Effect of genetic variants in FAS and let-7a on radiation-induced intestinal toxicity in the treatment of prostate cancer

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Abstract

Introduction: The present study aimed to explore the effects of pri-let-7a-1 rs10739971 and FAS-670 rs1800682 polymorphisms on the pathogenesis of radiation-induced intestinal toxicity in prostate cancer (PC) patients.
Material and methods: Three hundred eighty PC patients with or without signs of intestinal toxicity were enrolled in a study on the effects of let-7a rs10739971 and FAS-670 rs1800682 polymorphisms on rectal volume and the risk of intestinal toxicity. In addition, real-time PCR, Western blot analysis, immunohistochemistry (IHC), luciferase assays and computational analyses were performed to explore the mechanism underlying the role of let-7a rs10739971 polymorphism in radiation-induced intestinal toxicity.
Results: The let-7a rs10739971 polymorphism but not the FAS-670 rs1800682 polymorphism was closely associated with the risk of radiation-induced intestinal toxicity featured by a high rectal volume. In addition, there was no significant association between the rectal volume and the genotype and allele frequencies of FAS -670 rs1800682 and Pri-let-7a-1 rs10739971 polymorphisms. The GG genotype of let-7a rs10739971 polymorphism reduced let-7a expression but enhanced FAS expression. In addition, the intestinal toxicity (–) group showed a much higher level of let-7a and a much lower level of FAS than the intestinal toxicity (+) group. FAS was a virtual target gene of let-7a, which decreased FAS protein expression in a dose-dependent manner.
Conclusions: The GG genotype of pri-let-7a-1 rs10739971 polymorphism could increase the risk of radiation-induced intestinal toxicity in PC patients. Therefore, the pri-let-7a-1 rs10739971 polymorphism could be used as a putative marker to predict the risk of intestinal toxicity in PC patients undergoing radiotherapy.

Key words: prostate cancer, intestinal toxicity, radiotherapy, let-7a, FAS, rs1800682, rs10739971.

Introduction

At present, prostate cancer (PC) is regarded as a leading contributor of cancer-related mortality in males, while radiotherapy (RT) was applied as one of the treatment options [1]. However, the efficacy of RT in PC

treatment is limited by its rectal toxicity, which exerts a negative effect on patients' life quality [1]. Past studies showed that the prevalence and magnitude of RT-induced rectal toxicity vary significantly according to the characteristics of patients and radiation regimens, including the dose and volume of radiation [2]. In fact, as reported by a randomized study and a meta-analysis enrolling more than 2,000 patients, a higher delivered dose of total radioactivity to the prostate substantially elevated rectal toxicity [3, 4].

It was shown that single nucleotide polymorphisms (SNPs) located in the mRNAs of FASL and FAS increased the susceptibility to high-grade rectal and/or urinary toxicity in PC patients undergoing radiation therapies, indicating that the FASL -844 C>T SNP exerted a protective impact against the onset of high-grade toxicity during the RT of PC patients [5]. SNPs located in gene promoters were shown to affect the transcription of host genes, thus subsequently affecting the susceptibility to diseases [6]. Two SNPs located in the FAS promoter, i.e., rs2234767 1377 G>A and rs1800682670 A>G, were reported to affect the expression of STAT1 and SP1 genes, respectively, which in turn dysregulates the expression of FAS [7, 8].

MicroRNA (miRNA) can affect the expression of its target genes by interacting with the 3′ UTRs of these genes and subsequently altering their translation. The complementarity formed between the 3′ UTR of target mRNA and miRNA results in mRNA degradation and translation inhibition in both plants and mammals [9]. The miRNAs act as either oncogenes or tumor suppressors [10]. The miRNA let-7a was characterized as a tumor suppressor in several human cancers [11]. Dong et al. reported that let-7a expression was reduced in resected PC samples [12]. In addition, PC-3 and DU145 cells (androgen-independent) express less let-7a than LNCaP cells (androgen-dependent). Target prediction algorithms indicated that let-7a1 could target a number of oncogenes, such as IGF1R. A SNP located in the 559 bp position upstream of the coding sequence of let-7a-1, rs10739971, was shown to impact the susceptibility to gastric cancer. Furthermore, the rs10739971 SNP in pri-let-7a-1 could interact with the rs9471643 and rs6458238 SNPs in PGC to change the risk of atrophic gastritis [13].

The FAS/FASL signaling pathway is functionally involved in the pathogenesis of radiation-induced intestinal toxicity in prostate cancer [5, 14]. In addition, the rs1800682 SNP in the promoter region of FAS was shown to affect the expression of FAS, while the rs10739971 SNP in the flanking region of let-7a could alter the expression of let-7a, a negative regulator of FAS [15–17]. In this study, we collected samples from PC patients who have been treated with radiotherapy to investigate the role of the two SNPs in radiation-induced intestinal toxicity.

Material and methods

Human sample collection

A total of 380 PC patients were enrolled in our research, which consisted of 122 PC patients diagnosed with radiation-induced intestinal toxicity (the intestinal toxicity (+) group) and 258 PC patients free of intestinal toxicity (the intestinal toxicity (–) group). All subjects underwent RT prior to their enrollment in this study. The demographic and clinicopathological characteristics of all PC subjects, such as age at the initiation of RT, gender, PSA level upon the first diagnosis of PC, T1/T2 or T3/T4 stage of PC, grade 1–2 or 3 of PC, a history of any resection surgery prior to RT, a history of any adjuvant hormone therapy prior to RT, the administration of oral anticoagulants, a history of smoking (current smoker, non-smoker or past smoker), the presence of diabetes mellitus, the presence of hypertension, the levels of administered median total dose, mean rectal dose and mean V_{60} rectum dose, were collected and summarized. Student's *t* test was used to perform the statistical comparison between the demographic and clinicopathological characteristics of the PC subjects in these two groups.

In addition, the peripheral blood samples of all subjects were collected during fasting to analyze their expression of various target genes. At the same time, PC tissue samples were collected from 72 subjects during their surgical operation and were used for subsequent genotyping and immunohistochemistry assays.

