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Original Article

Effect of Rad18 suppresses cell proliferation of triple negative breast cancer through E2F3, FANCD2, Akt and cyclin D1 expression

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Abstract: In this study, we investigated the effect of Rad18 suppresses cell proliferation of triple negative breast cancer through E2F3, FANCD2, Akt and cyclin D1 expression. In triple negative breast cancer patients, the risk of RAD18 Gln/Gln (SNP) genotype was higher than that of RAD18 Arg/Arg (wild type, WT). The cell survival of cisplatin-induced pcDNA3.1-Rad18 WT MDA-MB-231 cells was lower than that of pcDNA3.1-Rad18 SNP MDA-MB-231 cells. The cytotoxicity, apoptosis rate and caspase-9/3 activities of cisplatin-induced pcDNA3.1-Rad18 WT MDA-MB-231 cells were very higher than those of pcDNA3.1-Rad18 SNP MDA-MB-231 cells. Meanwhile, the protein expressions of E2F3, FANCD2, NF-κB-p65, p-Akt/Akt rate and cyclin D1 in cisplatin-induced pcDNA3.1-Rad18 WT MDA-MB-231 cells were lower than those of pcDNA3.1-Rad18 SNP MDA-MB-231 cells. These results robustly demonstrate that the effect of Rad18 suppresses cell proliferation of triple negative breast cancer through regulating E2F3, FANCD2, Akt and cyclin D1 expression.

Keywords: Rad18 genotype, triple negative breast cancer, cisplatin, MDA-MB-231

Introduction

Breast cancer is one of the most common cancers for the women around the world. According to the statistics of 2008, new cases of breast cancer are up to 1.38 million, in which about 460,000 patients died of breast cancer [1]. In recent years, the incidence of breast cancer in China shows significant upward trend, and the incidence age gets smaller and smaller [2]. Among the immunohistochemical indices that are commonly used in breast cancer, estrogen receptor ER, progesterone receptor PR and human epidermal growth factor receptor-2 (HER-2) play a decisive role in guiding treatment options, so they are particularly important [3]. The so-called triple-negative breast cancer (TNBC) refers to the breast cancer of which the molecular markers ER, PR and Her-2 are all expressed negatively [4]. TNBC is prone to distant metastasis, which is one type of breast cancer with the worst prognosis, and the 5-year survival rate is significantly lower than that of non-TNBC [5]. As we all know, various organs of the body are subject to the aging for the elderly, and the systematic functions have been degraded; both single cell and the whole body are marked by time, and many older people even suffer from some chronic underlying diseases [6]. Unlike this, the body functions of young people are relatively active and energetic, with less basic chronic diseases consolidated, but the small age is a common risk factor for almost all the cancers [7]. Therefore, the same disease shows different clinical and biological characteristics between the elderly and young patients.

RAD18 is an E3 ubiquitin ligase, containing SAP, Zinc-finger and all the typical RING domains of many ubiquitin systems E3, with single-stranded DNA-dependent ATP activity; it is also a single-stranded DNA binding protein, which can protect the single strand produced by the damaged DNA and the integrity of linear DNA [8]. The phenomenon that a large number of DNAs

Table 1. Real time-PCR of Primers was as follows

	Primer	Amplify	
Forward primer-Gln allele	5'-ATACCCATCACCCATCTTC-3'	146-bp	RT-PCR
Reverse primer-Gln allele	5'-GTCTTCTCTATATTTTCG ATTTCTT-3'		
Forward primer-Arg allele	5'-TTA ACA GCTGCTGAA ATAGTT CG-3'	206-bp	
Reverse primer-Arg allele	5'-CTGAAATAG CCCATTAACATACA-3'		
Forward primer-Probes	5'-AGCCTGGGAAGCATCACATA-3'		Real time-PCR
Reverse primer-Probes	5'-CTGTGGCAACCAAAAGTACG-3'		
Forward primer-Fluorescein	5'-CGCTGAAAGTGCTGAGATTGAACCAAGAA-3'		
Reverse primer-LCRed640	5'-CAAGCGTAATAGGAATTAATGTGGGCTTTT GC		

of RAD18 bacterial strain damage cannot be copied caused by strain Y-rays, has proved this point [8].

