Original Article
Astragalus polysaccharide restores activation of NK cells in radiation therapy of tumors

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Abstract: Astragalus polysaccharide (APS) has been used as an adjuvant in the treatment of malignant tumors, however, most APS trials have been performed with chemotherapy of malignant tumors, the effect and mechanism of APS is unclear in radiotherapy for tumors. In the present study, the hepatocellular carcinoma H22 tumor-bearing mouse model was established. The H22 tumor-bearing mice received radiotherapy combined with APS treatment. Results showed APS could obviously up-regulate the expression of MHC class I chain-related molecule A (MICA) and B (MICB) on tumor cell surface, which is one of the major ligands of activating receptor NKG2D on natural killer cells (NK cells). The binding of MICA/B and NKG2D led to NK cell activation by improving the level of phosphorylated extracellular signal regulated kinase on NK cells. Activated NK cells released more IFN-γ, Granzyme B and Perforin, promoting the clearance of tumor cells. These results suggested that APS increased the sensitivity of tumors to radiotherapy by activating the extracellular signal regulated kinase pathway of NK cells. APS may be as an efficient adjuvant in radiotherapy of malignant tumors.

Keywords: Astragalus polysaccharide, NK cells, radiotherapy

Introduction

Radiotherapy is one of the main methods to treat malignant tumors. Although the therapeutic effects of radiotherapy of malignant tumors is noticeable, the radioresistance [1] and the side effects including oral mucositis, gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, hematopoietic system injury, cardiotoxicity, and neurotoxicity of post-cancer radiotherapy, limited the scope of the application of radiotherapy [2, 3]. In addition, these side effects often reduce the quality of life in cancer patients, and may lead to therapy discontinuation. Some researche suggested that radiotherapy damaged the functioning of immune cells, such as regulatory T cells [4], NK cells [5] and Macrophages [6], which probably resulted in the invasion of cancer cells and the development of metastases [7, 8].

Astragalus Membranaceus is one of Traditional Chinese Medicine. Astragalus membranaceus possesses multiple-effects including immuno-modulating, anti-oxidant, anti-inflammatory and anticancer effects. The major components of Astragalus membranaceus are polysaccharides, flavonoids, and saponins. Astragalus polysaccharides (APS) are one of the most important natural active components extracted from astragalus membranaceus and has multi-target biological activities including antioxidant, anti-inflammatory, anti-virus, and immune regulation effects. APS has anti-tumor function directly or indirectly [9]. APS can effectively slow tumor growth by regulating the immune system, and enhance CD4^{+} and CD8^{+} T-cell proliferation in mice [10] and promote the release of cytokines in various immune cells [11] in serum in the treatment of tumors. APS can enhance the sensitivity of HL-60 cells to cytotoxicity of natural killer cells (NK cells) [12]. Studies in animals and cells found that APS can increase the sensitivity of tumors cells to chemotherapy [13, 14]. So APS is used as a chemotherapy sensitizer [15], enhancing the anti-proliferative and apoptotic effect of cisplatin by

modulating expression of Bax/Bcl-2 ratio and caspases on nasopharyngeal carcinoma cells. It also can increase the sensitivity of SKOV3 cells to cisplatin potentially by activating the JNK pathway [13]. Many Clinical trials proved that APS was effective and safe in the adjuvant treatment of tumors; chemotherapy integrated with APS for patients with advanced non-small cell lung cancer significantly improved the quality of life and survival [16]. In addition, APS also have radioprotective action; it can ameliorate ionizing radiation-induced oxidative stress in mice [17] and inhibit bystander effects induced with ionizing radiation by regulating MAPK/NF-kB signaling pathway in bone mesenchymal stem cells.

NK cells are the predominant innate lymphocyte subset that mediate anti-tumor and anti-viral responses. Several studies have shown that the anticancer activity of APS is associated with NK cells [18]. Activated NK cells participate in the clearance of tumor cells. The activation of NK cells is regulated by the integration of signals from activating and inhibitory receptors of NK cells [19]. Activating receptors play a critical role in this process. If activating signals are enhanced, target cells will become highly sensitive to termination by NK cells [20]. NKp46 and NKG2D, as the activating receptors of NK cell, have been shown to be highly selective markers of all NK cells in mice and human. Studies have shown that NKp46 provided NK cells with the capacity to recognize and kill a variety of tumor target cells [21]. NKG2D is a lectin-like type 2 transmembrane receptor expressed in mice and humans in all NK cells [22]. Upon interaction with its ligands, NKG2D can trigger NK cell-mediated cytotoxicity against their targets [23]. The ligands for NKG2D are self-proteins related to MHC class I molecules [24]. Human NKG2D ligands include the MHC class I chain-related molecule A (MICA) and B (MICB) [22]. The high expression of NKG2D and NKp46 or their ligands will activate NK cells. Activated NK cells secrete cytokines or kill target cells directly. NK cells can secrete various cytokines, such as IFN-γ which exert antitumor functions in various manners including restricting tumor angiogenesis and stimulating adaptive immunity [25, 26]. In addition, in mice, the NK1.1 molecule has been an important marker for activated NK cells [27].

However, it is unclear whether APS has protective effects in radiotherapy of tumors and whether the effects involve NK cells. In the present study, H22 tumor-bearing mice were treated with radiotherapy combined with APS. We found that radiotherapy combined with APS increased the survival time of H22 tumor-bearing mice in an NK-dependent fashion. Notably, NK cells in tumors acquired a low response state, radiotherapy aggravated the injury in malignant tumor treatment process, the low response state was associated with dampened activation signals, specifically those mediating phosphorylation of extracellular signal regulated kinase 1 and 2 (ERK1/2). Importantly, the APS treatments prolonged survival time, and improved the low response state of NK cells within the tumors. Altogether, these results suggested a model in which NK cells infiltrating tumors are reset to a low response state in radiotherapy of tumors. Radiotherapy combined with APS can reverse the low response state of NK cells, increase the sensitivity of tumors to radiotherapy and inhibit the growth of tumors.

