

p27^{Kip1} and Ser10-phosphorylated p27^{Kip1} in breast cancer: clinical significance and expression

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Background: The protein p27 (p27^{Kip1}) is a member of the cyclin-dependent kinase inhibitor family, which negatively regulates cell cycle progression, and the phosphorylation of p27 has been proven to affect its stability and nuclear export. Clinical studies on the relation between p27 and phosphorylated p27 (p-p27Ser10) in breast invasive ductal carcinomas are still scarce.

Methods: We examined the expression of p27 and p-p27Ser10 using immunohistochemistry in 107 breast invasive ductal carcinomas and analyzed the relationship of these biomarkers and tumor characteristics.

Results: Of the 107 tumor samples, 38.3% (41 of 107) overexpressed p27 and 64.5% (69 of 107) overexpressed p-p27Ser10. Analysis of correlation with clinical characteristics showed that high expression level of p-p27Ser10 was linked to poor differentiation, advanced disease stage, and lymph node metastasis, whereas a contrary trend was observed for p27 (all $P < 0.05$). In addition, the expression of p-p27Ser10 was significantly higher in malignant tumors than in adjacent tissues, while p27 showed the opposite trend. Also, there were different levels of p27 and p-p27Ser10 in different types of breast cancer.

Conclusion: p27 and p-p27Ser10 are involved in the development of invasive ductal carcinoma and are potential indicators to judge the degree of malignancy as well as recurrence and metastasis.

Keywords: breast cancer, p27, p-p27Ser10, Ki67, ER α , PR, Her2

Introduction

Mammalian cell proliferation is strictly controlled by the ordered activation of cyclin-dependent kinases (Cdks).¹ The Cdk inhibitor p27 is a tumor inhibitor that regulates G0-to-S phase transitions by binding and inhibiting the activity of Cdks.^{1,2} The expression of p27 is high in quiescent cells and declines as cells enter the S phase.³ Multiple experiments have shown that p27 is an independent prognostic factor in a variety of human malignancies.⁴⁻⁶ In breast cancer, reduced p27 expression was associated with poor patient outcome.⁵ During the carcinogenic process, the expression of p27 gradually decreased: normal breast duct epithelia (95%), premalignant atypical ductal hyperplasia (85%), ductal carcinoma in situ (40%), and invasive cancer (34%).⁷ The activities of p27 are influenced by its intracellular concentration and subcellular localization.⁸ However, the phosphorylation of p27 can affect its nuclear-cytoplasmic localization.⁹ It has previously been confirmed that Ser10 was the principal phosphorylation site in p27, contributing to approximately 70% of the total phosphorylation of this protein.¹⁰ When p27 was phosphorylated at Ser10 by kinase interacting stathmin, it could form a functional complex by combining with Jun activation domain-binding protein-1 (JAB1) and then migrate from the nucleus to the cytoplasm by recruiting chromosome region maintenance 1 (CRM1).¹¹

Currently, only a small number of studies have focused on the expression of phosphorylated p27 (p-p27Ser10) in human malignancies. It has been proven that

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p-p27Ser10 overexpression is strongly associated with advanced stage and tumor grade in human epithelial ovarian carcinoma.¹² No studies have been reported to investigate the expression of p-p27Ser10 and the correlation between p27 expression and clinical characteristics in breast cancer. We detected the expression of p27 and p-p27Ser10 in 107 breast invasive ductal carcinomas and analyzed their relationships with tumor size, pathologic classification, and lymph node metastasis, as well as the expressions of estrogen receptor α (ER α), progesterone receptor (PR), human epidermal growth factor receptor-2 (Her2), and antigen identified by monoclonal antibody Ki-67 (Ki67). Moreover, the expression of p27 and p-p27Ser10 in malignant tumors and adjacent tissues, as well as in different types of breast cancer, was explored. Our study was designed to demonstrate the clinical significance of p27 and p-p27Ser10 in the development of breast cancer and to find new and effective predictors and therapeutic targets.

