Original Article

Positive Cofactor 4 as a Potential Radiation Biodosimeter for Early Assessment

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Abstract

During a major radiation event, a large number of people need to be rapidly assessed for radiation damage to ensure effective medical treatment and efficient use of medical resources. However, current techniques cannot meet the requirement of rapid detection of large quantities of samples in an emergency. It is essential to develop rapid and accurate radiation biodosimeters in peripheral blood. Here, we identified radiation sensitive genes in mice by RNA sequencing and evaluated their utility as radiation biodosimeters in human cell lines. Mice were subjected to gamma-irradiation with different doses (0–8 Gy, .85 Gy/min), and the tail venous blood was analyzed by RNA sequencing. We have identified 5 genes with significantly differential expression after radiation exposure. We found that positive cofactor 4(PC4) had well correlation with radiation dose in human lymphoblastoid cell line after irradiation. The relative expression of PC4 gene showed a good linear correlation with the radiation dose after 1–5 Gy irradiation (.85 Gy/min). PC4 gene can be rapidly recruited to the DNA damage sites faster than γ-H2AX after radiation in immunofluorescence detection. In conclusion, PC4 may be represented as new radiation biological dosimeter for early assessment.

Keywords

radiation dose, positive cofactor 4, γ-H2AX, biodosimeter

Introduction

People have been impressed by high dose of ionizing radiation damage caused by nuclear weapons explosions and nuclear power accidents.[1,2] Meanwhile, there is controversy over the long-term biological effects of radiation medical testing and treatment, as well as ongoing exposure to radiation in daily working life.[3] Hematopoietic system is one of the most sensitive systems to radiation damage.[4] High dose of ionizing radiation in the short-term or low-dose of radiation in the long-term may lead to abnormal hematopoietic function and even lead to the occurrence of malignant tumors in the hematopoietic system.[5] How to quickly and accurately evaluate the biological dose of radiation is of great significance for the prevention and treatment of hematopoietic dysfunction caused by radiation.

At present, radiation biological dose detection technology can be divided into epidemiological surveys of large populations, chromosome aberration analysis and cytokinesis-blocked method, and molecular biological detection technology.[6] All the above three methods have limitations. For example, traditional biological dosimetry methods, such as chromosome aberration analysis and cytokinesis-block method, which have some disadvantages such as manual operation, large difference between operators, time-consuming, and laborious.[7-9] Therefore, it is a scientific problem to search for an ideal method of biological dose assessment after radiation.

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Current studies on radiation-induced gene expression have focused on the early time interval 1–24 h after irradiation to identify genes involved in signaling, RNA and DNA synthesis, metabolism, DNA damage response and cell cycle arrest.[10-12] The expression of several genes has already been shown to be modulated in a dose-dependent manner, such as GADD45A, CDKN1A, SESN1, CCNG1 in human peripheral blood.[13-15] Budworth et al[16] reported that the level of 8 genes (BBC3, FDXR, CDKN1A, PCNA, XPC, GADD45A, DDB2, and POLH) were higher after total-body irradiation treatment in blood samples than that in the control group. The expression of the above genes was consistently higher after irradiation of 6 hours than 24 hours, suggesting a time-dependent decrease. Ghandhi et al[17] irradiated human whole blood with three doses, .56 Gy, 2.23 Gy, and 4.45 Gy, and showed that the expression of FDXR, AEN, DDB, PHLDA3, PCNA, GADD45A, PC4, and ZMAT3, was up-regulated. The ideal radiation biomarker should provide information about dose and time, and should be independent of environmental and confounding factors, such as smoking, drug therapy, and age[18] The purpose of this study was to identify potential biodosimeters and provide the dose and time relationship in vivo and vitro. Through a series of experiments, we found that the human positive cofactor 4 (PC4) might be a potential radiation biodosimeter.

The human positive cofactor 4 (PC4) is a highly conserved nuclear protein, which is composed of 127 amino acids and facilitates RNA polymerase II–driven gene transcription.[19,20]PC4 is involved not only in transcription, but also in DNA replication, damage repair, chromatin formation, and cell cycle regulation.[21-25] Mortusewicz O *et al*[26] found that the single-stranded DNA binding capacity of PC4 plays an important role in repairing DNA damage. Oliver et al[27] found that after laser-induced DNA damage in HeLa cells, PC4 played a role in the extremely early reaction of DNA damage by identifying single-stranded DNA damage, and could be recruited to the DNA damage site earlier than the traditional γ-H2AX, thus possibly initiating or promoting the subsequent steps of DNA damage and repair. Meanwhile, studies have shown that PC4 is involved in a variety of tumors.[28] PC4 is found to be up-regulated in lung cancer,[29] esophageal squamous cell carcinoma,[30] astrocytoma,[31] and prostate cancer,[32] which may play an important role in tumor development.[33,34] However, the dose-response relationship between PC4 and ionizing radiation has not been reported. Therefore, it is of great significance to evaluate the sensitivity and specificity of PC4 as a potential biodosimeter of ionizing radiation.

Methods

Animals

C57BL/6 mice that were 4–5 weeks old were obtained and followed the care and use of guidelines from Laboratory Animals of the AMU. All animal experimental procedures were approved by the AMU Animal Care and Use Committee.

Cell Lines and Cell Culture

Human lymphoblastoid cell line (CCRF-SD) was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in the recommended medium (Hyclone, USA), supplemented with 10% FBS (Gibco, USA) and 1% streptomycin/penicillin (Beyotime, Shanghai, China), and incubated in 5% CO_2 at 37°C. After the sample was irradiated, 0.2 mL cells and 2 mL RPMI 1640 culture solution were added into the 6-well culture plate, respectively, and put into the refrigerator at 4°C.

Irradiation Conditions

Medical electron linear accelerator, source skin distance 80 cm, field area 15 cm × 15 cm, absorbed dose rate .85 Gy/min. Absorbed doses were 0, l, 2, 3, 4, 5, 6 ,7, and 8 Gy.

Total RNA Extraction and Real-time quantitative PCR

Total RNA was collected from cells using Trizol (Cwbiotech, China). 1 μg RNA was reverse transcribed into cDNA according to the recommended protocol by the RevertAid First Strand cDNA Synthesis kit (#K1622, Thermo Fisher Scientific, Inc.). Quantitative RT-PCR was performed according to the recommended protocol by SYBR Green qPCR master mix (Takara). When the reactions were completed, the relative gene expression was calculated by the comparative threshold cycle (Ct) method. GAPDH expression was used as control.