Radiation therapy

All of the subjects enrolled in this study were treated with RT prior to their enrollment. Among these subjects, 40% of patients began their RT treatment in the prone position, while the remaining subjects began their RT treatment in the supine position. All subjects underwent periodic CT scans using an in-room CT system installed in the RT treatment room to follow up with their prognosis of PC. In addition, the position of the prostate in each subject was determined before the dosing in each RT cycle via the in-room CT image system to ensure the accurate delivery of RT. During the CT scan, the position of each subject was initially adjusted with label marks on the skin and lasers. In the next step, images were acquired using the in-room CT system. In the case of any major deviation in the position of a subject, the matching of pelvic bone location was performed using the in-room CT system, followed by the manual matching carried out by an RT therapist to make sure the

location of the prostate in each subject was at the planned location of RT treatment. Subsequently, feces and gas in the rectum, changes in bladder volume, and deviation of adjacent intestines (large and small intestines) from the planning CT images were checked. When the presence of feces and gas was noted in the rectum, defecation or passage of gas was encouraged. Nevertheless, if the situation of the subject did not improve, deaeration was performed by inserting a catheter into the rectum via the anus. Moreover, patients were encouraged to drink water when they had insufficient bladder volumes. At this moment, CT images were acquired once again. In the next step, irradiation was performed after there was enough geometric verification.

Definitions of target volumes

In this study, CTV, i.e., clinical target volume, was determined in subjects with a low risk of RT induced toxicity by calculating the volume of their prostate plus the volume of the base of the seminal vesicles. However, in subjects with a moderate to high risk of RT induced toxicity, CTV was determined by calculating the volume of their prostate plus the volume of 2/3 of seminal vesicles along the proximal direction. In addition, PTV, i.e., planning target volume, of each subject was calculated by adding 9 mm along the inferior direction, 9 mm along the left direction, 10 mm along the superior direction, 9 mm along the right direction, 6 mm along the posterior direction, and 6 mm along the posterior direction of the prostate with automatic expansion.

LSE scoring

The rectal LSE values were assessed by the LENT-SOMA scale.

Level of soluble Fas in serum

After the collection of peripheral samples from each subject, the serum component in each sample was isolated via centrifugation and the level of soluble Fas was measured using a commercially kit of sandwich ELISA (Diaclone, Besancon, France) in accordance with the instructions of the kit provider.

Genotyping by TaqMan assays

Using the peripheral samples collected from each subject, the genotypes of the rs1800682 SNP at the –670 position of FAS and the rs10739971 SNP in pri-let-7a-1 were determined using a TaqMan Gene Expression & Genotyping assay kit (Thermo Fisher Scientific, Waltham, MA) following the instructions of the kit. Subsequently, based on the results of rs10739971 genotyping, the subjects were allocated to three groups, i.e., AA, AG and GG groups.

RNA isolation and real-time PCR

Total RNA was first isolated from peripheral blood and tissue samples using Trizol reagents (Invitrogen, Carlsbad, CA). After converting isolated RNA to cDNA, real-time PCR was performed according to routine methods using a SYBR Green Master mix kit (ABI, Foster City, CA) on a PRISM 7900HT real-time PCR machine (ABI, Foster City, CA), so as to quantify the relative expression of let-7a and FAS. The expression of U6 and β-actin was used as the internal control during the calculation of mRNA relative expression.

Cell culture and treatment

DU145 and PC-3 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and appropriate antibiotics. Subsequently, the cells were divided into different groups and transfected with let-7a, let-7a inhibitor, or FAS siRNA, respectively, using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. The expression of target genes was measured at 48 h after transfection.

Vector construction and luciferase assays

The 3′ UTR of FAS containing the let-7a binding site was sub-cloned into a pcDNA3.1 luciferase reporter vector to generate the plasmid for the wild type FAS 3′ UTR. In the next step, the let-7a binding site located in the 3′ UTR of FAS was subjected to site-directed mutagenesis using a Stratagene gene mutagenesis kit (Stratagene, San Diego, CA) according to kit recommendations. The mutant FAS 3′ UTR was similarly sub-cloned into another pcDNA3.1 luciferase reporter vector to generate the plasmid for the mutant FAS 3′ UTR. Then, DU145 and PC-3 cells were seeded into 96-wells plates and grown to 50% confluence, followed by co-transfection with let-7a and wild type or mutant FAS 3′ UTR using Lipofectamine 3000. After 48 h of transfection, the luciferase activity of transfected cells was determined using a Bright-Glo Luciferase Assay kit (Promega, Madison, WI).

Western blot analysis

Western blot was carried out using a conventional method to determine the relative protein expression of FAS in the peripheral blood and tissue samples collected from each subject. The primary anti-FAS antibodies and HRP-labeled secondary antibodies were purchased from Ab-

cam (Cambridge, MA). The protein expression of β-actin was used as the internal control to calculate the expression of Fas. In addition, the protein bands on the PVDF membranes were developed using an ECL reagent kit (BD Biosciences, San Jose, CA) according to the kit manual. The gray values of protein blots were analyzed using Image J software (NIH, Bethesda, MD).

Immunohistochemistry assays

Collected tissue samples were routinely paraffin embedded, sectioned to 5 µm in thickness, deparaffinized, made transparent in xylene, rehydrated using gradient alcohol, blocked at room temperature for 10 min in 3% H_2O_2, and incubated with 0.1% trypsin for 30 min at 37°C to retrieve antigens. Then, the tissue sections were immersed in a solution of primary anti-FAS antibodies (1:200, Abgent, San Diego, CA) at 4°C overnight, followed by another 1 h of incubation at 37°C for 30 min with biotin-labeled secondary IgG antibodies (Invitrogen, Carlsbad, CA). After staining with 3,3-diaminobenzidine (VWR, Waltham, MA) and hematoxylin, the sample sections were assessed using Image-Pro Plus and a Leica image analyzer (Leica, Wetzlar, Germany) under a 400× magnification.

