Original Article

P16 Expression in Human Breast Carcinoma and its Relationship to Clinicopathological Parameters

Fereshteh Mohammadizadeh, Farnaz Nasri

Department of Pathology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: p16 is a cyclin-dependent kinase inhibitor and a cardinal regulator of the cell cycle. The relationship between p16 overexpression and poor prognosis of breast cancer has been reported in some studies. This study aimed to evaluate p16 expression in breast cancer in comparison to normal breast tissue and determine the association between p16 expression and clinicopathological parameters in breast cancer.

Materials and Methods: Paraffin blocks of 110 samples were studied. These included 40 invasive breast carcinoma (tumor group) and normal tissue adjacent to the tumor (tumor control), as well as 30 normal mammoplasty specimens (normal control). Samples were from the pathology archive of Alzahra Hospital, Isfahan, Iran, from 2016 to 2020. p16 expression was studied and compared in these three groups using the immunohistochemistry technique. Moreover, the relationship between p16 expression and age, tumor size, carcinoma subtype, tumor grade, and lymph node involvement was investigated in the tumor group. SPSS version 16 was used to analyze data.

Results: p16 expression showed a significant difference between the tumor group and the two control groups with a significantly higher expression in the tumor group. There was a significant direct relationship between the intensity of p16 expression and the number of involved lymph nodes ($P < 0.001$). No significant relationship was detected between p16 expression and other clinicopathological factors.

Conclusion: p16 seems to have a rather significant expression in breast cancer in comparison to normal breast parenchyma. However, among clinicopathological parameters, we found only a direct relationship between lymph node involvement and intensity of p16 expression.

Keywords: Breast cancer, cyclin-dependent kinase inhibitor p16, immunohistochemistry, prognosis

Address for correspondence: Dr. Farnaz Nasri, Department of Pathology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: fa.nasri.1368@gmail.com
Submitted: 31-May-2022; **Revised:** 26-Aug-2022; **Accepted:** 04-Oct-2022; **Published:** 28-Jun-2023

Introduction

Breast cancer is the most common non-cutaneous malignant tumor and is the second cause of cancer deaths among women.[1] Data reveal an increase in the incidence of this malignancy in Asia and a decrease in the mean age of Iranian patients in recent years.[2,3] Breast cancer is caused by the accumulation of mutations in drivers and other genes that provide cells a proliferative advantage.[4,5] The occurrence of genetic abnormalities in the major genes that control cell growth can lead to the onset of carcinogenesis.[6] One of the most common causes of human cancers is the disruption of cell cycle checkpoints. The p16INK4a is a tumor suppressor gene located on chromosome 9p21.[7] p16 protein, the product of that gene, is one of the negative regulators of the cell cycle which causes G1 arrest by blocking the G1 to S transfer in the cell cycle. p16 inhibits cyclin-dependent kinase 4/6 (CDKs 4/60).[8] CDKs 4/6 phosphorylate Retinoblastoma protein (pRb), resulting in pRb inactivation. pRb phosphorylation by CDKs 4/6 with subsequent inactivation of this protein is an important step in cell cycle progression. Deletions and mutations of p16 in human cancer cell lines point to the significant role of

Access this article online

Quick Response Code:

Website: www.advbiores.net

DOI: 10.4103/abr.abr_180_22

How to cite this article: Mohammadizadeh F, Nasri F. P16 expression in human breast carcinoma and its relationship to clinicopathological parameters. Adv Biomed Res 2023;12:154.

p16 in carcinogenesis.[9,10] With the activation of telomerase, cells may eventually progress to immortality and cancer.[11-13] The prognostic impact of p16 alteration has been reported in some human cancers.[14-18] Based on the functions of p16, it would be expected that p16 overexpression reduces the likelihood of cancer. However, when p16 mutation is associated with abrogation of p16 signaling, compromised Rb signaling will lead to overexpression of mutant p16 in cells, which continue to proliferate and bypass the senescence.[19] The majority of human cancers show dysregulation of p16.[20,21] Alterations of p16 expression have been found to be an early event in the transition from premalignant to malignant tumors.[8] Previous studies on p16 expression in breast cancer and its relationship with known prognostic factors are limited. The pattern of p16 expression is variable in breast tumors.[22] Estrogen receptor-negative breast cancers with a loss of p16 expression have been found to be resistant to treatment.[17]

Antibodies to p16 have been found to be increased in the circulation of patients with breast cancer and it has been shown that the levels of IgG antibody to this protein can be a promising marker for early detection of breast carcinoma.[23,24] Due to the limited results of previous studies in this field, we aimed to conduct this study to investigate the expression of p16 in invasive breast carcinoma and normal tissue adjacent to the tumor, as well as normal mammoplasty samples. We also investigated the relationship of p16 expression with some prognostic factors of breast cancer.