Primer design: According to the gene information in GenBank, the primer premier 5.0 software was applied to detect the primer sequences of PC4, NR4A1, CHAMP1, RFX7, c-REL, and internal reference GAPDH genes by real-time quantitative PCR. The primers were synthesized by Sangon biotech (Shanghai, China), and the purified primers were qualified for quality inspection.

Primer sequence: PC4 Forward 5'TGATTCTGACAGTG AGGTTGAC3', Reverse 5'TTATCATCTCTGCTGCTGC TG3'; GAPDH Forward 5'GCACCGTCAAGGCTGAGA AC3', Reverse 5'TGGTGAAGACGCCAGTGGA3'. NR4A1 Forward 5'ATGCCTCCCCTACCAATCTTC3', Reverse 5'CACCAGTTCCTGGAACTTGGA3'; CHAMP1 Forward 5'CCGGCAUAAUGAAGAGGCAAAUAAA3', Reverse 5'G GAAACACAGAAACUUGGUUCAGUU3'; RFX7 Forward 5'GTCACTCCCCAAACAGGAAGTCTATGATG3', Reverse 5'CCTCTAGTCCCTCCCATGTTTCTTGTC3'. c-Rel Forward 5'CCTCCTGTTGTCTCGAACCC3', Reverse 5'TGCCTTT TGCTTCCCAATCG3'.

Cell Apoptosis Analysis by Flow Cytometry

For apoptosis analysis, cells were stained with AnnexinV-FITC/PI (BD Biosciences) for 15 min at 37°C in the dark, and then analyzed by flow cytometry. Experiments were performed in triplicate.

Western Blotting Analysis

The cell lines were harvested, washed, and lysed with RIPA buffer (Beyotime, China) which contains protease inhibitor cocktail (Roche) for 30 min on ice. Total protein was collected and quantitated by a BCA kit (Beyotime, China) according to the recommended instruction. The protein samples were separated by electrophoresis in gel, and then transferred onto PVDF membranes (Millipore). Blotted membranes were incubated with primary antibodies overnight at 4°C. The membranes were washed 5 min for 3 times with TBST, and then incubated with HRP-linked secondary antibody (Cell Signaling Technology, USA) 1 h at room temperature. The band intensities were detected and visualized by an enhanced chemiluminescence detection system (Bio-Rad Laboratories). Primary antibodies against PC4 and β-actin were obtained from Cell Signaling Technology. Primary antibodies against PC4 were obtained from Sigma.

Flow Fluorescence Intensity Detection

For FCM analysis, data acquisition was set to analyze 10 000 lymphocytes. The level of PC4 was measured by relative PC4 fluorescence intensities, that is, all samples were analyzed on a BD FACS Calibur flow cytometer (BD Biosciences).

Immunofluorescence Staining

Cells were fixed in 4.0% formaldehyde for 10 min and permeabilized with .5% Triton X-100 or ice-cold methanol for 5 min. The following primary antibodies (diluted in PBS containing 4% BSA) were used: anti-γ-H2AX (Ser139) mouse monoclonal antibodies (Millipore), anti-PC4 rabbit polyclonal antibodies (SA2249; generated by standard techniques; Eurogentech). Primary antibodies were detected using secondary antibodies (diluted 1:200 in PBS containing 4% BSA) conjugated to AlexaFluor 488 or 555 (Invitrogen). Cells were counterstained with DAPI and mounted in Vectashield (Vector Laboratories). For mice bone marrow immunofluorescence, cryosections were fixed with 4% paraformaldehyde in PBS. After washing three times with PBS, tissues were permeabilized with .1% Triton X-100 and non-specific binding was blocked with 10% FBS in PBS for 1 h. Slides were then incubated with the primary antibody overnight at 4°C, followed by a 1.5 h incubation with the secondary antibody. Then nuclei were stained with DAPI. Images were captured using fluorescent microscope (Olympus BX51). The primary antibodies used were PC4 (1:200, Abcam). Secondary antibodies to different species IgG were Alexa Fluor® 488 (green) conjugated (1:500 for all, Invitrogen).

RNA Sequencing and Data Analysis

For RNA seq, total RNA was isolated from radiation groups or control. Poly(A) mRNA was subsequently purified from 100 ng total RNA using NEBNext Poly(A) mRNA Magnetic Isolation Module. NEBNext Ultra Directional RNA Library Prep Kit (New England BioLabs, Ipswich, MA) was used for library preparation. Each group was sequenced in duplicate by Illumine Hiseq 1000 with single end 50-bp read length. Sequence reads were aligned to the human genome version hg19 by using standard Illumina sequence analysis pipeline. To identify DEGs (differential expression genes) between two different samples, the expression level of each transcript was calculated according to the fragments per kilobase of exon per million mapped reads (FPKM) method. In addition, functional enrichment analyses including GO and KEGG were performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways at Bonferroni-corrected P value $\leq .05$ compared with the whole-transcriptome background. Gene ontology functional enrichment and KEGG pathway analysis were carried out by Goatools (https://github.com/tanghaibao/Goatools) and KOBAS 2.1.1 (http://kobas.cbi.pku.edu.cn/download.php).

Statistical Analysis

Statistical analysis was carried out using SPSS 22.0 software (SPSS Inc., Chicago, USA), and all data are presented as means ± SD. Comparisons between two groups were performed using the Student's t test. Comparisons among three or more groups were performed using a one-way analysis of variance (ANOVA).

Result

Identification of Different Gene Expression After γ-radiation Treatment in Mice

To evaluate different gene expressions after IR, we irradiated mice at a total dose of 2 Gy, 4 Gy, 6 Gy, and 8 Gy, respectively (each group n = 3). The non-irradiation gene expression profile was compared with that of gene expression from IR groups after 24 hours. A total of 2527 genes expression was significantly altered after IR exposure (The significant difference definition is determined by $P < .05$). The top 100 genes ranked according to their P-values converge to form Figure 1A. The rest of genes expression convergence heat maps were placed in Supplemental Figure 1. Figure 1A showed the different expression of genes in the normal group and irradiation groups. Venn analysis revealed 5 differentially expressed genes including CHAMP1 (Chromosome Alignment Maintaining Phosphoprotein 1), PC4, NR4A1 (Nuclear Receptor Subfamily 4 Group A Member 1), RFX7 (Regulatory Factor X7), and REL (REL Proto-Oncogene, NF-KB Subunit) between the normal group and radiation groups (Figure 1B). The violin plot (Figure 1C) revealed that the distribution of genes in each group was symmetrical and most of the gene expression was concentrated between log10(FPKM+1) 0–3, meanwhile, 4 Gy group have a significant higher median value

