

# Upregulated CDKN2A expression may be an independent protective factor in Luminal-like breast cancer

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## Research

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# Abstract

**Background** Previous studies revealed that CDKN2A (cyclin-dependent kinase inhibitor 2A) functioned as a tumour suppressor in various types of malignant tumours. The aim of the study was to clarify the value of CDKN2A expression in the prognosis of breast cancer.

**Method** Using the Cancer Genome Atlas (TCGA) database, we compared CDKN2A mRNA levels between breast cancer tissues and normal tissues and analyzed the relationship between clinical features and CDKN2A expression with the Wilcoxon test and the Kruskal-Wallis test. Kaplan-Meier and Cox analyses were performed to determine the correlation between CDKN2A expression and breast cancer prognosis. Gene set enrichment analysis (GSEA) was performed using the TCGA data set.

**Results** We first found that CDKN2A expression was markedly higher in breast cancer tissues than in normal tissues using the TCGA database ( $P=0.000$ ). In addition, CDKN2A mRNA expression in breast cancer was positively correlated with age ( $P=0.018$ ), histological types ( $P=0.028$ ), ER status ( $P=0.000$ ), PR status ( $P=0.000$ ) and molecular subtypes ( $P=0.000$ ). Kaplan-Meier analysis showed that increased CDKN2A expression was associated with increased survival time in breast cancer patients ( $P=0.000$ ), especially in Luminal-like subtype. Univariate and multivariate Cox analyses indicated that CDKN2A expression was an independent prognostic biomarker for breast cancer ( $P=0.037$ ). GSEA suggested that pathways involving cell adhesion molecules (CAMs), cytokine-receptor interactions, cytosolic DNA sensing, the cell cycle, and killer cell-mediated cytotoxicity were differentially enriched in the CDKN2A-high expression group.

**Conclusion** Our research demonstrated that high CDKN2A mRNA expression was an independent protective factor for improved prognosis in Luminal-like breast cancer. Additionally, the signaling pathways related to CAMs, cytokine-receptor interactions, cytosolic DNA sensing, the cell cycle, and killer cell-mediated cytotoxicity regulated by CDKN2A mRNA expression should be further studied.

## Background

Breast cancer (BC) has the highest incidence of any cancer not only in China (38.9% of total cases) but also worldwide (30% of total cases) and is also second most common female malignancy worldwide[1,2]. Based on the expression of different molecular biomarkers, breast cancer patients are grouped into four molecular subtypes: luminal A, luminal B, HER-2 enriched, and triple-negative BC(TNBC) [3-5]. Accordingly, dependent on molecular subtypes, there are numerous standard protocols and emerging treatments for breast cancer patients in the last few years[6,7]. However, treatment resistance, tumour metastasis and recurrence in breast cancer seriously influence patient survival and quality of life[8]. Therefore, it remains important to detect effective prognostic biomarkers and therapeutic targets for breast cancer patients.

Indeed, recent studies focus on an ideal target for breast cancer molecular targeting therapy. CDKN2A, which was situated on chromosome 9p21 and encoded the tumour suppressor protein p16[9], played a

vital role in the occurrence of melanoma in the earliest stage[10]. The function and importance of CDKN2A in different types of cancer have been frequently inferred. Steven et al. demonstrated that CDKN2A served as a tumour suppressor in mice and prevented plexiform neurofibroma progression[11]. Moreover, another study showed that increased CDKN2A immunostaining indicated increased survival time in cats with oral squamous cell carcinomas[12]. In addition, Ganjoo's study noted that CDKN2A deletion was associated with poor prognosis in histological subtypes of sarcoma[13]. Similarly, by inhibiting CDKN2A/p16, C-terminal binding protein-2 promoted proliferation and migration, as well as lower p16 expression was predictive of lower survival rate in BC patients[14]. In contrast, Lee et al. suggested that elevated CDKN2A expression in breast cancer predicted poor survival outcomes[15].

Given the controversy surrounding CDKN2A, we used TCGA data to identify the role of CDKN2A expression in breast cancer. Furthermore, GSEA was in order to recognize signalling pathways activated in breast cancer about the mechanism of CDKN2A. In our present study, we revealed that increased CDKN2A expression correlated with increased survival time in breast cancer. GSEA showed that signalling pathways associated with cell adhesion molecules (CAMs), the cell cycle, cytokine-receptor interactions, DNA replication, killer cell-mediated cytotoxicity and P53 were enriched in breast cancer patients with high CDKN2A expression.