Materials and methods

Animals

Fifty male Kunming (KM) mice (8 weeks old) were obtained from the Experimental Animal Center of Lanzhou University. The mice were kept in an animal house at 22 ± 2°C temperature, 65 ± 10% humidity, and 12 h light/dark cycle. The animals were provided with food and water ad libitum. All experimental protocols were approved by the Institutional Animal Ethics Committee, Lanzhou University. Efforts were exerted to minimize animal suffering and reduce the numbers of animals in the experiment.

Reagents

APS was purchased from Sainuo Pharmaceutical (Sano Pharmaceutical Co., Ltd, Tianjin, China), The anti-mouse CD16 FITC, anti-human CD335 (NKp46) APC, anti-mouse CD335 (NKp46) APC, anti-human/mouse phospho-ERK1/2 (T202/Y204) PE, anti-mouse NK1.1 FITC antibodies were purchased from eBioscience (eBioscience, CA, USA). PE anti-human MICA/B was purchased from Biolegend (Biolegend, CA, USA). The polyclonal mouse anti-goat IgG/Cy3, Cy3 conjugated antibody, the polyclonal rabbit anti-CD16 antibody, the polyclonal rabbit anti-perforin antibody, the poly-

clonal rabbit anti-granzyme B antibody were purchased from BIOSS (Biosynthesis Biotechnology Co., Ltd, Beijing, China). NKP46 (M-20) was purchased from Santa Cruz (Santa Cruz, IL, USA). Rabbit polyclonal anti-MICA antibody was purchased from Sigma (St. Louis, MO, USA); LDH cytotoxicity assay kit was purchased from Beyotime (Beyotime Biotechnology Co., Ltd., Shanghai, China). FIX & PERM kit, and flow cytometry Staining buffer was purchased from Multi Sciences (MultiSciences Biotech Co., Ltd, Hangzhou, China); Ficoll-hypaque solution was purchased from Solarbio (Solarbio Life Sciences, Beijing, China).

Preparation of H22 solid tumor-bearing mouse model

Under aseptic condition, ascites were taken from hepatoma H22 ascites tumor-bearing mice and diluted with normal saline into a suspended solution in a concentration of 1×10^7 cells/ml. The cell suspension (1×10^6 cells/mouse, 0.1 ml) was subcutaneously injected into the right armpit to establish a H22 solid tumor-bearing mouse model. Tumor growth s.c. was measured every day and tumor volume was calculated [28] as Tumor volume = length × (width^2)/2. Mice with tumor volume of ≥100 mm^3 were considered to be successful models and used in the present study.

Cell culture

Cell culture was performed at 37°C in a humidified atmosphere containing 5% CO_2. NK-92MI cells were cultured in complete Alpha Minimum Essential medium without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate containing 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, horse serum to a final concentration of 12.5%, and fetal bovine serum (FBS) to a final concentration of 12.5%. YAC-1 was cultured in complete RPMI-1640 containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. K562 cells were cultured in complete DMEM, containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Experimental procedures (treatment with APS)

Thirty H22 tumor-bearing mice were randomly divided into three groups, including the X-ray treatment group, the X-ray + APS treatment group, the NK cell depletion + X-ray + APS treatment group. All mice were irradiated by 4 Gy X-ray, then APS (100 mg/kg) [29] was given continuously for 10 days in the NK cell depletion + X-ray + APS treatment group and the X-ray + APS treatment group, the X-ray treatment group was given the same volume of normal saline continuously for 10 days. In the NK cell depletion + X-ray + APS treatment group, NK cells were depleted from mice by i.p. injection of 200 µg of PK136 mAb (specific for NKR-P1C) 1 day before the tumor received X-ray irradiation, repeated weekly for 4 weeks. The body weights and the volume of the tumors were measured. At the tenth day after the APS administration, blood samples were taken by ophthalmic artery. The tumor was removed and weighed for the next experiment. At the same time, the spleen was also removed and weighed for the calculation of organ indexes in the next experiment. The spleen index was calculated using the following formula: spleen index = spleen weight (mg)/mice weight (g).

Preparation of splenic cell suspension and tumor cell suspension

Mice were killed 11 days after X-ray irradiation and tissues were collected in sterile conditions. Spleens were crushed in RPMI 1640 medium, and the cell suspension was filtered with a 200-mesh stainless steel filter and centrifugated at 1500 rpm at 20°C for 3 min, and then washed 3 times with cell washing liquid. The cell fragments were removed by 500 rpm short time low speed centrifugation. At last, the cell mass was filtered with 200 mesh stainless steel filter again. The resulting single-cell suspensions were used for next experiments. The tumors were excised after separation of the skin, cut in pieces, and dissociated using a gentle MACS Dissociator (Miltenyi). Dissociated tumors were digested in RPMI containing 200 µg/ml collagenase IV (Sigma-Aldrich) and 20 µg/ml DNase I (Sigma-Aldrich) at 37°C for 25 minutes. The resulting single-cell suspensions were used for next experiments.

