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Original Article

Mechanism of cervical squamous cell carcinoma radioresistance conferred by Aurora-A

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Abstract: Cervical squamous cell carcinoma (CSCC) is one of the most common gynecological malignant tumor,the main reason of treatment failure is radiation resistance. Aurora kinase A (Aurora-A, AURKA) is a member of serine/threonine kinase family. The abnormal expression of Aurora-A induces tumorigenesis and radioresistance. This study was aimed to investigate the association of Aurora-A with CSCC radioresistance. Wecollect 98 cases of untreated patients diagnosed with CSCC and detect the expression of Aurora-A in biopsy specimens by immunohistochemistry. CSCCHCC94 cells were treated with Aurora-A kinase inhibitor MLN8237 and accept a certain amount of 4 Mv X-ray radiation (0, 2.0, 4.0, 6.0 Gy,) and then used to test cell proliferation, cell apoptosis, cell cycle, and cell cycle regulatory proteins respectively. The resultsshowed that Aurora-A was over-expression in CSCC and relate with cell invasion and metastasis. cell proliferation and cell apoptosis of MLN8237 treated group were increased after X-irradiation. And the expression of Cyclin D1, CDK2 and CDK6 were down-regulated to induce cell cycle arrest at G2/M phases. Therefore, we concluded that Aurora-A inhibitor can blocks cell proliferation and cell cycle progression, promote cell apoptosis, and increase the sensitivity of cervical cancercells to radiotherapy by regulating the expression of cell cycle related protein. Thus, Aurora-A may be used as one of therapeutic targets and an independent prognostic factor to increase the sensitivity of CSCC radiotherapy.

Keywords: CSCC, Aurora-A, cell cycle, cyclin D1, sensitivity, radiotherapy

Introduction

Cervical squamous cell carcinoma (CSCC) is one of the most common gynecological malignant which has a serious threat to the health and lives of women, and has been increasing in many developing countries. Definitive radiation therapy (RT) (with or without cisplatin-based chemotherapy) is one of the most useful treatments for cervical squamous cell carcinoma (CSCC), but the efficiency is limited due to resistance [1]. The different phases of cell division cycle are the important factors that affect the radiation sensitivity. Radiobiology has confirmed that there is much difference between different cell cycle phases in cell radiosensitivity. Aurora kinase A (Aurora-A, AURKA) is a kind of mitogen-activated protein kinase (MAPK), Its plays an important role in the process of cell mitosis. AURKA can promote cell cycle G2/M conversion, and interfere with cell cycle [2, 3]. Existing research has found that Aurora-A was over expression in Head and neck cancer [4], ovarian cancer [5], prostate cancer [6], oral squamous cell carcinoma [7]. And poor prognosis of high expression. And Aurora-A gene Silence can cause G2/M blocking, promotecell apoptosis, improve the sensitivity of tumors to X ray [8, 9]. Cell cycle arrests may have positive correlation to radiation sensibility. Cell apoptosis induced by irradiation has close relation to cell cycle arrest especially G2/M arrest. MLN8237 belongs to the second generation of Aurora-A selective inhibitors, which can significantly inhibit the expression of Aurora-A. Therefore, Aurora-A has been a promising therapeutic target of tumor treatment. Also the study on the sensitivity of radiotherapy in cervical carcinoma has got sufficient attention and recognition.

Table 1. The expression of Aurora kinase A in primary cervical squamous cell carcinoma (CSCC)

	-	+	++	+++
Aurora kinase A	26	26	25	21

According to the positive cells: - (<0%), + (<30%), ++ (30-60%) and +++ (>60%), ++ and +++ were strongly positive.

Materials and methods

Clinical data collection

Collected 98 cases of patients diagnosed with primary cervical squamous cell carcinoma (CSCC) in the Xinjiang Uyghur autonomous region people's hospital from January 2009 to December 2012. The expression of Aurora kinase A (AURKA) was detected by immunohistochemistry. All patients follow the principle of NCCN radical radiotherapy: Radiotherapy (EBRT) combined with brachytherapy (ICRT), The prescription dose of EBRT (6 MV X-rays) was 45 Gy, The prescription dose of ICRT (252Cf neutron), 40 Gy. Lifetime follow-up.