Materials and Methods

We did this study on paraffin tissue blocks. Samples included 40 invasive breast carcinoma (tumor group) and their adjacent normal tissue (tumor control), as well as 30 normal mammoplasty specimens (normal control) from the pathology lab of Al-Zahra Hospital, Isfahan, Iran, from 2016 to 2020. We had study approval from the ethical committee of Isfahan University of Medical Sciences, Isfahan, Iran (Ethical code: IR.MUI.MED.REC 1398-680).

Inclusion criteria for the tumor group were mastectomies or breast lumpectomies with invasive breast carcinoma having normal tissue adjacent to the tumor and dissected axillary lymph nodes. Data concerning age, tumor size, tumor grade, carcinoma subtype, and the number of axillary lymph nodes with metastasis were available from pathology reports. Mammoplasties with normal breast histology were included as the normal control group. Exclusion criteria for the tumor group were tumor specimens lacking normal breast tissue and/or axillary nodes. In the normal control group, mammoplasties showing various breast pathologies were excluded from the study.

We used the easy sampling method in this study. The following formula was used for sample size calculation with a 95% Confidence Interval (CI):

$$\frac{(Z1+Z2)^2[P1(1-P1)+P2(1-P2)]}{(P1-P2)^2}$$

Following sample collection, immunohistochemistry (IHC) was used to stain specimens with p16 antibody (Monoclonal Antibody, Master Diagnostica, Spain). The tissue block of a cervical conization specimen with cervical intraepithelial neoplasia 3 showing p16 block staining was used as a positive control. Areas of the normal squamous epithelium of the same block were used as a negative control. Sections with five-micron thickness were prepared from tissue blocks and stained by p16 antibody as follows:

They were incubated at 37°C in the oven for 48 h, dewaxed by 100% xylol, rehydrated by 100%, 85%, and 75% ethanol, rinsed in 10% phosphate-buffered saline (PBS) solution, incubated for 30 min in 10% H2O2 and methanol for blocking of endogenous peroxidase activity, rinsed in 10% PBS solution, incubated for 14 min in the microwave in citrate-buffered solution (PH = 6.1), rinsed in 10% PBS solution, exposed to blocking serum for 30 min for blocking of endogenous non-specific bindings, dried, exposed to the primary monoclonal antibody of p16 and incubated for 30 min at room temperature, rinsed in 10% PBS solution, exposed to a broad-spectrum secondary antibody for 30 min, exposed to horseradish peroxidase-streptavidin for 30 min, exposed to diaminobenzidine for 10 min, rinsed in 10% PBS solution, dehydrated by 75%, 85%, and 100% ethanol, and counterstained by hematoxylin.

Investigation and analysis of IHC samples

The intensity and percentage of p16 nuclear staining in carcinoma cells and normal breast epithelium were evaluated by a pathologist (supervisor) and a pathology resident according to the scoring system introduced in a previously published report.[25]

Each sample was scored grounded on the maximum intensity of nuclear staining as follows:

- 0: negative
- 1: weakly positive
- 2: moderately positive
- 3: strongly positive

Each sample was also scored based on the extent of nuclear staining (percentage of stained nuclei) as follows:

- 0: less than 5%
- 1: between 5% and 25%
- 2: between 25% and 50%
- 3: between 50% and 75%
- 4: more than 75%

To determine the final score of each sample, the staining intensity score was multiplied by the staining extent score. The lowest and highest final scores were 0 and 12, respectively. The final score was then semi-quantitatively divided into three groups:

- Negative: 0 to 4
- Weak: 5 to 8
- Strong: 9 to 12

We then compared the intensity and extent of p16 nuclear staining between the three groups. We also studied the relationship between p16 nuclear expression and age, tumor size, tumor grade, carcinoma subtype, and lymph node involvement in the tumor group.

