Niraparib restrains prostate cancer cell proliferation and metastasis and tumor growth in mice by regulating the lncRNA MEG3/ miR-181-5p/GATA6 pathway

Ji Cheng [1], **Yi Sun** [1], **Huacai Zhao** [1], **Wei Ren** [1], **Dan Gao** [1], **Zhigang Wang** [1], **Wei Lv** [1], **Qingchuan Dong** [Corresp. 1]

[1] Department of Urology Surgery, Shaanxi Provincial People's Hospital, Xi'an, China

Corresponding Author: Qingchuan Dong
Email address: dongqc169@163.com

Background: Poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi), have been approved for the treatment of PCa patients in castration resistant prostate cancer (CRPC) stage. LncRNA maternally expressed gene 3 (MEG3) can inhibit tumorigenesis through regulating DNA repair gene. This study investigated whether the anti-PCa effect of niraparib, a representative PARPi, was associated with MEG3 expression, and further explored their downstream pathway. **Methods:** The levels of MEG3, miR-181-5p, GATA binding protein 6 (GATA6) in clinical samples from PCa patients were accessed by RT-qPCR. PC3 cells were treated with niraparib, and MEG3, miR-181-5p, GATA6 expression was tested. PC3 cell proliferation, migration, and invasion were tested by CCK-8, wound healing, and Transwell assays, respectively. The binding between miR-181-5p and MEG3/GATA6 was determined by dual-luciferase reporter gene assay. Furthermore, we conducted rescue experiments to investigate the underlying mechanism of MEG3/miR-181-5p/GATA6 axis in PCa progression. Additionally, mice were injected with PC3 cells transfected with sh-MEG3 and treated with niraparib, and the xenograft tumor growth was observed. **Results:** MEG3 and GATA6 were upregulated and miR-181-5p was downregulated in PCa patients. Niraparib treatment substantially upregulated MEG3 and GATA6, and downregulated miR-181-5p expression in PCa cells. Niraparib restrained PC3 cell proliferation, migration, and invasion. MiR-181-5p targeted to MEG3, and the inhibitory effects of MEG3 overexpression on PC3 cell proliferation and metastasis were abrogated by miR-181-5p overexpression. Moreover, GATA6 was a target of miR-181-5p, and GATA6 silencing abolished the inhibitory effects of miR-181-5p inhibition on PC3 cell proliferation and metastasis. Besides, MEG3 silencing could abrogate niraparib-mediated tumor growth inhibition in mice. **Conclusions:** Niraparib restrains prostate cancer cell proliferation and metastasis and tumor growth in mice by regulating the lncRNA MEG3/miR-181-5p/GATA6 pathway

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Department of Urology Surgery, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi 710068, China

***Corresponding Author:** Qingchuan Dong

No.256 Youyi West Road, Xi'an City, 710068, Shaanxi, China

E-mail address: dongqc169@163.com

Abbreviations:

Prostate cancer (PCa); androgen deprivation (ADT); castration resistant prostate cancer (CRPC); poly (ADP-ribose) polymerase (PARP); PARP inhibitors (PARPi); maternally expressed gene 3 (MEG3); GATA binding protein 6 (GATA6)

Abstract

Background: Poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi), have been approved for the treatment of PCa patients in castration resistant prostate cancer (CRPC) stage. LncRNA maternally expressed gene 3 (MEG3) can inhibit tumorigenesis through regulating DNA repair gene. This study investigated whether the anti-PCa effect of niraparib, a representative PARPi, was associated with MEG3 expression, and further explored their downstream pathway.

Methods: The levels of MEG3, miR-181-5p, GATA binding protein 6 (GATA6) in clinical samples from PCa patients were accessed by RT-qPCR. PC3 cells were treated with niraparib, and MEG3, miR-181-5p, GATA6 expression was tested. PC3 cell proliferation, migration, and invasion were tested by CCK-8, wound healing, and Transwell assays, respectively. The binding between miR-181-5p and MEG3/GATA6 was determined by dual-luciferase reporter gene assay. Furthermore, we conducted rescue experiments to investigate the underlying mechanism of MEG3/miR-181-5p/GATA6 axis in PCa progression. Additionally, mice were injected with PC3 cells transfected with sh-MEG3 and treated with niraparib, and the xenograft tumor growth was observed.

Results: MEG3 and GATA6 were upregulated and miR-181-5p was downregulated in PCa patients. Niraparib treatment substantially upregulated MEG3 and GATA6, and downregulated miR-181-5p expression in PCa cells. Niraparib restrained PC3 cell proliferation, migration, and invasion. MiR-181-5p targeted to MEG3, and the inhibitory effects of MEG3 overexpression on PC3 cell proliferation and metastasis were abrogated by miR-181-5p overexpression. Moreover, GATA6 was a target of miR-181-5p, and GATA6 silencing abolished the inhibitory effects of miR-181-5p inhibition on PC3 cell proliferation and metastasis. Besides, MEG3 silencing could abrogate niraparib-mediated tumor growth inhibition in

mice.

Conclusions: Niraparib restrains prostate cancer cell proliferation and metastasis and tumor growth in mice by regulating the lncRNA MEG3/miR-181-5p/GATA6 pathway

Keywords: PCa, niraparib, PRAPi, lncRNA MEG3, miR-181-5p, GATA6

1. Introduction

Prostate cancer (PCa) is the second most common lethal cancer and ranks second in terms of mortality in males worldwide [1]. The aging population has ~~recently~~ significantly contributed to a ~~shape rise~~ sharp increase in the incidence and mortality of PCa. According to the cancer statistics ~~of 2023~~ 2023, ~~the~~ PCa incidence ~~of PCa~~ Traditional treatments like is increasing by 3% ~~every year~~ annually, ~~which is~~ equivalent to 99,000 new cases [2]. Surgery and radiation therapy have great limitations for PCa patients, ~~and a great many of patients died~~ with many succumbing to the disease or ~~developed~~ developing metastasis. Surgical treatment has high risk ~~and~~ of complications ~~and affect~~ , impacting patient's quality of life and ~~of life of patients, and~~ Meanwhile, ~~has the possibility of~~ carrying a potential for recurrence and distant metastasis. Radiation therapy ~~have~~ has a biochemical recurrence rate of approximately 40% and may cause side effects such as frequent urination and urgency. It ~~was also~~ Moreover, may not be effective for advanced PCa. The metastatic PCa has been linked ~~to the~~ with an increased risk of mortality. ~~The mortality rate of PCa accounts for~~ , contributing to 13% of all ~~cancers, which seriously affects the subsistence and~~ cancer-related deaths and significantly impacting patients' survival and life quality ~~of patients~~ [3]. At present, the ~~main~~ primary therapy for PCa is androgen deprivation (ADT) therapy, which can suppress tumor growth and delay clinical tumor progression [4]. However, the ~~generation~~ emergence of ADT resistance in PCa patients drives the disease to castration-resistant prostate cancer (CRPC) stage [5, 6]. Currently, continuous in-depth studies have developed various new drugs for CRPC. ~~However~~ Nonetheless, this poses a major challenge ~~for clinical treatment~~ clinical, including ~~selecting specific~~ the selection of tailored therapies for individual patients, ~~developing the best~~ the optimal combination of ~~new~~ these effective drugs, and ~~exploring the mechanisms of acquired resistance [7].~~ an exploration of the mechanisms underlying acquired resistance.

Drugs targeting poly (ADP-ribose) polymerase (PARP) to regulate cell proliferation and metastasis have been gradually applied for PCa treatment in clinic [8]. PARP inhibitors (PARPi) take effect through the synthetic lethality of homologous recombination repair gene defects such as BRAC to inhibit DNA damage repair and promote apoptosis in cancer cells [9, 10]. PARPi inhibit the catalytic activity of PARP1 through competitive binding with its catalytic domain, and then the single strand break can't be repaired and converse to double strand break. If homologous recombination (HR) repair gene defects exist in cancer cells, DNA damage can't be repaired and induce cancer cell apoptosis [11]. Moreover, PARPi enhance the binding strength of PARP-1 and damaged DNA, and induce PARP1 trapping, thus blocking the possible DNA repair pathway and finally killing cancer cells [11]. DNA repair pathway depend on PARP1 enzyme when HR repair

The manuscript has a few language and writing issues that could be improved for clarity and readability: long sentences, repetition, complex language of misuse of articles or transition words, organization (into clear sections or paragraphs for improve readability), and finally clarity - Some sentences are structurally complex and may require rephrasing for greater clarity.

This are minor issues that still need to be addressed before acceptance and production

gene defects, and PARPi will effectively impede DNA repair and ultimately kill cancer cells. However, the presence of HR repair gene can still repair DNA damage and make cells survive, so PARPi can be used as targeted drugs to selectively kill cells with HR repair gene defects [10]. PARPi have been approved for the treatment of breast and ovarian cancer [12, 13]. Olaparib and talazoparib, are already approved by the United States Food and Drug Administration (FDA) for BRCA-mutated breast cancer, based on positive outcomes in phase3 trials [12]. Niraparib treatment had significantly longer progression-free survival in patients with advanced ovarian cancer than placebo treatment [14]. The application of PARPi also has been expanded to treat advanced PCa. It has been confirmed that olaparib treatment improves the overall survival rate of metastatic CRPC patients with homologous recombination repair defects, which may be achieved by promoting DNA damage-induced cell death suppressed tumor growth [15]. Subsequent research further confirmed that CRPC patients with multiple DNA homologous recombination repair gene defects could also benefitted from PARPi with a comprehensive response rate of 46.7% [16]. Especially, it was reported that niraparib and talazoparib showed impressive performance in phase II trials for metastatic CRPC patients [17]. Moreover, niraparib treatment improved the objective response rate and progression-free survival in patients with biallelic BRCA1/2 alterations [18]. It is generally recognized that the regulatory mechanisms of PARPi mainly focus on DNA genetic variations and protein expression-mediated proliferation and apoptosis. There are numerous biomarkers have been explored, such as BRCA mutations and other genetic mutations related to HR. however, there are still no gold standards for determining patients who are candidates for PARPi therapy. At present, it's not clear that whether PARPi exert antitumor effects through regulating the transcriptome level. We need to consider the complex interactions among various genes and proteins in the underlying mechanisms to create more precise prognostic and therapeutic indicators and identify suitable candidates among the patient population for the use of PARPi.