Materials

The primary cervical squamous cell carcinoma HCC94 cells were purchased from American ATCC Corporation. As for the RPMU1640, fetal calf serum and trypsase, they were all bought from Gibco (USA). The thiazolyl blue kit (MTT) was purchased from Dingguo Biochemistry Ltd. (Beijing). The propidiumiodide (PI) was bought from Sigma (USA), whereas RNase A was from Fermentas (Canada). Lastly, the cell apoptosis kit was supplied by BD Corporation (USA). Aurora-A (CS-3042), p-Aurora-A (Thr288, CS-3079, Cyclin D1 (CS-2922), CDK2 (cyclin-dependent kinase2, CS-2546), CDK6 (cyclin-dependent kinase 6, CS-3136), β-actin were purchased from Sigma (USA). The inhibitors of AURKA MLN8237 were purchased from Selleck Corporation (USA).

Cell culture and radiation intervention

The primary cervical squamous cell carcinoma HCC94 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were cultured in RPMI-1640 (U2OS cells) and supplemented with 10% fetal bovine serum (FBS) 100 U/ml streptomycin and 100 mg/ml penicillin. The inhibitors of AURKAMLN8237 with the right amount of DMSO dissolved. And match the working concentration with the fresh medium before the experiment began. The cells were divided into 2 groups. Experience group (Inhibitor) cells were treated with MLN8237 with a working concentration of 2 μmol/L, Control group (Control) cells were treat with right amount of DMSO without MLN8237. Two groups of cell morphology were observed by inverted microscope. After 48 h, take the cells in the logarithmic growth phase in each group on treatment bed, and using 4 Mv X-ray irradiation with linear acceleratorfor irradiation, SSD was 100 cm, single dose were 0 Gy, 2.0 Gy, 4.0 Gy, 6.0 Gy, respectively. During the irradiation, alcohol was used to avoid cell pollution. After the end of the irradiation, the cell culture dish will be moved to the constant temperature box as soon as possible. Which would provide a platform for the further experimental study?

The cell proliferation were detected by MTT assay

The cells in the logarithmic growth phase in each group were digested and counted. The cell concentration was adjusted to 1×10^5/ml and seeded in 96-pore plate, with 5 complex pores for each group. After examining with microscope, the cells had their density adjusted and placed into a 5% CO_2 incubator at 37°C for a 5-day continuous culture. During each day, 20 μl of MTT with a concentration of 0.01 mol/L was added into the pores 4 hours prior to the end of daily culture. 4 hours later, the culture medium was completely discarded. Next, 150 μl demithyl sulfoxide (DMSO) dissolved Formazan granules were added in and shaken in oscillator for 5 to 10 minutes. Subsequently, microplate reader was utilized to measure its optical density (OD) at 490 nm of its wavelength, which is OD490. Lastly, the daily proliferation folds of cells in each groups compared to the first day (OD490/fold) were calculated and a bar chart of cell proliferation was created using time as abscissa as well as OD490/fold as ordinates.

The cell apoptosis were detected by flow cytometry

The cells in the logarithmic growth phase in each group were collected through sedimentation, while the supernatant was discarded and