Statistical analysis

All experimental data are shown as mean ± standard deviation. The comparisons among different groups were done using one-way ANOVA or Student's t test. A minimum of three repeated tests were done for each set of experiments. A p value of ≥ 0.05 was associated with statistical significance.

Results

Demographic, clinicopathological and genotypic parameters of the participants recruited in this study

The demographic and clinicopathological characteristics of all PC subjects are summarized in Table I. Student's t test was used to perform the

Table I. Demographic and clinicopathological characteristics of the participants of this study

Characteristics	Intestinal toxicity (+) (N = 122)	Intestinal toxicity (–) (N = 258)	P value
Age at initiation of radiotherapy [years]	64.5 ±5.9	63.8 ±6.5	0.624
PSA at first diagnosis	14.9 ±7.1	14.3 ±9.6	0.747
Stage:			0.425
T1/T2	73 (59.8)	143 (55.4)	
T3/T4	49 (40.2)	115 (44.6)	
Grade:			0.526
1–2	83 (68.0)	185 (71.7)	
3	39 (32.0)	73 (28.3)	
Surgery prior to radiotherapy:			0.934
Yes	108 (88.5)	225 (87.2)	
No	14 (11.5)	33 (12.8)	
Adjuvant hormonal therapy:			0.753
Yes	33 (27.0)	61 (23.6)	
No	89 (73.0)	197 (76.4	
Smoking status:			0.848
Yes	10 (8.2)	24 (9.3)	
No	112 (91.8)	234 (90.7)	
Diabetes mellitus:			0.927
Yes	31 (25.4)	69 (26.7)	
No	91 (74.6)	189 (73.3)	
Hypertension:			0.721
Yes	71 (58.2)	159 (61.6)	
No	51 (41.8)	99 (38.4)	
Mean rectal dose (range) [Gy]	73 (64–78)	75 (63–78)	
Mean rectal dose ± SD [Gy]	38.8 ±7.2	39.7.5 ±6.7	0.842
Mean V60 rectum ± SD [cm^3]	21.6 ±4.2	22.5 ±6.4	0.781

statistical comparison and the results revealed no significant differences between the two groups in terms of the above demographic, clinicopathological and genotypic parameters.

Pri-let-7a-1 rs10739971 was associated with radiation-induced intestinal toxicity in PC

Multivariate logistic regression analysis was used to evaluate the role of the pri-let-7a-1 rs10739971 SNP and FAS -670 rs1800682 SNP in the risk of radiation-induced intestinal toxicity in PC. The results showed that the FAS-670 rs1800682 SNP was not associated with the risk of radiation-induced intestinal toxicity in PC, while the pri-let-7a-1 rs10739971 SNP was closely associated with the risk of radiation-induced intestinal toxicity in PC (Table II).

Pri-let-7a-1 rs10739971 was associated with a high rectal volume

Rectal volume of radiotherapy was determined for all participants. Among all subjects, 190 PC patients had a high rectal volume and 190 patients had a low rectal volume. Then, the role of the genotype and allele frequencies of the pri-let-7a-1 rs10739971 SNP and FAS –670 rs1800682 SNP in the risk of radiation-induced intestinal toxicity was evaluated. As shown in Table III, we observed a significant association between the genotype distribution of the Pri-let-7a-1 rs10739971 SNP and a high rectal volume. Both the heterozygous GA (p = 0.011, OR = 2.24, 95% CI: 1.15–4.37) and homozygous AA (p = 0.0062, OR = 4.92, 95% CI: 1.57–15.38) variants were more frequent among patients with a high rectal volume. The A allele (p = 0.0005, OR = 2.37, 95% CI: 1.45–3.88) was also significantly more frequent in patients with a high rectal volume. However, there were no significant associations between the genotype and allele frequencies of the FAS –670 rs1800682 SNP and a high rectal volume. As shown in Table IV, there was no significant association between a low rectal volume and the genotype and allele frequencies of the FAS -670 rs1800682 and Pri-let-7a-1 rs10739971 SNPs.

Pri-let-7a-1 rs10739971 was involved in radiation-induced intestinal toxicity by affecting let-7a and FAS expression

Real-time PCR and IHC were performed to compare the expression of let-7a and FAS in prostate tissue and peripheral blood samples collected from the PC patients in the intestinal toxicity (+) and intestinal toxicity (–) group, as well as in patients carrying different genotypes (AA, AG and GG). The expression of let-7a was much higher in the prostate tissue (Figure 1 A) and peripheral blood (Figure 2 A) samples collected from the intestinal toxicity (–) group compared with the intestinal toxicity (+) group. In addition, the prostate tissue (Figure 1 B) and peripheral blood (Figure 2 B) samples collected from subjects carrying the GG genotype exhibited a lower level of let-7a

Table II. Comparison of genotype and allele of rs10739971 and rs1800682 between radiation-induced intestinal toxicity (+) and radiation-induced intestinal toxicity (–) groups

Genotype	Radiation-induced intestinal toxicity (+) (N = 122)	Radiation-induced intestinal toxicity (–) (N = 258)	OR (95% CI)	P value
FAS -670 rs1800682:				
AA	34 (0.28)	67 (0.26)	reference (1.00)	
AG	62 (0.51)	129 (0.50)	1.05 (0.63–1.76)	0.835
GG	26 (0.21)	62 (0.24)	1.21 (0.65–2.24)	0.544
AA vs. AG + GG	88	191	1.10 (0.67–1.78)	0.695
AA + AG vs. GG	96	196	1.16 (0.69–1.96)	0.557
A	130	263	reference (1.00)	
G	114	253	1.09 (0.80–1.48)	0.552
Pri-let-7a-1 rs10739971:				
GG	58 (0.43)	95 (0.37)	reference (1.00)	
GA	46 (0.41)	113 (0.44)	1.50 (0.93–1.98)	0.093
AA	16 (0.16)	50 (0.19)	1.99 (0.99–3.65)	0.051
AA + AG vs. GG	62	163	1.58 (0.99–2.48)	0.054
AA vs. AG + GG	104	208	1.56 (0.84–2.87)	0.151
G	162	303	reference (1.00)	
A	82	213	1.38 (1.01–1.90)	0.047