Statistical analysis

We used SPSS software, version 16, to analyze data concerning p16 staining, age, tumor size, tumor grade, carcinoma subtype, and the number of involved lymph nodes. A *P* value less than 0.05 was considered significant. Data were reported as frequency, mean, and standard deviation (SD). Fisher's exact test and Spearman correlation coefficient were used to examine the relationship between p16 expression and prognostic variables in the tumor group.

Results

In this study, we examined 40 specimens of invasive breast carcinoma (tumor group) and normal tissue adjacent to these carcinoma specimens (tumor control group), as well as 30 normal control specimens from mammoplasty surgery (normal control group).

The mean age in the carcinoma group was 47.52 ± 1.52 years with a minimum age of 32 years and maximum age of 76 years. The mean age in the mammoplasty group was 33.97 ± 6.55 years with a minimum age of 23 years and maximum age of 45 years. Data concerning clinicopathological prognostic factors in the carcinoma group have been presented in Table 1.

Data concerning intensity, extent, and final score of p16 nuclear expression in the three groups have been presented in Table 2.

The intensity, extent, and final score of p16 nuclear staining showed a statistically significant difference between the tumor group and the tumor control group. In general, it was found that the intensity, extent, and final score of staining were all higher in the tumor group than the tumor control group [Table 3 and Figures 1-3].

Similarly, the intensity, extent, and final score of p16 nuclear staining showed a statistically significant difference between the tumor group and the normal control group. In general, it was found that the intensity, extent, and final score of staining were all higher in the tumor group than in the normal control group [Table 4].

Table 1: Distribution of clinicopathological prognostic factors in invasive breast carcinoma specimens

Variable	Maximum	Minimum	Mean±SD
Age	76	32	47.52±1.52
Tumor size (cm)	10	1	3.14±0.31
Variable	**Percentage**	**Frequency**	**Type**
Number of involved nodes	67.5	27	0
	10	4	1-4
	22.5	9	≥5
Carcinoma subtype	2.5	1	lobular
	2.5	1	medullary
	5	2	mixed type
	90	36	NOS

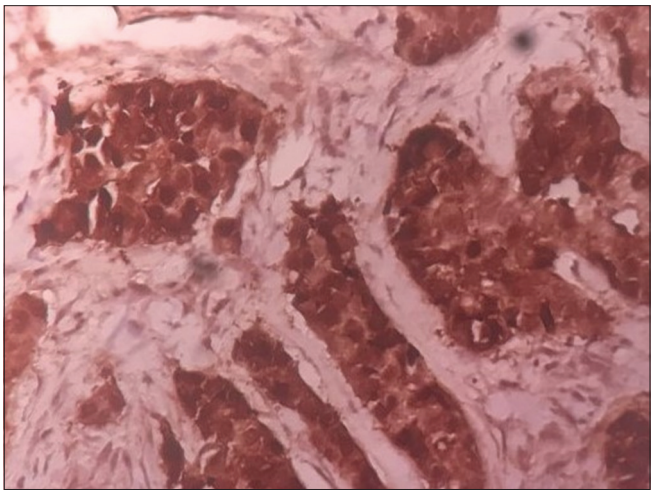

Figure 1: p16 immunohistochemistry. Strong intensity of p16 expression in invasive breast carcinoma (x400 magnification)

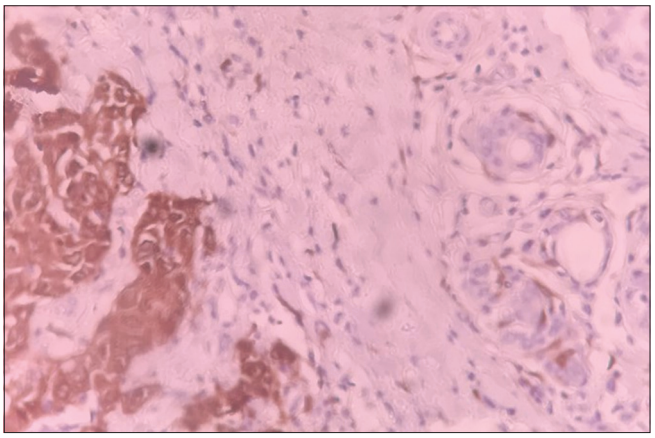

Figure 2: p16 immunohistochemistry. p16 expression with moderate intensity is seen in invasive breast carcinoma (left), while normal breast tissue adjacent to carcinoma has no p16 expression (right) (x400 magnification)

