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Up-regulation of eEF1A2 promotes proliferation and inhibits apoptosis in prostate cancer

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ABSTRACT

Background: eEF1A2 is a protein translation factor involved in protein synthesis, which possesses important function roles in cancer development. This study aims at investigating the expression pattern of eEF1A2 in prostate cancer and its potential role in prostate cancer development.
Methods: We examined the expression level of eEF1A2 in 30 pairs of prostate cancer tissues by using RT-PCR and immunohistochemical staining (IHC). Then we applied siRNA specifically targeting eEF1A2 to down-regulate its expression in DU-145 and PC-3 cells. Flow cytometer was used to explore apoptosis and Western-blot was used to detect the pathway proteins of apoptosis.
Results: Our results showed that the expression level of eEF1A2 in prostate cancer tissues was significantly higher compared to their corresponding normal tissues. Reduction of eEF1A2 expression in DU-145 and PC-3 cells led to a dramatic inhibition of proliferation accompanied with enhanced apoptosis rate. Western blot revealed that apoptosis pathway proteins (caspase3, BAD, BAX, PUMA) were significantly up-regulated after suppression of eEF1A2. More importantly, the levels of eEF1A2 and caspase3 were inversely correlated in prostate cancer tissues.
Conclusion: Our data suggests that eEF1A2 plays an important role in prostate cancer development, especially in inhibiting apoptosis. So eEF1A2 might serve as a potential therapeutic target in prostate cancer.

1. Introduction

Prostate cancer (PCa) is the second most frequently diagnosed cancer of men and the fifth most common cancer overall in the world [1]. Surgery and androgen ablation therapy remain two major treatments after diagnosis of PCa. Prostate cancers are usually androgen-dependent tumors, susceptible to growth arrest/apoptosis induced by androgen ablation therapy [2]. But unfortunately, androgen ablation will ultimately develop castration-resistant tumors, which is characteristically associated with marked increases in resistance to apoptosis [3,4]. And no standard therapy is effective for patients recurrence with advanced prostate cancer. So new molecules that might contribute to better outcome for prostate cancer patients should be developed.

Eukaryotic elongation factor 1-α (eEF1A) is a member of the G protein family and one of the four subunits that constitute the eukaryotic elongation factor 1 [5,6]. And it has two identified isoforms, namely eEF1A1 and eEF1A2. Different from eEF1A1 expressed almost ubiquitously, eEF1A2 is normally only present in the heart, brain, and skeletal muscle [7–9]. eEF1A2 is considered as an oncogene maker as it is highly expressed in a subset of cancers, such as ovarian cancer [10,11], breast cancer [12], and pancreatic cancer [13,14]. It has been reported that eEF1A2 exert pro-proliferation [15], anti-apoptosis [16], pro-metastasis [17] functions on cancer development. And mechanism studies reveal that PI3K/Akt and JAK/STAT signaling pathway play an important role in mediating the effects of eEF1A2 on cancers [12,17,18]. A previous study show a rise of eEF1A2 in PCa [19], but the mechanism underlying the different expression remains unclear.

In this study, we demonstrated that the expression level of eEF1A2 in prostate cancer tissues was much higher than in their corresponding normal tissues. Suppression of eEF1A2 significantly inhibited the growth rate and promoted apoptosis rate. More importantly, apoptosis proteins, such as caspase3, BAD, BAX, PUMA, were changed after altering the expression of eEF1A2 in PCa cells.

2. Methods and patients

2.1. Patients and samples

PCa tissues enrolled in this study were obtained from 30 patients who underwent radical prostatectomy in our center (First

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Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China). For frozen specimens, surgical pathologists examined the clinicopathological features of freshly frozen sections after HE staining and then the samples were snap frozen in liquid nitrogen. Only those samples with >70% tumor content were included as PCa tissues in this study. This study was approved by the Ethic Committee of Zhejiang University and was performed after obtaining written informed consent of patients.

2.2. Quantitative reverse transcription polymerase chain reaction (qPCR)

Total RNA was extracted from PC cells or tissues, and 2 μg RNA was used to synthesize cDNA. And the expression levels of eEF1A2 and other genes were determined by qPCR (ABI 7500fast system, Applied Biosystems, CA, USA). The GAPDH was used as the internal control. The detailed primers' sequences were listed in Table 1.