Multiple abnormal expressed lncRNAs play an important role in PCa development, and which has been identified as promising therapeutic target for PCa [19]. lncRNAs can serve as the prognostic and diagnostic markers in clinic [20]. Moreover, lncRNAs regulate the drug resistance and immune evasion of PCa cells [21]. Notably, microarray and RNAseq technologies have determined numerous predictive lncRNAs involved in biologically pathways including ADT therapy and PARP inhibition [22]. But it is still unclear ~~that~~ whether the roles of lncRNAs in PARPi-mediated anti-PCa effect. There is evidence that lncRNA maternally expressed gene 3 (MEG3) ~~was~~ downregulated in PCa tissues and cells, and MEG3 overexpression could mitigate the abilities of PCa cell proliferation, migration, and invasion through regulating the miR-9-

5p/QKI-5 axis [23]. Another study also proposed that MEG3 overexpression restrained the viability, clonogenicity, invasion and migration of PC3 cells, as well as the tumorigenic effects of PC3 cells in mice [24]. More importantly, MEG3 was found to be involved in the regulation of some DNA repair gene. For instance, it was previously reported that MEG3 impeded ovarian cancer cell proliferation and via promoting the DNA repair gene PTEN expression [25]. Also, it was showed that MEG3 restrained bladder cancer cell progression and tumor growth by promoting PTEN expression via sponging miR-494 [26]. MEG3 suppressed the proliferation and metastasis of gastric cancer by increasing p53 transcription and expression, which can protect the genome by coordinating various DNA damage response mechanisms [27]. In addition, MEG3 expression was significantly upregulated after ischemia-reperfusion, which decreased intact PARP1 level and increased cleavage PARP1 level, thus promoting cell apoptosis [28]. These studies suggested that PARP targeted CRPC therapies may require the activation of MEG3 to regulate DNA repair gene to exert anti-PCA effects. However, whether PRAPi can affect the expression of MEG3 in PCa cells is not clear.

In this study, we confirmed that MEG3 was conspicuously downregulated in PCa patients and cell lines. We further found that MEG3 was upregulated in PCa cells after PRAPi (niraparib) treatment, which may be associated with PRAPi-mediated anti-PCa effect. Therefore, we further investigated niraparib/MEG3-mediated downstream pathways in PCa.

By now, it remains unclear what knowledge gap you are addressing and WHY? Also why you used, eg, PC3 cells and not LNCap?

2. Materials and Methods

2.1 Clinical specimens

PCa patients (n=20, average age=51.4±8.6 years) were recruited from Shaanxi Provincial People's Hospital. The inclusion criteria were listed below: (a) patients were diagnosed as PCa by pathological investigations. (b) clinical information is comprehensive and tissue samples are available for use in experiments. (c) patients were not received any anti-tumor medications and treatments. Patients with other prostatic diseases, other malignant tumors, and severe complicated diseases of heart, lung, kidney and other organs or severe infectious diseases or received any anti-tumor treatment were excluded. PCa tissues and non-tumor adjacent tissues were excised from the patients during survey. All collected tissues were frozen in liquid nitrogen immediately and stored at -80°C before use. All samples obtained in this study were approved by the ethics committee of Shaanxi Provincial People's Hospital and abided by the ethical guidelines of the Declaration of Helsinki, and ethics committee agreed to waive informed consent.

patients were diagnosed as PCa??? language!

approval number??

2.2 Cell culture and treatment

PCa cell line PC3 (article number: CRL-3471) was acquired from ATCC (Manassas, VA, USA). Cells were maintained in DMEM containing 10% fetal bovine serum and antibiotics under 5% CO_2 at 37°C. Cells

why DMEM instead of RPMI?

which antibiotics? at which concentrations?

passage 3, or frozen eg at p=10 and then used at p=13?

were used for subsequent experiments after three passages. For niraparib treatment, PC3 cells were incubated with different final concentrations of niraparib (0, 1, 2, 4, 8 μM) for different ~~times~~ durations (0, 30, 60, 120, 240 min).

INCOMPLETE: needs sequences and source of DNA fragments, exact restriction enzymes, details on transformation (protocol, selection process and criteria for positive transformants + conditions for E. coli), verification of the construct (specific methods and conditions?), transfection protocol, control groups, and altogether the culture conditions!

2.3 Cell transfection

Ribobio (Guangzhou, China) provided pcDNA-MEG3, small ~~hairbin~~ hairpin RNA targeting MEG3 (sh-MEG3), miR-181-5p mimic, miR-181-5p inhibitor, sh-GATA6 and their corresponding negative controls. Refs#? For pcDNA vector construction, the pcDNA.3.1 vector and the DNA fragment containing the target gene were double-digested with restriction endonuclease BamH I and Age I, and then the two digested products were linked with T4-DNA ligase. The recombinant vector was transformed into E. coli DH5α competent cells. monoclonal colonies were selected for culture and positive transformants were screened. The constructed vector was verified by double digestion and sequencing analysis. They were transfected into PC3 cells with Lipofectamine 3000 regent (Invitrogen, USA). The transfection concentrations were as follows: pcDNA-MEG3 (2 μg), mimic (50 nM), inhibitor (100 nM), and shRNA (1 μg). Cells were harvested for further experiments after 48 h of transfection.

kit? products catalog numbers?

italics

how?

how? details?

specifics???

cat #?

what about the rest of the protocol? culture conditions? incubation times, etc?

2.4 RT-qPCR analysis

PC3 cells were incubated with TRIzol reagent (Invitrogen, USA) to extract total RNAs, whilch were quickly frozen in -80°C until used. The RNA concentration was tested using NanoDrop 2000 (Thermo Fisher, USA). RNAs were then subjected to synthesize complementary DNA by using a cDNA Reverse Transcription Kit (Invitrogen, USA) with temperature protocol: 70°C for 5 min, 37°C for 5 min and 42°C for 60 min. RT-qPCR reaction was conducted with SYBR Green PCR Kit (Applied Biosystems, USA) under the reaction condition: 95°C for 10 min, and 40 times repeat of 95°C for 30 s and 60°C for 1 min. The reaction system included 12.5 μL of SYBR Green PCR Mix, 1.0 μL of primer (Final concentration 0.5 μM), 1 μL of cDNA sample, and 10.5 μL of double distilled H_2O. Finally, the specificity of primer was verified by dissolution curve analysis, and the amplification specificity was considered to be better when the melting curve was single peak and Tm>80℃. LncRNA MEG3 and GATA6 expression levels were normalized to GAPDH and miR-181-5p were normalized to U6, and calculated by the $2^{-\Delta\Delta CT}$ method. The following primer sequences were used: MEG3 (forward, 5'-AGT CCA TCG CAG ATA CTG GC-3' and reverse, 5'-GGG AAT AGG TGC AGG GTG TC-3'), GATA6 (forward, 5'-TGC AAT GCT TGT GGA CTC TA-3' and reverse, 5'- GTG GGG GAA GTA TTT TTG CT-3'), GAPDH (forward, 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and reverse, 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'), miR-181-5p (forward, 5'-GAA CAT TCA ACG CTG TCG GTG-3' and reverse, 5'-. ATC CAG TGC AGG GTC CGA GGT A-3′), and U6 (forward, 5'-CTC GCT TCG GCA GCA CA-3' and reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3').

pure?? cat#?

cat #?

cat#?

cat#?

To replicate the experiment accurately, researchers would need these specific details to ensure that the methods and conditions are consistent with the original study. Unsure clarity and transparency! As well as accuracy and completeness!

2.5 Co-expression network analysis not comprehensive or clear enough!

The interaction between MEG3 and miRNAs, as well as miRNA and mRNA were predicted by TargetScan, miRTarBase and miRDB databases. The predicted target genes were then compared with the data set. The differentially expressed miRNAs and mRNAs take the intersection to obtain candidate target genes. Based on the regulatory relationship among MEG3, miRNA and mRNA, the MEG3-miRNA-mRNA regulatory network was established.

2.6 CCK-8 assay exact data sources? target prediction methods/algorithms? Differential expression analysis? intersection and selection criteria? Did you consider experimentally validated interactions or only predicted ones? validation? biological context?

Cell counting kit 8 (CCK-8) assay was employed to access cell proliferation. After transfection and Niraparib treatment, PC3 cells were inoculated in a 96-well plate (5×10^3/well). Cells were then cultured for 0, 24, 48 and 72 h respectively before adding 10 μL of CCK-8 (Beyotime, Jiangsu, China) solution into the culture medium in each well. After 2 h of incubation, the absorbance at 450 nm was accessed with a microplate reader (Bio-Rad, USA).

cat #? company?? still in DMEM? concentration? which was? final volume per well? model? specifics of the program?

2.7 ~~Wound healing assay~~ Cell Migration Assay, would be a better name here!