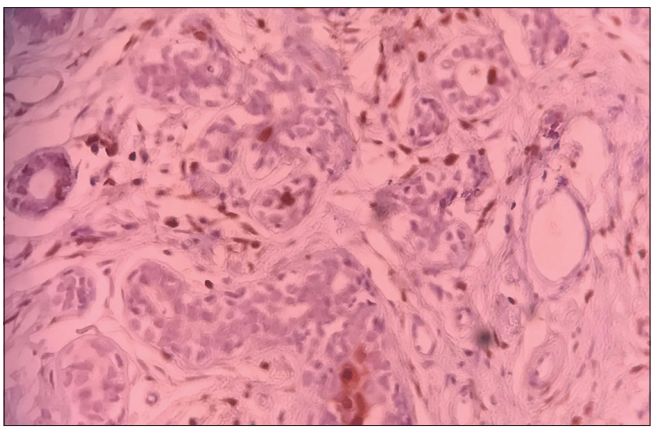

Figure 3: p16 immunohistochemistry. Focal p16 expression with moderate intensity is seen in normal breast tissue of mammoplasty specimen (x400 magnification)

Table 2: p16 nuclear expression (intensity, extent, and final score) in the three groups

	Normal control group	Tumor control group	Tumor group	p16 nuclear expression
Staining intensity	17 (56.7%)	26 (65%)	11 (27.5%)	Negative
	1 (3.3%)	0 (0%)	2 (5%)	Weakly positive
	8 (26.7%)	9 (22.5%)	16 (40%)	Moderately positive
	4 (13.3%)	5 (12.5%)	11 (27.5%)	Strongly Positive
Extent of staining	23 (76.7%)	30 (75%)	13 (32.5%)	<5
	6 (20%)	8 (20%)	6 (15%)	5-25
	0 (0%)	1 (2.5%)	5 (12.5%)	25-50
	1 (3.3%)	0 (0%)	3 (7.5%)	50-75
	0 (0%)	1 (2.5%)	13 (32.5%)	>75
Final score	29 (96.7%)	38 (95%)	23 (57.5%)	Negative
	0 (0%)	2 (5%)	8 (20%)	weak
	1 (3.3%)	0 (0%)	9 (22.5%)	strong

Table 3: Comparison of p16 nuclear expression in the tumor group and tumor control group

	Average	*P*	*Z* score
Intensity			
Tumor group	12.93	0.002*	−3.15
Tumor control group	10.38		
Extent			
Tumor group	13.63	<0.001*	−4.11
Tumor control group	5.75		
Final score			
Tumor group	9.74	<0.001*	−3.59
Tumor control group	5.50		

*Significant

Table 4: Comparison of p16 nuclear expression in tumor group and normal control group

	Average	*P*	*Z* score
Intensity			
Normal control group	29.22	0.017*	−2.38
Tumor group	40.21		
Extent			
Normal control group	27.80	<0.001*	−3.58
Tumor group	41.28		
Final score			
Normal control group	24.53	<0.001*	−4.23
Tumor group	43.73		

*Significant

Intensity, extent, and final score of p16 nuclear staining showed no statistically significant difference between tumor control and normal control groups [Table 5].

In the tumor group, there was a significant direct relationship between the intensity of p16 nuclear staining and the number of involved lymph nodes. No significant relationship was found between tumor size and age and p16 nuclear staining [Table 6].

Tumor grade and carcinoma subtype showed no significant relationship with p16 nuclear staining [Tables 7 and 8].

Discussion

This study was designed to compare p16 nuclear expression in invasive breast carcinoma (tumor group) with normal tissue adjacent to carcinoma (tumor control group) and normal mammoplasty specimens (normal control group). There was a significant difference in intensity, extent, and final score of p16 nuclear expression between the tumor group and normal control group ($P < 0.001$) and between the tumor group and tumor control group ($P < 0.001$). However, p16 nuclear expression showed no significant difference between tumor control and normal control groups ($P = 0.48$). We also found a significant direct relationship between the intensity of p16 nuclear expression and the number of involved lymph nodes ($P < 0.001$). However, significant relationship was not found between p16 nuclear expression and age, tumor size, tumor grade, and carcinoma subtype ($P > 0.05$).

