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Original Article

Prognostic value of PD-L1 for invasive breast cancer and its miR-34a-related mechanism of regulation

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Abstract: The goal of this study was to explore the relationship between expression of programmed death ligand-1 (PD-L1) protein and commonly assessed clinicopathological features as well as its prognostic value for invasive breast cancer patients. The effect of miR-34a on PD-L1 both *in vitro* and *in vivo* was also investigated. Among the 287 patients included in this study, 165 showed PD-L1 overexpression. PD-L1 was significantly correlated with ER, PR, Her-2, Ki-67, and molecular subtypes. ER+, PR+, Her-2-, Ki67>14% and Ki67≤14% patients with PD-L1 overexpression had a poorer prognosis. PD-L1 expression decreased along with the addition of a miR-34a mimic but increased along with the addition of a miR-34a inhibitor both at mRNA and protein level. miR-34a mimics can downregulate the luciferase activity of 231 cells transfected with wild-type PD-L1 plasmids. CCK-8 and transwell tests show that cell proliferation and invasion ability decreased after transfection with a miR-34a mimic. Xenografts with intra-tumoral injection of miR-34a agomir had a significantly lower growth rate. miR-34a expression when treated with miR-34a agomir was significantly higher and PD-L1 protein expression when treated with miR-34a agomir was significantly lower. Patients with overexpressed PD-L1 have a relatively poorer prognosis. miR-34a can negatively regulate PD-L1. miR-34a may be a promising therapy target for triple-negative breast cancer patients with PD-L1 overexpression.

Keywords: PD-L1, invasive breast cancer, prognosis, miR-34a

Introduction

As the most common malignant tumor and the second leading cause of cancer-related death in women, breast cancer draws a lot attention worldwide [1]. In China, breast cancer has been the most malignant type of tumor among women in terms of both incidence and mortality during recent years [2]. The prognosis of breast cancer is mainly evaluated based on histological grade, lymph node staging, pathological tumor staging (TNM) and four major protein molecular biomarkers: estrogen receptor (ER), progesterone receptor (PR), Ki67 and human epidermal growth factor receptor 2 (Her-2) [3, 4]. Some researchers have begun to study immunotherapy strategies and drugs that are applicable for breast cancer as they can be used on immune pathways to escape the anti-tumor immune response, in order to maintain tumor cell proliferation and metastasis [5]. However, more effective prognosis biomarkers and individualized treatment strategies for invasive breast cancer remain to be identified.

As a member of the B7 family, programmed cell death 1 (PD-1) is a immunoregulatory cell surface proteins that has two cognate ligands, PD-L1 and PD-L2. Expression of PD-1 and PD-L1 in the tumor microenvironment plays a major role in tumor immune evasion [6]. PD-L1 is found to be expressed not only in tumor infiltrating lymphocytes, but also in some tumor cells, including that of breast, prostate, lung and gastrointestinal cancers and malignant melanoma [7-11]. Additionally, high expression of PD-L1 in breast, non-small cell lung, pancreatic, and renal cell carcinoma has been found to be associated with a poor prognosis [12-14].

Scientists first detected miR-34 in *C. elegans* in 2001, and a total of 83 miR-34 members have

been found [15]. In simple creatures such as *C. elegans*, miR-34 has only one transcript; while normal human tissue contains miR-34a, miR-34b, and miR-34c [16]. Recent studies have reported that miR-34a can affect cancer cell proliferation [17] and promote tumor cell apoptosis [18] in pancreatic cancer, ovarian cancer and retinoblastoma [19-22].

In this study, the value of PD-L1 was evaluated in order to predict the prognosis of invasive breast cancer patients through the analysis of tissue microarrays. Then, the regulatory relationship between PD-L1 and miR-34a, as well as their effect on the proliferation, migration and invasion abilities of triple-negative breast carcinoma cell line MDA-MB-231 was investigated.

Material and methods

Samples from breast cancer patients and immunostaining of tissue microarrays

The tissue arrays purchased from the Shanghai Outdo Biotech co, Ltd contained 300 samples from invasive breast carcinoma patients. Expression of PD-L1, ER, PR, Ki-67, Her2, CK5/6, P53, and epidermal growth factor receptor (EGFR) in the samples were detected through immunohistochemistry (IHC).

Scoring, evaluation and statistical analysis

IHC staining was evaluated by two experienced pathologists, who were blinded from the clinical information. PD-L1 staining intensity in the cytoplasm of tumor cells was scored from 0-3 and the percentage of PD-L1-positive cells was scored 0-4 (0-5, 6-25, 26-50, 51-75 and 76-100%, respectively). The sum of staining intensity and positive cell percentage scores was taken as the final PD-L1 expression score, which when ranged between 0 and 3 was defined as negative, and when between 4 and 7 as positive. This expression criterion that takes into account staining intensity and staining percentage was selected by referring to other similar research studies [23]. ER and PR expression was mainly found in the nucleus, with their expression criteria compared with that of international standards [24], for which a positive staining ratio of >1% was positive, while a ratio of <1% is negative. Positive Ki-67 expression was >14%. For the Her-2 immunohistochemical results, 0 and + were defined as a negative result and +++ was defined as a positive result, while the PathVysion HER-2 DNA Probe kit (Abbott Pharmaceutical Co., Ltd., Lake Bluff, IL, USA) was used to determine the expression of ++ samples. Her2 expression was designated as either weak (IHC grade 0-1+ or FISH-) or strong (IHC grade 3+ or FISH+).

Cell culture and transfection

The human breast cancer cell line MDA-MB-231 was grown in Leibovitz's L-15 medium (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin solution at 37°C without extra CO_2. Well cultured cells were seeded at a density of 1×10^5 cells per well into six-well culture plates and cultured in Opti-MEM (Gibco, USA), overnight. Transfection with the miR-34a mimic, miR-34a inhibitor, miR-34a mimic NC (negative control) and miR-34a inhibitor NC (GenePharma, Shanghai, China) were conducted using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) and continued for about 6 hours. Cells were harvested at 48 or 72 hours after transfection, depending on the condition of cell proliferation.

Western blotting

Extracted protein was electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked with laboratory skim milk and then incubated at 4°C overnight with primary antibodies. Primary antibodies against PD-L1 (Cell Signaling Technology, Boston, USA) and GAPDH (Cell Signaling Technology, Boston, USA) were used at 1:1000 and 1:2000 dilution, respectively. Then, secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology, Boston, USA) were incubated with the samples for 2 hours at room temperature. The target protein bands were visualized using a chemiluminescence enhanced chemiluminescence kit (Guge Biological Technology, Wuhan, China).