Table III. Comparison of genotype and allele of rs10739971 and rs1800682 between radiation-induced intestinal toxicity (+) and radiation-induced intestinal toxicity (–) groups stratified by rectal volume exposed to radiation (high volume)

Genotype	radiation-induced intestinal toxicity (+) (*N* = 61)	radiation-induced intestinal toxicity (–) (*N* = 129)	OR (95% CI)	*P* value
FAS -670 rs1800682:				
AA	17 (0.28)	33 (0.26)	reference (1.00)	
AG	31 (0.51)	65 (0.50)	1.21 (0.66–1.86)	0.765
GG	13 (0.21)	31 (0.24)	1.28 (0.76–2.34)	0.612
AA vs. AG + GG	44	96	1.65 (0.68–1.98)	0.712
AA + AG vs. GG	48	98	1.32 (0.79–2.12)	0.627
A	65	131	reference (1.00)	
G	57	127	1.54 (0.91–1.76)	0.671
Pri-let-7a-1 rs10739971:				
GG	37 (0.43)	47 (0.37)	reference (1.00)	
GA	20 (0.41)	57 (0.44)	2.24 (1.15–4.37)	0.011
AA	4 (0.16)	25 (0.19)	4.92 (1.57–15.38)	0.0062
AA + AG vs. GG	24	82	2.68 (1.43–5.03)	0.002
AA vs. AG + GG	57	104	3.42 (1.13–10.33)	0.028
G	94	151	reference (1.00)	
A	28	107	2.37 (1.45–3.88)	0.0005

Table IV. Comparison of genotype and allele of rs10739971 and rs1800682 between radiation-induced intestinal toxicity (+) and radiation-induced intestinal toxicity (–) groups stratified by rectal volume exposed to radiation (low volume)

Genotype	radiation-induced intestinal toxicity (+) (*N* = 61)	radiation-induced intestinal toxicity (–) (*N* = 129)	OR (95%CI)	*P* value
FAS -670 rs1800682:				
AA	18 (0.28)	35 (0.26)	reference (1.00)	
AG	33 (0.51)	65 (0.50)	1.32 (0.74–1.99)	0.642
GG	10 (0.21)	29 (0.24)	1.32 (0.79–2.83)	0.423
AA vs. AG + GG	43	94	1.76 (0.72–2.18)	0.802
AA + AG vs. GG	51	100	1.42 (0.82–2.63)	0.512
A	69	135	reference (1.00)	
G	53	123	1.61 (0.82–1.90)	0.712
Pri-let-7a-1 rs10739971:				
GG	21 (0.43)	48 (0.37)	reference (1.00)	
GA	26 (0.41)	56 (0.44)	0.94 (0.47–1.88)	0.886
AA	12 (0.16)	25 (0.19)	0.91 (0.38–2.15)	0.832
AA + AG vs. GG	38	81	0.93 (0.49–1.77)	0.831
AA vs. AG + GG	47	104	0.94 (0.43–2.03)	0.878
G	68	152	reference (1.00)	
A	50	106	1.05 (0.67–1.63)	0.814

compared to the samples in the AA and AG groups (Figure 1 B). Meanwhile, the mRNA (Figures 1 C and 2 C) and protein (Figure 1 E) expression of FAS in the prostate tissue (Figures 1 C, E) and peripheral blood (Figures 2 C) samples collected from the intestinal toxicity (+) group was much higher than those in the intestinal toxicity (–) group, while the FAS mRNA expression in the prostate tissue (Figure 1 D) and peripheral blood (Figure 2 D) samples collected from the GG group was higher than that in the AA and AG groups. We also evaluated the effect of the FAS –670 rs1800682 SNP on the ex-