The key role of p16 as a regulator of the cell cycle results in a significant impact of its altered expression on pathological variables and the clinical course of a variety of human cancers.[15,18,26] However, there is not much data on p16 expression in normal breast tissue and various kinds of breast lesions and the relationship between p16 expression in breast cancer and significant clinicopathological variables of this tumor. Feriancová *et al*. examined COX-2, p16, and Ki67 expression in ductal intraepithelial neoplasia (DIN), invasive breast cancer, benign breast lesions, and normal tissue adjacent to breast cancer. They found p16 overexpression in 37% of invasive breast carcinomas and 8% of normal tissue adjacent to carcinoma.[19] Golmohammadi *et al.* found P16 overexpression in 82% of breast cancers. No p16 overexpression was seen in normal breast samples. Overexpression of p16 had a significant association with higher tumor grade and tumor stage.[27] The study of Bazarov *et al.* on two malignant human breast cancer cell lines showed individual RB family proteins to be sufficient for p16-initiated senescence establishment. Although we found a significant direct relationship between the intensity of p16 nuclear expression and lymph node involvement in breast cancer, Dublin *et al.* did not find any relationship between p16 staining and histopathological

Table 5: Comparison of p16 nuclear expression in tumor control group and normal control group

	Average	*P*	*Z* score
Intensity			
Normal control group	36.87	0.575	−0.561
Tumor control group	34.48		
Extent			
Normal control group	35.20	0.76	−0.304
Tumor control group	35.73		
Final score			
Normal control group	35.10	0.849	−0.191
Tumor control group	35.80		

Table 6: The relationship between intensity, extent, and final score of p16 nuclear staining with the number of involved lymph nodes, tumor size, and age

	Number of involved lymph nodes	Tumor size	Age
Extent of p16 nuclear staining			
Correlation	−0.12	0.054	0.063
P	0.460	0.739	0.698
Intensity of p16 nuclear staining			
Correlation	0.884	0.042	0.060
P	<0.001*	0.798	0.714
Final score of p16 nuclear staining			
Correlation	0.00001	0.016	0.078
P	0.998	0.920	0.631

*Significant

Table 7: The relationship between intensity, extent, and final score of p16 nuclear staining with tumor grade

Tumor grade	Grade I	Grade II	Grade III	*P*
Intensity of p16 nuclear staining				
Negative	0 (0%)	8 (34.8%)	3 (18.8%)	0.333
Weak	0 (0%)	1 (4.3%)	1 (6.3%)	
Moderate	1 (100%)	6 (26.1%)	9 (56.3%)	
Strong	0 (0%)	8 (34.8%)	3 (18.8%)	
Extent of p16 nuclear staining				
>5	0 (0%)	9 (39.1%)	4 (25%)	0.099
5-25	1 (100%)	1 (4.3%)	4 (25%)	
25-50	0 (0%)	4 (17.4%)	1 (6.3%)	
50-75	0 (0%)	3 (13%)	0 (0%)	
>75	0 (0%)	6 (26.1%)	7 (43.8%)	
Final score of p16 nuclear staining				
Negative	1 (100%)	13 (56.5%)	9 (56.3%)	0.9
Weak	0 (0%)	4 (17.4%)	4 (25%)	
Strong	0 (0%)	6 (66.7%)	3 (33.3%)	