Real-time PCR

Total RNA from the transfected 231 cells and control group were extracted using the TRIzol Reagent (Invitrogen). The primer pairs used for RT-PCR were: PD-L1 forward 5'-GAACTACCTC-

TGGCACATCCTC-3' and reverse 5'-GTATCACTTTGCTTCTTTGAGTTTGT-3'; miR-34a forward 5'-TGGCAGTGTCTTAGCTGGTTGT-3'; GAPDH forward 5'-TGTTGCCATCAATGACCCCTT-3' and reverse 5'-CTCCACGACGTACTCAGCG-3'; U6 forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'. PD-L1 expression was normalized with GAPDH and miR-34a expression with U6, and the different expression levels were evaluated relative to that of the control group. For each sample, experiments were performed in triplicate to confirm the results.

Luciferase reporter assay

The luciferase reporter plasmid contains 3'UTR of PD-L1 WT and PD-L1 MU oligonucleotides that correspond to the miR-34a binding site that was made by Ribobio. MDA-MB-231 cells were seeded into 12-well plates that were transfected with 50 ng of the pMIR-REPORT™ or each reporter construct, 1 ng of the Renilla luciferase reporter (pRL-CMV vector, Ribobio), and 100 nmol/l of the miR-34a mimic, miR-34a mimic negative control, miR-34a inhibitor and miR-34a inhibitor negative control, respectively, using Lipofectamine™ 2000. Firefly and Renilla luciferase activities were measured sequentially using dual-luciferase assays (Promega) 24 hours post transfection. The experiments were performed in triplicate for each transient transfection assay.

Cell proliferation and invasion assays

Proliferation of the MDA-MB-231 cells was elevated using a CCK8 cell proliferation assay. The MDA-MB-231 cells treated with the miR-34a mimic and miR-34a mimic negative control were seeded into a 96-well plate at a density of about 4×10^3 cells/well and cultured for 24 hours. Then a CCK8 reagent (Dojindo, Japan) was added to the cells 24, 36, 48, 60 and 72 hours after incubation. The absorbance at 450 nm represented cell proliferation. The experiments were performed in triplicate. MDA-MB-231 cells with a miR-34a mimic and its negative control were cultivated without fetal bovine serum overnight, then put on the upper chamber of the 24-well pates and covered with BD Matrigel. L-15 medium with 20% fetal bovine serum was added to the lower chamber. After 24-36 hours of incubation, cells in the lower chamber were fixed in 4% paraformaldehyde. The number of cells in the lower chamber was calculated using microscopy and represents invasive ability. Additionally, transwell chambers without Matrigel were used to evaluate migration ability. All experiments were performed in triplicate.

Xenograft mouse model

All animal experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of Laboratory animals. Thirty female nude mice were divided equally into three groups and 5×10^5 MDA-MB-231 cells were subcutaneously injected into the breast fat pad of the right or left chest of each nude mouse. Tumor volumes were measured every 3 days (volume = 0.5 × length × width^2). MiR-34a agomir, miR-34a agomir negative and a mock were injected when tumor volume reached 25 mm^3. Injection was performed every 3 days and each mouse was injected with 1 nmol of the reagent at 3 or more sites each time. The mice were sacrificed after 54 days and tumor tissues were collected for RNA and protein extraction, as well as immunostaining.

Statistical analysis

The experimental data were analyzed using SPSS 22.0 software (Chicago, IL, USA). Differences between groups were compared using a t-test. Student's t test or ANOVA statistical analysis was applied to the mean value and to differences between groups for continuous variables. Correlation between variables was analyzed using the Pearson X^2 test. Patient lifetime analysis and log-rank test were performed using Kaplan-Meier survival analysis. Univariate and multivariate regression analyses were performed using Cox proportional hazards regression analyses. All *p* values are two-tailed, with a *p* value of <0.05 indicating a statistical difference.

Results

PD-L1 expression is associated with breast cancer clinicopathological variables

A total of 300 invasive breast cancer patients were involved with our tissue microarrays and 286 cases were included in this study. The other 14 cases were excluded due to missing