After transfection and Niraparib treatment, PC3 cells were inoculated in a 6-well plate. On the back of the 6-well plate, uniform horizontal lines were scratched with a marker pen at approximately 0.5-1cm intervals. At least five lines were passed through each hole. Cell were incubated under 5% CO_2 at 37°C until confluence reached ~~to~~ 60–70%. Next, the cell surface was lightly scratched with a sterile micropipette tip, and the detached cells were removed through PBS flushing. Afterwards, serum-free medium was added into plates and cultured for 24 h. Wound healing area was monitored under a light microscope (Nikon, Japan) at different ~~points of time~~, and the wound healing distance was analyzed by ImageJ software.

inoculated or seeded? so, scratched or drawn? ??? Model? software? intervals? what were these time intervals?? version? -> this protocol also needs to be in the manuscript!

2.8 Transwell invasion assay

Transwell chamber (8 μm pore size; Corning, NY, USA) precoated with 50 μL Matrigel were used in Transwell invasion assay. The transfected PC3 cells suspended in FBS-free DMEM were seeded in the upper chamber, followed by addition of DMEM containing 10% FBS into the lower chamber. After 24 h culture at 37°C, the invading cells in the lower chamber were stained by 0.1% crystal violet, and then observed under and analyzed under a light microscope (Nikon, Japan).

cat#? concentration? based on a Ref? model? did you just take the pictures or did an image analysis protocol?

2.9 Western blot analysis

Protein s in PC3 cells or tissues were extracted using RIPA assay (Invitrogen, USA). The protein samples were mixed with loading buffer at a 4:1 ratio and then boiled at 95°C for 5 min. Afterwards, proteins (30 μM) were added to 10% SDS-PAGE and then transferred to a PVDF membrane (Millipore, Bedford, MA, USA). After blocked with 5% skimmed milk, the membranes were incubated with primary antibodies (Abcam) including GATA6 (1 mg/mL, 1:1000; ab175349), E-cadherin (0.294 mg/mL, 1:1000; ab40772), ICAM-1 (0.624 mg/mL, 1:1000; ab109361), CD44 (1 mg/mL, 1:1000; ab243894) and GAPDH

? cat #? concentration? quickly describe the extraction which loading buffer ? concentration? ? gel? cat# and company? cat#?

(1 mg/mL, 1:2500; ab9485), overnight at 4˚C, and then incubated with secondary antibody (2 mg/mL, 1:2000; ab6721) at 37˚C for 2 h. Protein bands were developed with the enhanced chemiluminescence system (Amersham, UK) and analyzed with ImageJ software (NIH, Bethesda, USA).

model? how? version?

2.10 Dual-luciferase reporter assay

The binding sites of miR-181-5p in MEG3 and GATA6 was searched in the Starbase v3.0 software (http://starbase.sysu.edu.cn/). We clicked the item of miRNA target and chose miRNA-lncRNA/miRNA-mRNA, and entered miR-181-5p in the miRNA item, and all lncRNAs/mRNAs have potential binding relationship with miR-181-5p would appear. Then we searched MEG3/GATA6 to get corresponding binding sites. For dual-luciferase reporter assay, the 3' UTR sequence of the predicted target lncRNA/mRNA was inserted into the 3' UTR of the firefly luciferase vector. Then the constructed vector was co-transfected with miRNA into cells. If miRNA can bind to the inserted 3' UTR sequence of lncRNA/mRNA, the translation of firefly luciferase is inhibited, resulting in a decrease in fluorescence value. renilla luciferase was used as an internal reference. The ratio of fluorescence values between firefly luciferase and renilla luciferase was taken as the relative luciferase activity. The MEG3-wild type (WT), MEG3 mutant type (MUT), GATA6-WT and GATA6-MUT reporter vectors were constructed by Transgen Biotech (Beijing, China). The fragments of MEG3 or GATA6 containing the wild or mutated miR - 181 - 5p binding site were synthesized and cloned into pmirGLO vector (Promega, Madison, WI, USA). Next, these plasmids were co-transfected into PC3 cells with NC mimic or miR-2113 mimic using Lipofectamine 3000 reagent for 48 h at 37°C. The relative luciferase activity was tested with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

accessed on…? package? you can cite PMID: 21037263 here if needed

we need more details here, of these constructs…

designation incomplete. cat #?

2.11 Animal studies

why do you need in vivo studies here?

Healthy male BALB/c nude mice (20 ± 2 g) were provided by the experimental animal center of Xi'an Jiaotong University. Animal experiments were approved and supervised by the Animal Ethics Committee of Shaanxi Provincial People's Hospital. All methods were carried out in accordance with relevant guidelines and regulations. Mice were maintained in cages under a standard experiment environment (12 h light/dark cycle, 22-25°C temperature, 55-60% humidity) with free access to standard food and water. Mice were divided into four groups based on the random number table method: PBS, Niraparib, Niraparib+sh-NC, Niraparib+sh-MEG3 (n=8 per group). After 7 days of acclimatization, PC3 cells (1×10^6, 200μL) were subcutaneously injected into the left flanks of mice to establish a xenograft tumor model. For niraparib treatment, niraparib was diluted in PBS (200 μL) and administered intraperitoneally into mice five days per week for four weeks. The same volume of PBS was used as control. For Niraparib+sh-NC and Niraparib+sh-MEG3 groups, PC3 cells transfected with sh-NC or sh-CENPA were injected into mice, followed by niraparib treatment. All mice were carefully nursed after treatment. Afterwards, we measured the length and width of tumors every 7 days, and tumor volume was calculated by the formula: volume = [length × width^2]/2. 28

how many?

approval number?

? 1X, 10X? what? Concentrations?? what's this?

in PBS , NaCl 0.7%, or DMEM?

Treatment and control groups need to be properly described! Doses need to be registered, etc

days later, mice were euthanized with an intraperitoneal injection of 100 mg/kg pentobarbital sodium, and tumors were excised, imaged by a camera (Z5; Nikon, Japan), and weighed. The tests were conducted by 2 independent researchers blinded to the experimental groups.

what tests?

2.12 Immunohistochemistry assay

Tumor tissues were fixed in 10% formaldehyde and embedded in paraffin, and cut to prepare 4 μm thick slices. Slices were microwaved with sodium citrate solution and inactivated with 3% H_2O_2 for 10 min. Next, slices were incubated with Ki-67 antibodies (1:200; Abcam, ab16667) or negative control IgG (1:300; Abcam, ab109489) overnight at 4°C and then secondary antibody (1:1000; Abcam, ab6721) for 1 h. Afterwards, the slices were stained by using a DAB kit (Beyotime, China) and captured images with a light microscope.

what about dehydrated? ethanol? which?
details of this solution?
in?
which IgG?
you used an anti-human secondary with a rabbit-raised primary??
very incomplete description!

2.13 Statistical analysis

Experimental data from at least triplicate experiments were presented as mean± deviation (SD). The cell sample size is N=6, and the animal sample size is N=8. SPSS 22.0 software was used for Statistical analysis. The normal distribution of data was verified by the Shapiro-Wilk test, and the homogeneity of variances was verified by the Levene's test. Student's t test was used for comparations between two groups, and one-way analysis of variance (ANOVA) followed by Tukey-Kramer correction was used for comparations among multiple groups. Non-parametric tests (Kruskal-Wallis test/Mann-Whitney test) were used if data were not normally distributed or variances were not homogeneous. $P<0.05$ was considered statistically significant.

where were made triplicate experiments?? you raised 8x4groups mice three times?
cell sample size? animal sample size?
look up how to properly cite this!
why Shapiro's and Levene's?

3. Results

3.1 LncRNA MEG3/miR-181-5p/GATA6 was intimately related in PCa

We first access MEG3 expression in tumor tissues of PCa patients, and our RT-qPCR results illustrated that MEG3 was dramatically downregulated in tumor tissues compared with non-tumor tissues (Fig. 1A). Then, we found that the MEG3/miR-181-5p/GATA binding protein 6 (GATA6) axis was intimately related in PCa by Co-expression network analysis (Fig. 1B). Additionally, our results implied that miR-181a-5p was obviously upregulated (Fig. 1C) and GATA6 mRNA was downregulated (Fig. 1D) in tumor tissues of PCa patients. As expected, MEG3 expression was negatively correlated with miR- miR-181-5p expression (Fig. 1E), and miR-181-5p expression was negatively correlated with GATA6 mRNA (Fig. 1F) in our recruited PCa patients.

3.2 Niraparib treatment upregulated MEG3 and GATA6, and downregulated miR-181-5p expression in PCa cells

PARPi take effect through the synthetic lethality of homologous recombination repair gene defects such as BRAC to inhibit DNA damage repair and promote apoptosis in cancer cells [9, 10]. It was reported that niraparib showed impressive performance in phase II trials for metastatic CRPC patients [17]. Recent studies suggested that PARPi therapy may exert anti-PCa effects through activating MEG3 and thereby

promoting PARP cleavage [25, 26, 28]. Thus, we explored whether the anti-PCa effect of niraparib is related to the change of MEG3 expression. We first treated PC3 cells with different concentrations of niraparib. CCK-8 assay showed that niraparib treatment restrained PC3 cell proliferation in a dose dependent manner, and 4 μM 4 μM and 8 μM niraparib had comparable inhibitory activity against PCa cell proliferation (Fig. 2A). Then, we treated PC3 cells with niraparib for 0, 30, 60, 120 min. It was observed that niraparib-mediated PC3 cell proliferation inhibition effect enhanced with incubation time (Fig. 2B). Next, we investigated whether niraparib affect MEG3 expression in PC3 cells. RT-qPCR results revealed that niraparib treatment substantially upregulated MEG3 expression in a dose dependent manner, and which reached peak value at 4 μM (Fig. 2C). Moreover, the promoting effect of niraparib on MEG3 expression intensified with incubation time, and which reached peak value at 120 min (Fig. 2D). Besides, we also found that niraparib treatment downregulated miR-181-5p expression (Fig. 2E, 2F). and upregulated GATA6 mRNA expression (Fig. 2G, 2H). Thus, our results implied that the anti-PCa effects of niraparib was associated with the MEG3/miR-181-5p/GATA6 axis.