## Methods

### Data mining

We downloaded CDKN2A expression data and corresponding clinical information from TCGA database (<https://portal.gdc.cancer.gov/>)[16].

### GSEA

GSEA is a powerful processor that catches on several cancer-related datasets and reveals many common biological pathways[17]. In our study, GSEA was implemented to distinguish signalling pathways activated in breast cancer between high and low CDKN2A groups. Gene sets with a normal P-value <0.05 and a false discovery rate (FDR) <0.25 were considered to be significantly enriched.

### Statistical analysis

Most statistical analyses were conducted using R (v.3.6.0). Differential expression of CDKN2A between normal tissues and tumour tissues in breast cancer patients was evaluated by Wilcox test. The relationship between clinicopathological parameters and CDKN2A mRNA expression was analysed with Wilcox test and Kruskal-Wallis test. Patients were assigned into two groups (high and low CDKN2A level) according to median value. Chi-square test was used to analyze associations of CDKN2A expression with clinicopathological data clinicopathological characteristics through IBM SPSS 24.0 (SPSS Inc, Chicago, USA) software. Survival analysis was displayed by Kaplan-Meier method and log-rank test. Univariate

and multivariate analysis was implemented to distinguish prognostic factors with the Cox proportional hazard regression test. P-values of  $< 0.05$  were regarded as statistically significant.

## Results

### Differential expression of CDKN2A in breast cancer patients based on TCGA data

First, the CDKN2A expression data of 122 normal tissues and 1,066 tumor tissues from breast cancer patients were downloaded from TCGA in March 2019. We detected that CDKN2A levels were markedly higher in tumor tissues ( $P= 0.000$ ) (Figure 1A). Besides, we compared CDKN2A mRNA levels in normal tissues and matched tumor tissues in breast cancer patients. Similarly, CDKN2A levels were higher in the tumor tissues ( $P= 0.000$ ) (Figure 1B), which implies that CDKN2A may play a relevant role in breast cancer.

### Clinicopathological parameters

As displayed in Table 1, we analysed Clinicopathological parameters of 832 female breast cancer patients according to clinical data downloaded from TCGA after excluding incomplete clinical prognostic information. The median age of confirmed diagnosis was 58 years old. Most of pathologic types (73.9%,  $n= 615$ ) were of infiltrating ductal carcinoma, 19.6% ( $n= 163$ ) were infiltrating lobular carcinoma, least were mixed histology ( $n= 25$ ) and another specific carcinoma ( $n= 29$ ). Moreover, clinical stage I of breast cancer was found in 143 patients (17.19%), stage II was found in 482 patients (57.93%), stage III was found in 193 patients (23.20%), and stage IV was found in 14 patients (1.68%). Besides, there were four different subtypes of breast cancer, including Luminal A (73.6%,  $n= 613$ ), Luminal B (7.7%,  $n= 64$ ), HER2-enriched (17.1%,  $n= 142$ ) and Triple negative (1.6%,  $n= 13$ ). The mean and median follow-up times after surgery were 3.2 and 2.3 years respectively. During the 23 years of follow-up in this survey, 109 (13.1%) patients died.

**Table 1.** Clinicopathological parameters of the breast cancer patients.

Clinicopathological parameters		N
Age		
≤58		416
>58		416
Histological type		N
Infiltrating ductal carcinoma		615
Infiltrating lobular carcinoma		163
Mixed histology		25
Another specific carcinoma		29
Tumor classification		
T1		220
T2		486
T3		104
T4		22
Lymphatic classification		
N0		396
N1		281
N2		105
N3		50
Distant classification		

M0	818
M1	14
Clinical stage	
I	143
II	482
III	193
IV	14
Estrogen receptor	
Positive	666
Negative	166
Progesterone receptor	
Positive	583
Negative	249
HER2	
Positive	77
Negative	755
Molecular subtypes	
Luminal A	613
Luminal B	64

Triple negative	142
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HER2-enriched	13
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Survival status	
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Death	109
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Survival	723
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### **CDKN2A expression in breast cancer tissues according to clinical characteristics**

We further investigated CDKN2A expression between tumour tissues and normal tissues in breast cancer patients based on clinical characteristics (Figure 2). Our results revealed that there were differences in CDKN2A expression among four molecular subtypes ( $P= 0.000$ ). In addition, we found that as the clinical stage increased, CDKN2A expression decreased inversely ( $P= 0.491$ ). However, no differences were observed according to TNM stage, distant metastasis and HER2 status ( $P= 0.917$ ,  $P= 0.458$ ,  $P= 0.5$  and  $P= 0.579$  respectively).