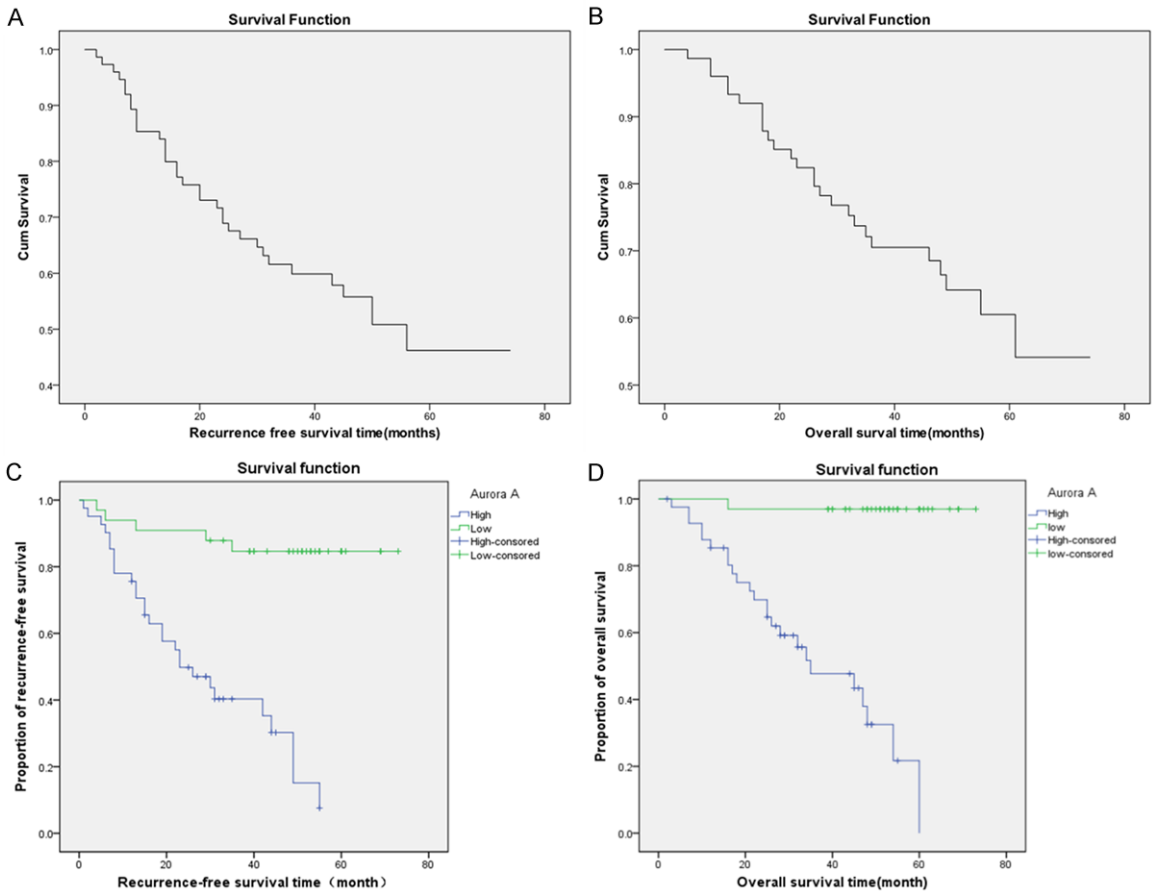


Figure 1. Recurrence-free survival (RFS) and overall survival (OS) curves, and the association between AURKA expression and survival. RFS curve (A) and OS curve (B), respectively, and the 5-year RFS and OS were 9.18% and 14.29%, respectively. Kaplan-Meier plots of (C) RFS and (D) OS in 98 patients with CSCC according to AURKA expression. The *P* values for survival comparison, which were obtained by log-rank test, were all less than 0.05.

washed twice with pre-cooled phosphate-buffered saline (PBS). Subsequently, the cells were gathered in 5 ml centrifugal tubes with 3 complex pores assigned per group. After centrifugal action, the supernatant was discarded, whereas the cell precipitation was washed with PBS once and centrifuged again before resuspended. Next, the cell suspension was supplemented with Annexin V-APC for staining and properly hid away from light at room temperature for 10 to 15 minutes. Lastly, flow Cytometry was used to detect the cell apoptosis in each group, and the treatment results were analyzed with ModFit (Verity Software House, Chula Vista, CA, USA), the DNA analysis software.

The cell cycle were detected by flow cytometry

The cells in the logarithmic growth phase in each group were digested and counted. The cell concentration was adjusted to 1×10^5/ml and seeded in 6-well plates. Each well was provided with 2 ml cell solution and the cells were cultured in a 5% CO_2 incubator at 37°C for 48 h, the fluid culture was collected in a centrifuge tube where the cells were digested and collected at 4°C. The cells were centrifuged at a rate of 1,000× g for 5 min. The supernatant was discarded and the cells were washed once with ice cold PBS. The cells were collected in a centrifuge tube at 4°C and centrifuged at 1,000× g for 5 min. The supernatant was discarded, while the ice cold PBS cells were re-suspended, transferred to the Eppendorf tube at 4°C and centrifuged at 1,000× g for 5 min. The supernatant was discarded again and 1 ml PBS was added. The re-suspended cells were pre-cooled and fixed with 70% ethanol at 4°C. The cells were centrifuged at 1,000× g for 5 min and the ethanol solution was discarded. Next, the cells were washed twice with PBS and filtered th-