3.3 Niraparib treatment restrained PCa cell proliferation, migration and invasion

We then investigate the exact effects of niraparib on Pca cell behaviors. PC3 cells were incubated with 4 nM niraparib for 120 min. Wound healing assay suggested that niraparib treatment remarkably restrained PC3 cell migration (Fig. 3A, 3B). Meanwhile, the invasion abilities of PC3 cells were suppressed by niraparib (Fig. 3C, 3D). Furthermore, it was obviously showed that niraparib incubation decreased E-cadherin protein level and increased ICAM-1 and CD44 protein levels in PC3 cells (Fig. 3E, 3F), indicating that niraparib inhibited PCa cell metastasis.

3.4 MiR-181-5p and GATA6 were downstream genes of MEG3 in PCa cells

We next perfected the molecular mechanisms of MEG3/miR-181-5p/GATA6 axis. As searched by Starbase software, miR-181a-5p had putative complementary binding sites with the 3′-UTR of MEG3 and 3′-UTR of GATA6 (Fig. 4A). Dual-luciferase reporter assay demonstrated that miR-181-5p mimic substantially suppressed the luciferase activity of wild MEG3 but not mutant MEG3, while NC mimic had no effects on the luciferase activity of wild and mutant MEG3 (Fig. 4B). Also, the luciferase activity of wild GATA6 was obviously inhibited by transfection of miR-181-5p mimic, but the mutant GATA6 group was not affected in PC3 cells (Fig. 4C). Afterwards, we confirmed that transfection of pcDNA-MEG3 obviously facilitated MEG3 expression in PC3 cells compared with transfection of empty vector, while transfection of sh-MEG3 restrained MEG3 expression compared with transfection of sh-NC (Fig. 4D). Notably, pcDNA-MEG3 transfection remarkably inhibited miR-181-5p expression compared with empty vector, while sh-MEG3 transfection facilitated miR-181-5p expression compared with sh-NC transfection (Fig. 4E). Additionally, transfection of miR-181-5p mimic increased miR-181-5p expression compared with NC mimic, while transfection of miR-181-5p inhibitor suppressed miR-181-5p expression compared

with NC inhibitor (Fig. 4F). Moreover, miR-181-5p mimic transfection prominently reduced GATA6 mRNA and protein levels compared with NC mimic, but they were elevated after miR-181-5p inhibition while transfection of miR-181-5p inhibitor elevated GATA6 mRNA and protein levels compared with NC inhibitor (Fig. 4G-4I). These above results confirmed that the miR-181-5p and GATA6 were downstream genes of MEG3 in PCa cells.

3.5 MiR-181-5p overexpression reversed MEG3 overexpression-mediated inhibition of PCa cell progression

We then adopted rescue experiments to determine the roles of MEG3/miR-181-5p/GATA6 axis in PCa cell progression. PC3 cells were co-transfected with pcDNA-MEG3 and miR-181-5p mimic. First, we observed that MEG3 overexpression suppressed miR-181-5p expression in PC3 cells, whereas miR-181-5p mimic transfection increased miR-181-5p level (Fig. 5A). Then, MEG3 overexpression prominently restrained PC3 cell proliferation, which were abolished by miR-181-5p overexpression (Fig. 5B). Furthermore, MEG3 overexpression suppressed PC3 cell migration (Fig. 5C, 5D) and invasion (Fig. 5E, 5F), whereas miR-181-5p overexpression retarded these effects. Besides, MEG3 overexpression decreased E-cadherin protein level and increased ICAM-1 and CD44 protein levels in PC3 cells (Fig. 3G, 3H), while this expression pattern was reversed by miR-181-5p overexpression. These results illustrated that MEG3 overexpression mediated PCa cell biological functions via regulating miR-181-5p expression.

3.6 GATA6 silencing abrogated the effects of miR-181-5p inhibition on T24/DDP cell behaviors

Next, PC3 cells were transfected with miR-181-5p inhibitor and si-GATA6. Western blot results proposed that miR-181-5p inhibition markedly enhanced GATA6 expression, while si-GATA6 transfection decreased GATA6 expression (Fig. 6A). MiR-181-5p inhibition suppressed PC3 cell proliferation (Fig. 6B), while GATA6 silencing retarded this effect. Also, miR-181-5p inhibition mitigated PC3 cell migration (Fig. 6C, 6D) and invasion (Fig. 6E, 6F), while these effects were abrogated by miR-181-5p inhibition. Additionally, our results suggested that E-cadherin level was reduced and ICAM-1 and CD44 levels were increased after miR-181-5p inhibition, while these effects were reversed by GATA6 silencing (Fig. 7G, 7H). The rescue experiment results implicated that MEG3 could attenuated PCa cell progression through the miR-181-5p/GATA6 axis.

3.7 Niraparib mitigated PCa tumor growth in vivo through regulating the MEG3/miR-181-5p/GATA6 axis.

We finally investigated the correction between niraparib and the MEG3/miR-181-5p/GATA6 axis in vivo. PC3 cells were injected into mice to establish a xenograft tumor model. It was clearly observed that tumor volume and weight were conspicuously decreased after niraparib injection compared with injection of PBS, whereas MEG3 silencing could retarded niraparib-mediated tumor inhibition (Fig. 7A-7C). Next, Immunohistochemistry assay suggested that niraparib

treatment intensified MEG3 and GATA6 expression and decreased miR-181-5p expression in tumor tissues, while MEG3 silencing abolished these effects (Fig. 7D-7F). In addition, immunohistochemistry assay illustrated that niraparib injection reduced Ki67 protein level in tumors, which were then reversed by MEG3 silencing (Fig. 7G, 7H). Therefore, our results proposed that niraparib mitigated PCa tumor growth in vivo through regulating the MEG3/miR-181-5p/GATA6 axis.

4. Discussion

The development of PARPi therapy has prominently improved the treatment outcomes of metastatic PCa patients with certain genetic mutations [29]. It was reported that niraparib and talazoparib showed impressive performance in phase II trials for metastatic CRPC patients [17]. A phase 2 clinical trial demonstrated that niraparib is relatively safe and exhibits anti-tumour activity in patients with metastatic CRPC [30]. Moreover, a recent study illustrated that niraparib offered better tissue exposure and more potent tumor growth suppression in PCa bone metastasis mice, compared with other PARPi [31]. The present study investigated niraparib-mediated anti-PCa molecular mechanisms.

Current evidence revealed that lncRNA MEG3 was downregulated in PCa tissues. MEG3 overexpression mitigated PCa cell proliferation and metastasis and induce apoptosis, and attenuated tumor development in mice [23, 24]. Notably, MEG3 was found to be involved in the progression of multiple cancers through regulating some DNA repair gene, such as PTEN [25, 26] and p53[27]. Importantly, it was found that MEG3 overexpression could decreased intact PARP level and increased cleavage PARP level, thus promoting cell apoptosis [28]. Based on these findings, we hypothesized that PARPi therapy may require the activation of MEG3 to regulate DNA repair gene to exert anti-PCA effects. The effect of PRAPi on MEG3 expression has not been studied to date. Therefore, to explore more targets for PRAPi therapy, it's of great significance to investigate the impact of MEG3 expression on PARP1 targeted CRPC treatment. As expected, our results showed that niraparib treatment upregulated MEG3 expression in PCa cells. Additionally, niraparib administration restrained tumor growth in a PCa xenograft mouse model, while MEG3 silencing treatment retarded these effects. Thus, niraparib mediated-MEG3 upregulation is a crucial mechanism for tumor inhibition.

Our study screened out miR-181-5p that showed high expression in PCa and was negatively correlated to MEG3 expression. A previous miRNA-microarray analysis identified that miR-181-5p was associated with drug resistance and efflux, and epithelial to mesenchymal transition in PCa [32]. MiR-181-5p could also lead to cisplatin resistance in PCa cells through complementary interactions with the 3 'UTR of the proapoptotic protein BAX transcript [33]. Moreover, MiR-181 facilitated PCa cell proliferation and tumor

development in mice through regulation of an androgen receptor negative regulator, DAX1 [34]. We can see that miR-181-5p is closely related to the natural course, drug resistance, and androgen receptor resistance of PCa. Our study implied that miR-181a-5p was obviously upregulated in PCa patients, and its expression was negatively correlated with MEG3 expression. Subsequently, we confirmed miR-181a-5p as a target of MEG3 in PCa cells through Starbase database prediction and dual-luciferase reporter assay validation. Rescue experiments implicated that miR-181a-5p overexpression reversed MEG3 overexpression-mediated suppression of PCa cell proliferation and metastasis, implying that MEG3 exerted anti-PCa effect through reducing miR-181-5p expression.

Our study found that the MEG3/miR-181-5p/GATA6 axis was intimately related in PCa. GATA6 is a member of the gene family with the promoter GATA core conserved sequence. An RNA-sequence analysis of tumor tissue samples from PCa patients revealed that GAGT6 was a downregulated gene in PCa [35]. Moreover, lncRNA LINC00261 could intensify GATA6-mediated transcriptional inhibition and then suppressed PCa tumorigenesis [36]. GATA6 was identified as a downstream of the Linc00518/miR-216b-5p axis, and intimately related to paclitaxel resistance in PCa [37]. Our further study confirmed that GATA6 mRNA was downregulated in PCa patients. GATA6 was a target gene of miR-181-5p, and its expression was suppressed by miR-181-5p. Furthermore, miR-181-5p inhibition restrained PCa cell proliferation, migration, and invasion, whereas these effects were abrogated by GATA6 silencing. Therefore, we proposed that MEG3 participated in PCa progression through the miR-181-5p/GATA6 pathway.