RAD18 is very conservative in eukaryotes, which is expressed in a variety of cells, such as Schizosaccharomyces pombe (KGY444), fruit Mei (S2), mouse (NIH3T3), rats (A10), monkey (C0S-7), human (293) and various tissues of rats [9]. RAD18 has high expression in mouse embryonic stem cells (mESCs) and spermatocytes mouse testis pachytene, and located in the XY body. RAD18 plays an important role in maintaining cell genome stable, the mESCs that knock RAD18 gene have increased sensitivity to various genetic toxin agents, and abnormal recombination frequency is increased [10]. Gene therapy is developed with the mature DNA recombinant technology as a new research field of contemporary medicine and biology, which can correct the defective gene or play a therapeutic role by guiding DNA sequence with therapeutic effect to target cells through a certain way, so as to achieve the purpose of curing disease [10].

Gene therapy is a new measure clinically for the treatment of various diseases, and cancer is the main application area [11]. Over the past decade, gene therapy research has undergone rapid development and made great progress. Partial gene therapy protocols have entered clinical trials.

Materials and methods

Cell lines culture

Human breast cancer MDA-MB-231 cells, MCF-7 cells and lung carcinoma cell lines EBC1 cells were used in this study and DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 to 95% air.

Real time-PCR and RT-PCR

Total RNA of tissue samples, MDA-MB-231 or bloods samples cells were isolated by using Trizol reagent or PURESCRIPT RNA Isolation Kit (Gentra systems) according to the instruction manual (Invitrogen). Complimentary DNA, 1-2 μg Total RNA was used to compound cDNA using One Step PrimeScript miRNA cDNA Synthesis kit (Takara) according to the manufacturer's instructions. Real time-PCR was conducted using SYBR® Premix Ex Taq™ II (Takara). The PCR amplification was initiated, 94°C for 5 min, followed by 40 cycles at 95°C for 45 sec, 60°C for 1 min, 72°C for 45 min and a final extension step at 72°C for 10 min. Real time-PCR of Primers was as follows at **Table 1**.

Construction of Rad18 wild type (WT) and Rad18 SNP (Arg302Gln)

Full lengths of Rad18 forward and reverse primers were amplified: 5'-ATTTCGAGT GGTGTTGGAGC-3' and 5'-TGGTACCTGTGTGAAATGTC-3', respectively. MCF-7 cells and EBC1 cells were used as a template for Rad18 WT and Rad18 SNP. Then, DNA fragments were ligated into plasmid vector pcDNA3.1/V5-His-TOPO (Invitrogen) to structure pcDNA3.1-Rad18 WT and pcDNA3.1-Rad18 SNP.

Transfection

MDA-MB-231 cells were transfected with either pcDNA3.1-Rad18 WT or pcDNA3.1-Rad18 SNP using lipofectamine 2000 (Invitrogen). Then, Dulbecco's Modified Eagle Medium (GIBCO) containing G418 (400 μg/ml) was used to structure stable cell lines for 8 weeks.

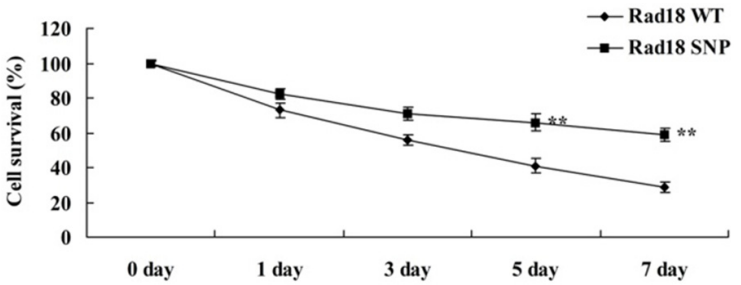

Figure 1. Effects of Rad18 WT and Rad18 SNP on cell survival of MDA-MB-231 cells-induced by cisplatin **P<0.01 compared with Rad18 WT group.