Natural killer (NK) cell activity

Experimental grouping was divided into: effector cells group, experimental group, target spontaneous group, target maximum group, volume correction control group, and back-

ground control group. Effector cells (NK cell) and target cells (YAC-1/K562) at the ratio of 40:1, 30:1 and 20:1, 10:1, 5:1, 1:1 were added to 96-well plates with RPMI media. The cells were then incubated for 4 h at 37°C in a humidified 5% CO_2 incubator. Lactate dehydrogenase (LDH) release reagent was then dispensed to the target maximum group and the volume correction control group. The plate was again incubated for 1 h at 37°C in a humidified 5% CO_2 incubator. The LDH concentration in cell supernatant was measured by LDH Cytotoxicity Assay Kit. The assay is based upon a coupled enzymatic assay involving the conversion of a tetrazolium salt 2-p-(iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), into a formazan product. The reaction was catalyzed by LDH released from cells and Diaphorase present in the assay substrate mixture. Absorbance was read at 490 nm. The following formula was used to calculate % cytotoxicity: % Cytotoxicity = $(OD_{experimental\ group} - OD_{effectors\ spontaneous\ group} - OD_{target\ spontaneous\ group})/(OD_{target\ maximum\ group} - OD_{target\ spontaneous\ group}) \times \%$. Experimental/effector and target cells in ratio (40:1, 30:1, 20:1, 10:1, 5:1, 1:1).

Flow cytometry and cell sorting

All NK cells were incubated for 20 minutes with the 2.4 G2 hybridoma supernatant to block FcγRII/III receptors, then NK cells were stained with the specified antibodies in 100 µl of flow cytometry staining buffer for 30 minutes. Intracellular staining of NK cells was performed after surface staining with the FIX & PERM Kit according to manufacturer's instructions, and data were analyzed with FlowJo software. For sorting experiments Single-cell suspensions coming from tumor and spleen were stained with CD3, CD19, NKp46 and sorted on a FACSAria II (BD Biosciences). NK cells were gated as viable CD3- CD19- NKp46+ cells [30].

IFN-γ ELISA

Blood was collected from the ophthalmic artery of mice. The blood samples were standed for two hours and centrifuged at 4000 r/min for 10 min to collect the serum, the amount of IFN-γ was determined by sandwich ELISA.

Western blot analysis

Four mice were randomly selected from each group and then anesthetized with pentobarbi-

tal. The tumor tissues of the mice were separated on the ice-cold glass plates and were frozen in liquid nitrogen. The samples were homogenized and total protein was extracted using RIPA buffer that contains protease inhibitors. Proteins (50 µg) were fractionated on 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and then transferred into polyvinylidene fluoride membranes [31]. The membranes were blotted with anti-Granzyme B (1:100), anti-Perforin (1:100) and anti-GAPDH (1:5000) antibodies, as well as with goat anti mouse IRDye 800CW and donkey anti rabbit IRDye 800CW second antibody (1:5,000). Immunoreactive protein bands were visualized by Odyssey ® Infrared Imaging Systems (LI-COR Biosciences, East Carolina, USA). Densitometry analysis of the protein bands was performed by using ImageJ (Rawak software, Inc., Stuttgart, Germany).

Statistical analysis

All data were expressed as Mean ± SD and analyzed with GraphPad Prism 6.0 (Graphpad software Inc., San Dieo, CA, USA), and the significance level was set at $p < 0.05$. Survival experiments were analyzed with the Log-Rank (Mantel-Cox) test. Comparisons between two groups were analyzed with Student's t-test. Comparisons of multiple samples were analyzed with One-way ANOVA and two way ANOVA with Bonferroni tests.

Results

APS improves the survival of the H22 tumor-bearing mice via the NK cell-dependent fashion following the radiotherapy

To determine whether injection of APS was beneficial to radiotherapy in the H22 tumor-bearing mice we established an H22 tumor-bearing mouse model. When tumor volume reached a size of 100 mm³ (**Figure 1A**), mice were treated with the 4 Gy x-ray irradiation one time, concomitantly, APS was administered by intraperitoneal injection continuously for 10 days. The parameters of tumor volume, the body weight of mice, and the survival ratio was observed after treatment. Radiotherapy combined with APS significantly prolonged the average survival time in the H22 tumor-bearing mice (**Figure 1B**), and a higher fraction of mice that survived long term (the survivors at 90 days still were live). The benefits of APS in mice with X-ray ther-
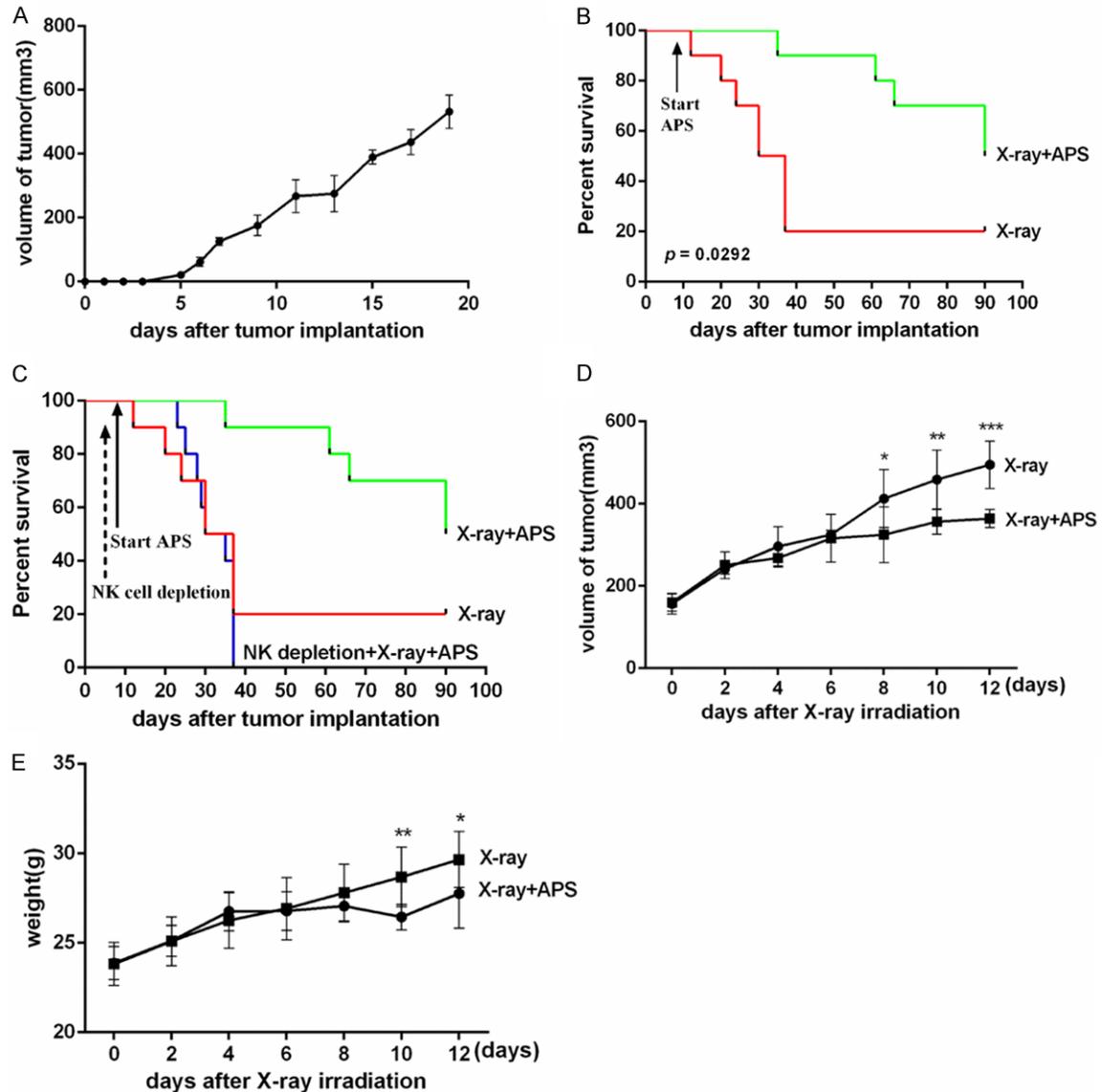
Figure 1. APS improves the survival of the H22 tumor-bearing mice following radiotherapy. A. 1×10^6 H22 cells were transplanted s.c. in KM mice, tumor growth was measured by caliper. B. Kaplan-Meier analyses of the H22 tumor-bearing mice treated or not with APS 10 day continuously after receiving X-ray irradiation. C. NK cells were depleted in one group of mice by injection of PK136 antibody weekly, starting 1 day before receiving X-ray irradiation. NK depletion of the APS-treated group resulted in significantly reduced survival (p = 0.0034) according to the log-rank (Mantel-Cox) test. D, E. The growth rate of the tumor and the weight of the H22 tumor-bearing mice treated or not with APS 10 day continuously were recorded after receiving X-ray irradiation. n = 10, *; p < 0.05, **; p < 0.01, ***; p < 0.001 vs. X-ray treatment group.