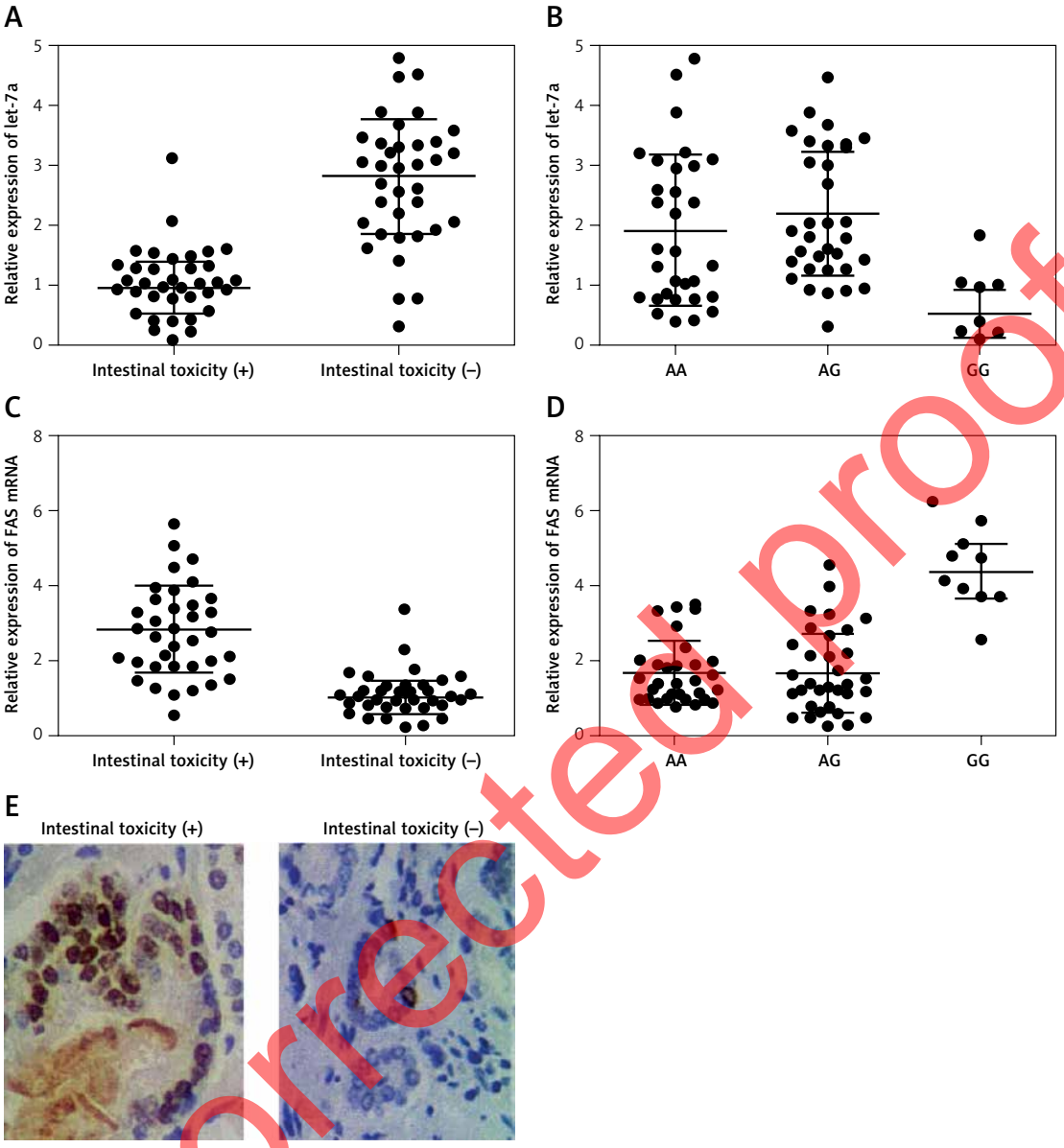

Figure 1. Differential expression of let-7a and FAS in the prostate tissues collected from different groups. **A** – let-7a level in the intestinal toxicity (+) group was lower than that in the intestinal toxicity (–) group. **B** – The GG genotype of the let-7a rs10739971 SNP was associated with lower let-7a expression compared with the AA or AG genotype. **C** – FAS mRNA expression in the intestinal toxicity (+) group was higher than that in the intestinal toxicity (–) group. **D** – The GG genotype of the let-7a rs10739971 SNP was associated with higher FAS mRNA expression compared with the AA or AG genotype. **E** – FAS protein in the intestinal toxicity (+) group was much higher than that in the intestinal toxicity (–) group

pression of let-7a and FAS, and no difference was noted among patients carrying different genotypes of rs1800682 SNP (data not shown).

FAS was confirmed as a direct target of let-7a

Based on the computational analysis, we identified let-7a as a direct target of FAS (Figure 3 A). To further confirm whether let-7a and FAS can interact with each other, we conducted a luciferase assay to show that FAS is a direct target of let-7a, since the luciferase activity of the wild-type FAS 3' UTR in DU145 and PC-3 cells over-expressing let-7a was significantly lower compared with that in cells expressing the mutant FAS 3' UTR or the scramble control (Figure 2 B). Real-time PCR and Western blot analysis were carried out to investigate the effect of let-7a on FAS expression. In DU145 and PC-3 cells, the transfection with let-7a dose-dependently increased let-7a expression, while the transfection with FAS siRNA had no effect on let-7a expression (Figure 3 C). In addition, the transfection of DU145 and PC-3 cells with let-7a