Materials and methods

Ethical approval

The study was approved by the Ethics Committee of Jinling Hospital, Southern Medical University, People's Republic of China. All patients provided written informed consent.

Study subjects

We studied 107 breast cancer patients who underwent radical or modified radical mastectomy in the Ganzhou Cancer Hospital, Jiangxi Province, People's Republic of China, from January 2008 to January 2013. Inclusion criteria of breast cancer patients included a set of clinical symptoms, imaging examinations, and pathological diagnoses. We also obtained 15 paired primary breast carcinomas and corresponding adjacent noncancerous tissues among 107 cases. Immunohistochemistry for ER α , PR, Her2, and Ki67 was conducted. ER α and PR were scored as a percentage of cells staining positive, using 10% as a positive cutoff point. The cutoff value for Ki67 index was set at 14% considering the optimum threshold determined by Cheang et al.¹³ Scoring of Her2 was done on a scale of 0–3 (–, no staining; +, weak or barely perceptible membranous staining in >10% of the tumor cells; ++, moderate membranous staining in >10% of the tumor cells; +++, strong complete membranous staining in >10% of the tumor population). Furthermore, the uncertain Her2 score (++) was confirmed by fluorescence in situ hybridization (FISH). Score of +++ and positive expression by FISH of ++ cases were defined as Her2-positive expression, and the remaining scores were considered negative.¹⁴ On the basis of the 2013 St Gallen Consensus, molecular subtypes of breast cancer (Luminal A, Luminal B,

Her2-overexpressing, basal-like, and normal breast-like) were categorized according to the immunohistochemical profiles of ER α , PR, Ki67, and Her2.¹⁵ Patient information consisted of age, menopausal status, and lymph node metastasis, and the tumor-node-metastasis (TNM) stage was obtained from clinical inquiry, medical records, and relevant inspections. The TNM classification was performed independently by two different physicians in accordance with the *TNM Classification of Malignant Tumors, Seventh Edition*, published by the Union for International Cancer Control. The present research was approved by the Ethics Committee of Jinling Hospital, Southern Medical University, People's Republic of China.

Immunohistochemistry of p27 and p-p27Ser10

To evaluate the expression of immune cell antigens using immunohistochemistry, 3 μ m tumor sections of paraffin-embedded, formalin-fixed tissues were deparaffinized in xylene and rehydrated in a series of graded ethanol. Endogenous peroxidase activity was blocked by immersing sections in 3% H₂O₂ for 5 minutes. The sections were blocked in 10% fetal bovine serum for 10 minutes at room temperature and then incubated with primary antibodies to p27^{Kip1} (rabbit polyclonal, ab7961, 1:100; Abcam) and p-p27Ser10 [EP233(2)Y] (rabbit polyclonal, ab62364, 1:200; Abcam) for 60 minutes of staining. Immunostaining was carried out using the MaxVision™ HRP-Polymer anti-Rabbit IHC Kit (KIT-5005; Maxim) for 15 minutes according to the manufacturer's protocol and finally visualized with diaminobenzidine. In addition, sections were then counterstained with hematoxylin. The testis tissue served as the positive control.

Evaluation of p27 and p-p27Ser10 immunoreactivity

Two investigators separately evaluated the staining patterns of the tumor and control tissue samples in a blinded, randomized manner and scored the same. For each slide, five random fields were evaluated; p27 immunoreactivity was scored for the percentage of nucleus-staining tumor cells and p-p27Ser10 immunoreactivity was scored for the percentage of cytoplasm-staining tumor cells (<10%, –; >10%, +).

Statistical analyses

Statistical analyses were conducted with SPSS 19.0 version software (IBM Corporation, Armonk, NY). The Wilcoxon signed-rank test was used for comparison of expression levels of p27 and p-p27Ser10 between breast tumor tissues and the corresponding adjacent normal tissues in 15 pairs of samples. The χ^2 test or the Fisher's exact test was used to compare the

expression of each index for different factors. The correlation between indicators and the molecular subtypes of breast cancer associated with Ki67 was analyzed using the χ^2 test or the Fisher's exact test. Kappa test was used to analyze the relevance between the expression of p27 and p-p27Ser10 and the correlation between the expression of p27 and p-p27Ser10 and clinicopathological characteristics in invasive ductal cancers. All statistical tests were two-sided, and the statistical significance threshold was set at $P < 0.05$.