2.3. Transfection

PC cells were seeded into 6-wells plates and transfected with si-eEF1A2 or its all-star negative control (both bought from QIAGEN) accompanied with lipofectamine2000 transfection reagent (Invitrogen, CA) according to manufacturer's instructions. After transfection for 48 h, the cells were collected for confirming silencing efficiency or further functional assays. The sequences of si-eEF1A2 were listed as follows: si-eEF1A2-1: AATGCGGAGGTATT GACAAAA, si-eEF1A2-2: TACGACGAGATCGTCAAGGAA.

2.4. Cell proliferation assay

The PCa cells were transferred to 96-wells plates with 3000 cells per well after transfected in 6-well plates for 24 h. Then the relative numbers of viable cells were detected by Cell Counting Kit-8 reagents (Dojindo Laboratories, Kyushu Island, Japan) after 24, 48, 72 h cultivation. The results were reflected in the form of the absorbance optical density at 450 nm using a microplate reader (Elx800; BioTek, VT, USA).

2.5. Colony formation assay

DU-145 cells and PC-3 cells were seeded and transfected with si-eEF1A2 or si-nc in 6-well plates. After 24 h, the transfected cells were seeded into another 6-well plate with a concentration of 1000 cells per well and were cultured for additional 14 days. Then the cells were fixed with methanol for 15 min and then stained by 0.1% crystal violet for 15 min. The clones of more than 50 cells were manually counted. The experiments were independently triplicated.

2.6. Flow cytometry assay

Apoptosis and cell cycle assays were performed by flow cytometry. For apoptosis assays, si-eEF1A2 or si-nc cells were stained with Annexin V-FITC and propidium iodide (BD Bioscience, San Jose, CA, USA) according to manufacturer's instructions to detect early and late apoptosis of cells. For cell cycle assays, si-eEF1A2 or si-nc cells were harvested and fixed with 75% ethyl alcohol at 4 °C overnight and then were stained with DNA PREP kit (Beckman Coulter, Fullerton, CA, USA). Then the cells were applied to flow cytometry detection. The experiments were performed in independent triplicates.

Table 1
Sequences of primers for qRT-PCR.

Gene	Forward sequence	Reverse sequence
eEF1A2	CCATGTGTGTGGAGAGCTTCTC	TCTCCACGTTCTTGATGACGCC
Caspase3	GGAAGCGAATCAATGGACTCTGG	GCATCGACATCTGTACCAGACC
BAD	CCAACCTCTGGGCAGCACAGC	TTTGCCGCATCTGCGTTGCTGT
BAX	TCAGGATGCGTCCACCAAGAAG	TGTGTCCACGGCGGCAATCATC
PUMA	ACGACCTCAACGCACAGTACGA	CCTAATTGGGCTCCATCTCGGG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

2.7. Western blot and immunohistochemistry (IHC)

The detailed procedures of western blot and immunohistochemistry were carried out as described [20]. In brief, Western blot: the cells were lysed in RIPA buffer and the lysates were centrifuged (12,000×*g* for 15 min) for proteins collections. Then the extracted proteins were separated by 10% SDS–PAGE and transferred to PVDF membranes. After blocked by 5% non-fat milk for 2 h, the membranes were immunoblotted by antibodies overnight. After washed by TBST for 3 times, membranes were exposed to horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence reagent ECL kit and exposed to X-ray films; Immunohistochemistry (IHC): paraffin-embedded clinical tissue specimens were cut into 4-μm sections and mounted on slides. Then the slides of tissue sections were deparaffinized, rehydrated and applied to microwave for antigen renovation. Subsequently, the slides were washed by PBS for twice and blocked by 5% FBS (fetal bovine serum) for 60 min in room temperature and then incubated with anti-eEF1A2 (1:400, 8056-1, epitomics) overnight at 4 °C. After wished by PBS, the slides were applied to horseradish peroxidase-conjugated goat anti-mouse immunoglobulin for 30 min at room temperature. Diaminobenzidine (DAB) was used to visualize the binding results and the sections were counterstained with hematoxylin.