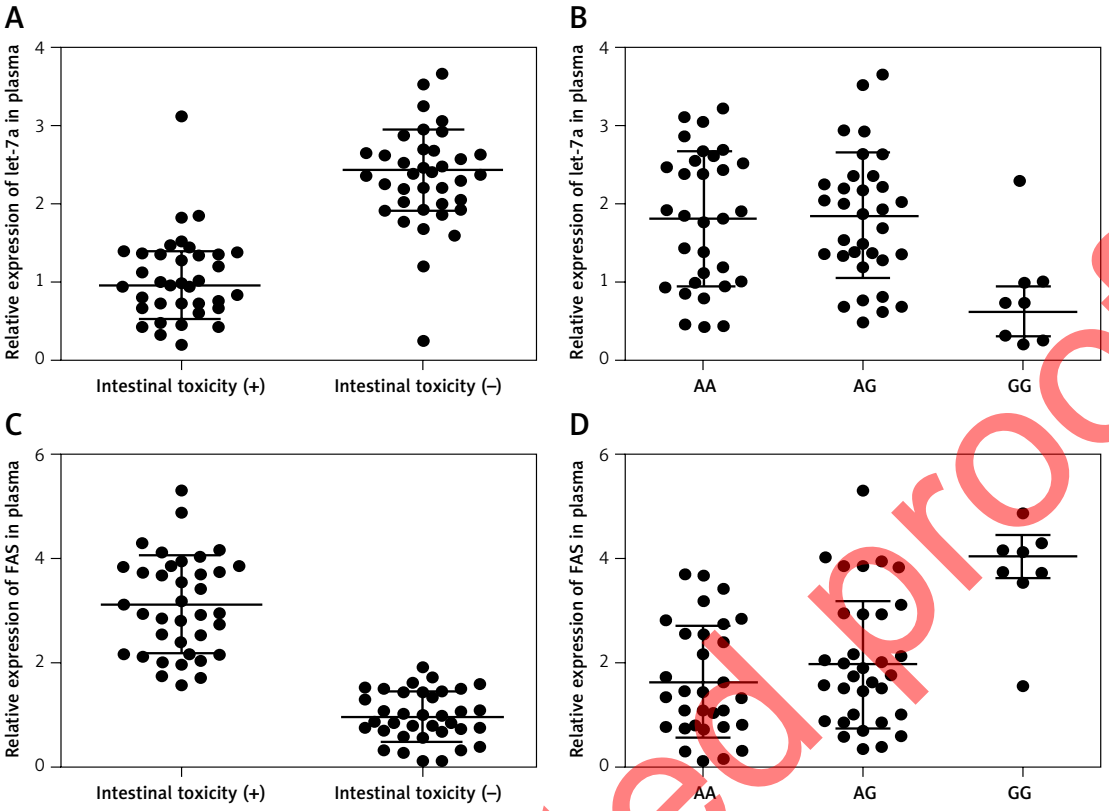

Figure 2. Differential expression of let-7a and FAS in the peripheral blood collected from different groups. **A** – let-7a expression in the intestinal toxicity (+) group was lower than that in the intestinal toxicity (–) group. **B** – The GG genotype of the let-7a rs10739971 SNP was associated with lower let-7a expression compared with the AA or AG genotype. **C** – FAS mRNA expression in the intestinal toxicity (+) group was higher than that in the intestinal toxicity (–) group. **D** – The GG genotype of the let-7a rs10739971 SNP was associated with higher FAS mRNA expression compared with the AA or AG genotype

reduced the levels of FAS mRNA (Figure 3 D) and protein (Figure 3 E) in a dose-dependent manner. In contrast, transfection with let-7a inhibitor dose-dependently decreased let-7a expression (Figure 3 F) and up-regulated mRNA (Figure 3 G) and protein (Figure 3 H) expression of FAS.

Discussion

In this study, we enrolled 380 PC patients with or without signs of radiation-induced intestinal toxicity to investigate the association between the risk of radiation-induced intestinal toxicity and the let-7a rs10739971 and FAS-670 rs1800682 SNPs using a multivariate logistic regression analysis. The results showed that the let-7a rs10739971 SNP but not the FAS-670 rs1800682 SNP was closely associated with the risk of radiation-induced intestinal toxicity. In addition, based on their rectal volume, we stratified the subjects into a high rectal volume group and a low rectal volume group, and found a significant association between the genotype distribution of the let-7a rs10739971 SNP and high rectal volume. In contrast, in the low rectal volume group, neither of the two SNPs was associated with radiation-induced intestinal toxicity.

As a family member of let-7 containing 12 different miRNAs, including miR-98 and let-7a-1, -7a-2, -7a-3, -7b, -7e, -7f-1, -7f-2, -7g, and -7i, let-7a was demonstrated in previous studies on clinical specimens to affect the development of human cancers, such as lung cancer, medulloblastoma, breast cancer, ovarian cancer, and ES [18, 19]. Nevertheless, it was also demonstrated that miRNAs could act as oncogenes according to different cellular contexts. Under certain conditions, miRNAs could switch from their repressive role to an activating role via upregulating their targets, including let-7a [20]. Lu et al. (2007, 2011) found that ectopic expression of let-7a substantially activated the expression of IGF-II, which plays an essential role in the onset of ES [21–24]. Let-7a could also be used as a marker for the diagnosis and treatment of PC [25]. A recent study demonstrated that let-7a1 could suppress the levels of IGF1R by targeting the 3' UTR of its mRNA [26]. Moreover, let-7a1-induced downregulation of IGF1R resulted in reduced expression of c-fos and Elk1 activity, thus leading to inhibited proliferation of PC-3 cells and enhanced apoptosis, while the suppression of let-7a1 expression up-regulated IGF1R expression and increased Elk1 activity to promote cell