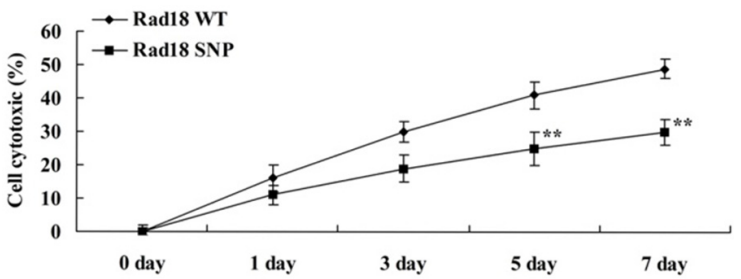

Figure 2. Effects of Rad18 WT and Rad18 SNP on cytotoxicity of MDA-MB-231 cells-induced by cisplatin **P<0.01 compared with Rad18 WT group.

Cell survival assay

pcDNA3.1-Rad18 WT or pcDNA3.1-Rad18 SNP MDA-MB-231 cells (1.5 × 10^4 cells per well) were seeded on 6-well plate and incubated at 37°C for 7 days. From 1 day to 7 days, 25 of μM cisplatin were added into every well. At day 1, 3, 5, 7, the cells were washed twice with PBS, 3 wells from every group were used to measure cell survival using 150 μl of 3-(4,5-dimethylthylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) for 4 h. Then, 20 μl of DMSO were added into every well for 20 min at room temperature. The absorbance was read at λ=570 nm.

Lactate dehydrogenase (LDH) assay

PcDNA LacZ, pcDNA3.1-Rad18 WT or pcDNA3.1-Rad18 SNP MDA-MB-231 cells (1.5 × 10^4 cells per well) were seeded on 6-well plate and incubated at 37°C for 7 days. From 1 day to 7 days, 25 of μM cisplatin were added into every well. At day 1, 3, 5, 7, the cells were washed twice with PBS, 3 wells from every group were used to measure cell survival using 100 μL LDH solutions for 30 min. Then, 20 μl of DMSO were added into every well for 20 min at room temperature. The absorbance was read at λ=490 nm.

Apoptosis assay

pcDNA3.1-Rad18 WT or pcDNA3.1-Rad18 SNP MDA-MB-231 cells (1.5 × 10^4 cells per well) were seeded on 6-well plate and incubated at 37°C for 7 days. From 1 day to 7 days, 25 of μM cisplatin were added into every well. At day 7 day, 10 μl of Annexin-V FITC (Bestbio. Co. Ltd., Shanghai, China) were added into each well and incubated for 30 min at 4°C in the dark. Then, 10 μL PI (Bestbio. Co. Ltd., Shanghai, China) were added into each well and incubated for 10 min in the dark. Apoptosis was analyzed using Flow cytometric (FACSCalibur; BD Biosciences, San Jose, USA).