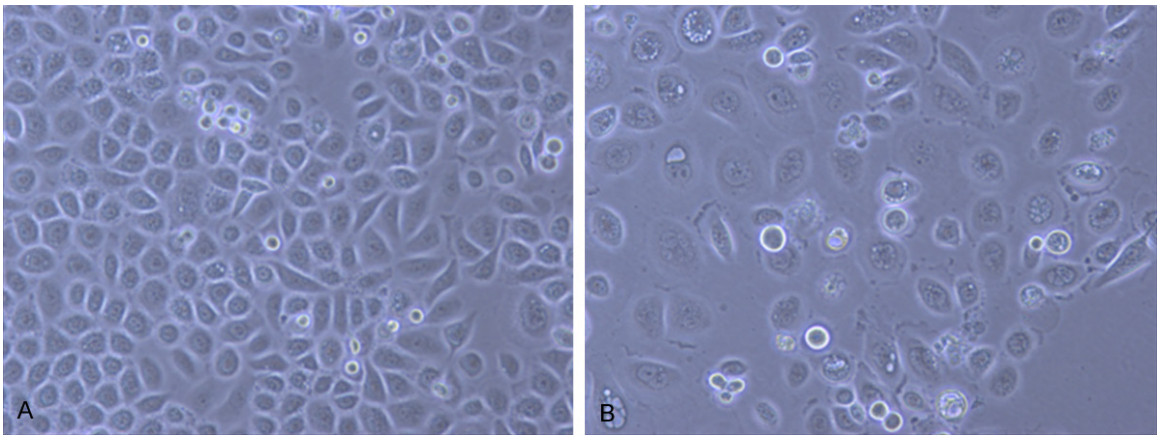


Figure 2. The Cell growth and morphological changes under inverted microscope. Control group (A), Inhibitor (B).

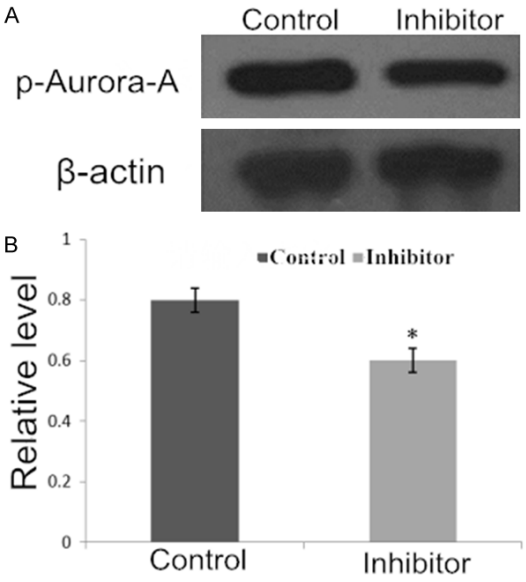


Figure 3. The expression of p-Aurora-A protein in primary cervical squamous cell carcinoma HCC94 cells-detected by Western blot. A. Protein blotting stripe. B. The relative content of p-Aurora-A protein. **P<0.01* versus the control group.

rough a 400-mesh sieve, prior to propidium iodide dye being added at 4°C in darkness for staining for 30 min. The proportion of cells in the G0/G1 phase, S phase and G2 phase was then detected by flow cytometry.

The expression of p-Aurora-A, cyclin D1, CDK2 and CDK6 were detected by western blot

The cells in the logarithmic growth phase in each group were digested and counted. The total protein (TP) extracted by Trizol method. After electrophoresis, wet method is applied to transfer TP onto nitrocellulose membrane. After 1 h of TBS of blocking, primary antibodies of p-Aurora-A, Cyclin D1, CDK2 and CDK6 are used for overnight incubation under 4°C (with a dilution ratio of 1:200). After TBS washing, secondary antibody of horse radish peroxidase (HRP) is applied for overnight blocking under 37°C. Then, after TBS washing and ECL color developing are completed, film processing is finally done. As β-actin is taken as the internal reference, dilution with a ratio of 1:400 is achieved. After the film is shot, Image-pro plus 6.0 software is adopted for semi-quantitative analysis.

Statistical analysis

SPSS 17.0 statistics software was employed to undergo statistical analysis. Both groups of measurement data had their difference compared using two sample *t* tests, whereas the pairwise comparison of rates utilized ×2 test or Fisher exact test. In addition, Kaplan-Meier was used to carry out survival curve analysis, whereas log-rank was employed to test difference with *P<0.05* indicating the statistical significance of difference.