parameters of invasive breast carcinoma. Geradts *et al.* also found no significant correlation between abnormal p16 expression in invasive breast cancer and several histopathological parameters of this tumor. Gorgoulis *et al.* found aberrant expression of p16 in 47% of breast carcinomas. However, they found no significant relationship between p16 expression and tumor size, lymph node metastasis, tumor grade, tumor stage, estrogen receptor (ER), and progesterone receptor (PR). Grupka *et al.* found breast cancers negative for both pRb and p16 to be associated with a better prognostic phenotype.[28-32] Shin *et al*. found a significant correlation between p16 negativity and ER negativity, PR negativity, and higher Ki67 labeling index, all of which are linked to more aggressive breast cancer behavior.[16] In the study of Hui *et al.*, p16 overexpression showed a significant association with high tumor grade, metastasis to axillary lymph nodes, ER negativity, and increased risk of relapse. Milde-Langosch *et al.* found a significant association between p16 overexpression and unfavorable prognostic indicators.[33,34] According to the findings of Shan *et al*., p16 expression in luminal-A breast cancers is associated with progression from ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma, and p16 expression is important for the development of triple-negative breast cancers.[35] In the study of Zhang *et al*., p16 expression inhibited breast cancer cell-induced angiogenesis and suppressed breast tumor metastasis in a spontaneous metastasis model in mice.[36] Radisky *et al*. found that p16 overexpression does not significantly stratify breast cancer risk in women with atypical ductal hyperplasia.[37] In the study of Naji-Haddadi *et al*., p16 positivity in breast cancer was not associated with tumor grade, tumor size, neural and vascular invasion, and lymph node metastasis.[38] Salih *et al*. reported an association between p16 expression in breast cancer and high histologic grade and lymph node metastasis.[39] According to the findings of Jovanovic *et al*., p16 protein has an important role in proliferation and malignant transformation, as well as in the progression from non-invasive breast lesions to invasive breast cancer.[40]

The different results of various studies may be attributed to different specificity and sensitivity of various antibodies, duration of fixation with its impact on the results of p16 immunohistochemical staining, and genetic differences among different populations. Despite these discrepancies, almost all studies including our study show a considerable frequency of p16 overexpression in breast cancer. Although the existence of a significant relationship between p16 overexpression in breast cancer and clinicopathological prognostic factors is not confirmed by all studies, at least some of them including our study confirm the presence of such a relationship between p16 overexpression and some clinicopathological prognostic factors. These findings suggest p16 as a potential biomarker for targeted therapy of breast cancer in future. Further studies are needed to examine this possibility.

Conclusion

This study suggests p16 overexpression as an important step in the malignant transformation of normal breast epithelial cells. Concerning the relationship between p16 overexpression in

Table 8: The relationship between intensity, extent, and final score of p16 nuclear staining with carcinoma subtype

Tumor subtype	Lobular	Medullary	Mixed	NOS	*P*
Intensity of p16 nuclear staining					
Negative	0 (0%)	0 (0%)	1 (50%)	10 (28.6%)	0.847
Weakly	0 (0%)	0 (0%)	0 (0%)	2 (5.7%)	
Moderate	1 (100%)	0 (0%)	1 (50%)	14 (40%)	
Strong	0 (0%)	1 (100%)	0 (0%)	10 (27.8%)	
Extent of p16 nuclear staining					
>5	0 (0%)	0 (0%)	1 (50%)	12 (34.3%)	0.904
5-25	0 (0%)	0 (0%)	1 (50%)	5 (14.3%)	
25-50	0 (0%)	0 (0%)	0 (0%)	5 (14.3%)	
50-75	0 (0%)	0 (0%)	0 (0%)	3 (8.6%)	
>75	1 (100%)	1 (100%)	0 (0%)	11 (30.6%)	
Final score of p16 nuclear staining					
Negative	0 (0%)	0 (0%)	2 (100%)	21 (60%)	0.156
Weakly	1 (100%)	0 (0%)	0 (0%)	7 (20%)	
Strong	0 (0%)	1 (100%)	0 (0%)	8 (22.2%)	

invasive breast carcinoma and clinicopathological prognostic factors, we only found a significant direct relationship between overexpression of this marker and metastatic involvement of axillary lymph nodes.

Financial support and sponsorship

Nil.

Conflicts of interest

The Deputy of Research of Isfahan University of Medical Sciences, Isfahan, Iran, has approved and financially supported this study.