Western blotting

pcDNA3.1-Rad18 WT or pcDNA3.1-Rad18 SNP MDA-MB-231 cells (1.5 × 10^4 cells per well) were seeded on 6-well plate and incubated at 37°C for 7 days. From 1 day to 7 days, 25 of μM cisplatin were added into every well. After 7 days, all cells were extracted using RIPA buffer (Beyotime Biotech, Nanjing China) including protease inhibitor and phosphatase inhibitor. The concentrations proteins were determined with BCA Protein Assay Kit (Beyotime Biotech, Nanjing China). 50 μg of protein samples were suspended in 10-12% SDS loading buffer and transferred to nitrocellulose (Trans-Blot; Bio-Rad). The membranes were blocked for 1 h in 5% milk in PBS-T (PBS 0.1% Tween 20) and incubated with anti-E2F3 (1:2000, Santa Cruz, CA, USA), anti-FANCD2 (1:2000, Santa Cruz, CA, USA), anti-NF-κB-p65, anti-phosphorylation-Akt (1:4000, Santa Cruz, CA, USA), anti-Akt (1:4000, Santa Cruz, CA, USA), anti-cyclin D1 (1:2000, Santa Cruz, CA, USA) and anti-β-actin (1:2000, Beyotime Institute of Biotechnology, Jingsu, China) overnight at 4°C. After washes in PBST, the membranes were incubated with secondary antibody at 1:5000 in PBS-T with 0.5% milk for 1 hour at room temperature and then developed by chemiluminescence (Supersignal West Pico Kit; Pierce).

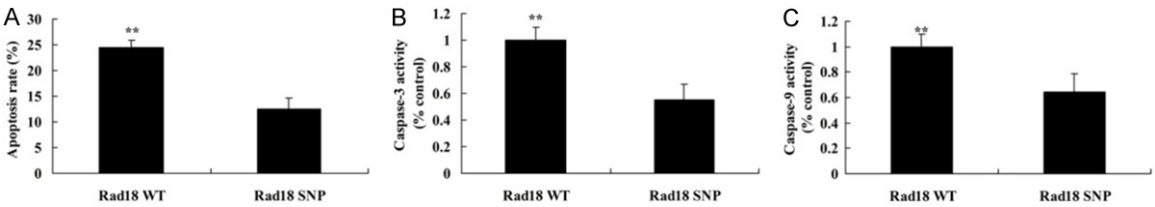

Figure 3. Effects of Rad18 WT and Rad18 SNP on apoptosis of MDA-MB-231 cells-induced by cisplatin. Effects of Rad18 WT and Rad18 SNP on apoptosis rate (A), caspase-3 activity (B) and caspase-9 activity (C) of MDA-MB-231 cells-induced by cisplatin. **P<0.01 compared with Rad18 WT group.

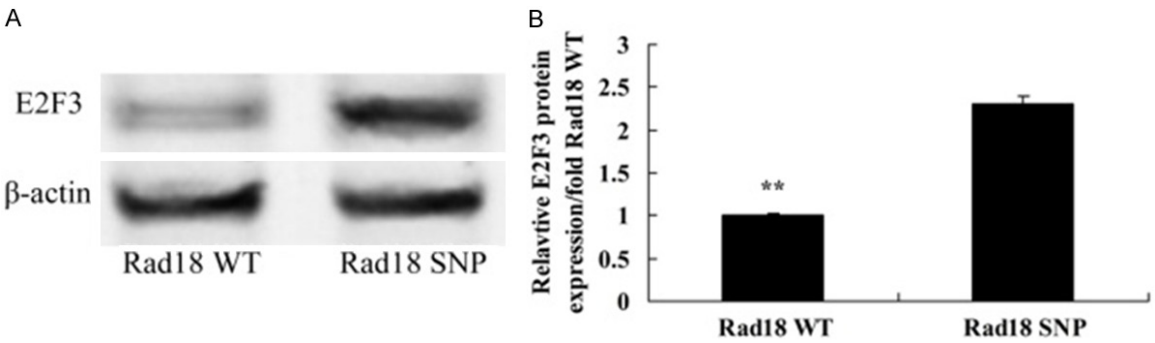

Figure 4. Effects of Rad18 WT and Rad18 SNP on E2F3 expression of MDA-MB-231 cells-induced by cisplatin. Effects of Rad18 WT and Rad18 SNP on E2F3 protein expression using Western blot analysis (A), statistical analysis of E2F3 protein expression (B) of MDA-MB-231 cells-induced by cisplatin. **P<0.01 compared with Rad18 WT group.