Results

AURKA was over-expression in CSCC

The immunohistochemical results and clinical data showed that AURKA was over expression in primary cervical squamous cell carcinoma (CSCC), The positive rate was 73.5%, The stro-

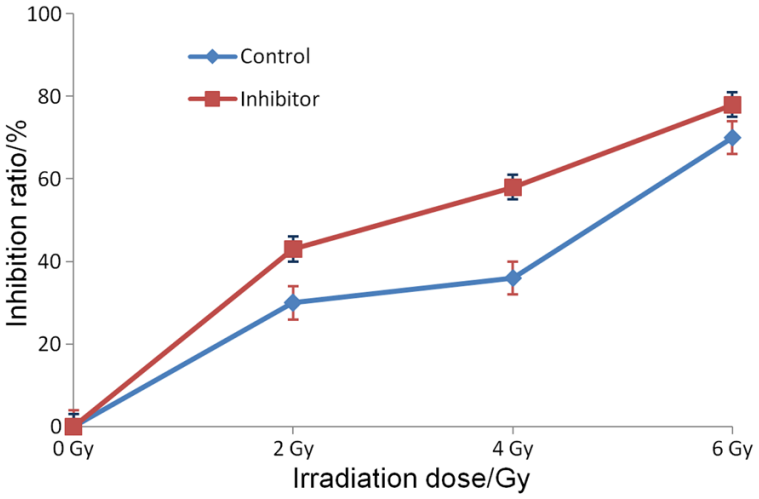


Figure 4. The inhibitory action of X ray on inhibitor group cells was significantly stronger than that of control group.