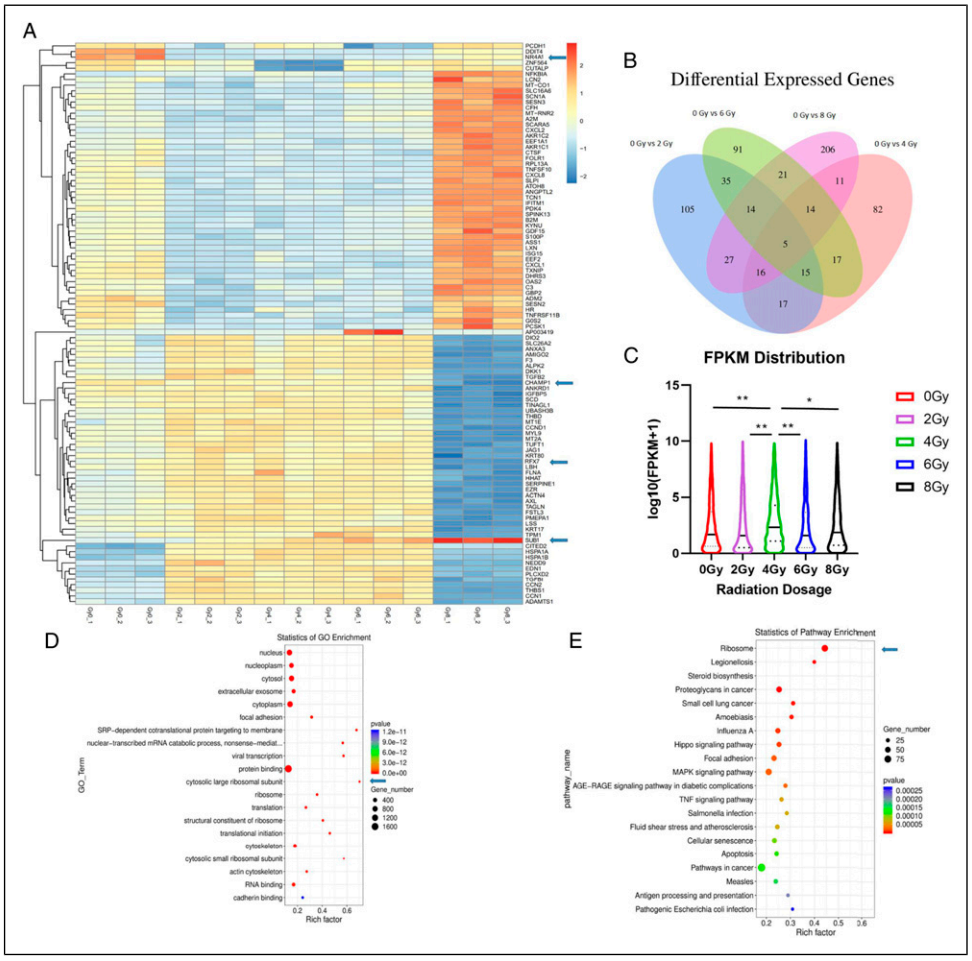

Figure 1. Identification of differentially expressed genes after γ-radiation treatment. (A) Clustered heatmap of differentially expressed genes between non-radiation control group and radiation groups. (B) Venny analysis of differentially expressed genes between pre-irradiation and post-irradiation samples in all IR groups. (C) Violin plot shows Avery expression levels of total analyzed genes in each group. (D) Gene ontology functional analysis between control group and IR groups. (E) Kyoto Encyclopedia of Genes and Genomes analysis between control group and IR groups. The blue arrow of Figure 1A refers to NR4A1, CHAMP1, RFX7, and SUB1 (PC4). The blue arrow of Figure 1D refers to cytosolic large ribosome subunit. The blue arrow of Figure 1E refers to ribosomal related pathways.*$P < .05$, **$P < .01$.

(2.847 ± .43) than the others (0 Gy, 2.416 ± .18; 2 Gy, 2.363 ± .24; 6 Gy 2.356 ± .35; 8 Gy 2.482 ± .42). Except for the 4 Gy group, there was no significant difference in median value among other groups.

To explore the functional roles of the differentially expressed genes, we conducted GO analysis and KEGG analysis by a union of differentially expressed genes from different dose groups (Figure 1D and 1E), the KEGG and GO analysis showed that the target genes were most riched in cytosolic large ribosome subunit (rich factor .71) and ribosomal related pathways (rich factor .44), which suggested that the target genes might be involved in transcription and translation.[35] In addition, the enrichment degree of the above pathway was positively correlated with radiation dose. We conducted the GO and KEGG analysis from different dose groups in Supplemental Figure 2 and Supplemental Figure 3. The cytosolic large ribosome subunit rich factor was .131, .274, .377 and .632 in 2 Gy, 4 Gy, 6 Gy, and 8 Gy, respectively. The ribosome pathway rich factor was .026, .057, .446, and .414 in 2 Gy, 4 Gy, 6 Gy, and 8 Gy, respectively. Among the above 5 genes, GO and KEGG are both enriched in DNA binding or nucleic acid binding and transcription-related function. CHAMP1, PC4, and NR4A1, were enriched in the cytosolic large ribosome subunit and ribosome-related pathway.

Identification of Potential Radiation Biodosimeter in Human Lymphocyte Cell Lines

We then validated the relationship between gene expression and radiation dose in human lymphocyte cell lines. The mRNA expressions of 5 genes (CHAMP1, PC4, NR4A1, RFX7, c-REL) were detected by RT-PCR in normal human lymphoblastoid cell line at 24 h after 2 Gy, 4 Gy, 6 Gy, and 8 Gy irradiation, respectively. We conducted linear regression