### **CDKN2A expression is associated with clinicopathological features**

To evaluate the clinical significance of CDKN2A, we analysed the associations between CDKN2A expression and the clinicopathological characteristics by chi-square test. CDKN2A expression was correlated with age ( $P= 0.018$ ), histological types ( $P= 0.028$ ), ER status ( $P= 0.000$ ), PR status ( $P= 0.000$ ) and molecular subtypes ( $P= 0.000$ ), respectively. However, no significant correlation was detected in TNM stage and HER2 status (Table 2).

**Table 2.** Relationships of CDKN2A expression with clinical features based on TCGA data.

Variable		CDKN2A (n)		
		low	high	<i>P</i> <sup>1</sup>
		n=416	n=416	
Age(years)	≤58	191	225	<b>0.018</b>
	>58	225	191	
Histological types	Infiltrating ductal carcinoma	315	300	<b>0.028</b>
	Infiltrating lobular carcinoma	69	94	
	Mixed histology	18	7	
	Other specific carcinoma	14	15	
T classification	T1	113	107	0.924
	T2	239	247	
	T3	52	52	
	T4	12	10	
Lymphatic metastasis	N0	195	201	0.051
	N1	135	146	
	N2	65	40	
	N3	21	29	
Distant metastasis	M0	409	409	1.000
	M1	7	7	
Clinical stage	I	69	74	0.831

	II	238	244	
	III	102	91	
	□	7	7	
ER	Positive	364	302	<b>0.000</b>
	Negative	52	114	
HR	Positive	316	267	<b>0.000</b>
	Negative	100	149	
HER2	Positive	41	36	0.550
	Negative	375	380	
Molecular subtypes	Luminal A	334	279	<b>0.000</b>
	Luminal B	35	29	
	Triple negative	41	101	
	HER2-enriched	6	7	

The bold number is on behalf of the P-values with significant differences. <sup>1</sup>P Value was calculated by  $\chi^2$  test. ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

### High CDKN2A expression is an independent protective factor for OS

Survival analysis showed that high CDKN2A expression was positively associated with overall survival (OS) (P=0.000, Figure 3A). We also found that increased CDKN2A expression was related to superior overall survival, in spite of age, lymphatic metastasis and HER2 status. Beyond that, high CDKN2A expression was correlated

with superior OS, especially in breast cancer patients with T2-T3 classification, clinical stage II-III and luminal-like subtype but without distant metastasis (Figure 3B-P).

Univariate Cox regression analysis showed that CDKN2A expression, age, TNM stage and HER2 status were significantly associated with OS (Table 3). Moreover, multivariate analysis confirmed that CDKN2A expression and age were independent predictors of OS in breast cancer patients (Table 3).

**Table 3.** Univariate and multivariate analyses in breast cancer patients

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value
CDKN2A	0.701	0.584-0.842	<b>0.000</b>	0.976	0.953-0.998	<b>0.037</b>
Age	1.033	1.018-1.049	<b>0.000</b>	1.030	1.014-1.046	<b>0.000</b>
T	1.624	1.280-2.059	<b>0.000</b>	1.260	0.964-1.645	0.091
N	1.624	1.334-1.978	<b>0.000</b>	1.267	0.999-1.607	0.051
M	3.251	1.961-5.391	<b>0.000</b>	1.885	0.925-3.842	0.081
STAGE	0.571	0.419-1.049	<b>0.000</b>	0.704	0.482-1.028	0.069
ER	1.067	0.697-1.632	0.765			
PR	1.225	0.831-1.806	0.304			
HER-2	0.501	0.312-0.811	<b>0.004</b>	0.743	0.441-1.253	0.265
Molecular types	1.143	0.939-1.391	0.182			

HR: Hazard ratio; CI: Confidence interval. The bold number is on behalf of P-values with significant differences.

## CDKN2A-related signaling pathway

We implemented GSEA to recognize the signalling pathways activated in breast cancer by comparing the low and high CDKN2A expression datasets. GSEA showed significant differences (FDR<0.25, NOM P-value <0.05) in the enrichment of the MSigDB Collection pathways. As shown in Table 4, we chose the signalling pathways that were most significantly enriched in breast cancer patients with high CDKN2A expression, including pathways related to bladder cancer, CAMs, the cell cycle, cytokine-receptor interactions, cytosolic DNA sensing, DNA replication, killer cell-mediated cytotoxicity and P53 (Figure 4).