Statistical analysis

Data are presented as the mean ± standard deviation. Statistical analysis was carried out with one-way ANOVA, followed by LSD's post hoc test. Values of $P<0.05$ were considered to be statistically significant.

Results

Effects of Rad18 WT and Rad18 SNP on cell survival of MDA-MB-231 cells-induced by cisplatin

Cisplatin-induced MDA-MB-231 cells at 1 day, cell survival of MDA-MB-231 cells began to inhibit without statistical significance (**Figure 1**). However, there was a significance increase in cell survival of cisplatin-induced MDA-MB-231 cells in Rad18 SNP group compared with Rad18 WT group (**Figure 1**).

Effects of Rad18 WT and Rad18 SNP on cytotoxicity of MDA-MB-231 cells-induced by cisplatin

We also explore the effects of Rad18 WT and Rad18 SNP on cytotoxicity of MDA-MB-231 cells-induced by cisplatin. As shown in **Figure 2**, the cytotoxicity of cisplatin-induced MDA-MB-231 cells was observable lower than that of Rad18 WT group.

Effects of Rad18 WT and Rad18 SNP on apoptosis of MDA-MB-231 cells-induced by cisplatin

To probe in the role of Rad18 genotype on apoptosis of MDA-MB-231 cells-induced by cisplatin, we measured apoptosis rate, and caspase-3 and caspase-9 activities. As shown in **Figure 3A**, the apoptosis rate in Rad18 SNP was higher than that of Rad18 WT in cisplatin-induced MDA-MB-231 cells. Meanwhile, caspase-3 and caspase-9 activities of Rad18 SNP group were also higher than those of Rad18 WT in cisplatin-induced MDA-MB-231 cells (**Figure 3B**, **3C**).

Effects of Rad18 WT and Rad18 SNP on E2F3 expression of MDA-MB-231 cells-induced by cisplatin

In order to explore the effects of Rad18 WT and Rad18 SNP on E2F3 expression of MDA-MB-231 cells-induced by cisplatin, we used

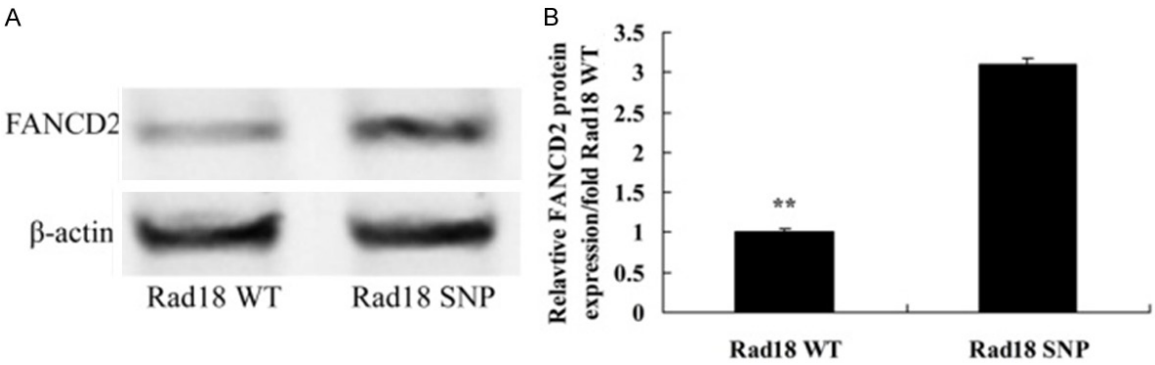

Figure 5. Effects of Rad18 WT and Rad18 SNP on FANCD2 expression of MDA-MB-231 cells-induced by cisplatin. Effects of Rad18 WT and Rad18 SNP on FANCD2 protein expression using Western blot analysis (A), statistical analysis of FANCD2 protein expression (B) of MDA-MB-231 cells-induced by cisplatin. **P<0.01 compared with Rad18 WT group.