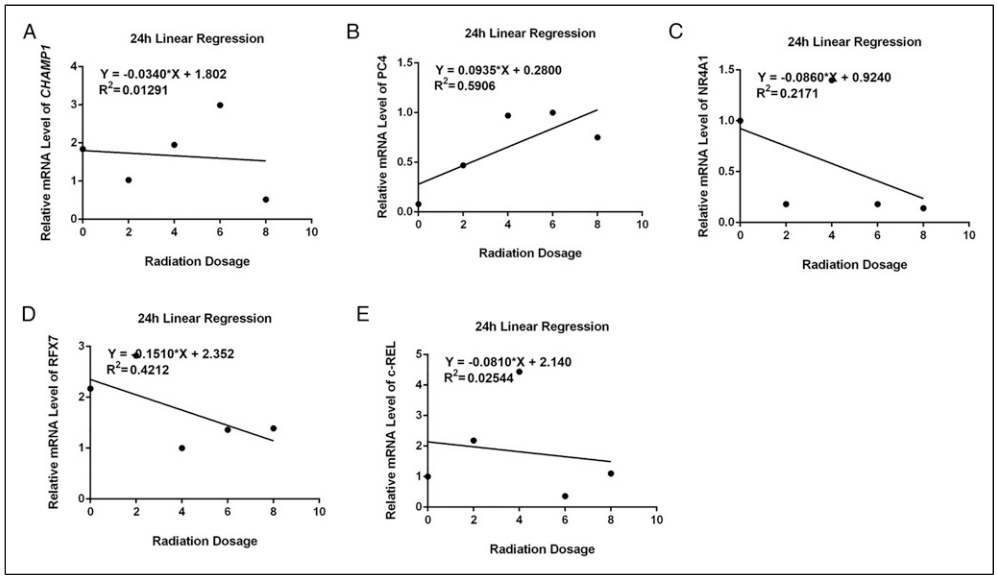

Figure 2. Identification of suitable dosimeter for human lymphoblastoid cell line at 24 h after irradiation. (A) Linear regression analysis of CHAMP1 gene expression at 24 h after irradiation. (B) Linear regression analysis of PC4 gene expression at 24 h after irradiation. (C) Linear regression analysis of NR4A1 gene expression at 24 h after irradiation. (D) Linear regression analysis of RFX7 gene expression at 24 h after irradiation. (E) Linear regression analysis of c-REL gene expression at 24 h after irradiation.

to assess the relationship between gene expression and radiation dose (Figure 2A–2E). The linear regression equation of CHAMP1 at 24 h: Y = −.0340X+1.802 (R^2 = .0129). The linear regression equation of PC4 at 24 h: Y = .09350X+.2800 (R^2 = .5906). The linear regression equation of NR4A1 at 24 h: Y = −.0860X+.9240 (R^2 = .2171). The linear regression equation of RFX7 at 24 h: Y = −.1510X+2.352 (R^2 = .4212). The linear regression equation of c-REL at 24 h: Y = −.0810X+2.140 (R^2 = .02544). Then, we conducted second order polynomial to assess the relationship between gene expression and radiation dose (Supplemental Figure 4 A-E). The second order polynomial equation of CHAMP1 at 24 h: Y = 1.345+.4231X–.05714X^2 (R^2 = .2092). The second order polynomial equation of PC4 at 24 h: Y = .03+.3435X–.03125X^2 (R^2 = .8703). The second order polynomial equation of NR4A1 at 24 h: Y = .7983+.03971X–.01571X^2 (R^2 = .2412). The second order polynomial equation of RFX7 at 24 h: Y = 2.486–.2853X–.01679X^2 (R^2 = .4209). The second order polynomial equation of c-REL at 24 h: Y = 1.109+.9504X–.1289X^2 (R^2 = .3799). In addition, we conducted third order polynomial to assess the relationship between gene expression and radiation dose (Supplemental Figure 5 A–E). The third order polynomial equation of CHAMP1 at 24 h: Y = 1.921–1.487X+.5986X^2–.05417X^3 (R^2 = .7234). The third order polynomial equation of PC4 at 24 h: Y = .0690+.2038X+.0175X^2–.2229X^3 (R^2 = .8936). The third order polynomial equation of NR4A1 at 24 h: Y = .8843–.2685X+.09179X^2–.008958X^3 (R^2 = .2920). The third order polynomial equation of RFX7 at 24 h: Y = 2.272–.4815X–.2507X^2+.02229X^3 (R^2 = .6186). The third order polynomial equation of c-REL at 24 h: Y = .7346–2.291X–.5964X^2+.03896X^3 (R^2 = .5132). In conclusion, the best correlation between gene expression and radiation dose was PC4 in human lymphoblastoid cell line.

Changes of PC4 mRNA Expression in Human Lymphocyte Cell Lines Induced by γ-Radiation

To evaluate the relationship between PC4 gene expression and radiation dose and radiation time, we conducted RT-PCR in human lymphoblastoid cell line at different radiation doses and different time. The relative expression of PC4 gene in human lymphoblastoid cell line in the 1-5 Gy groups was significantly higher than that in the control group, and increased with the increase of radiation dose. In the same dose group, at each time point, the expression of PC4 gene reached the peak at 12 h after radiation, and the expression of PC4 gene began to decline after 12 h (Figure 3A), while the 6 Gy and 7 Gy groups did not follow the above rules. The relative PC4 gene expression of 1–7 Gy groups is shown in (Table 1). We detected the apoptosis of lymphocytes in the 6 Gy and 7 Gy groups at 4 and 12 hours after radiation. After 6 Gy irradiation, the apoptosis rates of lymphocytes at 4 h and 12 h were 75.5% (P < .001) and 82.3% (P < .001), respectively. After 7 Gy irradiation, the apoptosis rates of lymphocytes at 4 h and 12 h were 88.1% (P < .001) and 92.3% (P < .001), respectively (Figure 3B–3C). The 6 Gy and 7 Gy dose groups did not follow the above rules. Considering that the radiation dose higher than 6 Gy would lead to massive lymphocyte death, PC4 gene could not be expressed in time. The linear regression equation of 1 h:Y = .1354X+.0008095 (R^2 = .9481,

