</tr>
<tr>
<td>Positive</td>
<td>17.4 ± 9.7</td>
<td>23.4 ± 15.1</td>
</tr>
<tr>
<td>ER</td>
<td>0.0002</td>
<td>0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>19.8 ± 8.5</td>
<td>29.1 ± 18.7</td>
</tr>
<tr>
<td>Positive</td>
<td>13.2 ± 9.2</td>
<td>17.5 ± 13.6</td>
</tr>
<tr>
<td>PR</td>
<td>0.036</td>
<td>0.038</td>
</tr>
<tr>
<td>Negative</td>
<td>17.4 ± 9.8</td>
<td>24.6 ± 17.7</td>
</tr>
<tr>
<td>Positive</td>
<td>13.6 ± 8.9</td>
<td>18.3 ± 14.4</td>
</tr>
</tbody>
</table>

*p Wilcoxon (two groups) or Kruskal-Wallis (3 or more groups) test.

disease-related death. Specifically, the time to the first adverse event tended to decrease as the expression of either marker increased. However, after adjusting for patient differences with respect to age, histological grade, clinical stage, and lymph node, ER, or PR status, neither marker was observed to be a significant independent predictor of time to untoward event.

Discussion

IDC is a very common human breast neoplasm, accounting for 70–80% of all breast malignancies. The use of several markers such as ploidy, ER, PR, c-erbB-2, p53, and proliferation indicators has been accepted as useful prognostic and predictive factors (8, 32). Among the proliferation markers detectable in vivo by immunohistochemical procedures, PCNA, Ki-67, and Mib-1 have been frequently used in breast cancer (33–34). The Ki-67 monoclonal antibody detects a nonhistone protein of unknown function that is present in all of the phases of cell cycle except G0 and very early G1. Its positivity correlates with other parameters of cell proliferation, such as thymidine-labeling index, S-phase fraction, and mitotic count (37). It also correlates well with other prognostic factors, such as histological grade and hormone status (32, 35). However, a disadvantage of the Ki-67 antibody is that it is reactive only in frozen sections. A newer antibody, Mib-1, which reacts with an antigenic epitope of Ki-67, is active on formalin-fixed, paraffin-embedded tissues. Several studies have shown that Mib-1 can be used as a prognostic/proliferative marker in breast cancer (32, 41–42).

PCNA is also a nonhistone nuclear antigen that is a cofactor for DNA polymerase delta. PCNA increases during G1 phase, reaches maximal value in S phase, and decreases in G2-M phase. Expression of PCNA in breast cancer has been correlated with thymidine-labeling index and weakly with S-phase fraction (37, 40). A relationship with histological grade and prognosis of breast cancer is controversial (43–44).

Immunohistochemistry of cyclin D1, one of the positive regulators of cell cycle, was considered as a potential prognostic/tumor marker in breast cancer (7, 8). Overexpression of cyclin D1 was observed in more than 50% of breast carcinomas (10, 11). Higher-grade tumors exhibited a more intense nuclear stain than the low-grade tumors (11). Overexpression of cyclin D1 was positively correlated with ER (12) and negatively correlated with histological grade and mitotic activity (45). Some studies could not find a relationship between cyclin D1 and prognosis (46–47). Interestingly, some investigators found that overexpression of cyclin D1 is associated with a better prognosis (48). There is lack of agreement as to the prognostic/predictive significance of the markers mentioned above in breast cancer. Thus, there is a need for reliable markers.

Because E2F-1 is an important downstream regulator in the cyclin-Cdk-Rb pathway of cell cycle, and its deregulation seems to be implicated in tumor development, E2F-1 could be useful as a prognostic marker. In the present study, we used a new E2F-1 monoclonal antibody to detect this important CCRG product in a series of breast IDCs and compared with Mib-1 index. Because we found a gradual increase of E2F-1 expression from normal to outright malignancy, these two sets of data based on in vivo and in vitro evaluation suggest that overexpression of E2F-1 may be involved in the development of breast cancer of ductal origin.

Furthermore, E2F-1 and Mib-1 indices increased with IDC histological grade and clinical stage. The histological grade, combining the aspects of tubular formation, nuclear atypia, and mitotic count, is accepted as a prognostic indicator of breast cancer. Higher histologic grade of breast cancer has poorer prognosis. The clinical stage is also an important prognostic indicator for breast cancer. The higher the stage, the poorer the prognosis (3, 33–34, 49). The ability of regional lymph node status to predict the outcome of breast cancer is well known. The breast cancer patients with positive axillary lymph nodes have a poorer disease-free and overall-survival rate after 5–10 years of follow-up compared with the patients without metastases (3, 33–34, 49). The primary tumors with lymph node metastasis had higher E2F-1 and Mib-1 expression than those without lymph node metastasis.

An inverse relationship between E2F-1 and Mib-1 expression and patient age was observed. This may reflect the fact that breast cancer in younger patients often progresses more rapidly.

An inverse relationship was also observed between E2F-1 expression and ER- and PR-positive tumors, respectively. Previous studies showed that a negative correlation between ER/PR status and different proliferation indices (including thymidine-labeling index, S-phase fraction, PCNA, and Ki-67/Mib-1) existed in breast cancer (3, 34, 37, 39). The breast cancer patients with ER- and/or PR-positive tumors have better prognosis or respond better to hormone therapy (3, 33–34). Supporting its potential role as a tumor marker, we have found parallel tendencies in the Mib-1 index and a strong positive correlation between the E2F-1 and Mib-1 indices.

The present study showed a similar pattern of immunostaining for E2F-1 and Mib-1. E2F-1 index and Mib-1 index had similar correlation with multiple clinicopathological parameters such as age, histological grade, clinical stage, lymph node status, and status of ER and PR. Furthermore, statistical analysis established a positive correlation between E2F-1 and the Mib-1 index. Taken together, these associations between E2F-1 and indicators of poor prognosis suggest that high E2F-1 expression in IDC is itself a possible predictive factor.

Our study suggests that deregulation of E2F-1 may be involved in the development of breast IDC, because E2F-1 expression was significantly higher in IDC and DCIS than in NB. Furthermore, E2F-1 expression could also be involved in...
tumor progression because the increased E2F-1 was a sensitive predictor of clinical stage and lymph node metastasis status.

Statistical analysis of our results showed that E2F-1 correlated quite well with Mib-1 indices. With a few exceptions, E2F-1 did not improve our capacity to prognostic outcome. The association between each marker and untoward event (i.e., death, metastasis, and local recurrence) could be explained to a significant degree, by the relationship of each to grade, stage, and so forth. Once these factors are accounted for, neither marker significantly improved our ability to predict a poor prognosis. Nevertheless, it has to be emphasized that, at this time, our survival observation period is in most cases between 20 and 30 months. It is possible that an improvement in prognostication will be established once our observation period reaches the more significant period of 5 years. Taken together, E2F-1 seems to be at least as useful as Mib-1 as a biomarker of breast lesion proliferation and as a prognostic marker, and it has the advantage over the latter in that it is a well-known cell cycle regulating protein. The Mib-1 antigen, Ki-67, is very popular as a marker of cell proliferation, but its role in the cell cycle is unknown. Thus, E2F-1, a gene product backed by a solid molecular rationale, is a promising candidate to become a new prognostic/predictive marker of breast cancer.

References


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