**Table 4.** Gene sets enriched in the high CDKN2A expression.

Gene set name	NES	NOM p-val	FDR q-val
BLADDER_CANCER	1.857	0.006	0.108
DNA_REPLICATION	1.853	0.013	0.094
CELL_ADHESION_MOLECULES_CAMS	1.763	0.032	0.062
CELL_CYCLE	1.780	0.040	0.058
CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	1.656	0.042	0.086
NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	1.634	0.043	0.085
P53_SIGNALING_PATHWAY	1.602	0.047	0.093
CYTOSOLIC_DNA_SENSING_PATHWAY	1.556	0.048	0.114

NES: normalized enrichment score; NOM: nominal; FDR: false discovery rate. Gene sets with NOM p-val $\leq$ 0.05 and FDR q-val $\leq$ 0.25 are regarded as significant.

## Discussion

Breast cancer (BC) accounts for approximately 2.1 million new cases and 62,000 deaths per year, with high mortality and morbidity rates in women worldwide[1]. Meanwhile, owing to gene expression profiles of breast cancer, there were four molecular subtypes as well as each requires different treatment[18]. Despite substantial research efforts devoted to exploring effective biomarkers for breast cancer, few basic research results have been applied in the clinical field. As a consequence, it is badly in need of

recognizing truly effective biomarkers and therapeutic targets for breast cancer. Recent studies have reported that CDKN2A functions as a tumour suppressor in different types of carcinoma, such as melanoma[10], ovarian cancer[19], lung cancer[20], atypical neurofibroma[11], malignant mesothelioma[21] and bladder cancer[22]. Several studies also showed that decreased CDKN2A expression correlated to poor prognosis in osteosarcoma[23], soft tissue sarcoma[13], and oral squamous cell carcinoma[24].

With respect to the roles of CDKN2A expression in breast cancer, Feng et al found that KDM2B promoted triple-negative breast cancer cell proliferation by suppressing CDKN2A/p16 expression[25]. Fu et al. also showed that C-terminal binding protein-2 motivated proliferation and migration in breast cancer via the inhibition of CDKN2A/p16[14]. The above results suggest that reduced CDKN2A expression contributes to poor outcomes in breast cancer. However, contrasting results showed that increased expression of CDKN2A/p16 was associated with poor prognosis in breast cancer [15].

Therefore, there still exists controversy regarding the prognostic value of CDKN2A in BC, and signalling pathways related to the regulatory mechanism have not yet been determined. In our study, we first found that CDKN2A expression was markedly elevated in BC tissues than in normal tissues using TCGA database, which implied that CDKN2A may play its part in the tumorigenesis of BC. Thus, we further analysed the relationship between CDKN2A expression and clinical features of BC patients. The results showed that the CDKN2A expression was positively related to age, ER status, PR status and molecular subtypes in breast cancer. Then, the Kaplan-Meier curves for OS also showed that reduced CDKN2A expression correlated to decreased survival time, especially in Luminal-like subtype, a finding that was in contrast to the findings of Lee's study[15] but was consistent with the previously identified roles of CDKN2A in breast cancer[14,25,23]. Then Univariate and multivariate Cox analyses indicated CDKN2A expression and age were prognostic biomarkers for BC patients. Additionally, to identify the potential signalling pathways, we implemented GSEA comparing the low and high CDKN2A expression datasets and observed that pathways related to bladder cancer, CAMs, the cell cycle, cytokine-receptor interactions, cytosolic DNA sensing, DNA replication, killer cell-mediated cytotoxicity and P53 were enriched in breast cancer. A previous study showed that loss of CDKN2A negatively regulated with the cell cycle and CDK4/6/RB pathway in Luminal-like breast cancer patients[26]. Meanwhile, another study pointed out that cancer gene therapy mediated by CDKN2A was beneficial for the induction of cell death in non-small-cell lung carcinoma[20]. In addition, we found CDKN2A mRNA expression was associated with bladder cancer, the cell cycle and DNA replication[27-29]. However, it is the first time that the results in this paper showing correlations between CDKN2A expression and signalling pathways involving CAMs, cytokine-receptor interactions, cytosolic DNA sensing and killer cell-mediated cytotoxicity are reported, as well as biological mechanism needs to be further clarified.