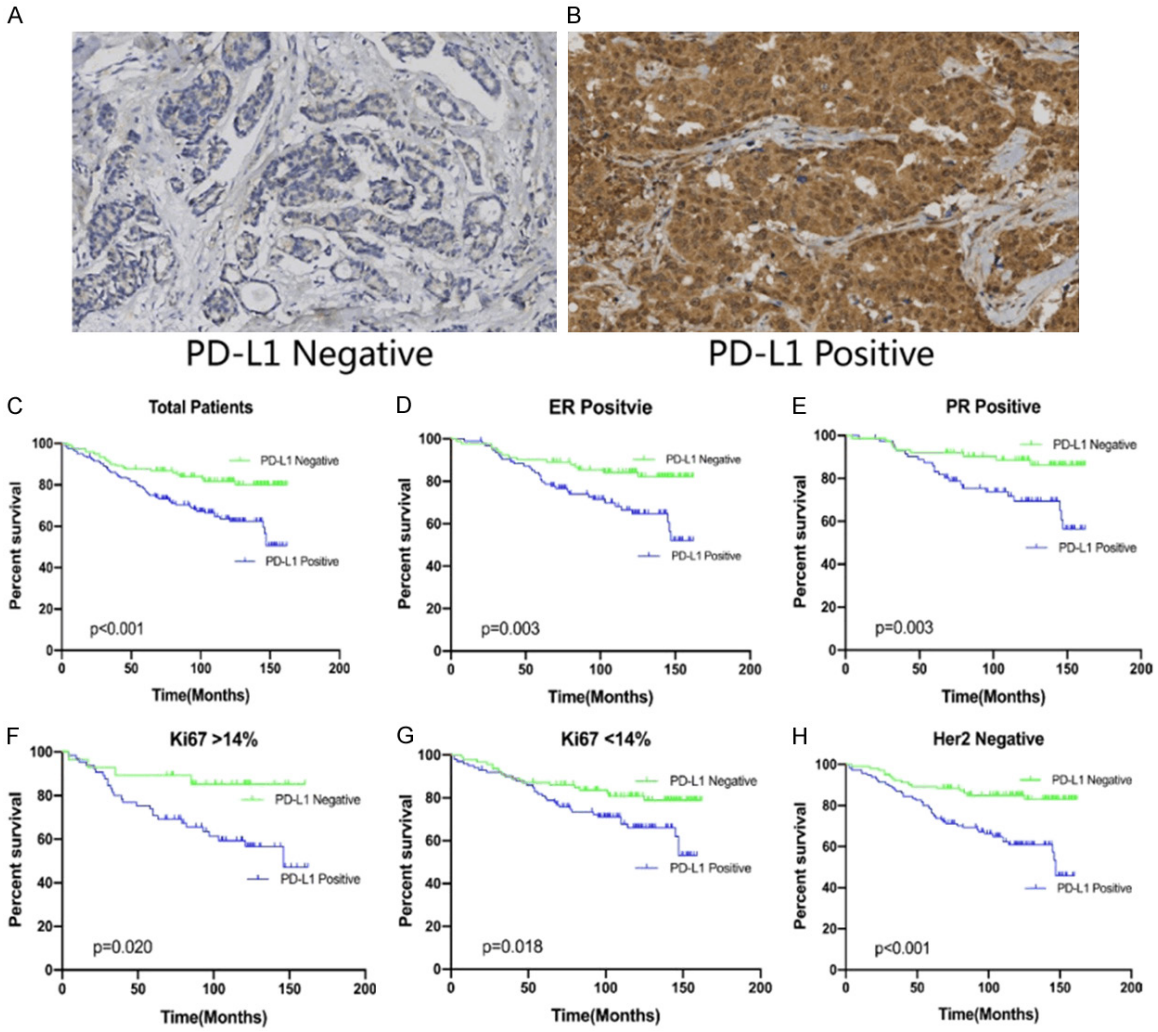

Figure 1. A. Example of negative immunohistochemistry staining of PD-L1 expression in breast cancer tissue. B. Example of positive immunohistochemistry staining of PD-L1 expression in breast cancer tissue. C. Kaplan-Meier analyses of PD-L1 expression and overall survival of invasive breast cancer patients. D. Kaplan-Meier analyses of PD-L1 expression and overall survival of ER positive patients. E. Kaplan-Meier analyses of PD-L1 expression and overall survival of PR positive patients. F. Kaplan-Meier analyses of PD-L1 expression and overall survival of Ki67>14% patients. G. Kaplan-Meier analyses of PD-L1 expression and overall survival of Ki67<14% patients. H. Kaplan-Meier analyses of PD-L1 expression and overall survival of Her-2 positive patients.

samples or because the stripping or immunohistochemistry results did not meet the inclusion criteria. All patients included in this study had received standardized surgery, chemotherapy, radiotherapy, endocrine therapy, and targeted therapy according to NCCN guidelines. As shown in **Figure 1**, 165 of the 286 (57.69%) patients had high expression of PD-L1. **Table 1** shows that there was significant correlation between PD-L1 and ER (p=0.002, X^2=10.507), PR (p=0.006, X^2=7.784), Her-2 (p=0.003, X^2=9.167), Ki-67 (p=0.005, X^2=8.404), and molecular classification (p<0.001 X^2=21.322). Among all subtypes, 42.4% (53/125) of Luminal A patients, 69.01% (49/71) of Luminal B patients, 68.75% (22/32) of Her-2 overexpression patients and 70.69% (41/58) of triple-negative patients had relatively high levels of PD-L1 expression. However, PD-L1 expression did not have a significant relationship with age (p=0.108, X^2=2.667), tumor site (p=0.185, X^2=2.016), histological grade (p=0.413, X^2=1.768), tumor diameter (p=0.326, X^2=2.243), lymph node metastasis (p=0.902, X^2=0.574), tumor clinical stage (p=0.897, X^2=0.217) CK5/6 level (p=0.183, X^2=2.154), P53 level (p=0.092, X^2=3.083) or EGFR level (p=0.058, X^2=3.726).

Table 1. Association between PD-L1 expression and clinical pathological factors of breast cancer

		PD-L1 expression		X^2	p
		Negative (121)	Positive (165)		
Age	<50	51	54	2.667	0.108
	≥50	70	111		
Tumor site	Right	74	87	2.016	0.185
	Left	47	78		
Histological grade	I	24	23	1.768	0.413
	II	88	129		
	III	9	13		
Tumor diameter (cm)	≤2	28	40	2.243	0.326
	>2, ≤5	76	111		
	>5	17	14		
Lymph node metastasis number	0	57	73	0.574	0.902
	1-3	33	46		
	4-9	24	33		
	≥10	7	13		
Clinical stage	0/1	14	21	0.217	0.897
	2	69	96		
	3	38	48		
ER	Negative	29	70	10.507	0.002
	Positive	92	95		
PR	Negative	48	93	7.784	0.006
	Positive	73	72		
Her-2	Negative	103	115	9.167	0.003
	Positive	18	50		
Ki-67 (%)	<14	93	100	8.404	0.005
	≥14	28	65		
CK5/6	Negative	101	126	2.154	0.183
	Positive	20	39		
p53	Negative	44	44	3.081	0.092
	Positive	77	121		
EGFR	Negative	88	102	3.726	0.058
	Positive	33	63		
Molecular classification	Luminal A	72	53	21.322	<0.001
	Luminal B	22	49		
	Her2 overexpression	10	22		
	Triple negative	17	41		

High expression of PD-L1is associated with a worse prognosis

Among these 286 invasive breast cancer patients, results of the Kaplan-Meier survival analysis showed that the expression of PD-L1 is significantly associated with OS (p=0.001). High PD-L1 expression patients had significantly shorter OS (**Figure 1**). Univariate COX regression analysis showed that T stage (p=0.003, HR=1.746, 95% CI: 1.210-2.520), N stage (p< 0.001, HR=1.493, 95% CI: 1.208-1.845), clinical tumor stage (p<0.001, HR=1.976, 95% CI: 1.379-2.793), molecular subtype (p=0.002, HR=1.319, 95% CI: 1.107-1.572), ER (p=0.027, HR=0.611, 95% CI: (0.395-0.945), PR (p= 0.002, HR=0.491, 95% CI: 0.314-0.767), PD-L1 (p=0.001, HR=2.317, 95% CI: 1.421-3.776) are the clinicopathological variants that had a significant HR value (**Table 2**). In the multivariate COX regressions, only PR (p=0.029, HR=0.518, 95% CI: 0.287-0.936) and PD-L1 (p=0.001,

Table 2. Univariate COX regression analysis of clinical pathological factors and prognosis of patients with invasive breast cancer