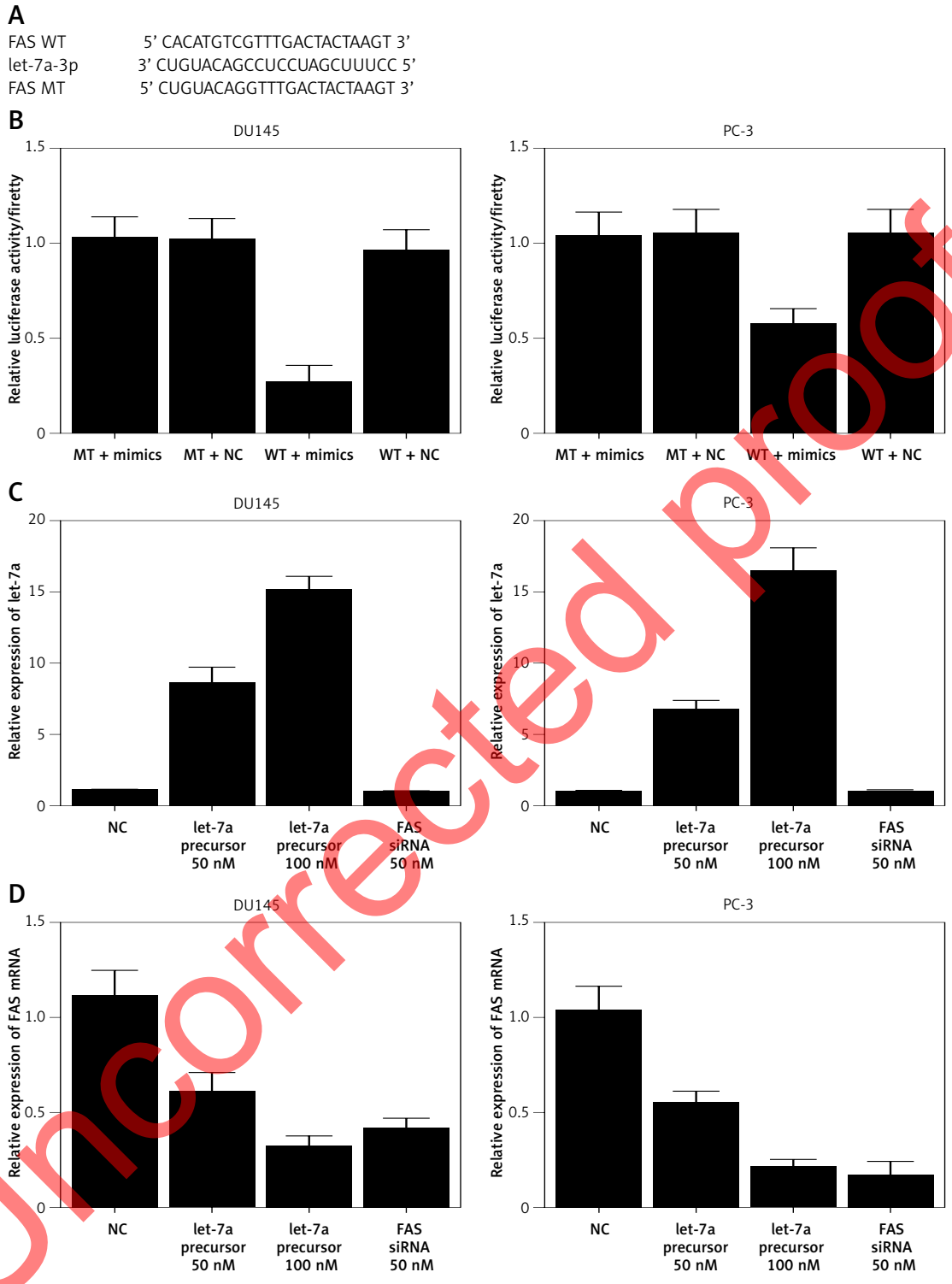

Figure 3. FAS was confirmed as a direct target of let-7a. **A** – A putative target of let-7a site was present on the FAS 3′ UTR. **B** – let-7a mimics significantly reduced the luciferase activity of the wild-type FAS 3′ UTR but not that of the mutant FAS 3′ UTR in DU145 and PC-3 cells. **C** – let-7a mimics dose-dependently increased let-7a expression in DU145 and PC-3 cells, while FAS siRNA had no effect on let-7a expression. **D** – let-7a mimics decreased FAS mRNA expression in DU145 and PC-3 cells in a dose-dependent manner

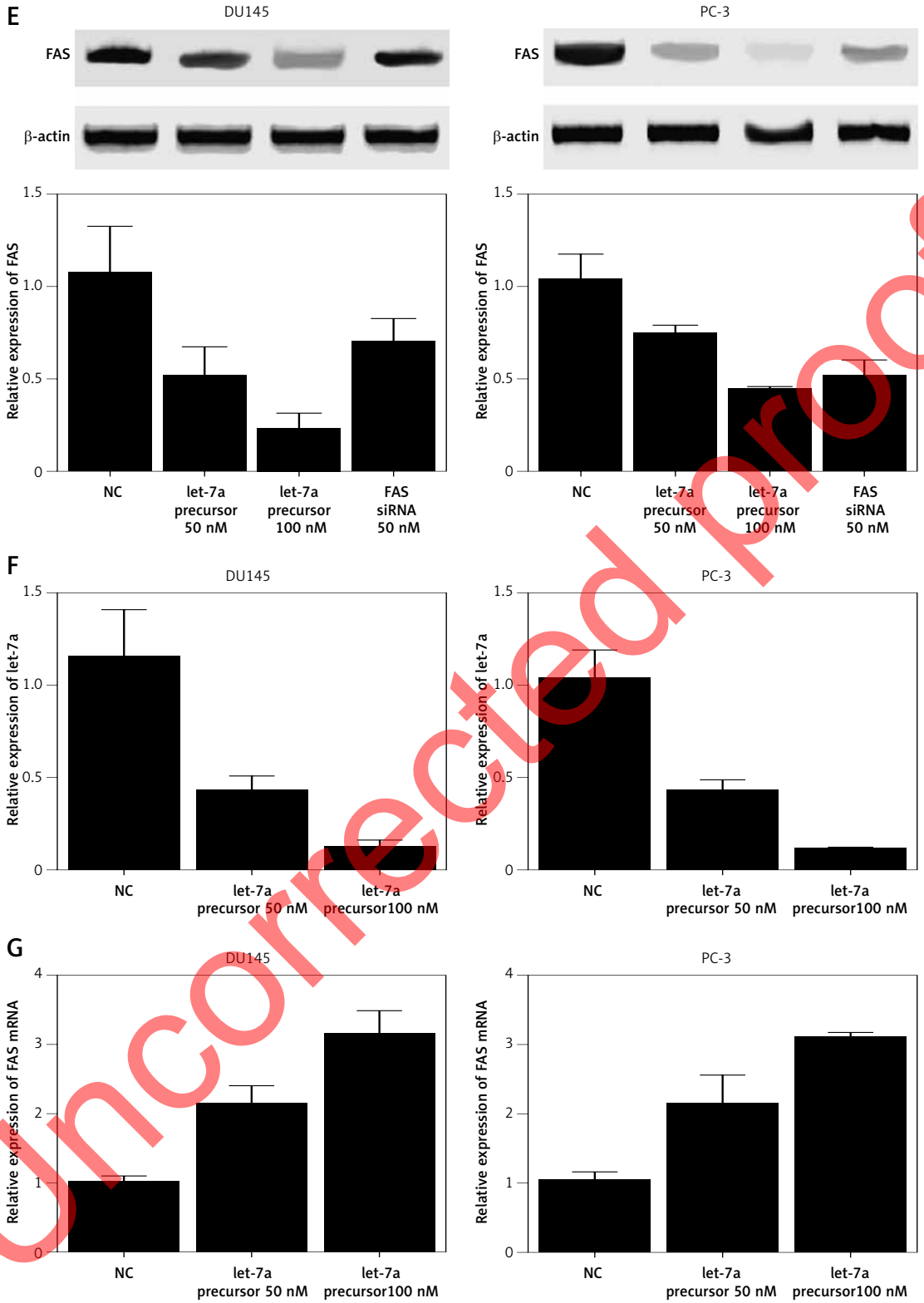

Figure 3. Cont. **E** – let-7a mimics decreased FAS protein expression in DU145 and PC-3 cells in a dose-dependent manner. **F** – let-7a inhibitors dose-dependently decreased let-7a expression in DU145 and PC-3 cells. **G** – let-7a inhibitors up-regulated FAS mRNA expression in DU145 and PC-3 cells in a dose-dependent manner