## Conclusions

In conclusion, upregulated CDKN2A expression may be an effective prognostic biomarker in luminal-like breast cancer suggesting longer survival times. However, we should focus on the association between

luminal-like subtypes and CDKN2A to inform the current controversial results. Additionally, signalling pathways related to CAMs, cytokine-receptor interactions, cytosolic DNA sensing and killer cell-mediated cytotoxicity may be pivotal pathways regulated by CDKN2A in breast cancer, and these relationships should be further clarified.

## Abbreviations

CDKN2A: cyclin dependent kinase inhibitor 2A; TGCA: The Cancer Genome Atlas; GSEA: Gene Set Enrichment Analysis; OS: overall survival; HR: Hazard ratio; CI: Confidence interval; NES: normalized enrichment score; NOM: nominal; FDR: false discovery rate

## Declarations

### Acknowledgements

None.

### Authors' contributions

YEQ and YJW participated in the study design. WJX and GWL contributed to data collection and analysis. All authors were involved in the writing of the article. YEQ critically reviewed the manuscript. All authors have read and approved the final manuscript.

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### Availability of data and materials

The datasets analyzed during the current study are available in the TGCA (<https://portal.gdc.cancer.gov/>).

### Ethics approval and consent to participate

None.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

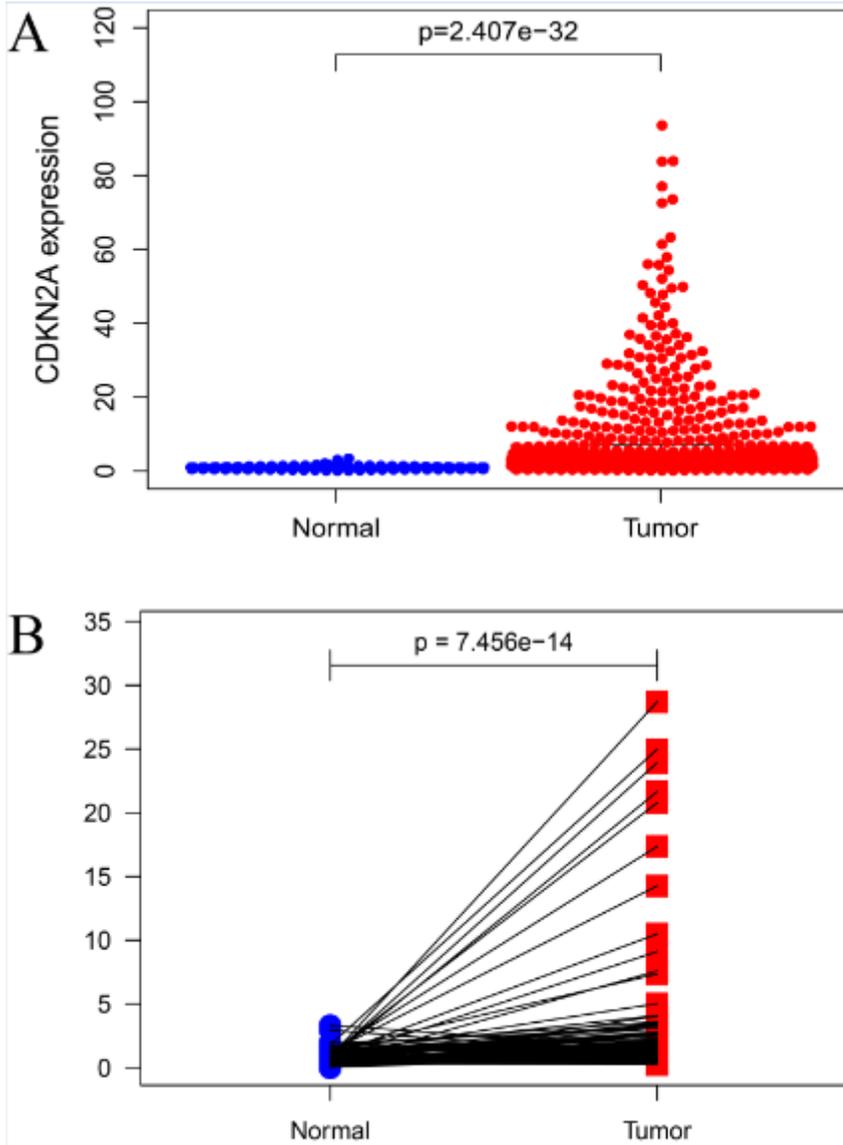
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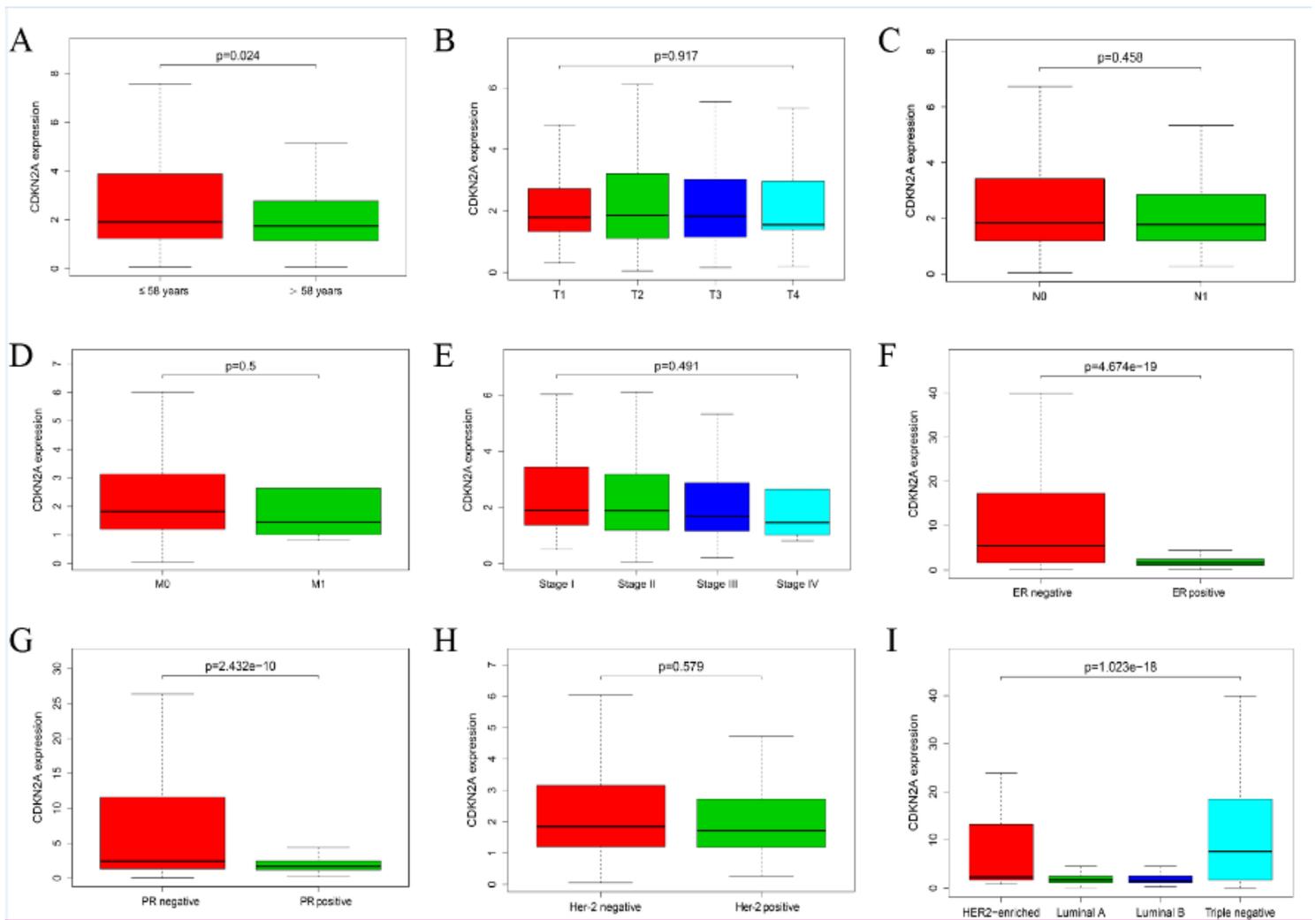
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## Figures