Variable	HR	95% CI	p
T stage	1.746	1.210-2.520	0.003
N stage	1.493	1.208-1.845	<0.001
Clinical tumor stage	1.976	1.379-2.793	<0.001
Molecular subtype	1.319	1.107-1.572	0.002
ER (positive vs. negative)	0.611	0.395-0.945	0.027
PR (positive vs. negative)	0.491	0.314-0.767	0.002
Her2 (positive vs. negative)	1.211	0.738-1.988	0.448
Ki67 (positive vs. negative)	1.377	0.881-2.151	0.160
CK5/6 (positive vs. negative)	1.351	0.815-2.239	0.243
EGFR (positive vs. negative)	1.316	0.843-2.056	0.227
p53 (positive vs. negative)	1.035	0.654-1.639	0.883
PD-L1 (positive vs. negative)	2.317	1.421-3.776	0.001

Table 3. Multivariate COX regression analysis of clinical pathological factors and prognosis of patients with invasive breast cancer

Variable	HR	95% CI	p
T stage	1.438	0.878-2.356	0.149
N stage	1.160	0.769-1.750	0.480
Clinical tumor stage	1.598	0.727-3.514	0.244
Molecular subtype	1.415	0.970-2.065	0.071
ER (positive vs. negative)	2.559	0.976-6.708	0.056
PR (positive vs. negative)	0.518	0.287-0.936	0.029
PD-L1 (positive vs. negative)	2.299	1.389-3.803	0.001

HR=2.299, 95% CI: 1.389-3.803) were statistically significant (**Table 3**).

Kaplan-Meier survival analyses also showed that high PD-L1 expression is related with a worse prognosis in ER+, PR+, Ki67>14%, Ki67< 14% and Her-2+ patients. As shown in **Figure 1**, ER+ patients with high expression of PD-L1 had a shorter survival time and higher mortality rate than ER+ patients with low expression of PD-L1. A similar trend in results (high PD-L1 expression related with worse prognosis) was observed in the other four subgroups.

miR-34a influences the expression of PD-L1 both at RNA and protein level in MDA-MB-231 cells

RT-PCR results showed that miR-34a expression was significantly increased and PD-L1 mRNA expression was significantly decreased in the miR-34a mimic group, while the expression of miR-34a was significantly reduced and PD-L1 mRNA expression was significantly increased in the miR-34a inhibitor group. All these results were of a statistically significant *p* value of <0.05. Additionally, miR-34a and PD-L1 expression showed no change in miR-34a mimic NC or miR-34a inhibitor NC group, as shown in **Figure 2**. The Western blotting results show that PD-L1 protein expression has a reduced statistical significance (p<0.05) in the miR-34a mimic group and increased statistical significance (p<0.05) in the miR-34a inhibitor group, whereas there was no significant change in PD-L1 protein expression in the miR-34a mimic NC group or the miR-34a inhibitor NC group, which is also shown in **Figure 2**. Thus, miR-34a expression was found to have a negative association with PD-L1 gene expression and protein level.

miR-34a directly targets PD-L1 3'UTR

The 3'UTR sequence of PD-L1 wild type (WT) and mutant (MU) mRNA was cloned into plasmid psiCHECK-2 (dual luciferase reporter vector). Double luciferase assay results, as shown in **Figure 2**, confirmed that compared with the control level, the miR-34a mimic can downregulate the luciferase activity of MDA-MB-231 cells transfected with the PD-L1 WT plasmid (p<0.05), but has no effect on PD-L1 MU infected cells. Additionally, the luciferase activity of cells with PD-L1 WT and PD-L1 MU plasmids were not affected by the miR-34a inhibitor. These results indicate that miR-34a could directly mediate transcriptional gene silencing by targeting the PD-L1 3'UTR complementary site.

High expression of miR-34a inhibits the proliferation, migration and invasive ability of MDA-MB-231 cells

CCK-8 assay was used to detect proliferation changes in MDA-MB-231 cells transfected with either miR-34a mimic or miR-34a inhibitor. As shown in **Figure 2**, cell proliferation ability decreased after transfection with the miR-34a mimic and was enhanced after transfection with a miR-34a inhibitor. That is, high expression of miR-34a had a negative effect on the proliferation ability of MDA-MB-231 cells.