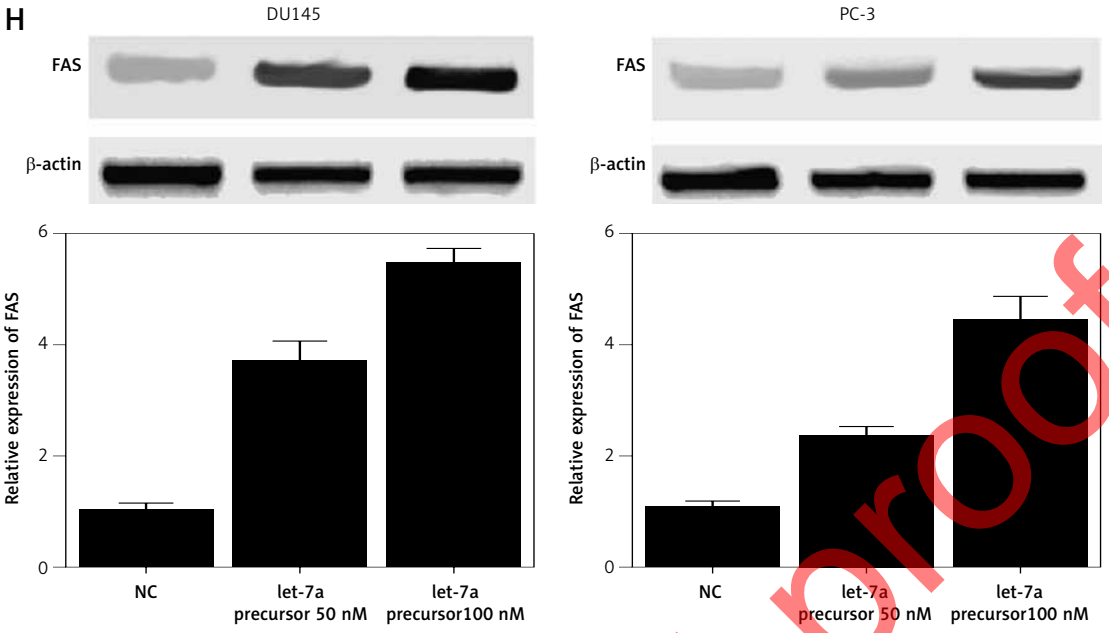

Figure 3. Cont. **H** – let-7a inhibitors up-regulated FAS protein expression in DU145 and PC-3 cells in a dose-dependent manner

proliferation [26]. It was proven that let-7a, one of the most conserved and abundant miRNAs in BMSCs, targets both Fas and Fasl mRNAs to suppress their expression [27]. Let-7a is also enriched in BMSCs and can suppress both Fas and FasL expression [27]. Therefore, let-7a is a crucial regulator of immunoregulatory properties in BMSCs. In this study, we carried out computational analyses, luciferase assays, real-time PCR and Western blot analyses to explore the interaction between let-7a and FAS. Our results identified FAS as a virtual target gene of let-7a, which inhibited the expression of FAS.

As a receptor of tumor necrosis factor, Fas could induce apoptosis and prevent the onset of autoimmune diseases and cancer [28, 29]. In addition, Fas could enhance cancer metastasis and growth by regulating the proliferation and survival of cancer cells [30]. Therefore, Fas is deemed essential for the development of cancer and has demonstrated some potential in clinical application, although the detailed mechanism underlying the role of Fas in cancer and cell apoptosis remains elusive [31, 32]. In addition, subjects carrying the -844CC allele of FASL showed much higher levels of FASL expression than carriers of the homozygous -844T genotype, which can significantly affect the toxicity induced by radiation [33]. Therefore, the -844T allele of FASL may protect its carriers against RT-induced urinary and/or rectal toxicity [5]. In this study, we performed real-time PCR and IHC to explore the mechanism underlying the role of the let-7a rs10739971 SNP in radiation-induced intestinal toxicity. The results showed that the GG genotype of let-7a rs10739971 SNP reduced let-7a expression while enhancing FAS expression. In addition, the intestinal toxicity (–) group showed a much higher level of let-7a and a much lower level of FAS than the intestinal toxicity (+) group.

An A to G SNP has been found at the -670 position of the Fas promoter and may affect the expression of STAT-1 [34]. In addition, this SNP was linked to the onset of PC, hepatocellular cancer, breast cancer, and esophageal cancer [35]. Recent studies also revealed that the SNPs located in some members of the let-7 family can affect the susceptibility to cancer. In particular, the interaction between the rs1917799 SNP in ERCC6 and the rs10739971 SNP in pri-let-7a-1 could substantially change the susceptibility to GC [13]. Moreover, the rs10877887 SNP in the let-7 promoter was associated with a substantially increased risk of hepatocellular cancer, while the rs629367 SNP located in pri-let-7a-2 could result in a poor prognosis of GC [36, 37]. However, few researchers have studied the correlation between the SNPs in pri-let-7a-1 and the prognosis of cancer, although a SNP in pri-let-7a-1 could alter the binding affinity between let-7a-1 and GATA to affect the expression of pri-miR-34 b/c [38]. It was also speculated that the rs10739971 SNP in pri-let-7a-1 could significantly affect the GC prognosis by regulating the expression of mature let-7a.

There are however limitations of this study. All subjects included in this study were of the Chinese population. Also, the signaling pathway established in this study should be verified not only in cell lines, but also in vivo in animal models.

In conclusion, the FAS rs1800682 and let-7a rs10739971 SNPs were involved in radiation-in-

duced intestinal toxicity in PC patients. In addition, the rs1800682 SNP in the promoter region of FAS changed the expression of FAS, while the rs10739971 SNP in the flanking region of let-7a altered the expression of let-7a, a negative regulator of FAS. Therefore, the pri-let-7a-1 rs10739971 polymorphism could be used in clinical practice as a putative marker for the prognosis of intestinal toxicity in PC patients undergoing radiotherapy.

Conflict of interest

The authors declare no conflict of interest.

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