The primary antibodies used were listed as follows: eEF1A2 (8056-1, epitomics), cleavage-caspase3 (1476-1, epitomics), BAD (1541-1, epitomics), BAX (1063-1, epitomics), BAK (1542-1, epitomics), β-actin (A5441, mAb; Sigma–Aldrich).

For IHC, Immunostaining of eEF1A2 and active caspase3 were semi-quantitatively scored using a composite score by multiplying the values of the mean staining intensity and the percentage of positive cells. The intensity was graded as absent (0), weakly positive (1), moderately positive (2), or strongly positive (3). The percentage of positive cells was assessed based on the proportion of positively stained cells (0%-100%). The final composite score ranged from 0 to 3.

2.8. Statistical analysis

Each value in this study was obtained from at least three independent experiments and presented as mean ± SD. Comparisons between two groups were analyzed by *t* test. The associations between of eEF1A2 level, active caspase3 and clinicopathological variables were analyzed by chi-square test. The association between of eEF1A2 level and cleavage-caspase3 was analyzed by Spearman correlation analysis regression. Statistical analysis was performed with SPSS 15.0 and GraphPad Prism 5.0. $P < 0.05$ was thought to be statistically different.

3. Results

3.1. The Expression of eEF1A2 is up-regulated in prostate cancer tissues

To assess the expression profile of eEF1A2 in PCa, we first performed RT-PCR in 30 pairs of PCa tissues. Among these 30 tissues, we found 26 of them (86.7%) revealed a higher expression level of

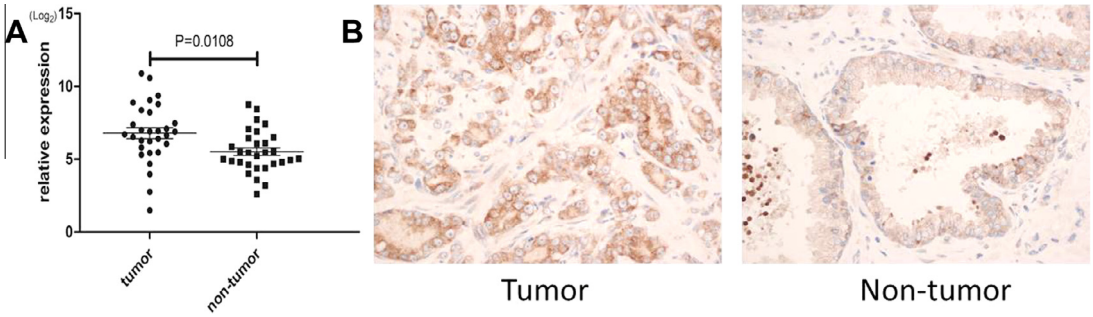

Fig. 1. The expression levels of eEF1A2 are up-regulated in PCa tissues. (A) RT-PCR detection of eEF1A2 in 30 pairs of PCa tissues and their corresponding non-tumor tissues. (B) Representative IHC images of PCa tumor tissues and non-tumor tissues from the same case. The images were shown at 400× magnifications.

eEF1A2 in tumor tissues compared to their corresponding normal tissues and the integral comparison reached a significant difference (Fig. 1A). Then we further validated the expression difference of eEF1A2 in the paraffin-embedded tissues of these 30 cases. The staining of eEF1A2 was mainly located in the cell cytoplasm and similar to the result of RT-PCR, most of tumors showed higher expression than their corresponding normal tissues (Fig. 1B). We then divided these cases into two groups by choosing the median IHC staining score of eEF1A2 as the cut-off point. Upon clinicopathological correlation analysis, the expression of eEF1A2 revealed no significant correlation with any single clinicopathological characteristic (Table 2). Taken together, the results above suggest that PCa tissues exhibit an up-regulation of eEF1A2.