Results

Correlation between clinicopathological features and p27 and p-p27Ser10 expression in 107 breast cancer cases

The positive rates of p27 and p-p27Ser10 were 38.3% (41 of 107) and 64.5% (69 of 107) in tumors, respectively. In breast invasive ductal carcinomas, the level of p27 varied in subgroups

with different states of tissue differentiation ($\chi^2=16.612$, $P < 0.001$), TNM stage ($\chi^2=14.755$, $P < 0.0001$), and lymph node metastasis ($\chi^2=23.017$, $P < 0.0001$). The p27 protein was higher in tissue differentiation G1 grade, I–II TNM stages, and lymph node-negative group. But p27 expression in various subgroups of age ($\chi^2=0.587$, $P=0.444$), menstrual status ($\chi^2=1.765$, $P=0.184$), and tumor size ($\chi^2=5.75$, $P=0.058$) had no difference in distribution. Expression of p27 protein was related with the level of ER α , Her2, and Ki67. The kappa values were 0.222, –0.281, and –0.300, respectively. The expression of p27 was positively correlated with the expression of ER α ($P=0.020$), but it was negatively correlated with the expression of Her2 ($P=0.002$) and Ki67 ($P=0.001$). No significant difference was found in the expression of p27 and PR ($P > 0.05$) (Table 1). The level of p-p27Ser10 in breast invasive ductal cancers was differentially regulated in subgroups with various stages of tissue differentiation ($\chi^2=7.688$, $P=0.021$), TNM ($\chi^2=4.408$,

Table 1 Correlation between clinicopathological features and p27 expression in 107 breast cancer cases

Clinicopathological features	n ^a	p27 ^{Kip1}		P (McNemar)	Kappa	χ^2	P ^b
		Negative (%)	Positive (%)				
All cases	107 (100%)	66 (61.7%)	41 (38.3%)				
Age (years)						0.587	0.444
≤44	55 (51.4%)	32 (29.9%)	23 (21.5%)				
>44	52 (48.6%)	34 (31.8%)	18 (16.8%)				
Menopausal status						1.765	0.184
Premenopausal	70 (65.4%)	40 (37.4%)	30 (28.0%)				
Postmenopausal ^c	37 (34.6%)	26 (24.3%)	11 (10.3%)				
Tumor size (cm)						5.750	0.058
≤2	27 (25.2%)	12 (11.2%)	15 (14.0%)				
2–5	72 (67.3%)	47 (43.9%)	25 (23.4%)				
>5	8 (7.5%)	7 (6.5%)	1 (0.9%)				
Histological differentiation						16.612	<0.0001
G1	29 (27.1%)	9 (8.4%)	20 (18.7%)				
G2	51 (47.7%)	35 (32.7%)	16 (15.0%)				
G3	27 (25.2%)	22 (20.6%)	5 (4.7%)				
TNM staging						14.755	<0.0001
I–II	48 (44.9%)	20 (18.7%)	28 (26.2%)				
III–IV	59 (55.1%)	46 (43.0%)	13 (12.1%)				
Metastatic lymph nodes						23.017	<0.0001
Negative	40 (37.4%)	13 (12.1%)	27 (25.20%)				
Positive	67 (62.6%)	53 (23.4%)	14 (13.1%)				
ER α				0.211	0.222		0.020
Negative	57 (53.3%)	41 (38.3%)	16 (15.0%)				
Positive	50 (46.7%)	25 (23.4%)	25 (23.4%)				
PR				0.451	0.154		0.110
Negative	60 (56.1%)	41 (38.3%)	19 (17.8%)				
Positive	47 (43.9%)	25 (23.4%)	22 (20.6%)				
Her2				0.052	–0.281		0.002
Negative	82 (76.6%)	44 (41.1%)	38 (35.5%)				
Positive	25 (23.4%)	22 (20.6%)	3 (2.8%)				
Ki67				0.120	–0.300		0.001
Negative	52 (48.6%)	24 (22.4%)	28 (26.2%)				
Positive	55 (51.4%)	42 (39.3%)	13 (12.1%)				

Notes: ^aNumber of cases in each group; ^bP for χ^2 test; ^cpostmenopausal status for natural menopause.