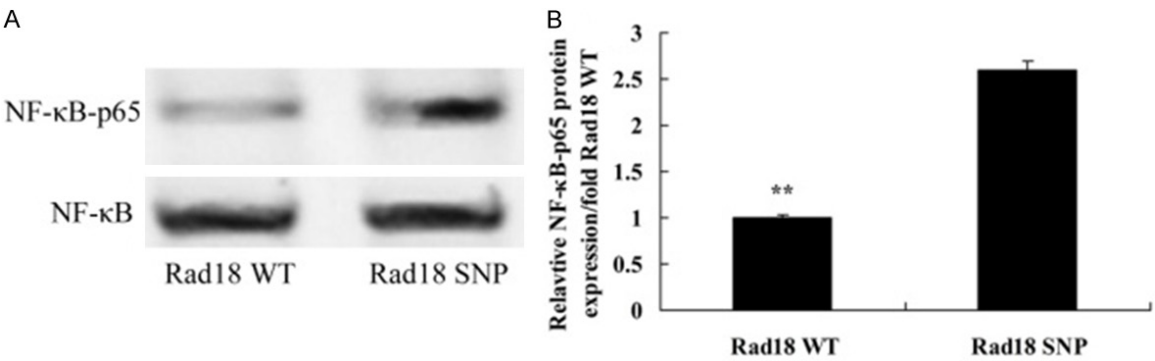

Figure 6. Effects of Rad18 WT and Rad18 SNP on NF-κB-p65 expression of MDA-MB-231 cells-induced by cisplatin. Effects of Rad18 WT and Rad18 SNP on NF-κB-p65 protein expression using Western blot analysis (A), statistical analysis of NF-κB-p65 protein expression (B) of MDA-MB-231 cells-induced by cisplatin. **P<0.01 compared with Rad18 WT group.

Western Blotting to analyze the E2F3 protein expression. We found that the E2F3 protein expression of Rad18 WT group was lower than that of Rad18 SNP in MDA-MB-231 cells-induced by cisplatin (**Figure 4A**, **4B**).

Effects of Rad18 WT and Rad18 SNP on FANCD2 expression of MDA-MB-231 cells-induced by cisplatin

To seek the effects of Rad18 WT and Rad18 SNP on FANCD2 expression of MDA-MB-231 cells-induced by cisplatin, FANCD2 protein expression of MDA-MB-231 cells-induced by cisplatin was measured using Western Blotting. FANCD2 protein expression of Rad18 WT group was very lower than that of Rad18 SNP group in MDA-MB-231 cells-induced by cisplatin (**Figure 5A**, **5B**).

Effects of Rad18 WT and Rad18 SNP on NF-κB-p65 expression of MDA-MB-231 cells-induced by cisplatin

We explored the effects of Rad18 WT and Rad18 SNP on NF-κB-p65 expression of MDA-MB-231 cells-induced by cisplatin. As shown in **Figure 6A**, **6B**, NF-κB-p65 expression of MDA-MB-231 cells-induced by cisplatin in Rad18 WT group was lower than that of Rad18 SNP group.

Effects of Rad18 WT and Rad18 SNP on Akt expression of MDA-MB-231 cells-induced by cisplatin

We probe into the effects of Rad18 WT and Rad18 SNP on Akt expression of MDA-MB-231 cells-induced by cisplatin. The p-Akt/Akt rate of Rad18 WT group was very lower than that of