apy was completely abrogated if the mice were NK-depleted (**Figure 1C**), demonstrating that the effect of the APS depended on NK cells. In addition, APS inhibited the growth of the tumor. The volume of the tumor was smaller in X-ray + APS treatment group than the X-ray treatment group starting at the eight days of APS treatment (**Figure 1E**), the body weight of the mice was also significantly different between the groups at the tenth days of APS treatment (**Figure 1D**), the superficial skin of tumor begin

to ulcerate early and a portion of the tumor appeared in the central cavitation in APS treatment group. The responsiveness of mice to external stimuli was poor in X-ray treatment.

The effect of APS on tumor and spleen in the H22 tumor-bearing mice following radiotherapy

To investigate the influence of APS, the H22 tumor-bearing mice received radiotherapy, we
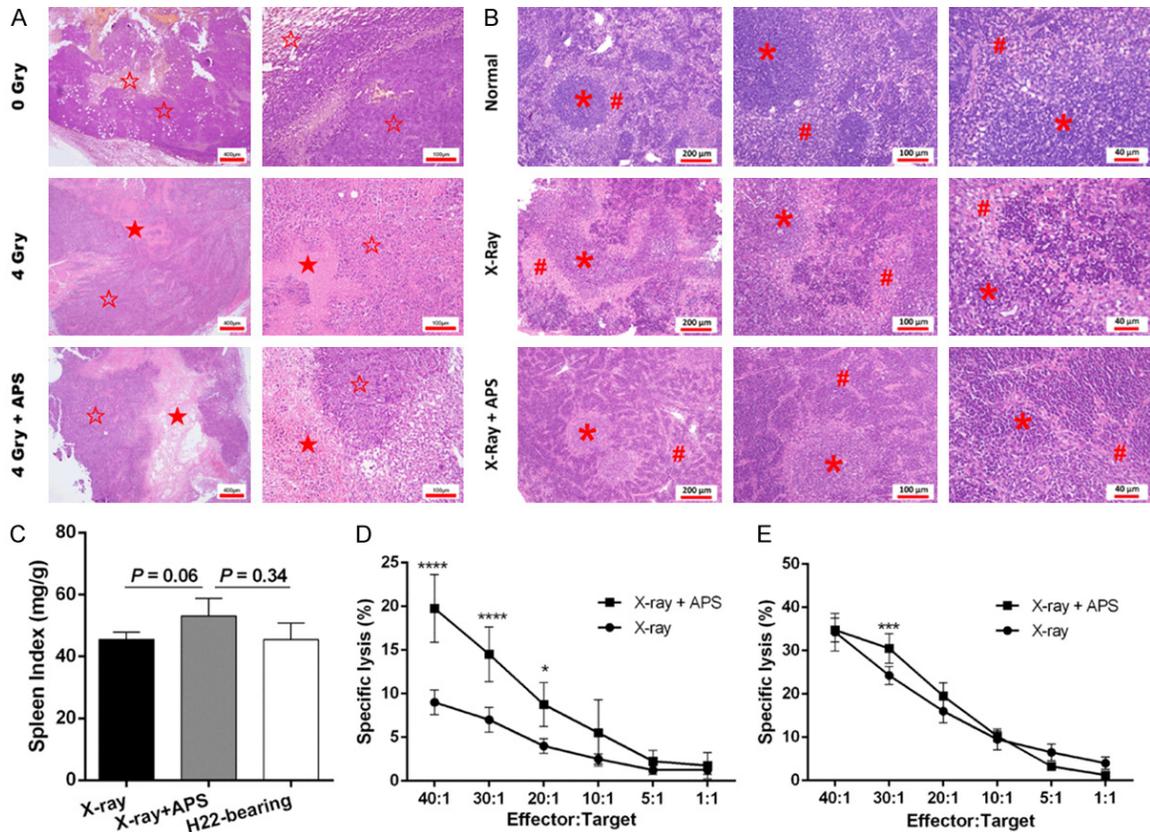
Figure 2. The effect of APS on tumors and spleen in the H22 tumor-bearing mice receiving radiotherapy. A, B. The morphological changes of tumor and spleen from the H22 tumor-bearing mice were observed by using H&E staining. ☆indicates the tumor tissues, ★indicates necrotic tissues, *indicates the white pulp, #indicates red pulp. C. The spleen index of the H22 tumor-bearing mice was evaluated. D, E. NK cell isolated from tumor and spleen tested for killing of YAC-1 target cells after incubation in 37°C, 5% CO_2 for 4 h by LDH Cytotox kit. n = 10, *; p < 0.05, **; p < 0.01, ***; p < 0.001, ****, p < 0.0001.