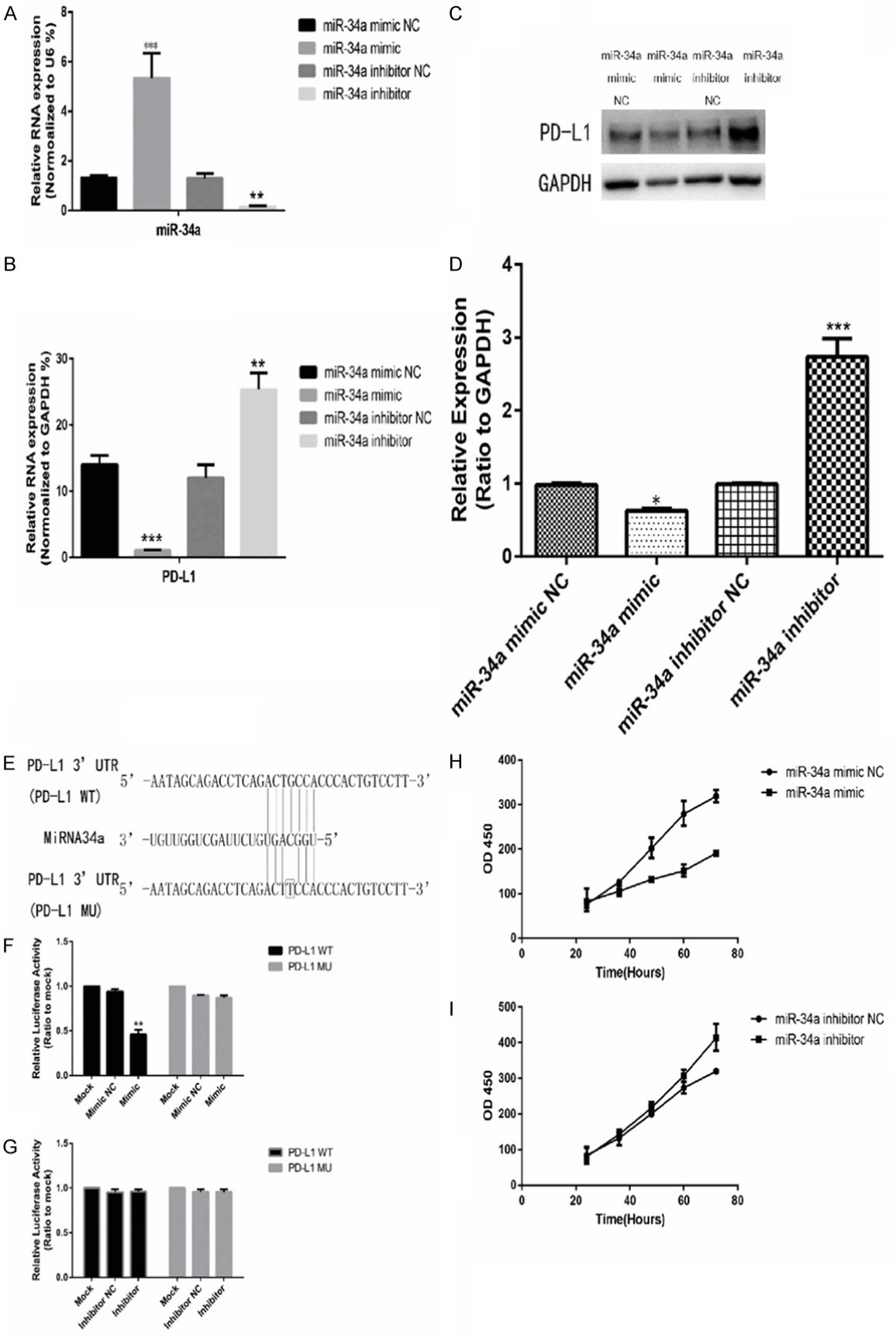
A
Relative RNA expression (Normoalized to U6 %)
miR-34a mimic NC
miR-34a mimic
miR-34a inhibitor NC
miR-34a inhibitor
miR-34a
B
Relative RNA expression (Normoalized to GAPDH %)
PD-L1
C
miR-34a mimic NC
miR-34a mimic
miR-34a inhibitor NC
miR-34a inhibitor
PD-L1
GAPDH
D
Relative Expression (Ratio to GAPDH)
E
PD-L1 3' UTR
5' -AATAGCAGACCTCAGACTGCCACCCACTGTCCTT-3'
(PD-L1 WT)
MiRNA34a 3' -UGUUGGUCGAUUCUGUGACGGU-5'
PD-L1 3' UTR 5' -AATAGCAGACCTCAGACTTCCACCCACTGTCCTT-3'
(PD-L1 MU)
F
Relative Luciferase Activity (Ratio to mock)
PD-L1 WT
PD-L1 MU
Mock
Mimic NC
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Figure 2. A. miR-34a expression was significantly increased in miR-34a mimic group and significantly reduced increased in miR-34a inhibitor group, $p<0.05$. B. PD-L1 mRNA expression was significantly decreased in miR-34a mimic group and significantly increased in miR-34a inhibitor group, $p<0.05$. C. PD-L1 protein expression was reduced with statistical significance ($p<0.05$) in the cells transfected with miR-34a mimic. D. PD-L1 protein expression was increased significantly ($p<0.05$) in the miR-34a inhibitor group. E. Sequence of 3'UTR of PD-L1 wild type (WT) and mutant (MU) mRNA as well as miR-34a. F. Luciferase activity of MDA-MB-231 cells transfected with PD-L1 WT plasmid was down-regulate by miR-34a mimic ($p<0.05$). G. Luciferase activity of cells with PD-L1 WT and PD-L1 MU plasmid were not affected by miR-34a inhibitor. H. The MDA-MB-231 cell proliferation ability was decreased after transfection of miR-34a mimic compared to negative control group. I. The MDA-MB-231 cell proliferation ability was enhanced after transfection of miR-34a inhibitor compared to negative control group.

Transwell insert experiments were used to detect the effect of miR-34a overexpression on migration and invasion of MDA-MB-231 cells. The results that are illustrated in **Figure 3** show that MDA-MB-231 cells transfected with a miR-34a mimic have a significantly weakened migration ability compared with those of the miR-34a mimic NC group ($p<0.05$). A similar result was observed in the invasive ability experiment, where the number of cells transfected with a miR-34a mimic was significantly less than that of the NC group ($p<0.05$). Therefore, high expression of miR-34a was able to weaken the migration and invasive ability of MDA-MB-231 cells.

In vivo experiment

After tumor challenge, drug injection, and observation, the volume and growth rate of the nude mice injected with the miR-34a agomir were analyzed and found to be significantly lower than those injected with the NC and the mock. RT-PCR results confirmed that miR-34a expression is significantly higher in tumors of the miR-34a agomir group, than that of the NC group and Mock group. As for the protein, Western blots shows that the expression level of PD-L1 in the miR-34a agomir group was significantly lower than that of the other two groups and are statistically significant ($p<0.05$), as shown in **Figure 4**. Additionally, expression of PD-L1 in the miR-34a agomir group was significantly lower than that of the NC and the Mock groups, while the IHC results of the peeled tumors in three groups are shown in **Figure 5**. In brief, negative regulation of miR-34 and PD-L1 expression were found using a xenograft mouse model.

Discussion

PD-L1 mRNA has been found in a variety of normal tissues in the body, including thymus, bone marrow, lymph nodes, heart, lungs, kidneys, liver, muscles, and nerve tissues. However, prevalence of its mRNA expression differs from that of its protein expression [25]. The PD-L1 protein is mainly expressed in antigen presenting cells (APCs), T cells, B cells, epithelial cells, myocytes, endothelial cells, as well as a variety of tumor cells and tumor infiltrating lymphocytes, and may participate in multiple tumor cell-associated immune responses [26]. Recent studies have shown that overexpression of PD-L1 may lead to immune escape in some tumors [27]. The binding of PD-L1 on tumor cells or APCs to the PD-1 receptor on T cells induces apoptosis, inhibits proliferation, and suppresses T cell IFN-γ, IL-4, and IL-2 cytokine release, impairing the function of T cells, while promoting the differentiation of $CD4^+/CD25^-/Foxp3^-$ T cells into $Foxp3^+$ regulatory T cells (Treg) and inducing apoptosis of tumor-specific T cells. Regulatory T cells have the ability to limit the growth of tumor cells, whereas the PD-1/PD-L1 signaling pathway in tumor cells can produce an immune escape by inhibiting regulatory T cells [28]. The expression profile of PD-L1 is slightly broader than that of PD-1 and is more involved in various links mediated by tumor cells. Therefore, immunohistochemistry was used to detect the expression level of PD-L1 protein in tissue microarrays and combine that with survival data of patients, in order to analyze the effect of PD-L1 on prognosis.