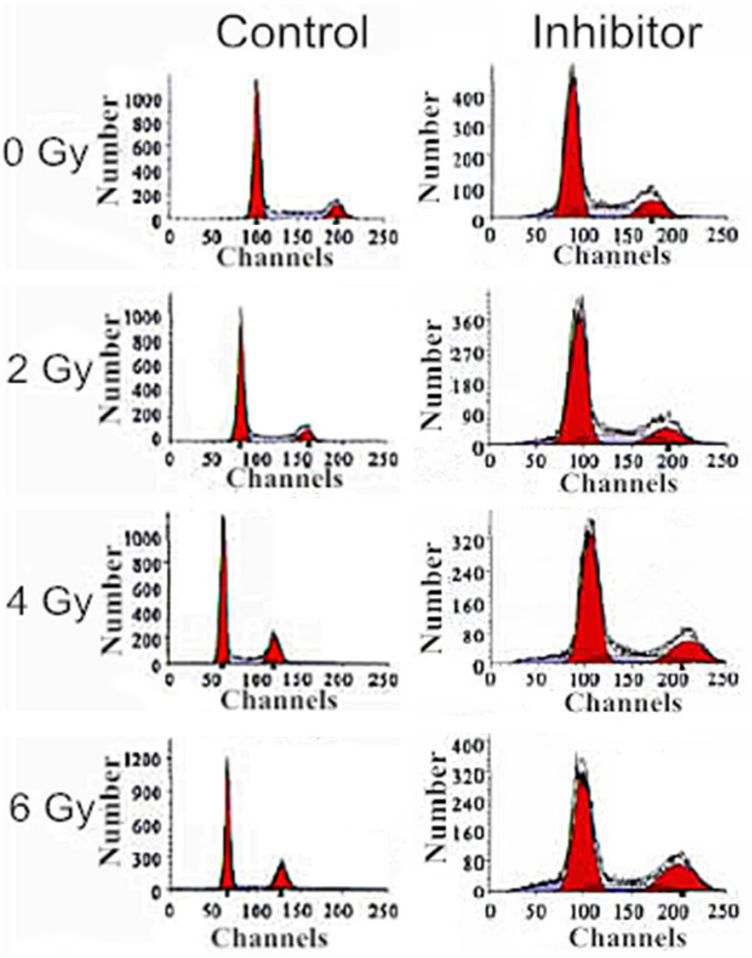
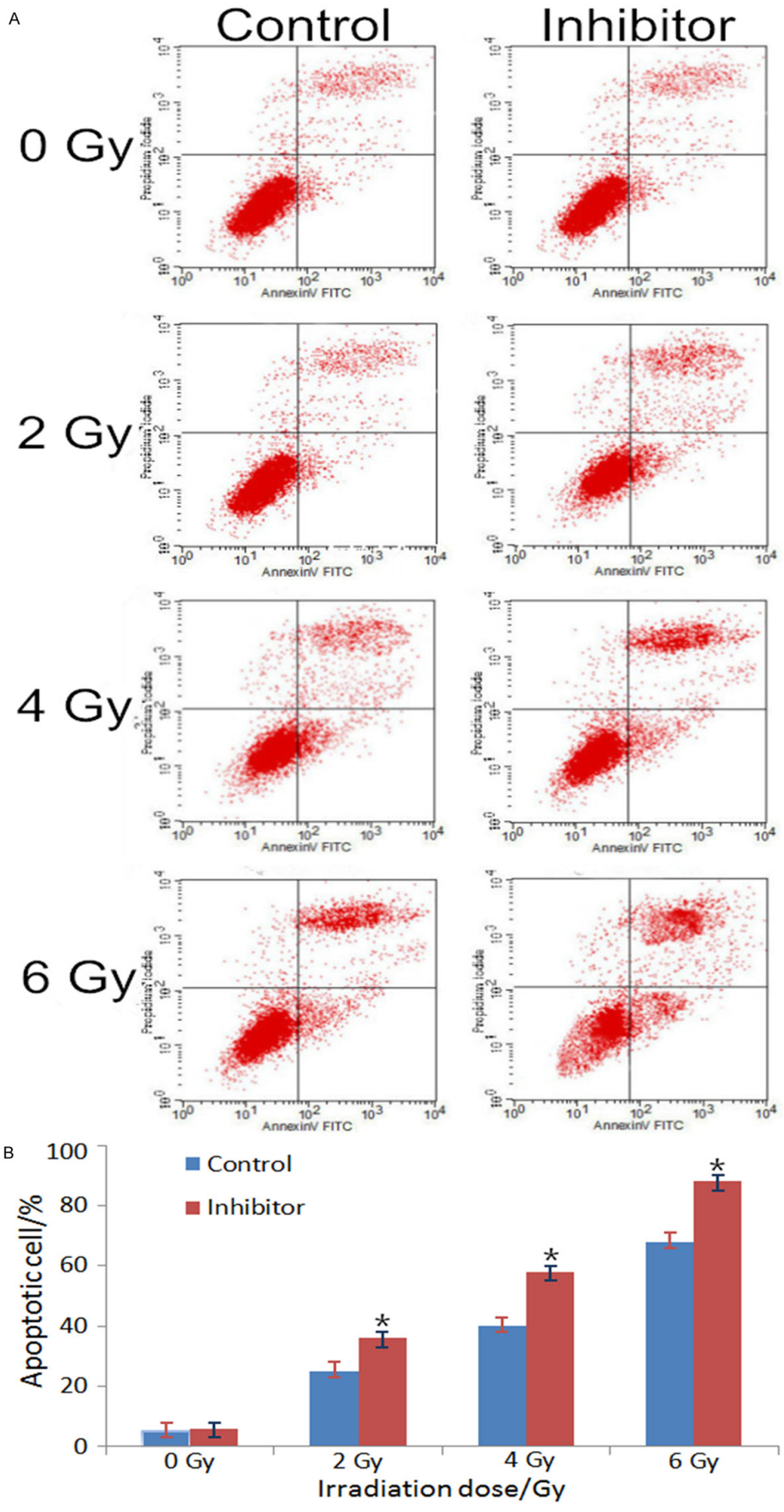


Figure 5. The effects of AURKA on cellcycle using flow cytometry. The effect of G2/M blocking became better with radiation dose increasing.

ngly positive rate was 47% (**Table 1**). And Lymph node metastasis (P=0.001), large tumor size (P=0.001), low hemoglobin (Hb) level (P=0.017) and recurrence event (P< 0.001) were significantly associated with AURKA overexpression. Univariate analysis showed that RFS was significantly influenced by lymph node metastasis (P<0.001), large tumor size (P=0.013), and AURKA overexpression (P<0.001). OS was significantly influenced by lymph node metastasis (P<0.001), large tumor size (P<0.001), low Hb level (P=0.011) and AURKA overexpression (P<0.001). However, only AURKA overexpression (P=0.036, average OR=14.525) was identified as an independent unfavorable prognostic factor for OS in multivariate analysis (**Figure 1**).