first observed the morphological changes of spleen and tumor. When the H22 tumor-bearing mice received 4 Gy X-ray irradiation alone, in the spleen, the changes included the clear atrophy of white pulp, the splenic corpuscle disappeared, the medullary sinus of red pulp became dilated and filled with blood and the proliferation of macrophages were observed on the spleen pathological slide. APS could relieve the atrophy of white pulp, keep the structure of the splenic corpuscle clear and lead to the proliferation of lymphocytes (**Figure 2B**), but the index of the spleen had not changed (**Figure 2C**). In the tumor, X-ray irradiation led to necrosis of tumor cells. APS significantly increased the extent of necrosis (**Figure 2A**). These data suggested that radiotherapy combined with APS induced the proliferation of lymphocytes in the spleen and necrosis of tumor cells. Then we wanted to know what happened to the NK cells. We investigated the functionality of NK cells. We analyzed the capacity of NK cells coming from the spleen and tumors to kill target cells.

The cytotoxic activity of NK cells coming from tumor toward YAC-1 cells was strongly impaired in the X-ray treatment group at 40:1, 30:1 and 20:1 of effector (NK cells)/target cell (YAC-1 cells). The cytotoxic activity of NK cells was improved in X-ray + APS treatment group compared with the NK cell in X-ray treatment group (**Figure 2D**). The cytotoxic activity of NK coming from the spleen had no significant difference between the two groups in addition to 30:1 of effector (NK cells)/target cell (YAC-1 cells) (**Figure 2E**). These results showed that NK cells in tumor were impaired in the X-ray treatment group. APS could improve the activity of the NK cells in tumors.

APS significant increased the activity of NK cells by enhancing the corresponding ligand expression in tumors

The activity of NK cells was reduced in X-ray treatment group. One possibility to explain the result was that NK cells were somehow exclud-
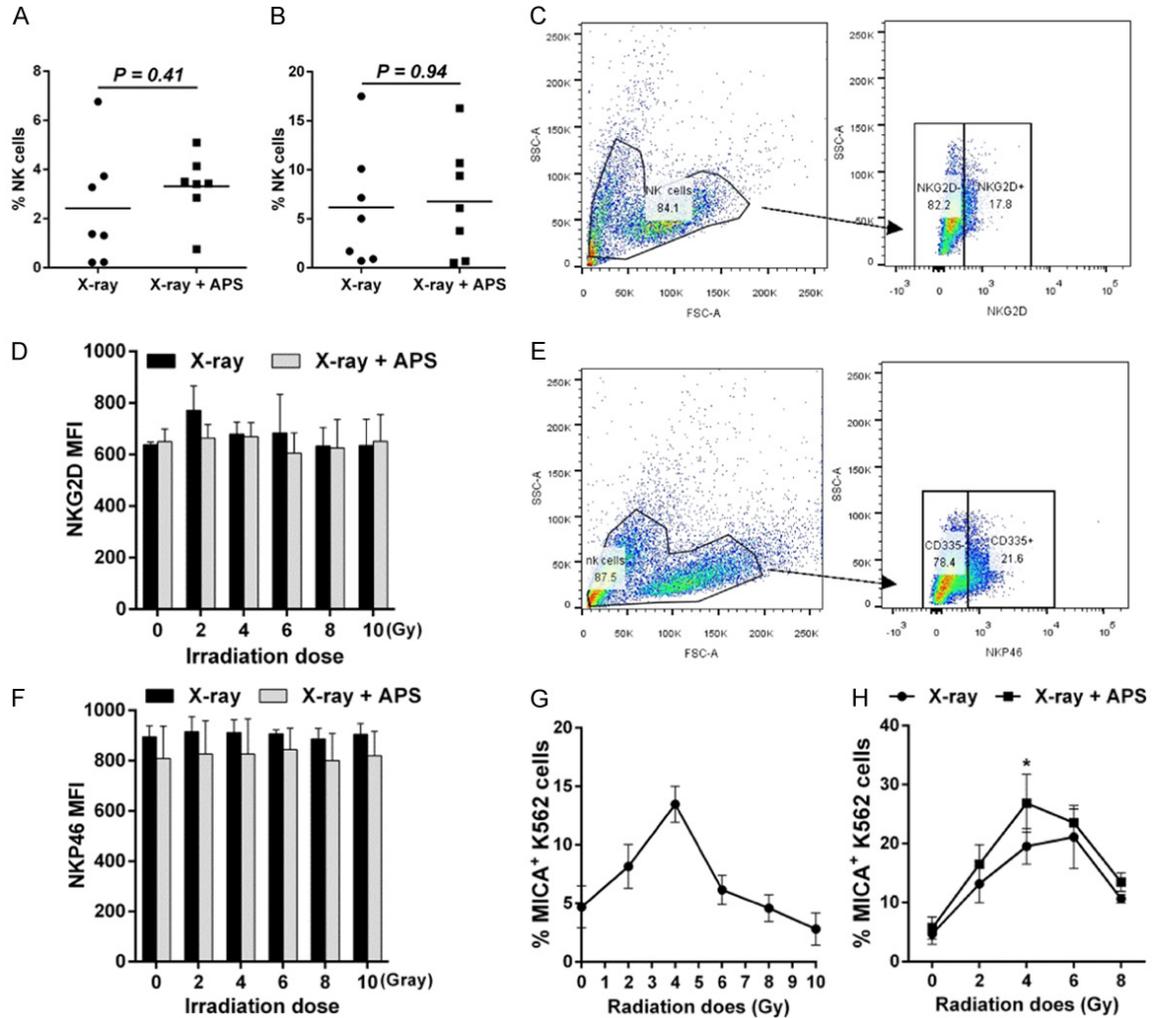
Figure 3. APS significantly increased the activity of NK cells by enhancing the corresponding ligand expression in tumors. (A, B) Ten days after APS treatment, the percentages of NK cells in the tumors and spleen; n = 7. (C, E) NK-92MI cells received X-ray radiation of various doses (0 Gy, 2 Gy, 4 Gy, 6 Gy, 8 Gy, 10 Gy), then the cells were treated with 100 mg/L APS for 24 hours. The expression of membrane NKG2D and NKP46 was assessed by flow cytometry; n = 4. (D, F) The mean fluorescence intensity (MFI) of NKG2D and NKP46 was analysed in NK-92MI cells as shown in (C) and (E), N = 4. (G) The expression of MICA after K562 cells received X-ray radiation of various doses by flow cytometry. (H) The expression of MICA after human K562 cells received X-ray radiation of various doses and incubated with APS or not for 24 hours by flow cytometry, *, P < 0.05.