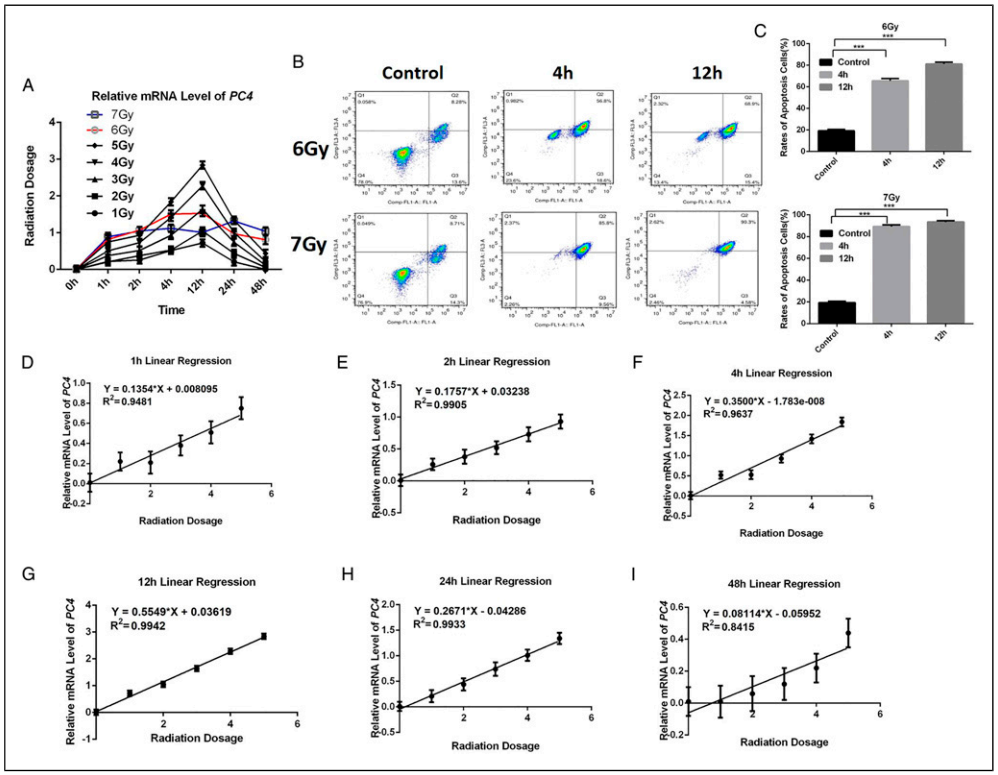

Figure 3. Changes of PC4 gene expression in human lymphoblastoid cell line induced by γ-radiation. (A) The mRNA level of PC4 in human lymphoblastoid cell line which were treated with 1-7 Gy radiation for different time points. (B) Cells were treated with 6 Gy and 7 Gy and apoptosis was detected using flow cytometry at 4 h and 12 h (Control groups were unirradiated cells). (C) A statistical analysis of the data derived from (B). All data indicate the mean ± SD. (D) Linear regression analysis of PC4 gene expression at 1 h. (E) Linear regression analysis of PC4 gene expression at 2 h. (F) Linear regression analysis of PC4 gene expression at 4 h. (G) Linear regression analysis of PC4 gene expression at 12 h. (H) Linear regression analysis of PC4 gene expression at 24 h. (I) Linear regression analysis of PC4 gene expression at 48 h. $^{**}P < .01$, $^{***}P < .001$.

Table 1. The relative PC4 gene expression after irradiated human lymphocytes with γ-radiation.

Time dose, Gy	0	1 h	2 h	4 h	12 h	24 h	48 h	P Value
1	.01 ± .01	.22 ± .02*	.26 ± .01*	.52 ± .01*	.72 ± .01***	.21 ± .03	0 ± .01	.0009
2	.01 ± .01	.21 ± .03	.38 ± .02*	.53 ± .01*	1.05 ± .03***	.44 ± .01*	.06 ± .01	.0002
3	.01 ± .01	.38 ± .01*	.52 ± .03*	.93 ± .04*	1.64 ± .06***	.74 ± .02*	.12 ± .02	<.0001
4	0 ± .01	.51 ± .04*	.73 ± .06*	1.42 ± .02*	2.28 ± .02***	1.01 ± .04*	.22 ± .05*	<.0001
5	.01 ± .01	.75 ± .02*	.93 ± .05*	1.84 ± .05*	2.84 ± .04***	1.34 ± .01*	.44 ± .02*	<.0001
6	.01 ± .01	.82 ± .01*	1.07 ± .11*	1.51 ± .12*	1.53 ± .02***	.97 ± .06*	.81 ± .12*	<.0001
7	.01 ± .01	.89 ± .03*	1.05 ± .09*	1.12 ± .10*	1.01 ± .11*	1.32 ± .07***	1.04 ± .10*	.0001

The relative expression value: mean ± standard deviation. * Represents statistical difference between the test groups and the control group. *** Represents the group with the greatest difference.The P value is the group with the most difference. Paired t test was performed.

$P < .05$) (Figure 3D); The linear regression equation of 2 h: Y = .1757X+.03238 ($R^2 = .9905$, $P < .05$) (Figure 3E); The linear regression equation of 4 h:Y = .3500*X—1.783e–008 ($R^2 = .9637$, $P < .05$) (Figure 3F); The linear regression equation of 12 h:Y = .554X+.036 ($R^2 = .994$, $P < .05$) (Figure 3G); Linear regression equation at 24 h:Y = .267X-.042 ($R^2 = .993$, $P < .05$) (Figure 3H). The linear regression equation of 48 h:Y = .08114X–.05952 ($R^2 = .8415$, $P < .05$) (Figure 3I). After γ-radiation, PC4 gene expression showed a good linear correlation with radiation dose at 1 h, 2 h, 4 h, 12 h, 24 h, and 48 h in human lymphoblastoid cell line.

The Expression of PC4 Protein in Hematopoietic System After γ-Radiation

In the next experiment, we verified the expression of PC4 protein in hematopoietic system after radiation. We conducted western blot at 24 h after 2 Gy, 4 Gy, 6 Gy, 8 Gy irradiation in bone marrow of