References

1. Farhood B, Geraily G, Alizadeh A. Incidence and mortality of various cancers in iran and compare to other countries: A review article. Iran J Public Health 2018;47:309-16.
2. Lam WW, Fielding R, Ho EY. Predicting psychological morbidity in Chinese women after surgery for breast carcinoma. Cancer 2005;103:637-46.
3. Golmohammadi R, Pejhan A. The prognostic value of the P53 protein and the Ki67 marker in breast cancer patients. J Pak Med Assoc 2012;62:871-5.
4. Skibinski A, Kuperwasser C. The origin of breast tumor heterogeneity. Oncogene 2015;34:5309-16.
5. Visvader JE, Stingl J. Mammary stem cells and the differentiation hierarchy: Current status and perspectives. Genes Dev 2014;28:1143-58.
6. Rivenbark AG, Coleman WB. Field cancerization in mammary carcinogenesis-Implications for prevention and treatment of breast cancer. Exp Mol Pathol 2012;93:391-8.
7. Sheng X, Guo Y, Lu Y. Prognostic role of methylated GSTP1, p16, ESR1 and PITX2 in patients with breast cancer: A systematic meta-analysis under the guideline of PRISMA. Med (Baltimore) 2017;96:e7476.
8. Ni J, Kabraji S, Xie S, Wang Y, Pan P, He X, *et al*. p16(INK4A)-deficiency predicts response to combined HER2 and CDK4/6 inhibition in HER2+breast cancer brain metastases. Nat Commun 2022;13:1473.
9. Langendijk JA, Psyrri A. The prognostic significance of p16 overexpression in oropharyngeal squamous cell carcinoma: Implications for treatment strategies and future clinical studies. Ann Oncol 2010;21:1931-4.
10. Peurala E, Koivunen P, Haapasaari KM, Bloigu R, Jukkola-Vuorinen A. The prognostic significance and value of cyclin D1, CDK4 and p16 in human breast cancer. Breast Cancer Res 2013;15:R5.
11. Danforth DN, Jr. Genomic Changes in normal breast tissue in women at normal risk or at high risk for breast cancer. Breast Cancer (Auckl) 2016;10:109-46.
12. Sun C, Wang G, Wrighton KH, Lin H, Songyang Z, Feng XH, *et al*. Regulation of p27(Kip1) phosphorylation and G1 cell cycle progression by protein phosphatase PPM1G. Am J Cancer Res 2016;6:2207-20.
13. Peng G, Cao RB, Li YH, Zou ZW, Huang J, Ding Q. Alterations of cell cycle control proteins SHP-1/2, p16, CDK4 and cyclin D1 in radioresistant nasopharyngeal carcinoma cells. Mol Med Rep 2014;10:1709-16.
14. Andrade DAPd, da Silva VD, Matsushita GdM, de Lima MA, Vieira MdA, Andrade CEMC, *et al*. Squamous differentiation portends poor prognosis in low and intermediate-risk endometrioid endometrial cancer. PLoS One 2019;14:e0220086.
15. Macha MA, Rachagani S, Pai P, Gupta S, Lydiatt WM, Smith RB, *et al*. MUC4 regulates cellular senescence in head and neck squamous cell carcinoma through p16/Rb pathway. Oncogene 2015;34:1698-708.
16. Shin E, Jung W-H, Koo J-S. Expression of p16 and pRB in invasive breast cancer. Int J Clin Exp Pathol 2015;8:8209-17.
17. Srivastava V, Patel B, Kumar M, Shukla M, Pandey M. Cyclin D1, retinoblastoma and p16 protein expression in carcinoma of the gallbladder. Asian Pac J Cancer Prev 2013;14:2711-5.
18. Tarakji B, Alenzi F, Al-Khuraif AA. Assessment of inverse correlation of p16 and pRb expression in carcinoma ex pleomorphic adenoma. Pol J Pathol 2013;64:144-8.
19. Feriancová M, Walter I, Singer CF, Gazdarica J, Pohlodek K. Expression of COX-2, p16, and Ki67 in the range from normal breast tissue to breast cancer. Neoplasma 2021;68:342-51.
20. Rana MK, Rana APS, Khera U. Expression of p53 and p16 in carcinoma breast tissue: Depicts prognostic significance or coincidence. Cureus 2021;13:e19395.
21. Hashmi AA, Naz S, Hashmi SK, Hussain ZF, Irfan M, Khan EY, *et al*. Prognostic significance of p16 & p53 immunohistochemical expression in triple negative breast cancer. BMC Clin Pathol 2018;18:9.
22. Goyal A, Sahu RK, Kumar M, Sharma S, Qayyum S, Kaur N, *et al*. p16 promoter methylation, expression, and its association with estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 subtype of breast carcinoma. J Cancer Res Ther 2019;15:1147-54.
23. Chen C, Huang Y, Zhang C, Liu T, Zheng HE, Wan S, *et al*. Circulating antibodies to p16 protein-derived peptides in breast cancer. Mol Clin Oncol 2015;3:591-4.
24. Zhao H, Zhang X, Han Z, Wang Y. Circulating anti-p16a IgG autoantibodies as a potential prognostic biomarker for non-small cell lung cancer. FEBS Open Bio 2018;8:1875-81.
25. Heidari Z, Mahmoudzadeh Sagheb H, Charkhat Gorgich EA.