Abbreviations: ER α , estrogen receptor α ; Her2, human epidermal growth factor receptor-2; Ki67, antigen identified by monoclonal antibody Ki-67; PR, progesterone receptor; TNM, tumor-node-metastasis.

Table 2 Correlation between clinicopathological features and p-p27Ser10 expression in 107 breast cancer cases

Clinicopathological features	n ^a	p-p27Ser10		P (McNemar)	Kappa	χ^2	P ^b
		Negative (%)	Positive (%)				
All cases	107 (100%)	38 (35.5%)	69 (64.5%)				
Age (years)						1.964	0.164
≤44	55 (51.4%)	23 (21.5%)	32 (29.9%)				
>44	52 (48.6%)	15 (14.0%)	37 (34.6%)				
Menopausal status						0.826	0.363
Premenopausal	70 (65.4%)	27 (25.2%)	43 (40.2%)				
Postmenopausal ^c	37 (34.6%)	11 (10.3%)	26 (24.3%)				
Tumor size (cm)						3.647	0.150
≤2	27 (25.2%)	13 (12.1%)	14 (13.1%)				
2–5	72 (67.3%)	24 (22.4%)	48 (44.9%)				
>5	8 (7.5%)	1 (0.9%)	7 (6.5%)				
Histological differentiation						7.688	0.021
G1	29 (27.1%)	14 (13.1%)	15 (14.1%)				
G2	51 (47.6%)	20 (18.7%)	31 (29.0%)				
G3	27 (25.2%)	4 (0.4%)	23 (21.5%)				
TNM staging						4.408	0.044
I–II	48 (44.9%)	22 (20.6%)	26 (24.3%)				
III–IV	59 (55.1%)	16 (15.0%)	43 (40.2%)				
Metastatic lymph nodes						10.591	0.001
Negative	40 (37.4%)	22 (20.6%)	18 (16.8%)				
Positive	67 (62.6%)	16 (15.0%)	51 (47.7%)				
ER α				0.016	–0.046		0.615
Negative	57 (53.3%)	19 (17.8%)	38 (35.5%)				
Positive	50 (46.7%)	19 (17.8%)	31 (29.0%)				
PR				0.005	–0.047		0.594
Negative	60 (56.1%)	20 (18.7%)	40 (37.4%)				
Positive	47 (43.9%)	18 (16.8%)	29 (27.1%)				
Her2				<0.0001	0.126		0.064
Negative	82 (76.6%)	33 (30.8%)	49 (45.8%)				
Positive	25 (23.4%)	5 (4.7%)	20 (18.7%)				
Ki67				0.049	0.0171		0.067
Negative	52 (48.6%)	23 (21.5%)	29 (27.1%)				
Positive	55 (51.4%)	15 (14.0%)	40 (37.4%)				

Notes: ^aNumber of cases in each group; ^bP for χ^2 test; ^cpostmenopausal status for natural menopause.

Abbreviations: ER α , estrogen receptor α ; Her2, human epidermal growth factor receptor-2; Ki67, antigen identified by monoclonal antibody Ki-67; PR, progesterone receptor; TNM, tumor-node-metastasis.