Maybe you'd like this opportunity to address the recent FDA approval of Niraparib + Abiraterone for mCRPC?

5 Conclusions

and ovarian! it started for epithelial ovarian/peritoneal tumours!

Our study illustrated that niraparib, a PRAPi drug for PCa patients, restrained PCa cell invasive and metastatic phenotypes and delayed tumor growth in mice by upregulating MEG3 expression, which in turn mediated the miR-181-5p/GATA6 pathway. The findings reveal a novel molecular mechanism by which the representative PRAPi drug niraparib exerts anti-tumor effects, and provide a theoretical basis for PCa patient treatment.

wasn't this shown before?

Conclusions should be reformulated. They are not accurate; there is no real novelty! what is it? theoretical? Scientific claims should always be evaluated within the context of the broader body of research in the field.

a quick search: https://www.iasj.net/iasj/article/223160

Funding Statement

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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

408

409 **Competing interests**

410 No conflicts of interest exit in the submission of this manuscript.

411

412 **References**

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Figure 1

LncRNA MEG3/miR-181-5p/GATA6 was intimately related in PCa.

(A) MEG3 expression in tumor tissues and non-tumor tissues from PCa patients (N=20) was accessed with RT-qPCR assay. (B) The MEG3/miR-181-5p/GATA6 axis was intimately related in PCa. (C) MiR-181a-5p and (D) GATA6 mRNA expression levels in tumor tissues and non-tumor tissues from PCa patients were accessed with RT-qPCR assay. (E) MEG3 expression was negatively correlated with miR- miR-181-5p expression in our recruited PCa patients. (F) MiR-181-5p expression was negatively correlated with GATA6 mRNA in our recruited PCa patients. N=6. Data from at least triplicate experiments were presented as mean ± SD. ***P*<0.01.

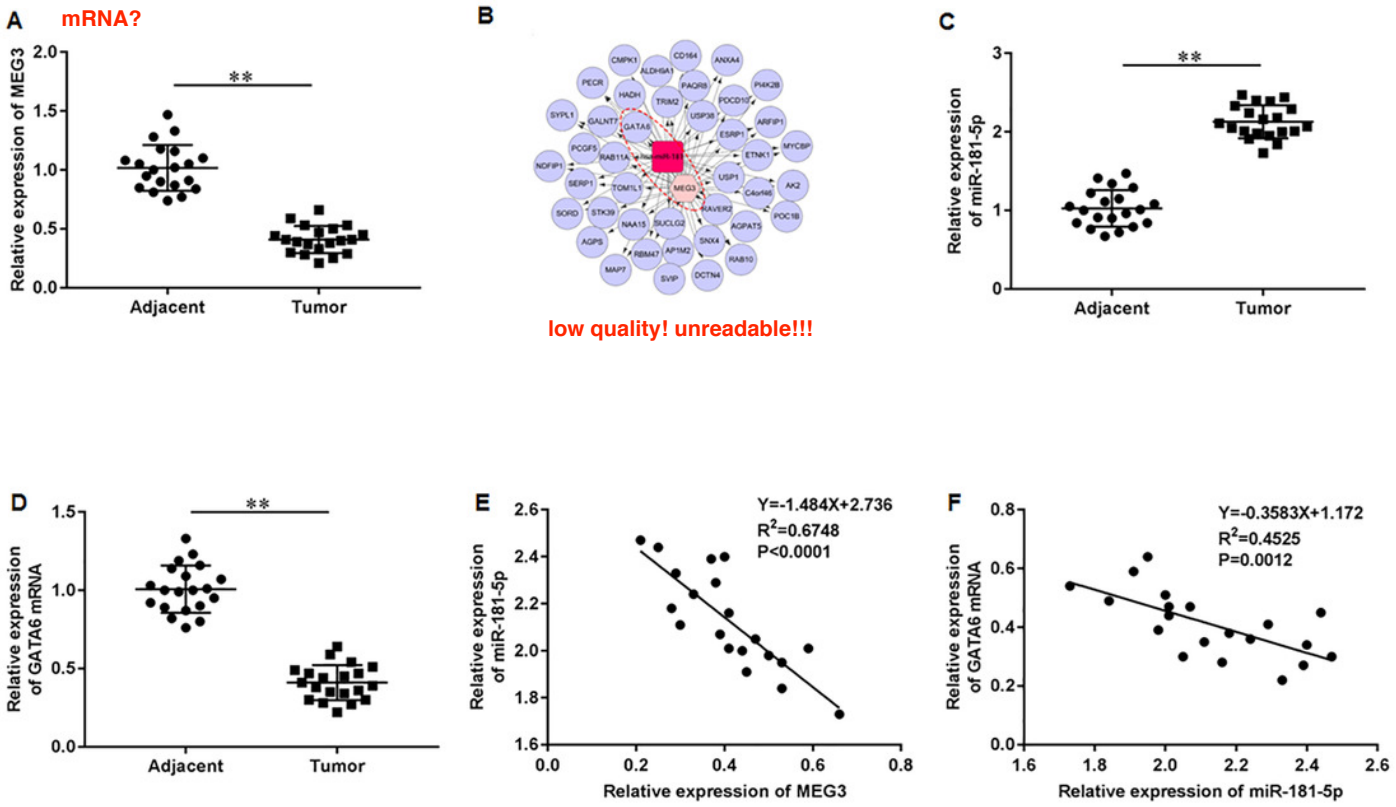
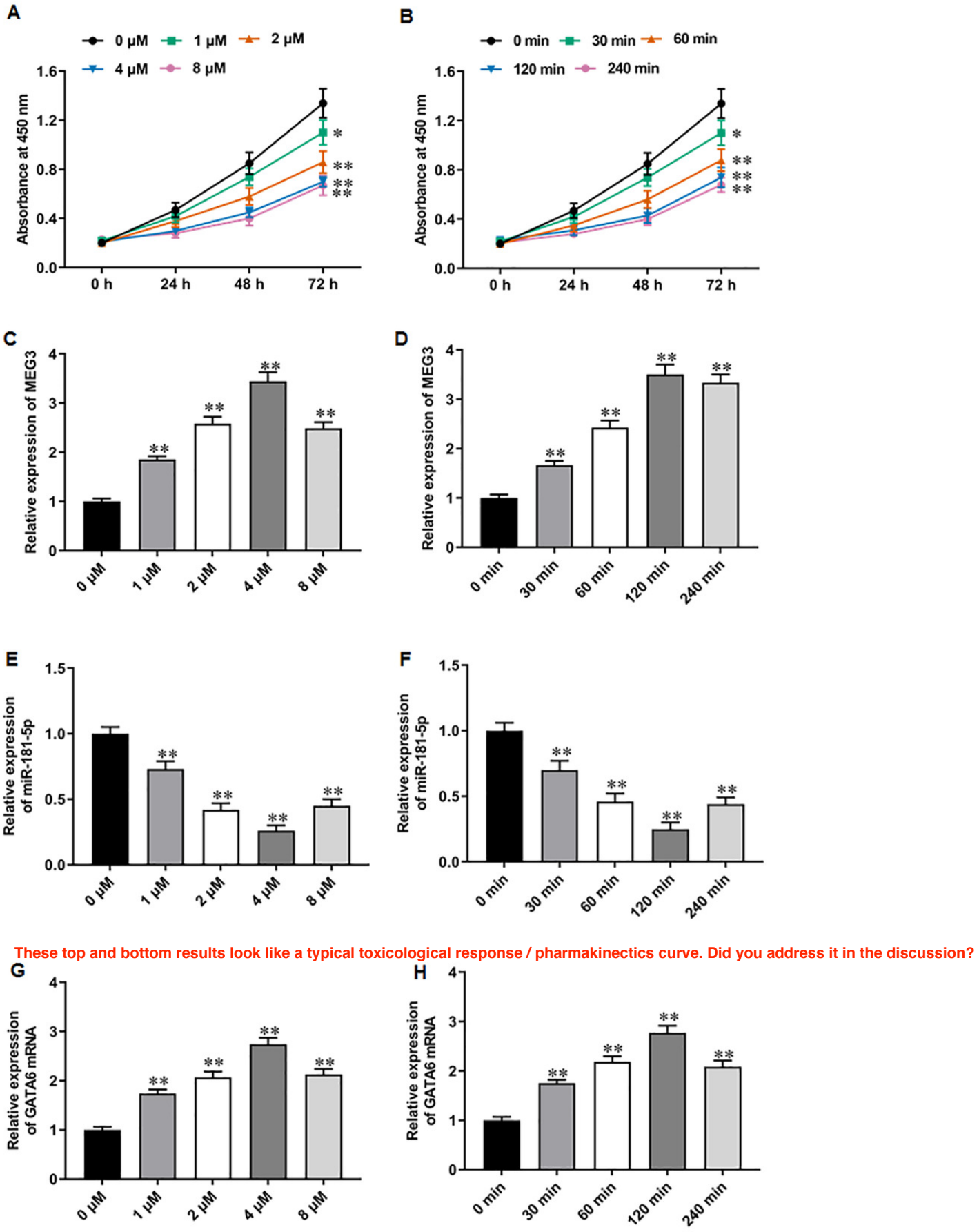


Figure 2

Niraparib treatment upregulated MEG3 and GATA6, and downregulated miR-181-5p expression in PCa cells.