Numerous studies indicate that miRNAs are closely related with the occurrence and progression of various diseases in humans [29]. miRNAs can suppress cancer by downregulating oncogene mRNA levels, as well as promote carcinogenesis through downregulation of anti-oncogene mRNA levels [30]. This phenomenon suggests that miRNAs may be deeply involved in the process of tumor development. Therefore, it is of great significance to explore the biological functions of miRNAs in various tumors. The

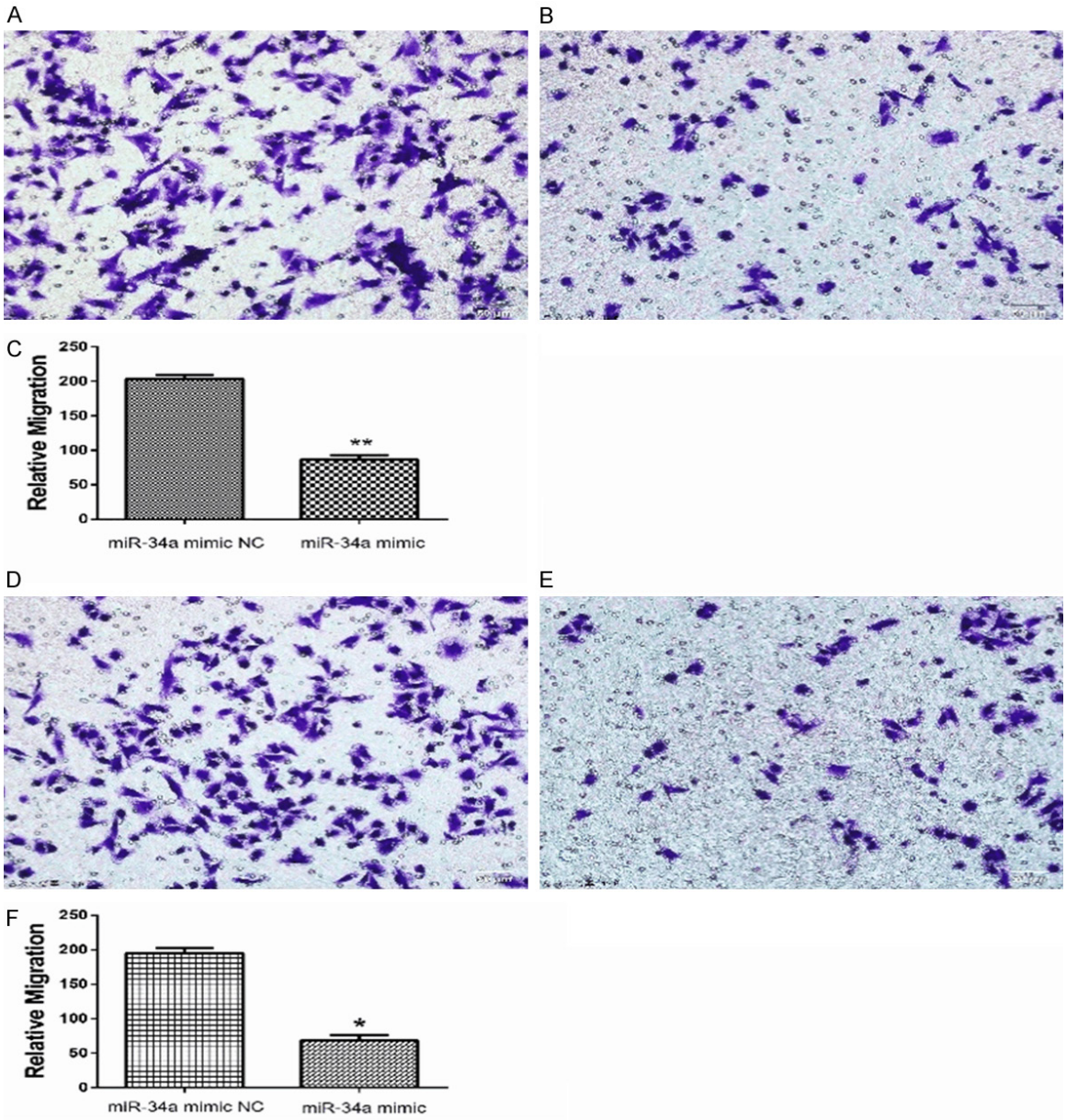

Figure 3. A. The example of cells with miR-34a mimic NC migrated to the lower chamber. B. The example of cells with miR-34a mimic migrated to the lower chamber. C. The number of cells with miR-34a mimic migrated to the lower chamber was significantly lower than the number of NC group, the *p* value <0.05. D. The example of cells with miR-34a mimic NC invaded to the lower chamber. E. The example of cells with miR-34a mimic invaded to the lower chamber. F. The number of cells with miR-34a mimic invaded to the lower chamber was significantly lower than the number of NC group, the *p* value <0.05.

miR-34 family contains three members, namely miR-34a, miR-34b and miR-34c. miR-34a is located on chromosome 1p36.23, while miR-34b and miR-34c are located on chromosome 11-q23. Both gene loci are susceptible to change and are associated with fragile sites of the tumor genome [31]. In normal tissues, miR-34a is widely found in most tissues and organs, while miR-34b and miR-34c are found only in the lung, trachea, ovary and prostate. miR-34a may be involved in targeting a variety of oncogenic genes that are associated with proliferation, apoptosis, and invasion [19, 32]. Numerous studies have demonstrated that miR-34a plays an important role in chemotherapy and chemotherapeutic resistance of tumors, as well as tumor immunotherapy and immune resistance [33, 34].