$P=0.044$), and lymph node metastasis ($\chi^2=10.591$, $P=0.001$). The expression of p-p27Ser10 protein in tissue differentiation G2 grade, III–IV TNM stages, and lymph node-positive group was higher. This difference was statistically significant ($P<0.05$). The expression of p-p27Ser10 protein in the various age ($\chi^2=1.964$, $P=0.164$) and menstrual status ($\chi^2=0.826$, $P=0.363$) subgroups showed no difference in distribution. Moreover, the expressions of p-p27Ser10 protein and ER α ($P=0.615$), PR ($P=0.594$), Her2 ($P=0.064$), and Ki67 ($P=0.067$) were not related (Table 2). Meanwhile, the authors analyzed the correlation between the expression of p27 and p-p27Ser10 in invasive ductal carcinomas, and the results suggested that there was an inverse correlation between p27 and p-p27Ser10 in invasive ductal carcinomas, which was statistically significant (Table 3; Figure 1) (kappa values = –0.611, $P<0.0001$).

Expression of p27 and p-p27Ser10 in 15 breast invasive ductal carcinomas and adjacent tissues

We further analyzed the paired specimens of tumor tissues and adjacent tissues. In 15 tumor tissues and corresponding benign tissues, p27 overexpression was detected in 40.0% (6 of 15) of malignant tumors and 86.7% (13 of 15) of

Table 3 Correlation between the expression levels of p27 and p-p27Ser10 in invasive ductal carcinoma

p-p27Ser10	p27		P (McNemar)	Kappa	P
	Negative	Positive			
Negative	6	32	0.005	–0.611	<0.0001
Positive	60	9			