(A) PC3 cell proliferation was accessed with CCK-8 assay after treatment with different concentrations of niraparib (0, 1, 2, 4, and 8 μM). (B) PC3 cell proliferation was accessed with CCK-8 assay after treatment with 4 μM niraparib for 0, 30, 60, 120 min. (C) MEG3 expression was accessed with RT-qPCR assay after treatment with different concentrations of niraparib (0, 1, 2, 4, and 8 μM). (D) MEG3 expression was accessed with RT-qPCR assay after treatment with 4 μM niraparib for 0, 30, 60, 120 min. (E) MiR-181-5p expression was accessed with RT-qPCR assay after treatment with different concentrations of niraparib (0, 1, 2, 4, and 8 μM). (F) MiR-181-5p expression was accessed with RT-qPCR assay after treatment with 4 μM niraparib for 0, 30, 60, 120 min. (G) GATA6 mRNA expression was accessed with RT-qPCR assay after treatment with different concentrations of niraparib (0, 1, 2, 4, and 8 μM). (H) GATA6 mRNA expression was accessed with RT-qPCR assay after treatment with 4 μM niraparib for 0, 30, 60, 120 min. N=6. Data from at least triplicate experiments were presented as mean ± SD. $^{**}P<0.01$.

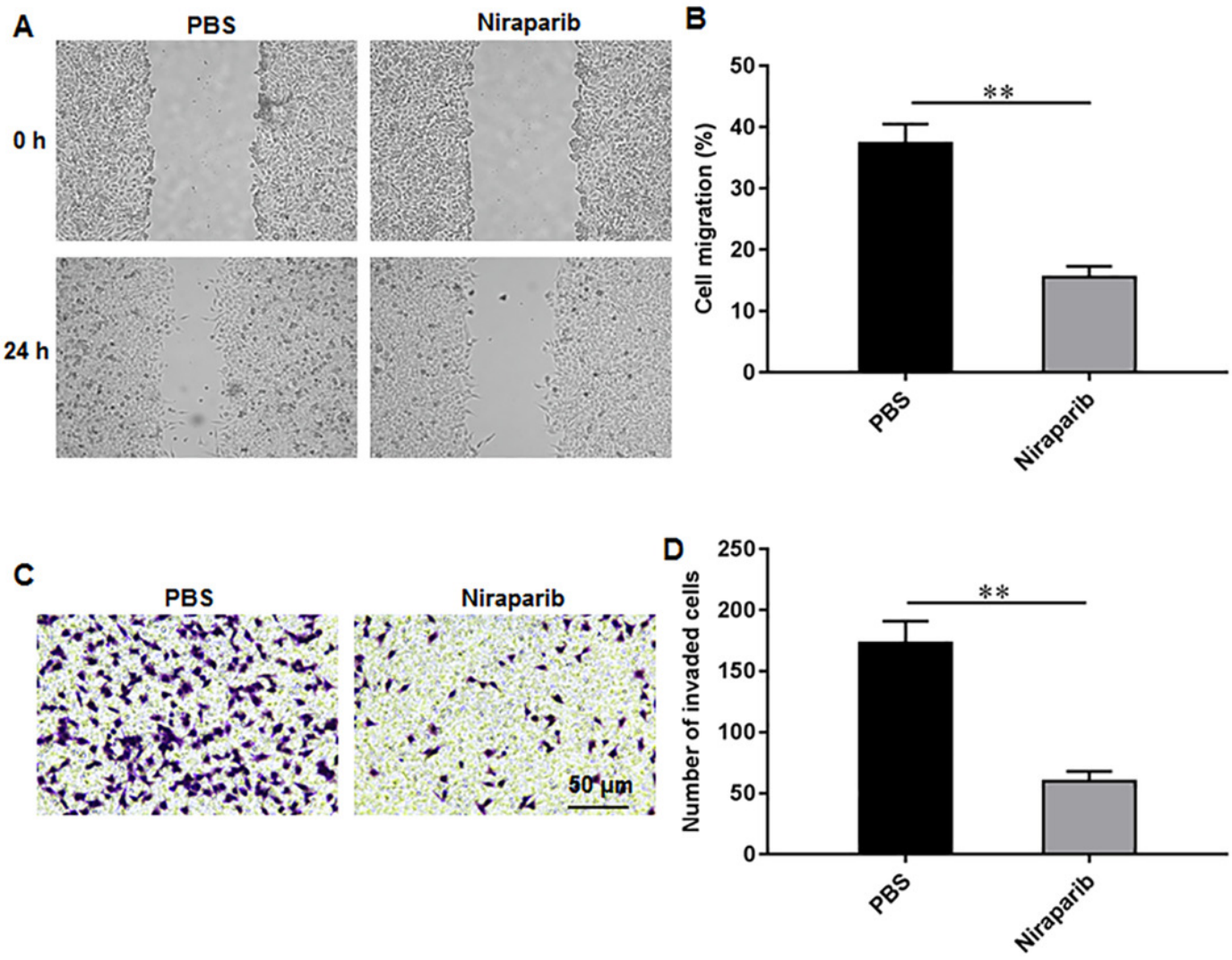


These top and bottom results look like a typical toxicological response / pharmakinectics curve. Did you address it in the discussion?

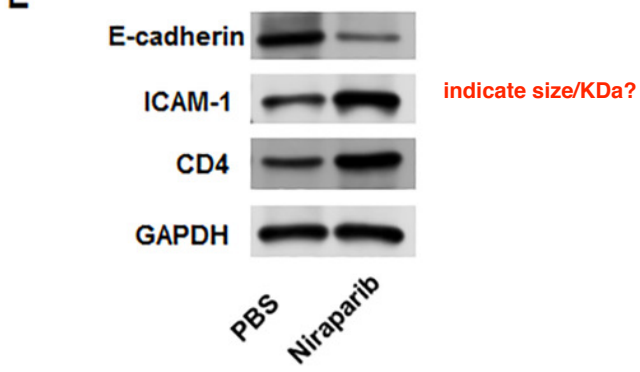
Figure 3

Niraparib treatment restrained PCa cell proliferation, migration and invasion.

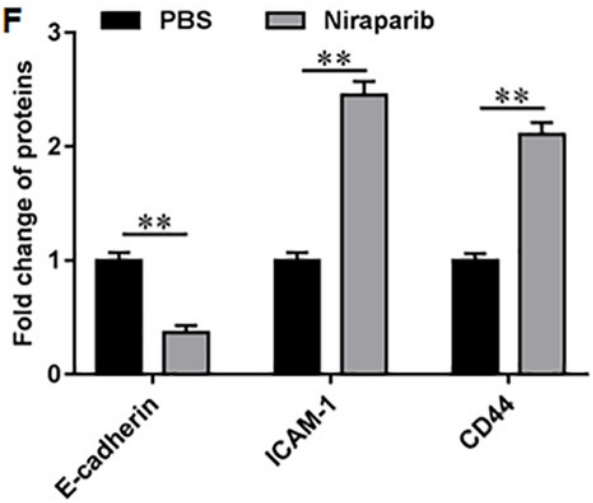
PC3 cells were incubated with 4 nM niraparib for 120 min. (A, B) PC3 cell migration was accessed with wound healing assay. (C, D) PC3 cell invasion was tested with Transwell assay. (E, F) E-cadherin, ICAM-1, and CD44 protein levels were gauged with Western blot assay. N=6. Data from at least triplicate experiments were presented as mean ± SD. $^{**}P<0.01$.



original WB? with protein ladder showing, please!



indicate size/KDa?



densitometry? where is the protocol described in the methods?

Figure 4

MiR-181-5p and GATA6 were downstream genes of MEG3 in PCa cells.

(A) StarBase software showed that miR-181a-5p had putative complementary binding sites with the 3′-UTR of MEG3 and 3′-UTR of GATA6. (B, C) Dual-luciferase reporter assay was applied to validate the binding between MEG3 and miR-181-5p, as well as miR-181-5p and GATA6. (D) MEG3 and (E) miR-181-5p expression was gauged with RT-qPCR assay after transfection of pcDNA-MEG3 or si-MEG3 in PC3 cells. (F) MiR-181-5p expression was gauged with RT-qPCR after transfection of miR-181-5p mimic or si- miR-181-5p inhibitor in PC3 cells. (G-I) The mRNA and protein expression of GATA6 was gauged with RT-qPCR or Western blot assay after transfection of miR-181-5p mimic or miR-181-5p inhibitor in PC3 cells. N=6. Data from at least triplicate experiments were presented as mean ± SD. $^{**}P<0.01$.

where were this designed?