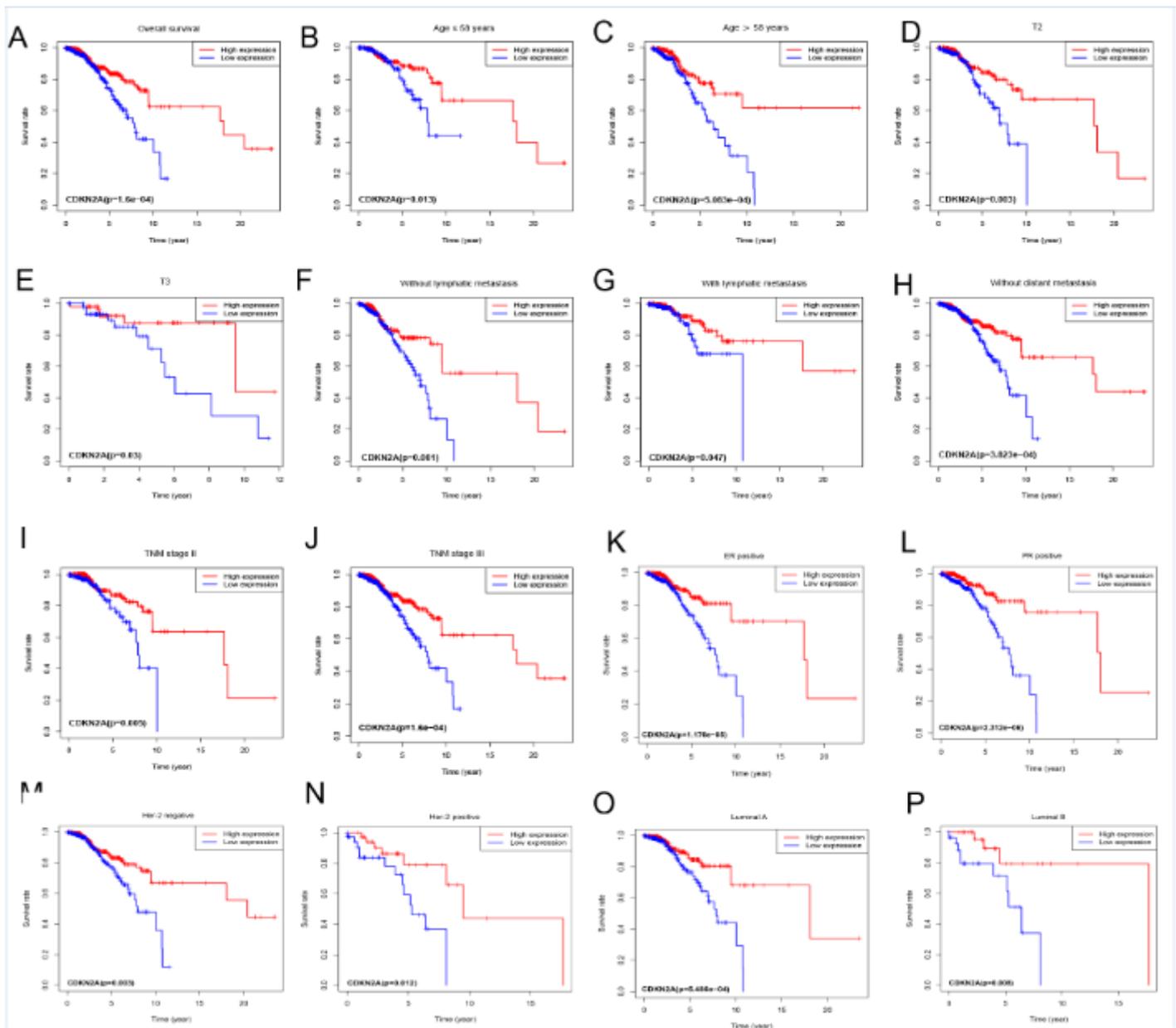
**Figure 1**

CDKN2A expression in breast cancer patients based on TCGA data. A: Scatter gram represents CDKN2A expressions from normal to tumor tissues, respectively; B: A dot plot with connecting lines represents CDKN2A expressions from normal to matched tumor tissues. P-values of  $< 0.05$  were considered significant.