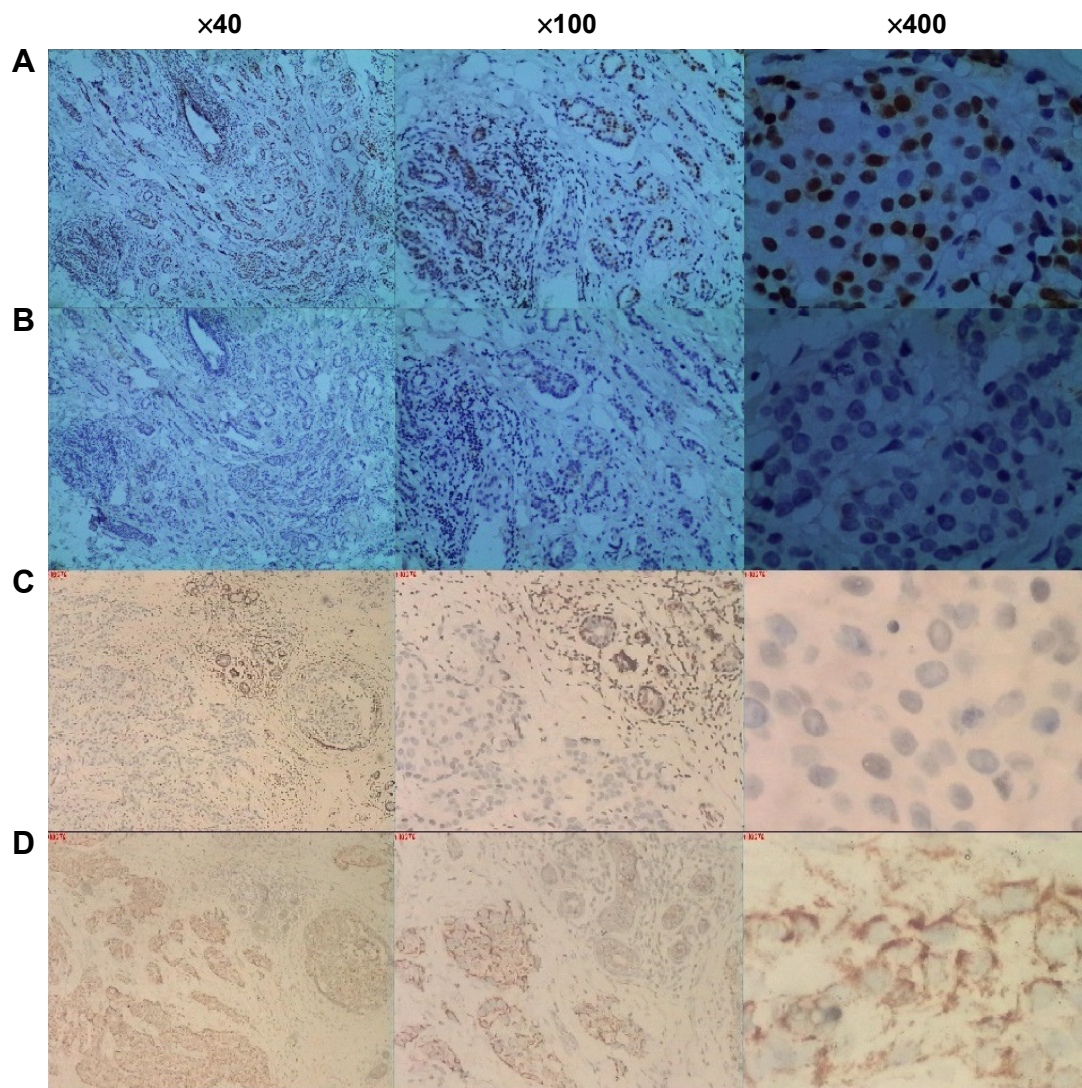


Figure 1 Representative examples of immunohistochemical analysis of p27 and p-p27Ser10 in breast invasive ductal carcinomas and adjacent tissues.

Notes: (A) Breast invasive ductal carcinomas shows uniform, nuclear positive staining for p27; (B) share the same vision fields with (A), p-p27Ser10 was negative expression; (C) p27 was negative expression in breast invasive ductal carcinomas; (D) share the same vision fields with (C), shows the cytoplasmic positive expression of p-p27Ser10.

adjacent tissues (Table 4). Furthermore, p-p27Ser10 overexpression was found in 73.3% (11 of 15) of malignant tumors and 26.7% (4 of 15) of adjacent tissues (Table 5). The expression of p27 was higher in adjacent normal tissues than in tumor tissues ($P=0.004$), while the expression of p-p27Ser10 was reverse. The expression of p27 and p-p27Ser10 was different between malignant tumors and tumor adjacent tissue ($P=0.037$). Difference between these groups had statistical significance ($P<0.05$).

The expression of p27 and p-p27Ser10 in different subtypes of breast cancer

Among the breast cancer samples, 31 (29.0%) cases were of Luminal A; 24 (22.4%) cases were of Luminal B; 20 (18.7%) cases showed Her2 overexpression; and 32 (29.9%) cases were of the basal-like subtype. The expression of p27 in different subtypes of breast cancers ($\chi^2=14.653$, $P=0.002$) was statistically significant ($P<0.05$) and the rate

Table 4 Expression of p27 in breast invasive ductal carcinomas and adjacent tissues

Sample number ^a	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	P ^b
Invasive ductal carcinoma	50%	0	0	10%	0	25%	0	0	25%	0	50%	0	10%	0	0	
Adjacent tissue	50%	25%	50%	50%	0	50%	50%	25%	10%	50%	50%	25%	50%	0	50%	0.004

Notes: ^aEach sample of breast cancer was collected with the corresponding adjacent tissue; ^bP for Wilcoxon signed-rank test.

Table 5 Expression of p-p27Ser10 in breast invasive ductal carcinomas and adjacent tissues

Sample number ^a	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	P ^b
Invasive ductal carcinoma	0	50%	25%	0	50%	0	25%	25%	10%	25%	0	50%	25%	25%	50%	
Adjacent tissue	0	10%	0	0	0	25%	0	0	0	0	25%	0	50%	0	0	0.037

Notes: ^aEach sample of breast cancer was collected with the corresponding adjacent tissue; ^bP for Wilcoxon signed-rank test.

of positive p27 expression was lower in breast cancers with Her2 overexpression. The expression of p-p27Ser10 in different subtypes ($\chi^2=6.442$, $P=0.090$) showed no significant difference ($P>0.05$) (Table 6).