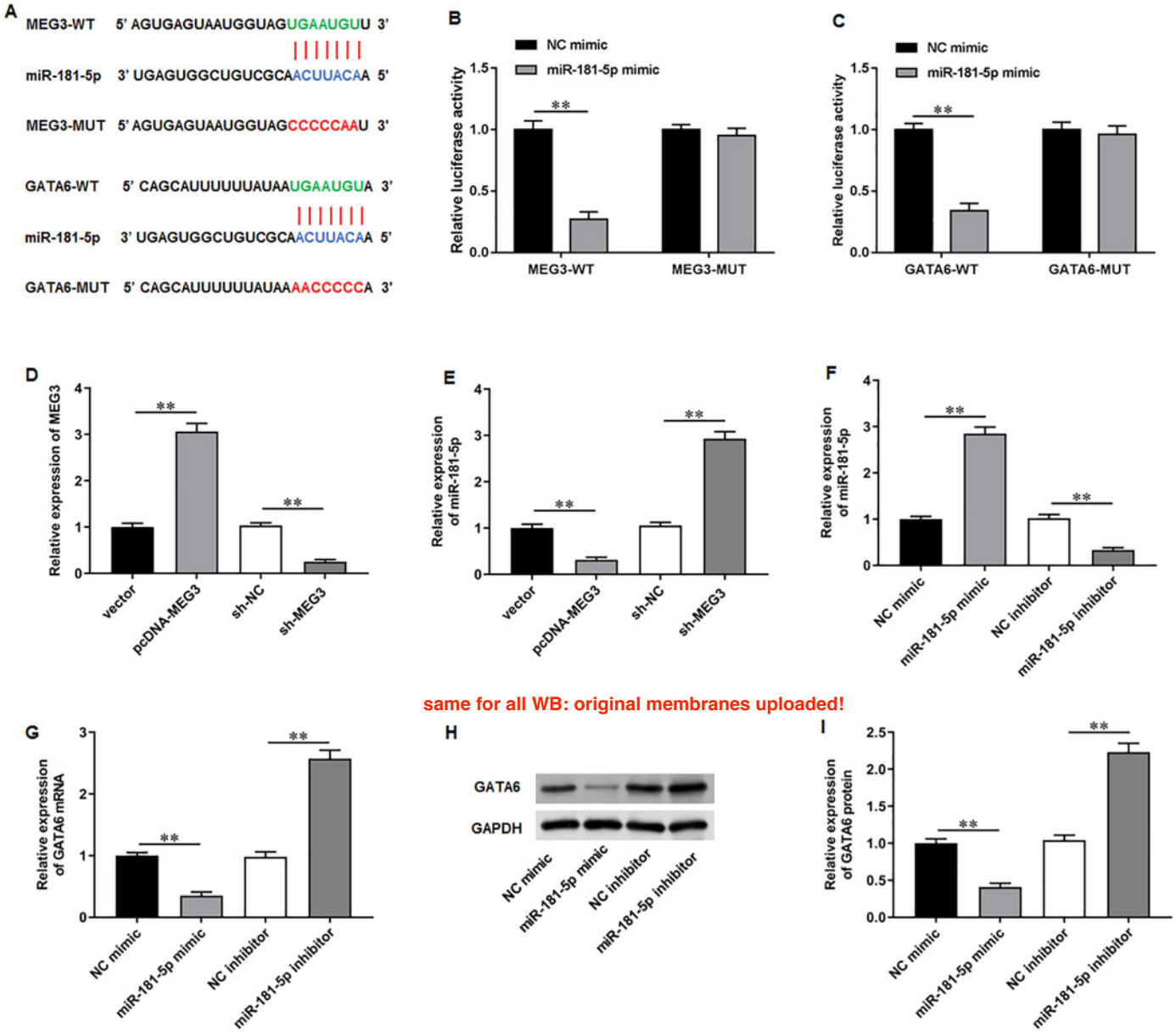


Figure 5

MiR-181-5p overexpression reversed MEG3 overexpression-mediated inhibition of PCa cell progression.

PC3 cells were co-transfected with pcDNA-MEG3 and miR-181-5p mimic. (A) MiR-181-5p level was tested with RT-qPCR analysis. (B) PC3 cell proliferation was accessed with CCK-8 assay. (C, D) PC3 cell migration was accessed with wound healing assay. (E, F) PC3 cell invasion was tested with Transwell assay. (G, H) E-cadherin, ICAM-1, and CD44 protein levels were gauged with Western blot assay. N=6. Data from at least triplicate experiments were presented as mean ± SD. $^{**}P<0.01$.

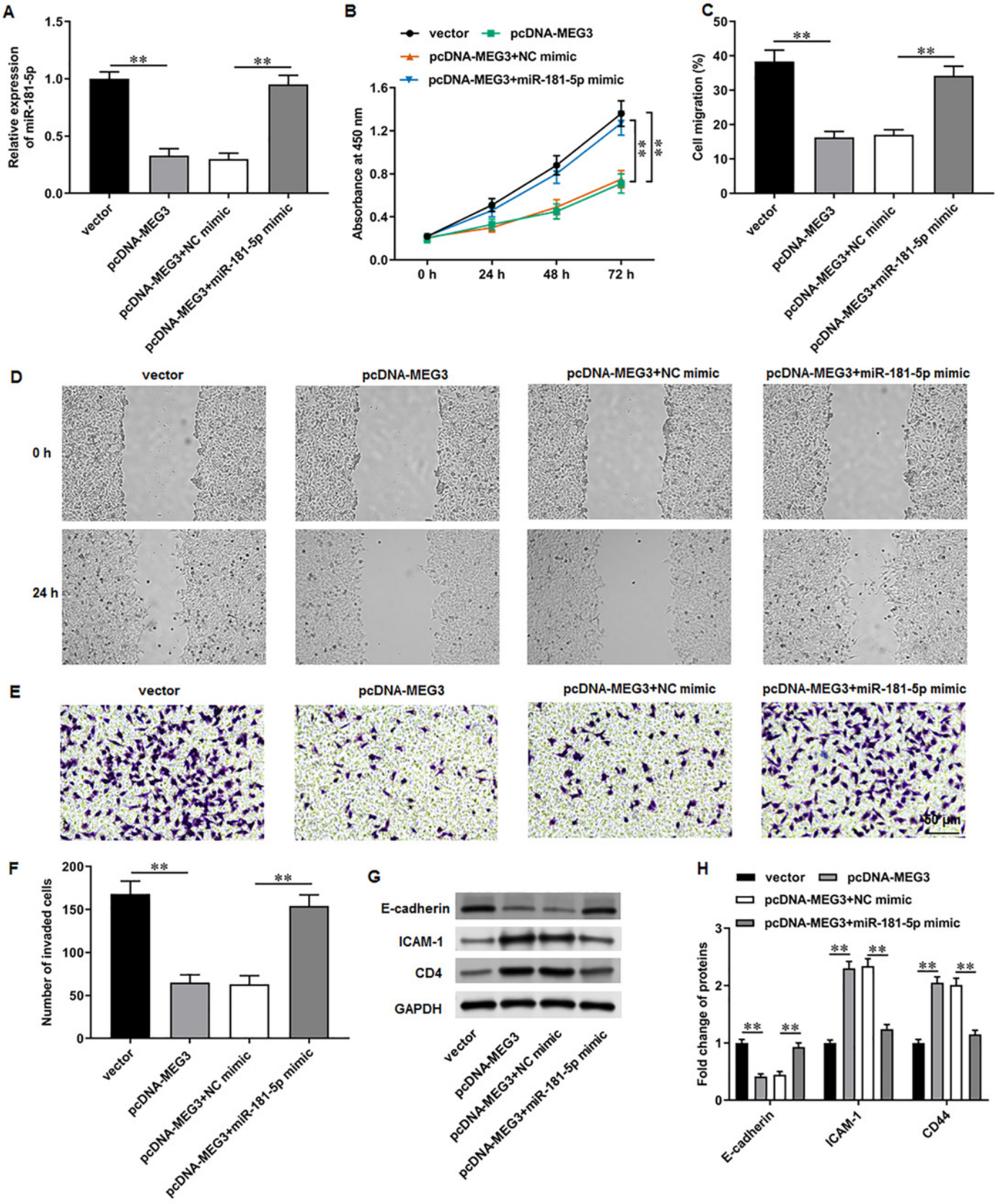

A
Relative expression of miR-181-5p
1.5
1.0
0.5
0.0
**
**
vector
pcDNA-MEG3
pcDNA-MEG3+NC mimic
pcDNA-MEG3+miR-181-5p mimic
B
vector
pcDNA-MEG3
pcDNA-MEG3+NC mimic
pcDNA-MEG3+miR-181-5p mimic
Absorbance at 450 nm
1.6
1.2
0.8
0.4
0.0
0 h
24 h
48 h
72 h
**
**
C
Cell migration (%)
50
40
30
20
10
0
**
**
vector
pcDNA-MEG3
pcDNA-MEG3+NC mimic
pcDNA-MEG3+miR-181-5p mimic
D
vector
pcDNA-MEG3
pcDNA-MEG3+NC mimic
pcDNA-MEG3+miR-181-5p mimic
0 h
24 h
E
vector
pcDNA-MEG3
pcDNA-MEG3+NC mimic
pcDNA-MEG3+miR-181-5p mimic
50 μm
F
Number of invaded cells
200
150
100
50
0
**
**
vector
pcDNA-MEG3
pcDNA-MEG3+NC mimic
pcDNA-MEG3+miR-181-5p mimic
G
E-cadherin
ICAM-1
CD4
GAPDH
vector
pcDNA-MEG3
pcDNA-MEG3+NC mimic
pcDNA-MEG3+miR-181-5p mimic
H
vector
pcDNA-MEG3
pcDNA-MEG3+NC mimic
pcDNA-MEG3+miR-181-5p mimic
Fold change of proteins
3
2
1
0
**
**
**
**
**
**
E-cadherin
ICAM-1
CD44

Figure 6

GATA6 silencing abrogated the effects of miR-181-5p inhibition on T24/DDP cell behaviors.

PC3 cells were transfected with miR-181-5p inhibitor and si-GATA6. (A) GATA6 level was tested with Western blot analysis. (B) PC3 cell proliferation was accessed with CCK-8 assay. (C, D) PC3 cell migration was accessed with wound healing assay. (E, F) PC3 cell invasion was tested with Transwell assay. (G, H) E-cadherin, ICAM-1, and CD44 protein levels were gauged with Western blot assay. N=6. Data from at least triplicate experiments were presented as mean ± SD. $^{**}P<0.01$.