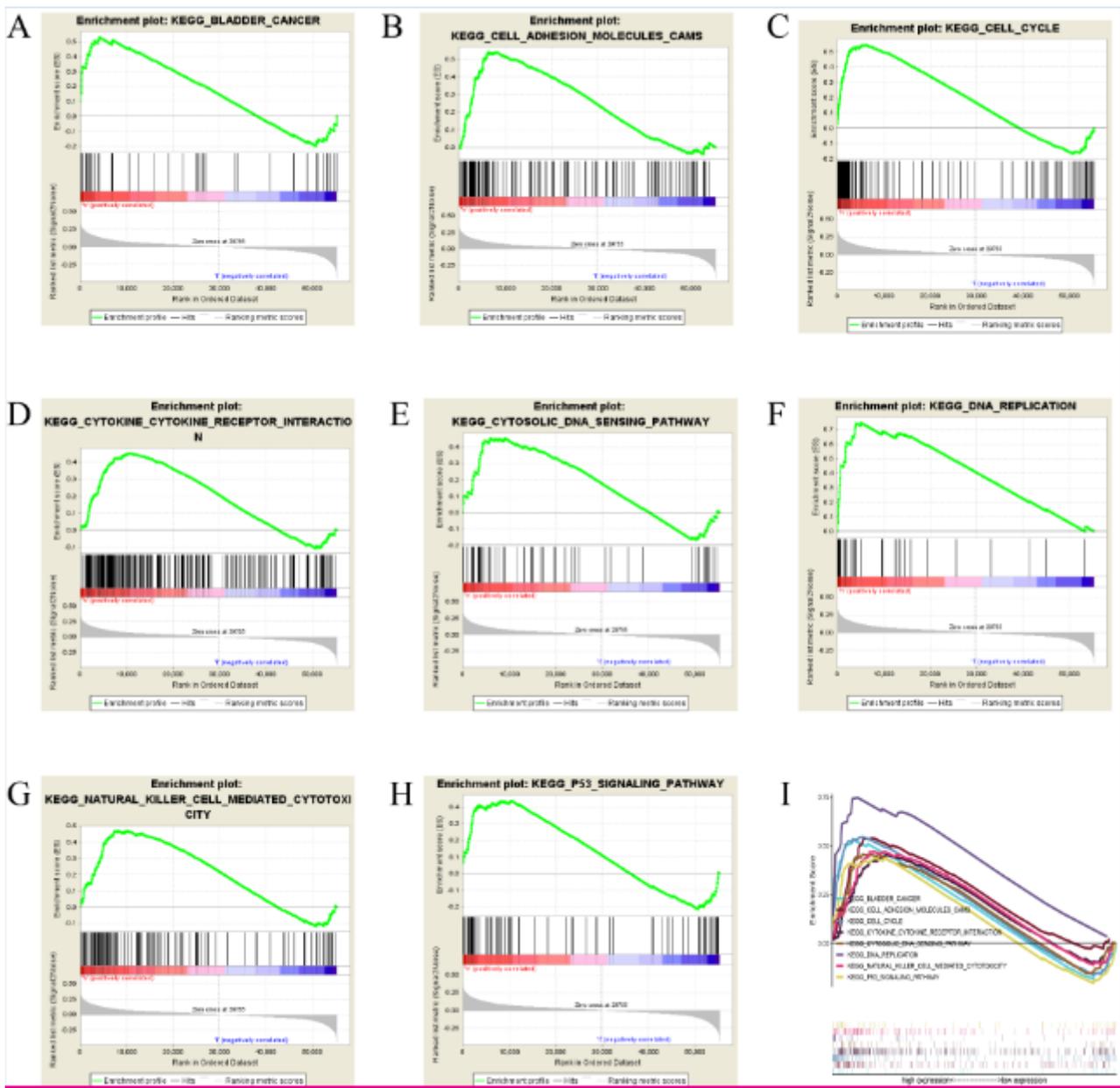
**Figure 2**

CDKN2A expression on the basis of TCGA data. A-F: CDKN2A expression was compared according to patient age, T classification, lymph node, distant metastasis and clinical stage, respectively.



**Figure 3**

Survival curves on TCGA data. (A): Comparisons of the overall survival duration between the low and high CDKN2A expression; (B-C): Independent of patient age; (D-E): Independent of T classifications; (F-G): Independent of lymphatic metastasis; (H): Independent of distant metastasis; (I-J): Independent of clinical stage; (K-P): Independent of ER status, PR status, HER2 and molecular subtypes.



**Figure 4**

Enrichment plots from GSEA. Different pathways including bladder cancer (A), cell adhesion molecules cams (B), cell cycle (C), cytokine receptor interaction (D), cytosolic DNA sensing cell cycle (E), DNA replication (F), killer cell mediated cytotoxicity (G) and P53 signaling (H) are enriched in breast cancer patients with high CDKN2A expression according to GSEA. (I): Sum of 8 enriched pathways in breast cancer patients with high CDKN2A expression.