Discussion

p27 is an important component of the cell cycle machinery.¹⁶ Quiescent normal epithelia of breast, prostate, ovary, and lung highly express nuclear p27.^{3,17,18} An overwhelming amount of data showed the inverse correlation between expression of p27 and prognosis in a variety of human neoplasms, including those of the breast, colon, stomach, and prostate.^{4–6,19} p-p27Ser10 could affect the nuclear–cytoplasmic localization of p27. A recent report provided convincing evidence for a serine 10-dependent regulatory mechanism affecting p27 tumor suppressive activity during epithelial ovarian cancer development, as well as increased p-p27Ser10 expression with enhanced malignancy.^{12,20} Point mutations in Ser10 and base mutations within the nuclear export sequence damaged p27 nuclear export and indicated that the induction of p27 expression was associated with declined cancer cell proliferation.^{12,20} In addition, the survival analyses showed that Jab1 and p-p27Ser10 expression is significantly associated with poor prognosis in hepatocellular carcinoma.²¹

Our research illustrated by immunohistochemistry that there was a correlation between p27 and p-p27Ser10 expression and invasive ductal breast carcinomas. Comparison between malignant tumors and benign tumors showed that the expression levels of p27 and p-p27Ser10 were significantly different. Results showed that p27 and p-p27Ser10 expression is obviously correlated with the degree of differentiation, TNM staging, and lymph node metastasis number. Higher expression of p-p27Ser10 was observed in tumors that

showed poor histological differentiation, high TNM staging, and incidence of lymph node metastasis. The negative correlation between the expression of nuclear p27 and cytoplasm p-p27Ser10 suggested that the degradation of nuclear p27, which p-p27Ser10 mediates, might be an important mechanism in breast cancer. The results concur with those of several previous studies in other human tumors.^{19–21}

Currently, the treatment and prognosis of breast cancer are based primarily on molecular subtypes.²² Effective prognostic markers that differentiate patient outcomes when analyzing an intermediate-stage disease are crucially important in making appropriate therapeutic evaluations. In human breast cancer, Her2 amplification is apparently correlated with reduced levels of p27, which is consistent with the results from previous reports.²³ Given the facts mentioned herein about the relation between p27 and p-p27Ser10 expression and different molecular subtypes of breast cancer, they can be used to guide the clinical treatment. For example, the correlation between p27 and the ER α and PR duo in invasive ductal carcinoma suggested that p27 was associated with endocrine therapy as a good prognostic factor. Because breast cancers often become chemotherapy resistant, p27 as a predictive factor of response to chemoprevention for malignancies requires further investigation.²⁴ So far, few large prospective randomized studies explicitly indicate that p27 predicts response to certain chemotherapeutic drugs. Further research in this area would be needed to make clear the prognostic value of p27 and p-p27Ser10 for effectiveness of treatment in different contexts.

In conclusion, our research provides evidence that p27 and p-p27Ser10 have correlation with clinicopathological characteristics in breast cancers. p27 and p-p27Ser10 are involved in the development of invasive ductal carcinoma and

Table 6 Expression of p27 and p-p27Ser10 in different subtypes of breast cancer

Subtype	p27 ^{Kip1}		χ^2	P	p-p27Ser10		χ^2	P
	Negative (%)	Positive (%)			Negative (%)	Positive (%)		
Luminal A	15 (14.0%)	16 (15.0%)			11 (10.3%)	20 (18.7%)		
Luminal B	12 (11.2%)	12 (11.2%)			12 (11.2%)	12 (11.2%)		
Her2 overexpressing	19 (17.8%)	1 (0.9%)			3 (2.8%)	17 (15.9%)		
Basal-like	20 (18.7%)	12 (11.2%)			13 (12.1%)	19 (17.8%)		
All cases	66 (61.7%)	41 (38.3%)	14.653	0.002	39 (36.4%)	68 (63.6%)	6.442	0.090

are potential biomarkers to judge the degree of malignancy as well as recurrence and metastasis. It is demonstrated that p27 and p-p27Ser10 potentially can be used as a clinical prognosis and therapeutic target.

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Disclosure

The authors report no conflicts of interest in this work.

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