ed from tumors. However, we found a similar number of NK cells within the tumor and spleen (**Figure 3A**, **3B**). The other possibility to explain the result was that the expression level of receptors on the surface of NK cells or its ligand on the target cells surface changed. So we first assessed the expression level of NKG2D and NKP46 of NK cells *in vitro* when the NK cells received X-ray irradiation. The level of NKG2D and NKP46 had no significant difference when NK cells received difference radiation doses of X-ray (**Figure 3C-F**). Then, we placed our focus on the expression of the NK cell's ligand on the

target cells surface. MICA that was the ligand of NKG2D on K562 cells surface. K562 cells received different radiation doses of X-ray in the presence or absence of APS. We found that the expression of MICA in K562 cells increased gradually and arrived at the peak when radiation doses reached 4 Gy, then descended gradually (**Figure 3G**). APS could clearly up-regulate the expression of MICA in K562 cells (**Figure 3H**), there was obvious improvements when radiation doses arrived 4 Gry. Together, these data suggested that APS clearly improved the expression of MICA in tumor cells with radio-

therapy, thus enhancing sensitivity of K562 cells to NK cells.

APS protected the NK cells from the radiotherapy by the activity of ERK signaling

To determine whether specific signaling pathways of NK cells were impaired after the H22 tumor-bearing mice received radiotherapy, we tested signal transduction resulting from aggregation of activating receptors ex vivo. Phosphorylation of ERK1/2 (p-ERK1/2) is important for promoting cytotoxicity and cytokine secretion in NK cells, so we initially examined p-ERK1/2 in isolated NK cells coming from tumor and spleen after the H22 tumor-bearing mice received radiotherapy combined with or without APS. The expression of p-ERK1/2 in isolated NK cells coming from tumor was significantly increased in X-ray + APS treatment group compared with the expression of p-ERK1/2 in X-ray treatment group (**Figure 4C**, **4D**). However, p-ERK1/2 in isolated NK cells coming from spleen was not significantly changed after the H22 tumor-bearing mice received radiotherapy combined with or without APS (**Figure 4A**, **4B**). These data show that the low response of NK cells in tumor receiving radiotherapy is imposed by mechanisms that dampen signaling upstream of ERK1/2 activation. After the NK cell was activated, it would usually secret cytokines or kill target cells directly. We then analyzed the level of IFN-γ in the serum and the expression of Granzyme B and perforin in NK cells coming from tumors. We found the mice produced less IFN-γ in X-ray treatment group than they did in X-ray + APS treatment group (**Figure 4E**). Similarly, the expression of Granzyme B and perforin were increased in X-ray + APS treatment group than in X-ray treatment group (**Figure 4F**, **4I**). These results suggest that the low response state of NK cells in tumors receiving radiotherapy is associated with reduced intracellular transmission of activating signals from activating cell surface receptors: APS reversed the low response state of NK cells and activated NK cells.

Taken together, these results show that the low response state of NK cells is induced during the process of tumor radiotherapy as a result of the growth of tumors, although some of the tumor cells can initially be killed by x-ray irradia-

tion treatment, radiotherapy would aggravate the situation of low response in NK cells and enable the tumor cells to escape from NK cell immune surveillance. Radiotherapy combined with APS might improve or prevent deterioration in NK cells by activating NK cells. Moreover, radiotherapy combined with APS in cancer treatment reversed the low response state of NK cells, providing therapeutic benefit to tumors.