Immunohistochemical expression of P16ink4a in colorectal adenocarcinoma compared to adenomatous and normal tissue samples: A study on southeast iranian samples. Iran Red Crescent Med J 2017;19.
26. Caponio MA, Addati T, Popescu O, Petroni S, Rubini V, Centrone M, *et al*. P16(INK4a) protein expression in endocervical, endometrial and metastatic adenocarcinomas of extra-uterine origin: Diagnostic and clinical considerations. Cancer Biomark 2014;14:169-75.
27. Golmohammadi R, Elyasi H, Golmohammadi M. Relationship between P16 expression and breast cancer using histology and immunohistochemistry. J Mazand Univ Med Sci 2017;27:181-6.
28. Bazarov AV, Lee WJ, Bazarov I, Bosire M, Hines WC, Stankovich B, *et al*. The specific role of pRb in p16 (INK4A) -mediated arrest of normal and malignant human breast cells. Cell Cycle 2012;11:1008-13.
29. Dublin EA, Patel NK, Gillett CE, Smith P, Peters G, Barnes DM. Retinoblastoma and p16 proteins in mammary carcinoma: Their relationship to cyclin D1 and histopathological parameters. Int J Cancer 1998;79:71-5.
30. Geradts J, Wilson PA. High frequency of aberrant p16(INK4A) expression in human breast cancer. Am J Pathol 1996;149:15-20.
31. Gorgoulis VG, Koutroumbi EN, Kotsinas A, Zacharatos P, Markopoulos C, Giannikos L, *et al*. Alterations of p16-pRb pathway and chromosome locus 9p21-22 in sporadic invasive breast carcinomas. Mol Med 1998;4:807-22.
32. Grupka NL, Bloom C, Singh M. Expression of retinoblastoma protein in breast cancer metastases to sentinel nodes: Evaluation of its role as a marker for the presence of metastases in non-sentinel axillary nodes, and comparison to p16INK4a. Appl Immunohistochem Mol Morphol 2006;14:63-70.
33. Hui R, Macmillan RD, Kenny FS, Musgrove EA, Blamey RW, Nicholson RI, *et al*. INK4a gene expression and methylation in primary breast cancer: Overexpression of p16INK4a messenger RNA is a marker of poor prognosis. Clin Cancer Res 2000;6:2777-87.
34. Milde-Langosch K, Bamberger AM, Rieck G, Kelp B, Löning T. Overexpression of the p16 cell cycle inhibitor in breast cancer is associated with a more malignant phenotype. Breast Cancer Res Treat 2001;67:61-70.
35. Shan M, Zhang X, Liu X, Qin Y, Liu T, Liu Y, *et al*. P16 and p53 play distinct roles in different subtypes of breast cancer. PLoS One 2013;8:e76408.
36. Zhang J, Lu A, Beech D, Jiang B, Lu Y. Suppression of breast cancer metastasis through the inhibition of VEGF-mediated tumor angiogenesis. Cancer Ther 2007;5:273-86.
37. Radisky DC, Santisteban M, Berman HK, Gauthier ML, Frost MH, Reynolds CA, *et al*. p16(INK4a) expression and breast cancer risk in women with atypical hyperplasia. Cancer Prev Res (Phila) 2011;4:1953-60.
38. Naji-Haddadi S, Elieh-Ali-Komi D, Aghayan S, Asghari R, Rasouli J. Investigation of p16 protein expression and its association with histopathologic parameters in breast cancer. Mol Biol Res Commun 2021;10:165-70.
39. Salih MM, Higgo AA, Eed EM. Prognostic significance of p16 protein expression in breast cancer. *In Vivo* 2022;36:336-40.
40. Jovanovic DV, Mitrovic SL, Milosavljevic MZ, Ilic MB, Stankovic VD, Vuletic MS, *et al*. Breast cancer and p16: Role in proliferation, malignant transformation and progression. Healthcare (Basel) 2021;9:1240.