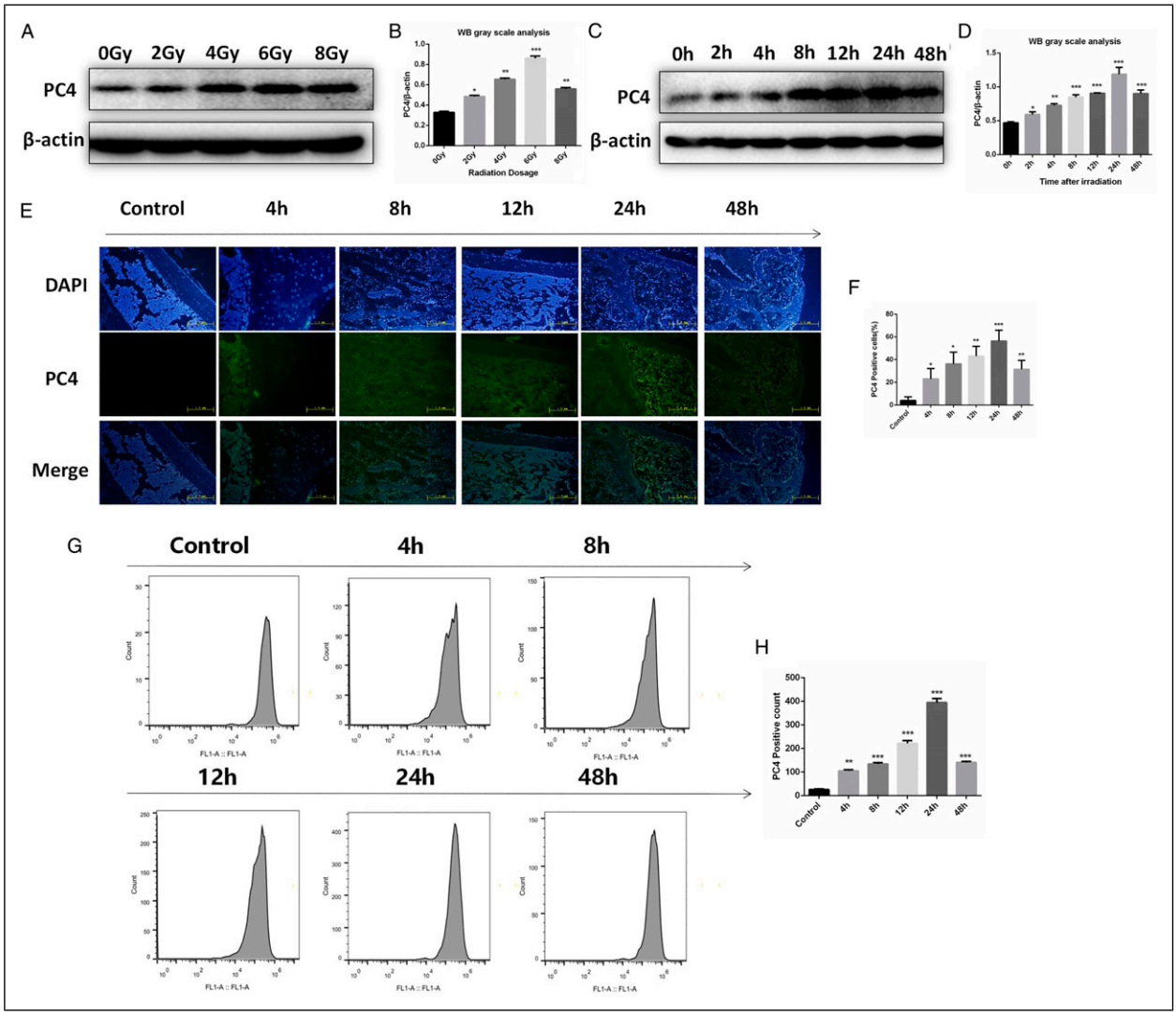

Figure 4. The expression of PC4 protein in hematopoietic system after radiation. (A) The protein level of PC4 after different dose irradiation in bone marrow from mice. (B) A statistical analysis of the WB gray scale derived from (A). (C) The protein level of PC4 after 6Gy irradiation at different time points in bone marrow from mice. (D) A statistical analysis of the WB gray scale derived from (C). (E) Immunofluorescent staining for PC4 after 6Gy irradiation at different time points in bone marrow from mice. Scale bar represents 1mm. (F) A statistical analysis of the data derived from (E). (G) Flow fluorescence intensity detection after 4Gy irradiation at different time points in human lymphoblastoid cell line. (H) A statistical analysis of the data derived from (G). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

mice. Compared with the control group (0 Gy), PC4 protein expression increased with the increase of radiation dose (Figure 4A and 4B). In addition, the expression of PC4 protein was detected by Western blot (Figure 4C and 4D) and immunofluorescence (Figure 4E) after 4 Gy irradiation at different time points in bone marrow of mice. The expression of PC4 protein reached the peak at 24 h after radiation, and the expression of PC4 protein began to decline after 24 h. Moreover, we analyzed PC4 protein fluorescence intensity by flow fluorescence detection after 4 Gy irradiation at different time points in human lymphoblastoid cell line. The average of PC4 expression was similar to the above results, which reached the peak at 24 h and also began to decline after 24 h (Figure 4G and 4H). The above results proved that PC4 protein also has a good correlation with radiation dose and radiation time.

PC4 Protein Could Be Recruited to the DNA Damage Site Faster Than γ-H2AX in Human Lymphocyte Cell Lines

Many kinds of literature have reported that PC4 is involved in a variety of cell processes, including transcription, replication, cell cycle, and DNA repair.[3-7] PC4 can quickly respond to DNA damage caused by radiation, so it may be a new biomarker for rapid and accurate assessment of radiation-induced hematopoietic damage. After radiation of human lymphoblastoid cell

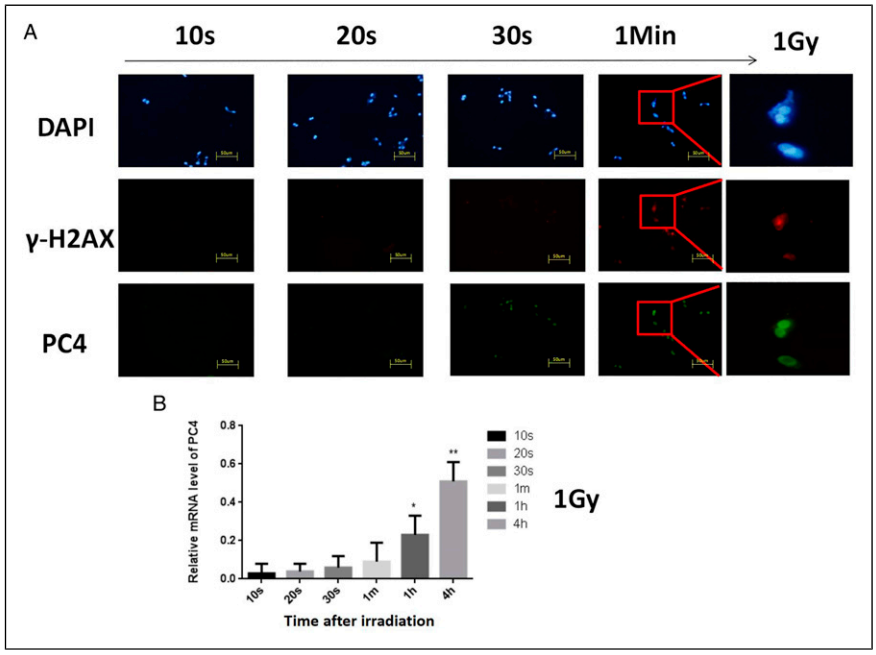

Figure 5. PC4 and γ-H2AX immunofluorescence double staining after radiation. (A) Immunofluorescent staining for PC4 and γ-H2AX expression after 1 Gy radiation at four time points. Scale bar represents 50um. (B) The mRNA level of PC4 in human lymphoblastoid cell line which were treated with 1 Gy radiation for different time points. All data indicate the mean ± SD.* $P < .05$, **$P < .01$.

line with 1 Gy γ-ray, PC4 and γ-H2AX immunofluorescence double staining was performed at four time points of 10 s, 20 s, 30 s, and 1 min. It was shown that PC4 gene could be recruited to the DNA damage site faster than γ-H2AX (Figure 5A). At the same time, we conducted RT-PCR analysis of PC4 at six time points of 10 s, 20 s, 30 s, 1 min, 1 h and 4 h after radiation of human lymphoblastoid cell line with 1 Gy γ-ray. It was found that the difference of PC4 mRNA expression at 10 s, 20 s, 30 s, and 1 min was not statistically significant, and it was significantly increased at 1 h and 4 h ($P < .05$, $P < .01$) (Figure 5B). Notably, PC4 protein increased prior to mRNA after radiation.