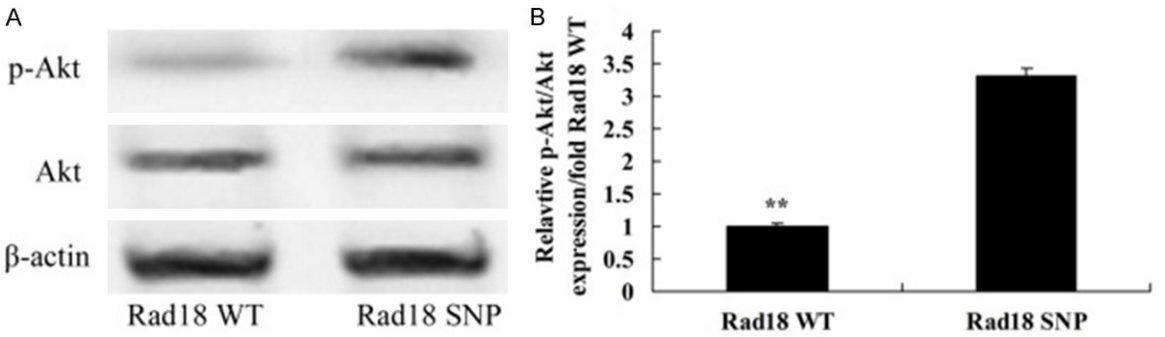

Figure 7. Effects of Rad18 WT and Rad18 SNP on Akt expression of MDA-MB-231 cells-induced by cisplatin. Effects of Rad18 WT and Rad18 SNP on p-Akt and Akt protein expression using Western blot analysis (A), statistical analysis of p-Akt and Akt protein expression (B) of MDA-MB-231 cells-induced by cisplatin. **P<0.01 compared with Rad18 WT group.

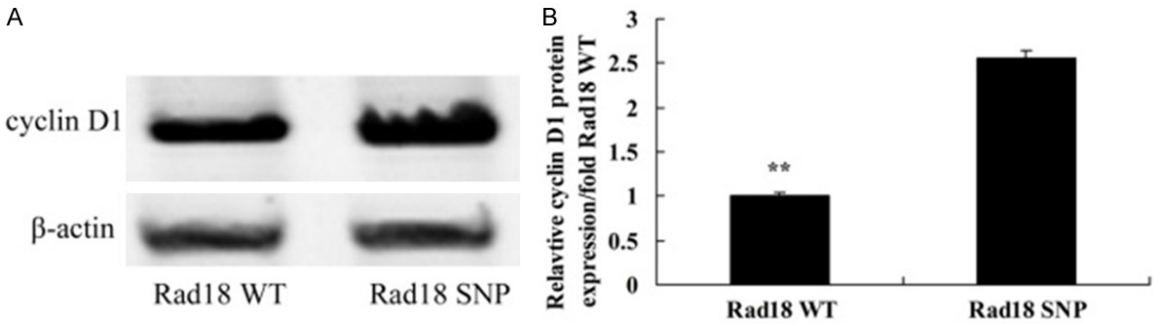

Figure 8. Effects of Rad18 WT and Rad18 SNP on cyclin D1 expression of MDA-MB-231 cells-induced by cisplatin. Effects of Rad18 WT and Rad18 SNP on cyclin D1 protein expression using Western blot analysis (A), statistical analysis of cyclin D1 protein expression (B) of MDA-MB-231 cells-induced by cisplatin. **P<0.01 compared with Rad18 WT group.

Rad18 SNP group in cisplatin-induced MDA-MB-231 cells (**Figure 7A**, **7B**).

Effects of Rad18 WT and Rad18 SNP on cyclin D1 expression of MDA-MB-231 cells-induced by cisplatin

We detected the effects of Rad18 WT and Rad18 SNP on cyclin D1 expression of MDA-MB-231 cells-induced by cisplatin. As shown in **Figure 8A**, **8B**, cyclin D1 protein expression of MDA-MB-231 cells-induced by cisplatin in Rad18 WT group was lower than that of Rad18 SNP group.