3.2. Suppression of eEF1A2 inhibits the proliferation and promotes apoptosis in prostate cancer cells

To further explore the biological significance of eEF1A2 in PCa, we suppressed the expression of eEF1A2 in DU-145 and PC-3 cells by siRNA (Fig. 2A). The more effective siRNA, both in mRNA and protein level, was applied for further functional study. Then we performed proliferation assay in these two PCa cells, which is a well-known hallmark of cancer [21]. As was shown in Fig. 2B, after transfection for 24 h, 48 h and 72 h, the group of si-eEF1A2 revealed a significant slower proliferation rate than the control group. Consistent with the results of CCK8, inhibition of eEF1A2 also obviously suppressed colony formation (Fig. 2C). As the change of proliferation is always accompanied with the alteration of cell-cycle and apoptosis, we further employed flow cytometric to perform cell-cycle and apoptosis assays. We revealed that, compared to si-nc, si-eEF1A2 enhanced apoptosis rates from 5.3 ± 0.7% to 10.9 ± 1.5% and 7.1 ± 0.9 to 15.5 ± 1.5% in DU-145 and PC-3 respectively (Fig. 3A), while no significant change was found in cell-cycle assay between two groups (data not shown). Taken together, our data supports the conclusion that suppression of eEF1A2 inhibits PCa cells growth and promotes PCa cells apoptosis.

Table 2
Clinicopathological correlation of eEF1A2 in PCa tissues.

Variables	eEF1A2 expression			Cleavage-caspase3 expression		
	Positive	Negative	*P*	Positive	Negative	*P*
Age (Years)			0.673			0.682
>65	8	7		6	5	
⩽65	4	5		6	7	
PSA (ng/ml)			0.098			0.219
>50	9	5		4	7	
⩽50	3	7		8	5	
Gleason score			0.346			0.014*
>7	10	8		3	9	
⩽7	2	4		9	3	
Clinical stage			0.206			
>T2	6	9		4	9	0.041*
⩽T2	6	3		8	3	

Statistical analyses were performed with chi-square test.
* $P < 0.05$.

3.3. Inhibition of eEF1A2 enhances the expression of apoptotic relevant proteins

To elucidate the anti-apoptosis mechanism of eEF1A2, we then applied Western blot to examine the level of apoptotic relevant proteins. The cleavage-caspase3, which is thought to be the key protein of apoptosis pathway, was demonstrated to increase after silencing of eEF1A2 (Fig. 3B). Additionally, we explored the mitochondria intrinsic apoptotic proteins, which are known as upstream of cleavage-caspase3 [22]. As was shown in Fig. 3B, si-eEF1A2 significantly enhanced the protein expression levels of BAD, BAX, PUMA in PCa cells. Collectively, these results suggest that inhibition of eEF1A2 activated the expression of cleavage-caspase3 as well as its upstream pro-apoptotic proteins.

3.4. The expressions of eEF1A2 and cleavage-caspase3 are inversely correlated in PCa tissues

Owing to the observation that the suppression of eEF1A2 could enhance the apoptotic rate and the expression of pro-apoptotic proteins in PCa cells, we then sought to explore the correlation between eEF1A2 and the key apoptotic protein, cleavage-caspase3, in human PCa tissues. By using IHC assays, we found that the expression of cleavage-caspase3 was significantly down-regulated in 25 cases of these 30 PCa tissues, compared to their corresponding normal tissues (Fig. 4A). Clinicopathological correlation analysis revealed that the expression of cleavage-caspase3, which was divided into two groups by choosing the median IHC score as the cut-off point, was significantly associated with Gleason score and clinical stage (Table 2). We further performed Spearman correlation analysis to assess the relationship between eEF1A2 and cleavage-caspase3. The result showed that there was a negative correlation between these two proteins (rho = −0.390, $P = 0.033$, Fig. 4B). In conclusion, the data suggests that eEF1A2 and cleavage-caspase3 also have an inverse correlation in PCa tissues.

4. Discussion

Several studies have demonstrated that eEF1A2 is significantly over-expressed in various cancers [13,18]. And it has been reported to be closely related to the process of cancer development and poor survival, especially in pancreatic cancer. A previous study shows that high level of eEF1A2 correlates with lymph node metastasis and decreased survival in pancreatic ductal adenocarcinoma [14].