Discussion

Radiotherapy has anticancer affects, but side effects and the resistance of radiotherapy limit clinical application. To improve radiotherapy sensitivity of tumors and reduce radiotherapy side effects or complications are critical to the success of radiation. A great deal of research showed that traditional Chinese medicine could increase the sensitivity to chemotherapy and reduce the side effects of chemotherapy, but the effect and mechanism of traditional Chinese medicine is unclear in radiotherapy for tumors. In this paper, we found the radiotherapy combined with APS prolonged the survival of H22 tumor-bearing mice in an NK cell-dependent fashion. So we hypothesize that APS improves the sensitivity of tumor cells to radiotherapy by activating NK cells in tumors. We found that the low response state of NK cells was induced in radiotherapy of tumors, radiotherapy combined with APS reversed the low response state of NK cells by improving the expression of phosphorylated extracellular regulated protein kinases (p-ERK1/2), The results were in line with this prediction.

Indeed, the therapeutic effects accompanying administration of APS in H22 tumor-bearing mice were striking. Notably, H22 tumor-bearing mice exhibit differences in tumor volume and the responsiveness to external stimuli in X-ray + APS treatment group compared with H22 tumor-bearing mice in X-ray treatment group, suggested the effectiveness and low toxicity of the APS treatments. Because the loss of weight and the growth of the tumor volume slowed down, the weight gain trend slows in APS treatment group.

The longer survival of H22 tumor-bearing mice receiving radiotherapy combined with APS was correlated with activated NK cells of the tumor. The activated level of NK cells damaging tumor
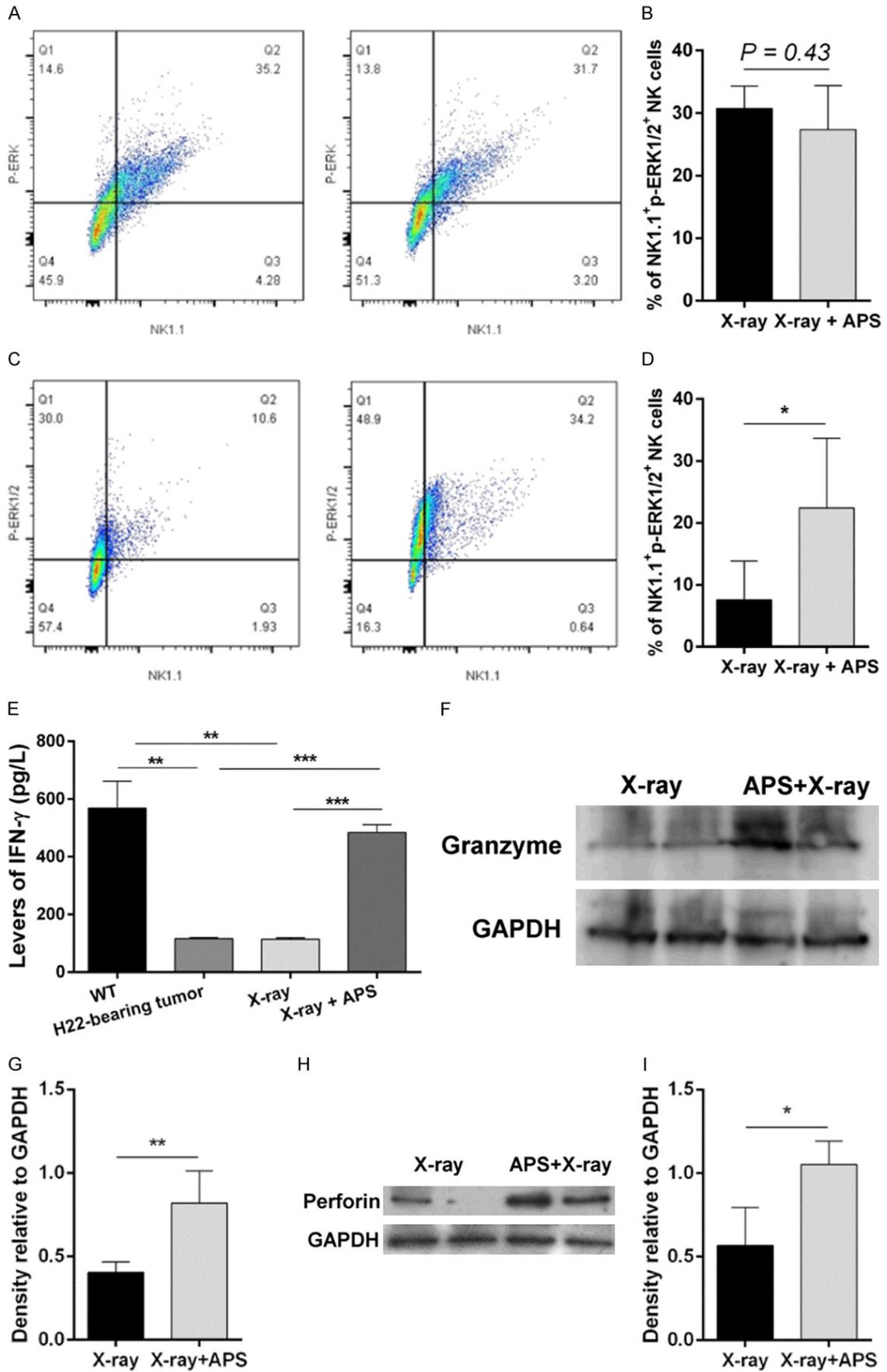
Figure 4. APS protected the NK cells from the radiotherapy by the activity of ERK signals. (A, C) NK cells isolated from the spleen or tumor were analyzed for p-ERK and NK1.1 expression by flow cytometry. n = 4. (B, D) Quantification of p-ERK and NK1.1 was analyzed in NK cells isolated from the spleen or tumor. *; P < 0.05. (E) Effects of APS on serum IFN-γ in the H22 tumor-bearing mice. n = 10. (F, H) The expression of Gramzy B and Perforin levels were evaluated in tumor by western blot; n = 4. (G, I) Quantification of the western blot as shown in (F) and (H) by densitometry; N = 4, *; P < 0.05, **; P < 0.01.