Discussion

TNBC accounts for about 15% of all breast cancers. Clinical features of TNBC include large tumor size, high histological grade, proneness to distant metastasis, and the ineffectiveness in anti-ER and PR endocrine therapy and anti-HER-2 targeted therapy [12]. Studies have shown that no matter which stage of TNM that TNBC is in, the prognosis is poor and 5-year survival rate is less than 15% [13]. Available information indicates that the patient with TNBC is more likely to have a family history of breast cancer than that with non-TNBC, and poor prognosis can often be seen in the patient with a family history [14]. In this study, we found that Rad18 SNP increase, reduced cytotoxicity and inhibited caspase-3 and caspase-9 activities of cisplatin-induced MDA-MB-231 cells, compared with Rad18 WT group.

E2F2 family members have an N-terminal DNA binding domain, dimerization domain and transcriptional activation domain containing the protein binding pocket region located at the C-terminus [15]. E2F family of proteins can be combined with cyclinDI to form a heterodimer to further activate a series of gene expression that plays a key role in the transition process

from G1 to S phase, with the effect of promoting cell division and proliferation [16]. Therefore, the over-expression of any pocket protein can lead to the arrest of cell cycle in G1 phase [17]. In the process of tumorigenesis, P16INK/pRb/E2F axis is one of the important pathways for controlling tumorigenesis [18]. Our study showed that Rad18 SNP increased the E2F3 protein expression in MDA-MB-231 cells-induced by cisplatin, compared with Rad18 WT group.

DNA crosslinking damage is a common DNA damage, of which the repair process is relatively complex, mainly realized by the FA/BRCA pathway [19]. D type of FA includes two types, D1 and D2, of which the corresponding proteins are respectively FANCD1 and FANCD2 [20]. The body is likely to conduct fine-tuning regulation for FANCD2 ubiquitination/de-ubiquitination in different signaling pathways to regulate FA/BRCA pathway to take part in a different DNA repair processes [21]. Studies have shown that FANCD2 is involved in cell cycle regulation, chromatin remodeling, DNA methylation and apoptosis in addition to DNA damage repair, and meanwhile plays an important role in maintaining normal operation of the body [22]. In this study, we found that the FANCD2 protein expression of Rad18 SNP group was higher than that of Rad18 WT group.

Some recent studies have shown that PI3K/Akt signaling pathway abnormality is an important mechanism of cell canceration [23]. The activation of this signaling pathway is related to the breast cancer progression characteristics such as high histological grade, basal-like correlation and poor prognosis [23]. Reports show that there may be some mutation or deletion of the key factors in PI3K/Akt signaling pathway of TNBC patients. Current study shows that PI3K/Akt signaling pathway is activated in many cancers, in which the activation of the signaling pathway in breast cancer is up to 70% [23, 24]. There have been some reports about the relation between PI3K/Akt signal pathway and breast cancer [25]. In present study, NF-κB-p65 and p-Akt/Akt rate protein expression of MDA-MB-231 cells-induced by cisplatin in Rad18 SNP group were higher than that of Rad18 WT group.

It has been confirmed by studies that the imbalance of cell cycle regulation is caused by the uncontrolled cell proliferation as a major factor, and cell cycle protein has important function in the regulation of cell cycle [26]. As a member of cyclin family, Cyclin D1 is a kind of protein with molecular weight of 34 kDa, located at llql3 and encoding the transcriptional expression of 295 kinds of oxyacid, which mainly regulates the cycle from cells to the cell proliferation and plays a very important regulatory role in the process of cell division from G1 to S phase [27, 28]. The binding of Cyclin D1 and cyclin kinase is the primary mechanism. The high expression of Cyclin D1 makes the G1 phase of the cell cycle shortened, decreases chromatin, weakens the dependence on mitogen, and leads to early entering into the S phase, so the growth, the proliferation, and the malignant transformation of the cells cannot be controlled [29]. We found that the cyclin D1 protein expression of MDA-MB-231 cells-induced by cisplatin in Rad18 SNP group was higher than that of Rad18 WT group. In conclusion, our data provide evidence that the effect of Rad18 suppresses cell proliferation of triple negative breast cancer through regulating E2F3, FANCD2, Akt and cyclin D1 expression.

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Disclosure of conflict of interest

None.

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