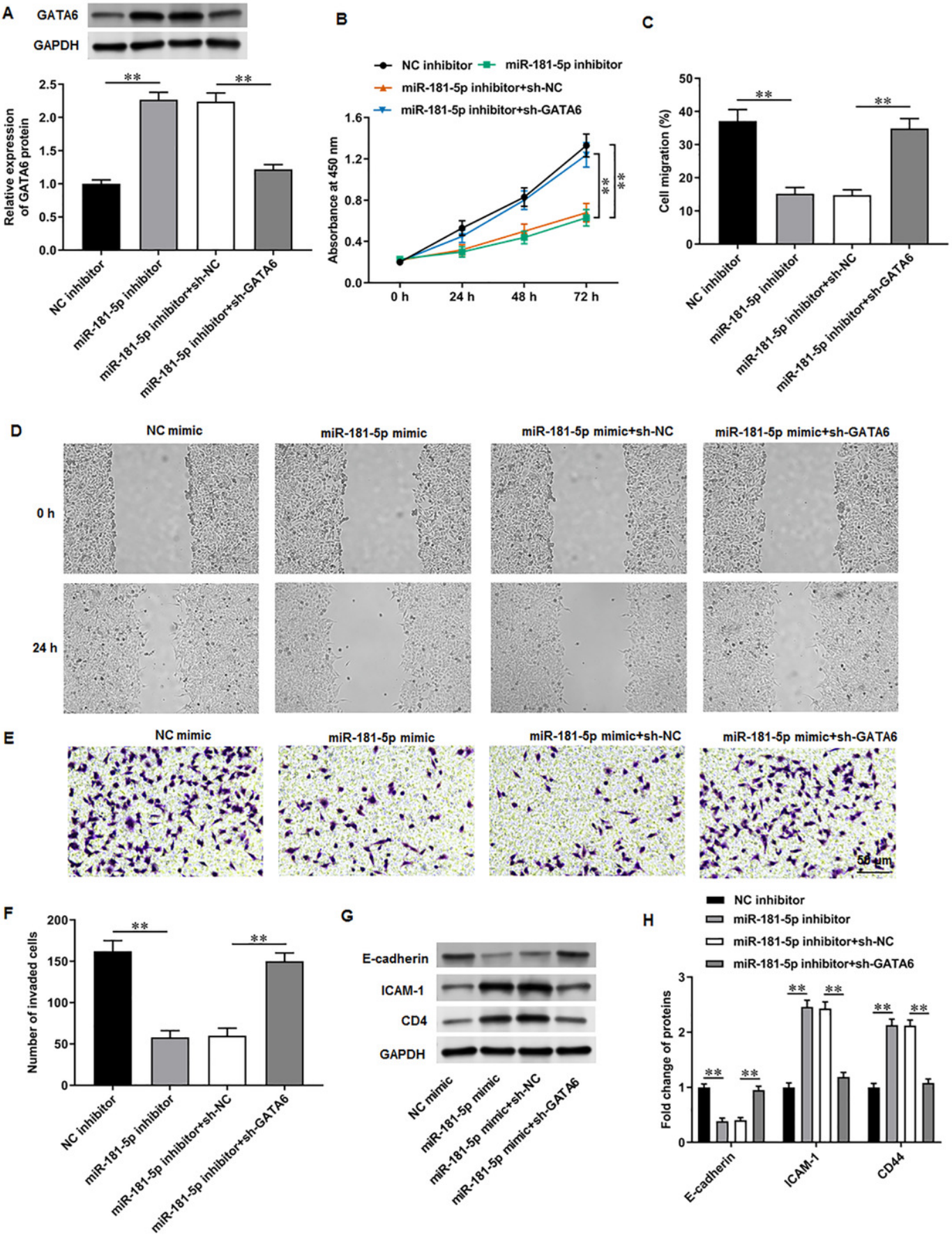
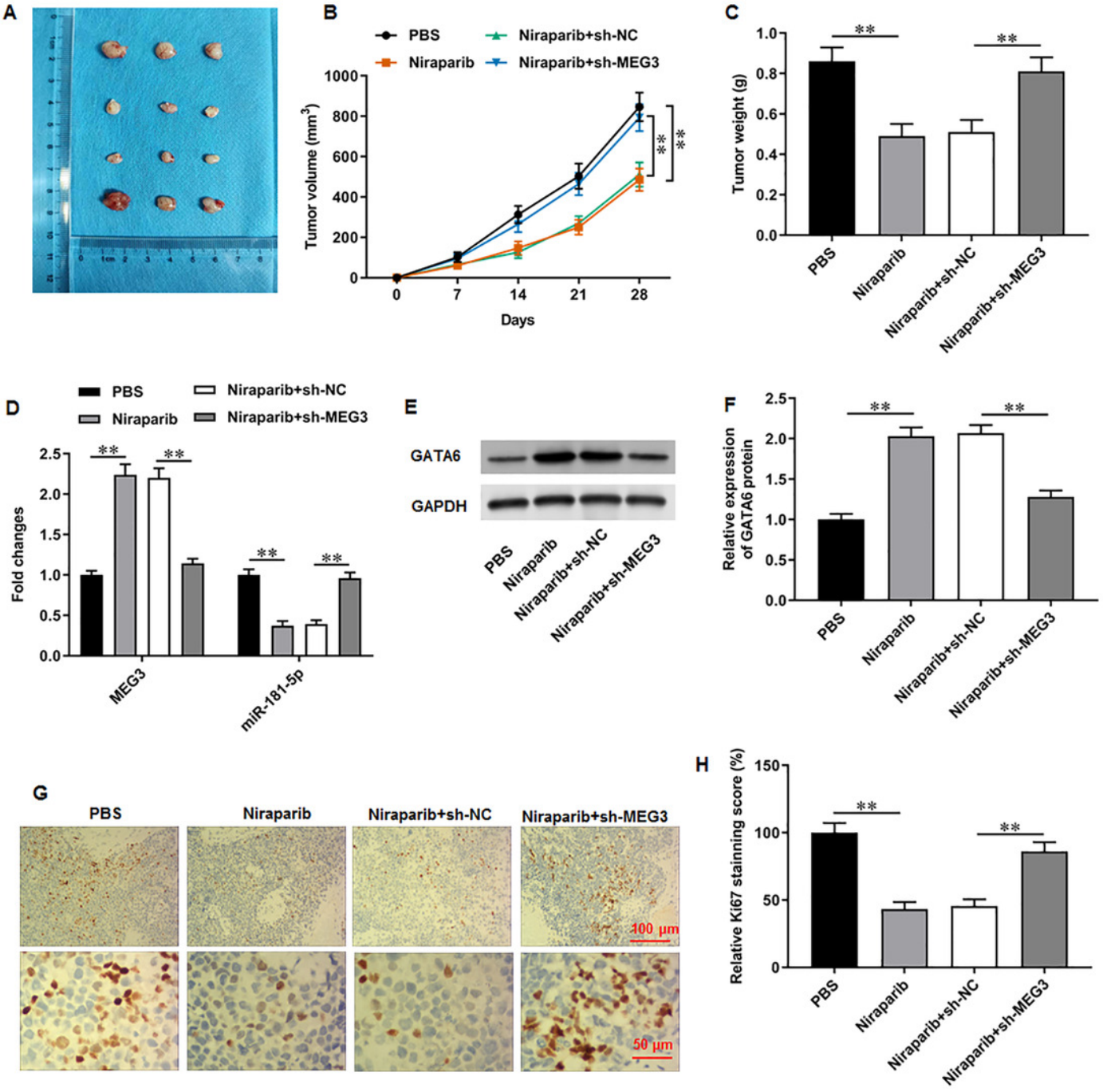

A
GATA6
GAPDH
Relative expression of GATA6 protein
NC inhibitor
miR-181-5p inhibitor
miR-181-5p inhibitor+sh-NC
miR-181-5p inhibitor+sh-GATA6
B
NC inhibitor
miR-181-5p inhibitor
miR-181-5p inhibitor+sh-NC
miR-181-5p inhibitor+sh-GATA6
Absorbance at 450 nm
0 h
24 h
48 h
72 h
C
Cell migration (%)
D
NC mimic
miR-181-5p mimic
miR-181-5p mimic+sh-NC
miR-181-5p mimic+sh-GATA6
0 h
24 h
E
50 μm
F
Number of invaded cells
G
E-cadherin
ICAM-1
CD4
GAPDH
H
Fold change of proteins
E-cadherin
ICAM-1
CD44

Figure 7

Niraparib mitigated PCa tumor growth in vivo through regulating the MEG3/miR-181-5p/GATA6 axis.

A xenograft PCa tumor mouse model was establishd, and mice were divided into four groups: PBS, Niraparib, Niraparib+sh-NC, Niraparib+sh-MEG3 (n=8 per group). (A-C) Tumor volume and weight were accessed. (D) MEG3 and miR-181-5p expression were gauged with RT-qPCR assay. (E, F) GATA6 protein level was gauged with Western blot assay. (G, H) Ki67 level was gauged with immunohistochemistry assay . N=8. Data from at least triplicate experiments were presented as mean ± SD. *P<0.01.


A
B
PBS
Niraparib
Niraparib+sh-NC
Niraparib+sh-MEG3
Tumor volume (mm3)
Days
C
Tumor weight (g)
D
Fold changes
MEG3
miR-181-5p
E
GATA6
GAPDH
F
Relative expression of GATA6 protein
G
100 μm
50 μm
H
Relative Ki67 stainning score (%)