cells is not only associated with the expression level of its activation receptors, but also closely related to the ligands' expression level of the target cells surface. NK cells depend on the activating receptors such as NKG2D or NKP46 to recognize and eliminate cancer cells that up-regulate ligands for these receptors [32, 24]. MHC class I chain-related molecule A (MICB) and B (MICB) are one of the major ligands of NKG2D receptor (natural killer group 2, member D) on natural killer cells, which is poorly expressed by normal cells but up-regulated in tumor cells [33]. Tumor cells are eliminated by the interaction of MICA/B with NKG2D. The present study showed that radiotherapy may induce the release of MICA/B in tumors [34]. Our results suggested that the tumor cells expressed the MICA/B and x-ray radiation lead to overexpression on the surface of tumor cells, this result is consistent with previous studies [35]. In addition, MICA/B would form soluble MICA during the process of tumor proliferation [36]. Numerous studies have suggested that soluble MICA/B reduced the expression of NKG2D from the cell surface and desensitized anti-tumor effector cells [37]. It has previously been observed that matrix metalloproteinases (MMPs) was up-regulated after human non small cell lung cancer cells were irradiated by ionizing radiation and MMPs might result in the shedding of soluble MICA [35]. APS is widely used as a kind of adjuvant of chemotherapy in cancer [38, 39]. Studies showed that APS exerted anticancer activities directly on tumor cells in various ways. APS can inhibit tumor cell growth in mice [40] by inducing the apoptosis of tumor cells and promoting the release of cytokines. APS also may modulate immunity of the host organism through activation of TLR4-mediated MyD88-dependent signaling pathway and inhibit tumor cells *in vitro* and *in vivo* [41]. Furthermore, chemotherapy combined with APS could improve the outcome of malignant tumors [42]. On the basis of our finding that the expression of MICA in tumor cells increased after the tumor cells were irradiated by X-ray, X-ray irradiation combined with APS further pro-

moted the expression of MICA and enhanced NK cell responsiveness and tumor rejection. It is possible that because APS inhibited the shed of MICA from tumor cells, this resulted in a continuous activation of NK cells. The effect of APS on soluble MICA needs to be studied in the future.

Activation of NK cells involves a variety of molecules. ERK1/2 plays an important role in the activation signaling pathway of NK cells. This common pathway involves the activation of Rac, which sequentially leads to activation of the mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase (ERK1/2) by way of p21-activated kinase (PAK) and mitogen-activated or extracellular signal-regulated protein of intracytoplasmic granules and their exocytosis at the interface between the NK cell and its target cells. In the present study, we found radiotherapy caused the low expression of p-ERK1/2 in NK cells coming from tumors, suggesting that tumor cells maybe reflect defective ERK1/2 [43] signaling, as also occurs in the case of NK cells in H22 tumor-bearing mice, which exhibit a similar low response [44]. Conversely, the level of p-ERK1/2 in NK cells coming from tumor is greatly elevated after the tumor received radiotherapy combined with APS. Presumably, this can be explained, at least in part by APS inducing the activity of NK cells by improving the level of p-ERK1/2 during the tumor receiving radiotherapy combined with APS.

In humans, there are two NK cell subsets based on CD56 and CD16 (FCRγIII) surface expression: $CD56^{bright}CD16^{-/+}$ and $CD56^{dim}CD16^{+}$. $CD56^{bright}$ NK cells [45, 46] are predominantly involved in immunoregulation by producing cytokines. $CD56^{dim}$ NK cells are mainly related to cytolytic function. The $CD56^{dim}$ NK cells are mainly located in blood and spleen, the $CD56^{bright}$ NK cells are distributed in lymphoid and non-lymphoid tissues, such as lymph nodes, tonsils, inflamed and tumor tissues, liver, and uterus [47]. Murine NK cells can be divided based on the expression of CD27 and

CD11 [48]. Jurgen R and colleague have suggested that in murine NK cells the activation resembles human CD56^{bright} NK cells in many aspects, producing cytokines such as TNF-a, IFN-γ, IL-10 and GM-CSF [49]. In the paper, we found radiotherapy combined with APS promoted the release of IFN-γ, perforin and Granzyme B in H22 tumor-bearing mice, suggesting that APS might activate all of NK cells in H22 tumor-bearing mice. Moreover, IFN-γ has extensive anti-tumor potential, IFN-γ can inhibit the proliferation of cancer cells by affecting the cell cycle or promoting apoptosis of cancer cells [50]. IFN-γ also inhibits tumor angiogenesis and promotes destruction of tumor-associated blood vessels [51]. Activated NK cells can produce various cytokines as well as IFN-γ, the relationship of APS and other cytokines needs further test to verify.

Indeed, the therapeutic effects of APS in H22 tumor-bearing mice were striking. Notably, some clinical trials found that the APS integrated with chemoradiotherapy had significantly improved quality of life of patients in physical capacity and several quality-of-life indices, including treatment-related symptoms such as fatigue, nausea and vomiting, pain, and loss of appetite [52]. In our test, H22 tumor-bearing mice treated with APS did not exhibit obvious differences in weight loss. Moreover, in the paper, we found that the cytotoxic activity of NK cells was unchanged in spleen. The morphological changes of spleen appeared early. These results may be due to the short course of treatment. In general, APS can be used for more courses of treatment to achieve satisfying therapeutic effect.

In conclusion, our study showed that the outcome of radiotherapy combined with APS treatment in H22 tumor-bearing mice largely depends on the level of expression of MICA/B in tumor cells, and is associated with the low response state that NK cells acquire within such tumors. For the first time, we demonstrated that the low response state of NK cell is caused by impaired signal transduction and that APS can reverse responsiveness of intratumoral NK cells.

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Disclosure of conflict of interest

None.

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