Discussion

In recent years, with the development of nuclear industry, nuclear explosions, and nuclear leakage events are more likely to cause mass radiation wounded personal in a short time. However, traditional radiation dose detection technology has their limitations. These techniques often require specialized researchers and cannot quickly examine large numbers of samples and draw accurate conclusions. Hematopoietic system is very sensitive to radiation. How to quickly and accurately evaluate the radiation damage of hematopoietic system is of great significance. So it is urgent to search for a rapid and accurate biodosimeter in peripheral blood. The research field of biological dosimeters is advancing rapidly within the last decade. γ-H2AX is a typical biodosimeter to analyze body exposures to ionizing radiation in humans.[36] Balog RP[37] also identified three protein biomarkers for radiation by using human and non-human primate fingerstick blood samples. Considerable effort has been put into the development of radiation exposure biomarkers, which would provide information about the effective dose of radiation.

We began our study by identifying radiation sensitive genes in mice after gamma-irradiation with different doses. 2 Gy, 4 Gy, 6 Gy, and 8 Gy were applied to mice respectively. We collected tail venous blood and harvested cells for high-throughput sequencing 24 hours after radiation. A total of 2527 genes were significantly altered after IR exposure ($P < .05$). We identified 5 genes by Venn analysis and Violin plot, including CHAMP1, PC4, NR4A1, RFX7, and REL, which were in good correlation with the change of radiation dose from 0 Gy to 8 Gy. It indicated that the above genes were enriched in DNA binding and transcription-related function through KEGG and GO analysis. It was reported that several circRNAs were differentially expressed after IR exposure.[38] Yang et al[39] found that several circRNAs were involved in gene transcription and translation regulation after IR exposure. Radiation might induce certain transcription factors and co-factors to promote transcription and translation, and participate in DNA damage and repair. In addition, we also found that the enrichment level of ribosome-related functions would be enhanced with the increase of radiation dose through KEGG analysis. It is well known that ribosome function is closely related to the expression of plasma proteins. Radiation-induced plasma protein expression has been reported as a possible radiometric dosimeter.[40]

In the following experiments, we performed linear regression, second order polynomial, and third order polynomial analysis on the expression of five candidate genes after radiation. The expression of PC4 had the best correlation with radiation dose. Moreover, the expression of PC4 decreased at 8 Gy irradiation compared with 6 Gy. It was suggested that 8 Gy irradiation might lead to apoptosis of lymphocytes in large numbers, which was the reason that PC4 could not timely transcribed to participate in DNA damage and repair. The expression of PC4 was well evaluated by linear regression analysis in 1-5 Gy, and the linear regression was the most accurate according to R^2 value. The detection range of PC4 could be extended to 1-8 Gy by third order polynomial analysis, which made up for the deficiency of dose detection range by linear regression. Finally, we found that PC4 protein could recruit to the DNA damage sites faster than γ-H2AX. These results suggested that PC4 might have a higher priority than H2AX in the whole DNA damage and repair process, which indicated that PC4 played a crucial role in the repair process. It would not predict all doses and time post-irradiation by one single dosimeter because the function of a single dosimeter is complex and can be affected by other diseases.[41] It will be necessary to use a panel of multiple-omic biomarkers[42] with appropriate time and dose windows to predict radiation dose. In addition, from the clinical point of view, the biological effect prediction seems to be more important for the triage than mere information of the received dose, even though if it would be accurate. PC4 as a potential biodosimeter needs to be further evaluated in combination with radiation in large populations, as well as in diabetes, hypertension, tumors and other common diseases.

Conclusion

In our study, we detected the different gene expressions after irradiation in mice and identified 5 genes (CHAMP1, PC4, NR4A1, RFX7, c-REL) which were associated with radiation dose. Furthermore, we found that the expression of PC4 was best correlated with radiation dose in hematopoietic system. Finally, through immunofluorescence detection, we found that PC4 could recruit to the DNA damage site faster than γ-H2AX in human cell lines. In conclusion, PC4 could serve as a potential biodosimeter for early assessment.

Declaration of Conflicting Interests

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Supplemental Material

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References

1. Gonzalez AJ, Lauriston S. Taylor lecture: radiation protection in the aftermath of a terrorist attack involving exposure to ionizing radiation. *Health Phys*. 2005;89:418-446.
2. Conklin WC, Liotta PL. Radiological threat assessment and the Federal response plan–a gap analysis. *Health Phys*. 2005;89:457-470.
3. Santovito A, Cervella P, Delpero M. Increased frequency of chromosomal aberrations and sister chromatid exchanges in peripheral lymphocytes of radiology technicians chronically exposed to low levels of ionizing radiations. *Environ Toxicol Pharmacol*. 2014;37(1):396-403.
4. Kuefner MA, Grudzenski S, Schwab SA, et al. DNA double-strand breaks and their repair in blood lymphocytes of patients undergoing angiographic procedures. *Invest Radiol*. 2009;44:440-446.
5. Agrawala PK, Adhikari JS, Chaudhury NK. Lymphocyte chromosomal aberration assay in radiation biodosimetry. *J Pharm Bioallied Sci*. 2010;2(3):197-201.
6. Reisz JA, Bansal N, Qian J, Zhao W, Furdui CM. Effects of ionizing radiation on biological molecules – mechanisms of damage and emerging methods of detection. *Antioxid Redox Signal*. 2014;21(2):260-292.
7. Ramalho AT, Costa ML, Oliveira MS. Conventional radiation biological dosimetiy using frequencies of unstable chromosome aberration. *Mutat Res*. 1998;404(1/2):97-100.
8. Bonassi S, Fenech M, Lando C, et al. Human Micronucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes:I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *E nviron M ol M utagen*. 2001;37(1):31-45.
9. Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S. The human micronucleus project-an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat Res*. 1999;428(1/2):271-283.
10. Khodarev NN, Park JO, Yu J, et al. Dose-dependent and independent temporal patterns of gene responses to ionizing radiation in normal and tumor cells and tumor xenografts. *Proc.Natl.Acad.Sci. U.S.A*. 2001;98:12665-12670. doi:10.1073/pnas.211443698.
11. Kis E, Szatmari T, Keszei M, et al. Microarray analysis of radiation response genes in primary human fibroblasts. *Int J Radiat Oncol Biol Phys*. 2006;66:1506-1514. doi:10.1016/j.ijrobp.2006.08.004.
12. Koenen J, Bachelerie F, Balabanian K, Schlecht-Louf G, Gallego C. Atypical chemokine receptor 3 (ACKR3): a comprehensive overview of its expression and potential roles in the