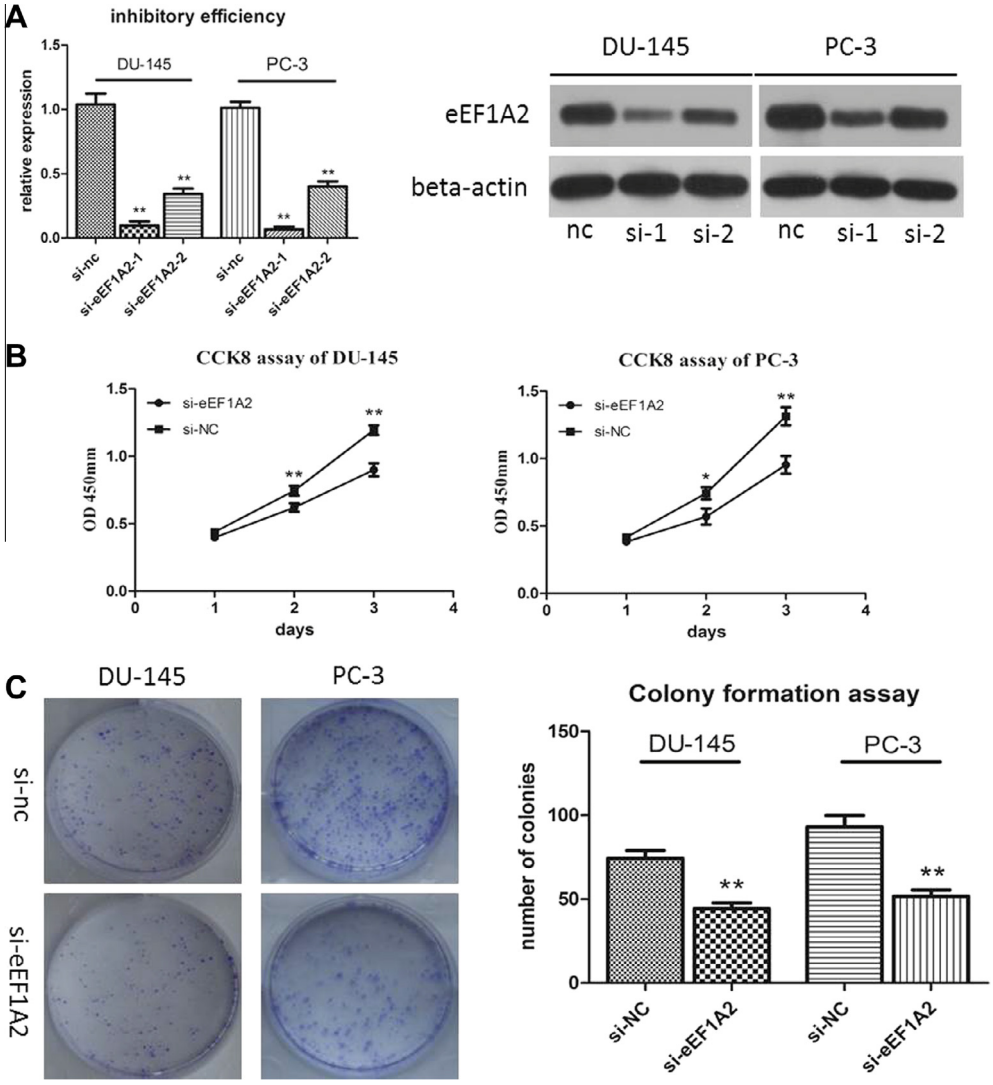

Fig. 2. Knockdown of eEF1A2 significantly inhibits proliferation in PCa cells. (A) The efficiency of si-eEF1A2 was confirmed by RT-PCR in DU-145 and PC-3 cells. (B) Viable cell numbers were detected by CCK8 assays after transfected for 24 h, 48 h and 72 h in PCa cells. (C) Transfected cells were seeded in 6-well plated and incubated for for 2 weeks. The colonies of si-eEF1A2 groups were much less and smaller. The data are presented as the mean of triple assays with standard deviation of the mean. Representative images are shown. ($^{*}p < 0.05$, $^{**}p < 0.01$).