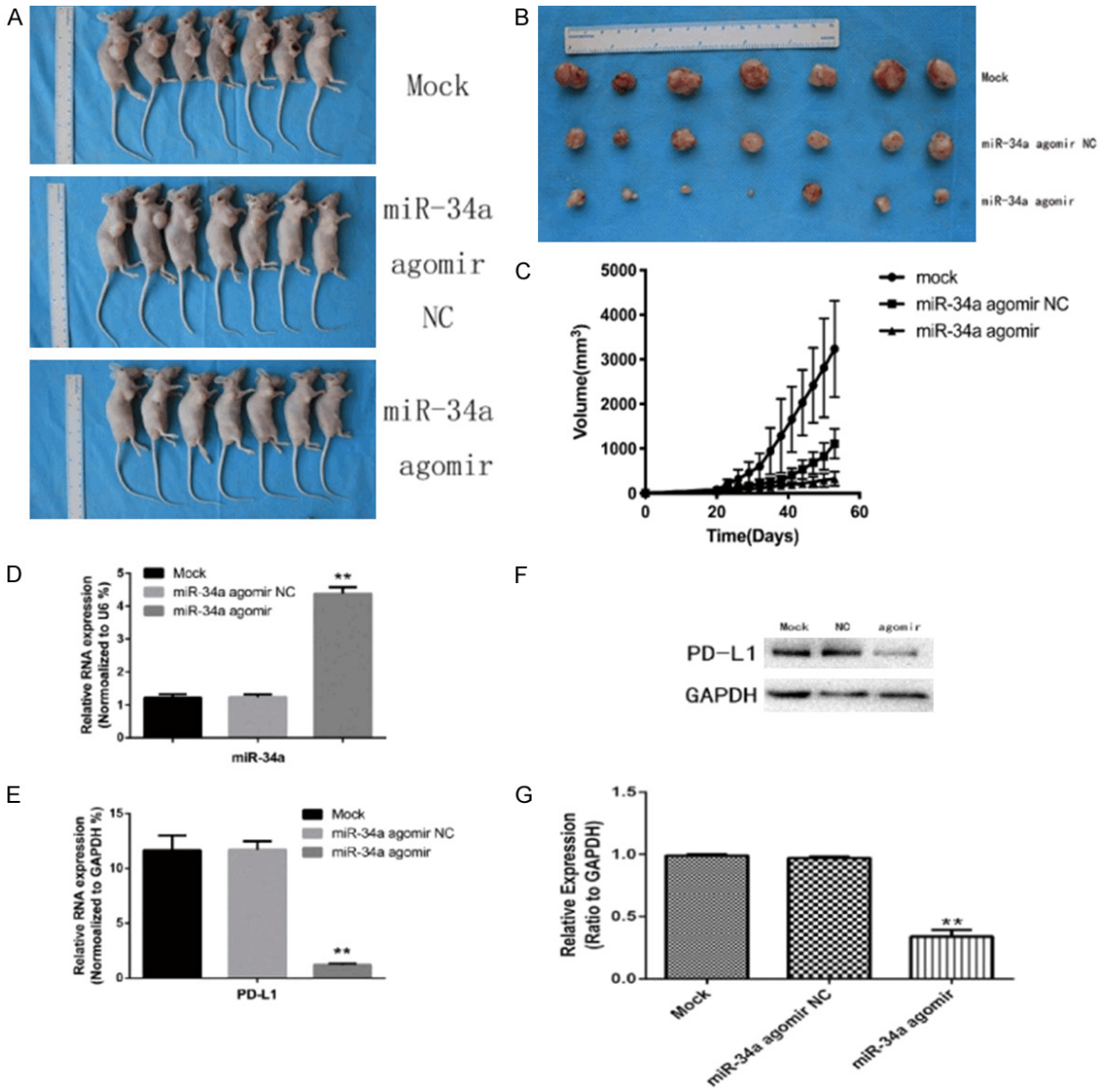

Figure 4. Tumor growth was monitored every 3 days after breast cancer cells subcutaneously injection and mice were sacrificed after 54 days, those had not formed tumor were excluded. A. The final mice of each group. B. Peeled off tumors of three groups. C. Growth curve of mean tumor volume of each group. D. RT-PCR result of miR-34a expression level in tumors from miR-34a agomir, miR-34a agomir NC and Mock group. E. RT-PCR result of PD-L1 expression level in tumors from miR-34a agomir, miR-34a agomir NC and Mock group. F. Western blot result of PD-L1 protein expression in the tumor samples of miR-34a agomir, miR-34a agomir NC and mock group. G. Expression of PD-L1 protein in miR-34a agomir was significantly lower than other two groups with p value <0.05.

As for the regulatory association between miR-34a and PD-L1, there are studies conducted on acute myeloid leukemia that demonstrating that miR-34a can target PD-L1 mRNA to regulate PD-L1 expression [35]. Interaction between PD-1 and its ligands, PD-L1 and PD-L2, can control the magnitude and duration of T cell responses, inhibit T cell proliferation, activation, and cytokine secretion. Therefore, it can mediate tumor immune escape [36]. However, the role and regulatory mechanism of miR34a and PD-L1 in invasive breast cancer are still unclear.

In this study, high expression of PD-L1 was observed in 57.69% of invasive breast carcinoma patients. There was significant correlation between PD-L1 expression and ER, PR, Her-2, Ki-67 and molecular classification, but no significant relation between age at diagnosis, tumor location, tumor stage, histological grade,

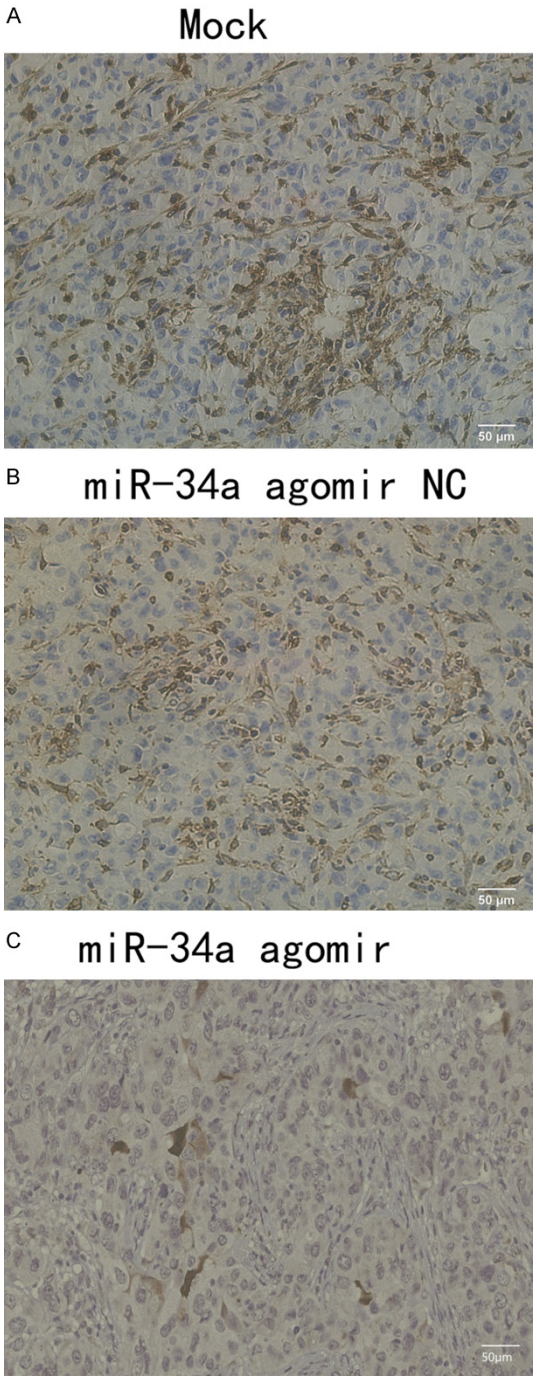

Figure 5. Immunohistochemistry graph presenting the PD-L1 expression in Xenograft tumors of miR-34a agomir, miR-34a agomir NC and Mock group.