immune system. *Mol Pharmacol*. 2019;96:809-818. doi:10.1124/mol.118.115329.
13. Amundson SA, Do KT, Shahab S, et al. Identifcation of potential mRNA biomarkers in peripheral blood lymphocytes for human exposure to ionizing radiation. *Radiat Res*. 2000;154:342-346.
14. Dressman HK, Muramoto GG, Chao NJ, et al. Gene expression signatures that predict radiation exposure in mice and humans. *PLoS Med*. 2007;4:690-701.
15. Kultova G, Tichy A, Rehulkova H, Myslivcova-Fucikova A. The hunt for radiation biomarkers: current situation. *Int J Radiat Biol*. 2020;96:370-382.
16. Budworth H, Snijders AM, Marchetti F, et al. DNA repair and cell cycle biomarkers of radiation exposure and inflammation stress in human blood. *PLoS One*. 2012;7:e48619.
17. Ghandhi SA, Smilenov LB, Elliston CD, Chowdhury M, Amundson SA. Radiation dose-rate effects on gene expression for human biodosimetry. *BMC Med Genomics*. 2015;8:22.
18. Pernot E, Hall J, Baatout S, et al. Ionizing radiation biomarkers for potential use in epidemiological studies. *Mutat Res*. 2012; 751(2):258-286.
19. Ge H, Roeder RG. Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. *Cell*. 1994;78:513-523.
20. Kretzschmar M, Kaiser K, Lottspeich F, Meisterernst M. A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. *Cell*. 1994;78: 525-534.
21. Malik S, Guermah M, Roeder RG. A dynamic model for PC4 coactivator function in RNA polymerase II transcription. *Proc Natl Acad Sci U S A*. 1998;95:2192-2197.
22. Wang Z, Roeder RG. DNA topoisomerase I and PC4 can interact with human TFIIIC to promote both accurate termination and transcription reinitiation by RNA polymerase III. *Mol Cell*. 1998;1:749-757.
23. Calvo O, Manley JL. The transcriptional coactivator PC4/Sub1 has multiple functions in RNA polymerase II transcription. *EMBO J*. 2005;24:1009-1020.
24. Tavenet A, Suleau A, Dubreuil G, et al. Genome-wide location analysis reveals a role for Sub1 in RNA polymerase III transcription. *Proc Natl Acad Sci U S A*. 2009;106:14265-14270.
25. Gao J, Zybailov BL, Byrd AK, et al. Yeast transcription coactivator Sub1 and its human homolog PC4 preferentially bind to G-quadruplex DNA. *Chem Commun (Camb)*. 2015;51: 7242-7244.
26. Mortusewicz O, Evers B, Helleday T. PC4 promotes genome stability and DNA repair through binding of ssDNA at DNA damage sites. *Oncogene*. 2016;35:761-770.
27. Mortusewicz O, Roth W, Li N, Cardoso MC, Meisterernst M, Leonhardt H. Recruitment of RNA polymerase II cofactor PC4 to DNA damage sites. *JCB:REPORT*. 2008;183(5):769-776.
28. Shi C, Mai Y, Zhu Y, Cheng T, Su Y. Spontaneous transformation of a clonal population of dermis-derived multipotent cells in culture. *Vitro Cell Dev Biol Anim*. 2007;43:290-296.
29. Peng Y, Yang J, Zhang E, et al. Human positive coactivator 4 is a potential novel therapeutic target in non-small cell lung cancer. *Cancer Gene Ther*. 2012;19:690-696.
30. Qian D, Zhang B, Zeng X-L, et al. Inhibition of human positive cofactor 4 radiosensitizes human esophageal squmaous cell carcinoma cells by suppressing XLF-mediated nonhomologous end joining. *Cell Death Dis*. 2014;5:e1461.
31. Chen L, Du C, Wang L, et al. Human positive coactivator 4 (PC4) is involved in the progression and prognosis of astrocytoma. *J Neurol Sci*. 2014;346:293-298.
32. Chakravarthi BVSK, Goswami MT, Pathi SS, et al. MicroRNA-101 regulated transcriptional modulator SUB1 plays a role in prostate cancer. *Oncogene*. 2016;35:6330-6340.
33. Zhang T, Liu X, Chen X, et al. Inhibition of PC4 radiosensitizes non-small cell lung cancer by transcriptionally suppressing XLF. *Cancer Med*. 2018;7:1326-1337.
34. Du Z, Luo Q, Yang L, et al. Mass spectrometric proteomics reveals that nuclear protein positive cofactor PC4 selectively binds to cross-linked DNA by a trans-platinum anticancer complex. *J Am Chem Soc*. 2014;136:2948-2951.
35. Pemsel A, Rumpf S, Roemer K, Heyne K, Vogt T, Reichrath J. Tandem affinity purification and nano HPLC-ESI-MS/MS reveal binding of Vitamin D receptor to p53 and other new interaction partners in HEK 293T Cells. *Anticancer Res*. 2018; 38(2):1209-1216.
36. Redon CE, Nakamura AJ, Gouliaeva K, Rahman A, Blakely WF, Bonner WM. Q(γ-H2AX), an analysis method for partial-body radiation exposure using γ-H2AX in nonhuman primate lymphocytes. *Radiat Meas*. 2011;46:877-881.
37. Balog RP, Bacher R, Chang P, et al. Development of a biodosimeter for radiation triage using novel blood protein biomarker panels in humans and non-human primates. *Int J Radiat Biol*. 2020;96:22-34.
38. He N, Sun Y, Yang M, et al. Analysis of circular RNA expression profile in HEK 293T cells exposed to ionizing radiation. *Dose Response*. 2019;17:1559325819837795.
39. Yang Y, Fan X, Mao M, et al. Extensive translation of circular RNAs driven by N6-methyladenosine. *Cell Res*. 2017;27(5): 626-641.
40. Ge C, Liang Y, Zhang Y, et al. Plasma proteins As biodosimetric markers of low-dose radiation in mice. *Dose Response*. 2021;19: 15593258211016257.
41. Kim D, Marchetti F, Chen Z, et al. Nanosensor dosimetry of mouse blood proteins after exposure to ionizing radiation. *Sci Rep*. 2013.
42. Policastro Connor G, Jay S, Martins Francisco E, et al. A multi-institutional critical assessment of dorsal onlay urethroplasty for post-radiation urethral stenosis. *World J Urol*. 2020. undefined: undefined.