Additionally, another study indicates that overexpression of eEF1A2 promotes metastasis through AKT pathway [17]. In prostate cancer, others have reported the overexpression of eEF1A2 in 4 pairs of PCa tissues [19]. In this study, we performed RT-PCR and IHC assays in 30 pairs of PCa tissues and found that most tumor tissues revealed higher expression levels compared to their corresponding normal tissues, which verified the overexpression of eEF1A2 in PCa. Further, for the first time, we elucidated the underlying mechanism of eEF1A2 in prostate cancer development. Proliferation assay showed that si-eEF1A2 suppressed the proliferation potential of PCa cells. Because inhibition of cell proliferation is always induced by increased apoptosis or cell cycle arrest, we further explored the effect of eEF1A2 knockdown on apoptosis and cell cycle by flow cytometry. As a result, eEF1A2 knockdown obviously enhanced apoptotic rate rather than affecting cell cycle.

Intrinsic apoptotic pathways have been demonstrated to play a vital role in initiation and maintenance of apoptosis [23,24]. Caspase3 is well known to act as a key protein in the apoptotic pathway [25]. Here we showed that eEF1A2 known could obviously enhance the protein level of cleavage-caspase3, which is thought to be the active form of caspase-3. Moreover, intrinsic apoptotic pathway vital proteins, such as BAD, BAX, PUMA, which are upstream proteins of caspase3 [26], were demonstrated to increase after suppression of eEF1A2 in this study. This pathway finding could explain the phenomenon that the apoptotic rate was increased after eEF1A2 suppression in PCa cells. In support of our finding of eEF1A2 affecting apoptosis, a similar phenomenon has also been described in hepatocellular carcinoma [18]. So we believe that eEF1A2 is an important molecular in cancer development. Based on the correlation between eEF1A2 and cleavage-caspase3 in PCa cell lines, we wondered whether they were also correlated in PCa tissues. In the 30 PCa tissues, we found that the expression level of cleavage-caspase3 was significantly decreased in PCa tissues compared to normal tissues and was significantly associated with Gleason score and clinical stage, which is consistent to previous studies [27]. Then we applied Spearman correlation analysis to explore the potential relationship between eEF1A2 and cleavage-caspase3, and we revealed a negative correlation between them. Therefore, we further confirmed the vital role of eEF1A2 in affecting apoptotic pathway.

In summary, our study demonstrated that eEF1A2 expression was up-regulated in PCa tissues, and that inhibition of eEF1A2