tumor size, lymph node metastasis, CK5/6, p53, EGFR or other indicators. Subsequent Kaplan-Meier survival analysis demonstrated that high PD-L1 expression patients had a lower overall survival rate, compared with low expression level patients. Thus, high PD-L1 expression was found to be significantly associated with a poor prognosis of invasive breast carcinoma patients. Additionally, PD-L1 expression was found to be statistically associated with patient prognosis both in the single-factor COX regression and multi-factor COX regression analysis, which included T stage, N stage, tumor clinical stage, molecular classification, ER, PR, PD-L1 and 7 other indicators. Therefore, high PD-L1 expression may be a specific predictor of poor prognosis for patients with invasive breast cancer. To further analyze the relationship between PD-L1 and other common clinicopathological features, a subgroup analysis was performed to explore the influence of PD-L1 expression on prognosis under different conditions. A significant association between high PD-L1 expression and a poor survival rate in groups of patients with ER+, PR+, Ki67>14%, Ki67≤14%, and Her-2+ was found. Therefore, high PD-L1 expression in hormone receptor-positive and Her-2 positive patients may imply poor prognosis, which means that PD-L1 targeted therapy may be a new treatment choice for patients with high PD-L1 expression in order to overcome endocrine treatment resistance and inability of performing Herceptin therapy.

The human breast cancer cell line MDA-MB-231 was chosen for basic experimental research because of its relatively high expression of PD-L1. After transfection of miR-34a mimic, miR-34a mimic NC, miR-34a inhibitor and miR-34a inhibitor NC to MDA-MB-231 cells, PD-L1 mRNA was found to be downregulated when miR-34a is upregulated, while when PD-L1 protein was found to be downregulated when miR-34a is upregulated, and vice versa. Detection of mRNA and protein changes implies that there is an inverse relationship between them. To further demonstrate whether miR-34a directly regulates PD-L1, the 3'UTR ends of mutant and wild type PD-L1 were cloned into psiCHECK-2 and simultaneously transfected MDA-MB-231 cells with miR-34a mimics. Luciferase activity of cells transfected with PD-L1 WT plasmids decreased significantly, which indicates that PD-L1 is a direct target gene of miR-34a. Additionally, transfection of a miR-34a mimic into MDA-MB-231 cells attenuated the proliferation, migration and invasive abilities of the cell, while transfection of a miR-34a inhibitor enhanced the same. The regulatory relationship between miR-34a and PD-L1 is in accord with

our hypothesis that mRNA-34a can target PD-L1 and that these changes can affect important biological processes of the breast carcinoma cell line, which may be an important mechanism behind breast tumor cell immune evasion.

Based on the above findings using a tumor burden model of nude mice there can be several conclusions. First, the tumor volume and growth rate of the nude mice injected with a miR-34a agomir were significantly lower than that of those injected with a miR-34a agomir NC or Mock. It was confirmed that miR-34a expression in the agomir group was found to be significantly higher than that of the agomir NC and Mock groups, while the PD-L1 mRNA expression level was found to be significantly lower than that of the miR-34a agomir NC group and Mock group. Second, the agomir group showed lower expression of PD-L1 protein compared with that of the agomir NC and Mock groups. This indicates that miR-34a can downregulate mRNA and protein expression of PD-L1 in a xenograft model, as well as inhibit the growth of tumors *in vivo*.

Although the analysis of our samples found that high expression of PD-L1 is associated with a poor prognosis for invasive breast carcinoma, there have been several references in recent literature that claim that high expression of PD-L1 is associated with a good prognosis for breast cancer patients [23, 37-39]. However, several other reports have indicated that high PD-L1 expression is associated with a poor prognosis for breast cancer, since it is also combined with the downregulation of infiltration of lymphocytes [40] and upregulation of $Foxp3^+$ regulatory T cells [41]. The difference between conclusions may be related to a variety of factors, including tumor heterogeneity and patient inclusion criteria. This study included patients with various molecular types, while most of these other studies only included either triple-negative breast cancer patients or Her2 overexpression patients. The choice of experimental subjects and reagents may lead to different results. For example, the choice of antibodies from different companies and the criteria of the PD-L1 high expression may lead to different conclusions [42]. Race and ethnic differences can also bring about differences in results. More large-scale, multi-center research and clinical trials related to the treatment of PD-L1 inhibitors are needed to determine not only gold standard antibodies and the most appropriate cut-off value, but also to further clarify the relationship between PD-L1 and the prognosis of breast cancer. On the other hand, the regulatory relationship between miR-34a and PD-L1 has now been demonstrated, while the specific mechanisms of downstream genes of PD-L1 and signaling pathways still need to be explored. Furthermore, the precise mechanism of miR-34a in inhibiting breast tumor cell proliferation, migration and invasion has not been studied in detail. Last but not the least, the *in vivo* experiment was only conducted on the upregulated expression of miR-34a, and the specificity of downregulation of miR-34a expression was not investigated. Therefore, in subsequent research plans, future studies will address these inadequacies and deepen comprehension of the regulatory mechanism of miR-34a on PD-L1, so as to further explore how to apply tumor immunotherapy targeting of PD-L1 and miR-34a for the treatment of invasive breast cancer patients.

In summary, this study found that PD-L1 overexpression is associated with a poor prognosis for invasive breast cancer patients and can be used as an independent hazard predictor of prognosis. It shows that there is a reverse correlation between the expression of PD-L1 and miR-34a, while upregulation of miR-34a can inhibit the proliferation and invasive ability of the MDA-MB-231 cell line. Considering these results, miR-34a may serve as a promising treatment target for invasive triple-negative breast cancer patients with overexpressed PD-L1, but a large number of experiments are still required still require to verify its specific mechanism of action and efficacy.

Disclosure of conflict of interest

None.

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