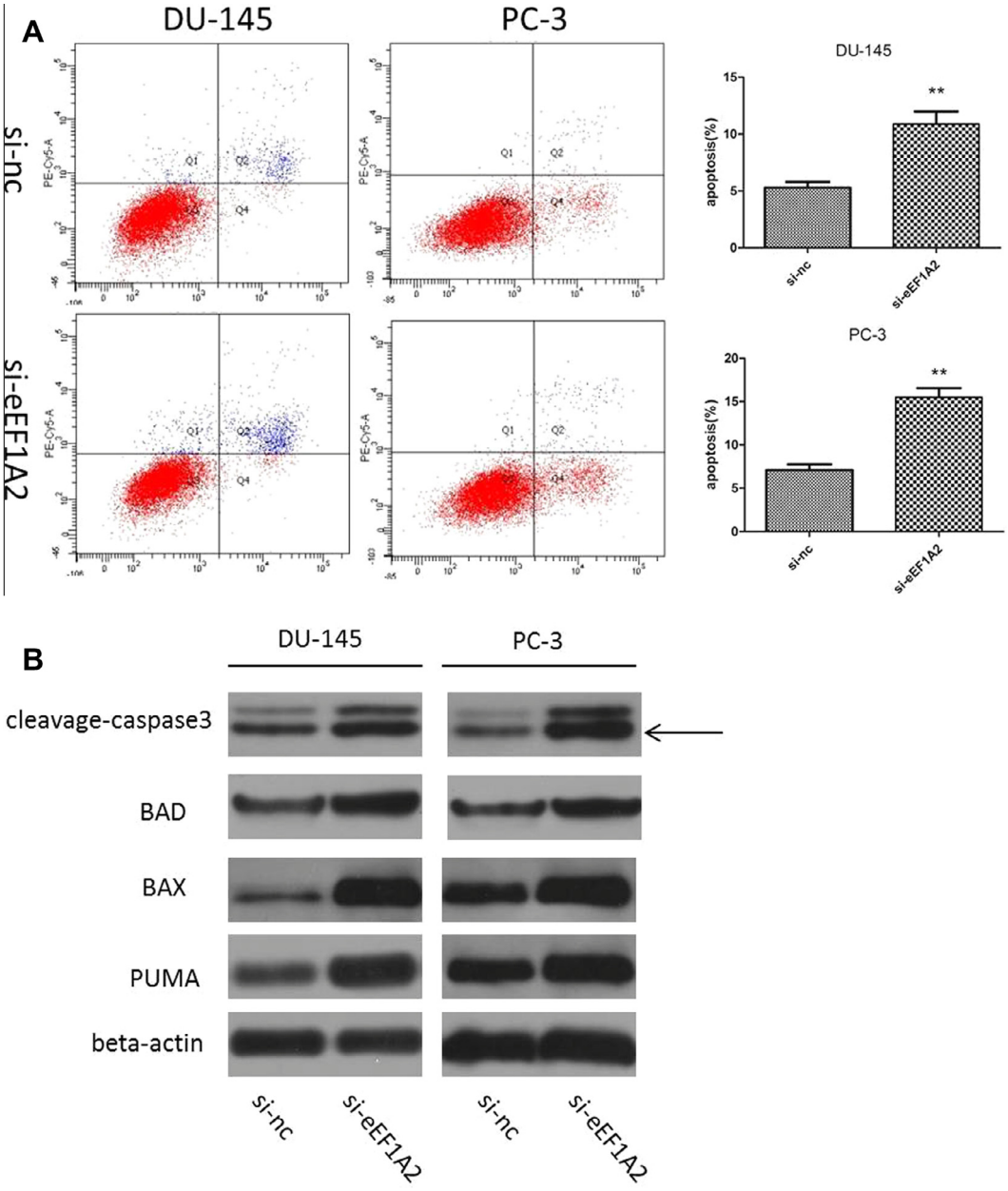

Fig. 3. Inhibition of eEF1A2 enhances the apoptosis rate and protein levels in PCa cells. (A) Flow cytometer assays showed that the apoptosis rate was obviously increased after eEF1A2 knockdown. (B) Western blot indicated that the protein expression levels of cleavage-caspase3, BAD, BAX, PUMA were obviously enhanced after eEF1A2 knockdown. ($^{**}p < 0.01$).

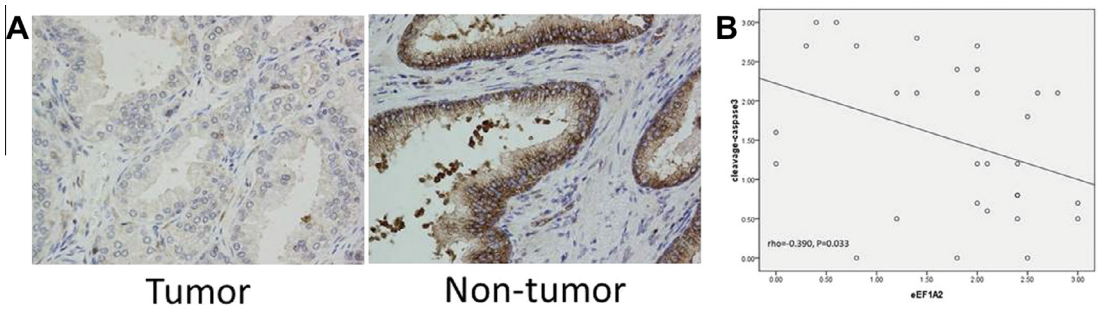

Fig. 4. IHC assay of cleavage-caspase3 in PCa tissues and the correlation of eEF1A2 and cleavage-caspase3. (A) Representative IHC images of cleavage-caspase3 staining in PCa tumor tissues and non-tumor tissues. The images were shown at 400× magnifications. (B) Spearman correlation analysis indicated the inverse correlation of eEF1A2 and cleavage-caspase3 in 30 PCa tissues. (rho = −0.390, $P = 0.033$).

suppressed proliferation and promoted apoptosis by enhancing intrinsic apoptotic proteins. Therefore suppression of eEF1A2 might prompt new effective therapeutic strategies in PCa.

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