Cell growth and morphological changes were observed by inverted microscope

Results it was seen under the microscope that the control group cells grew well with round or fusiform appearance and clear outline, and the cellular plasma plenty and bright (**Figure 2**). The cells treated with MLN8237 exhibited characteristics of apoptosis including increased intra-cellular granules, increased vacuoles in size, decreased cytoplasm and condensed nucleus with smaller, rounded cells shape. More cells detached from the adherent state and suspended in culture medium. The cells treated with DMS exhibited characteristics of apoptosis including increased intracellular granules, increased


A
Control
Inhibitor
0 Gy
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6 Gy
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Apoptotic cell/%
100
80
60
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20
0
0 Gy
2 Gy
4 Gy
6 Gy
Irradiation dose/Gy
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Figure 6. The effects of AURKA on apoptosis using flow cytometry. the apoptosis cells in the inhibitor group was significantly higher than the control group,and with the increase of radiation dose, the inhibition effect is more obvious. *$P<0.01$ versus the control group.

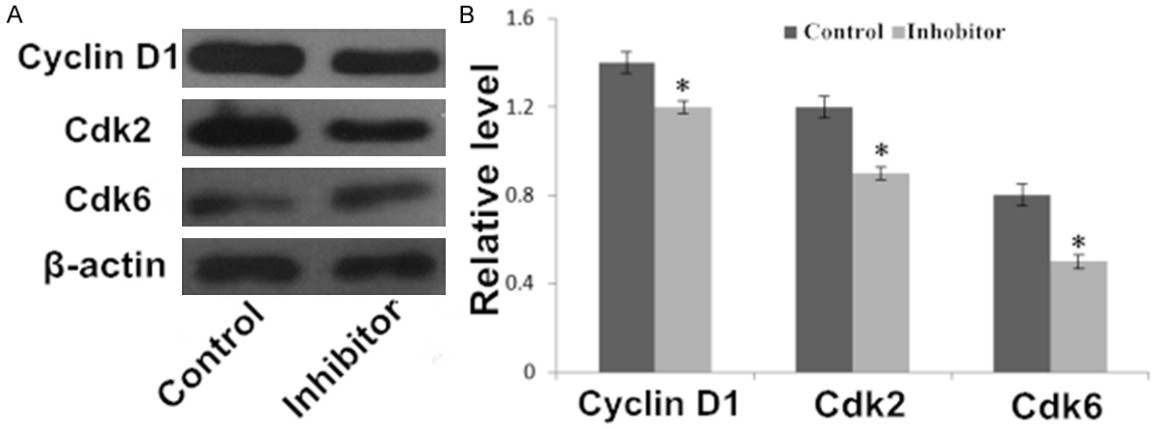

Figure 7. The expression of Cyclin D1, CDK2 and CDK6 protein in cervical carcinoma HCC94 cells detected by Western blot. A. Protein blotting stripe. B. The relative content of Cyclin D1, CDK2 and CDK6 protein. *$P<0.01$ versus the control group.

vacuoles in size, decreased cytoplasm and condensed nucleus with smaller, rounded cells shape. More cells detached from the adherent state and suspended in culture medium (**Figure 2**).

Thep-Aurora-A proteinswere low expressionsin inhibitor groups

The Western-blot results showed that the expression of p-Aurora-A protein in the inhibitor group was significantly lower than the control group (P<0.01) (**Figure 3**). It was suggested that the p-Aurora-A expression can be inhibited by MLN8237.

MTT result

The result of MTT assay demonstrated that X rayhad significant inhibitory effect onthe two group cells,and showed dose positive correlation. Butthe inhibitory action of X ray on inhibitor group cells was significantly stronger than that of control group (**Figure 4**), the difference was statistically significant (*P<0.05*). Such result implied that the low expression of AURKA can improve the sensitivity of tumors to X ray.

G2/M phase blocking

The flow cytometry results showed that the cells of G2/M phase in the inhibitor group and control groupat 2 Gy dose point were (69.25± 2.58)% and (40.12±1.23)% respectively, and with statistically significant difference (P<0.05). Andthe effect of G2/M blocking became better with radiationdose increasing (**Figure 5**). It was suggested that the low expression of AURKA canarrest cellsat G2/M phase, Cell cycle arrests may have positive correlation to radiation sensibility.

Cell apoptosis

The results of flow cytometry demonstrated that the apoptosis percentage of cells in the inhibitor group was significantly higher than the control group (*P<0.01*), and with the increase of radiationdose, the inhibition effect is more obvious (**Figure 6**). It was suggested that the low expression of AURKA can improve the sensitivity of tumors to X ray, and promote cells apoptosis. Cell apoptosis induced by irradiation has close relation to cell cycle arrest especially G2/M arrest.

Down-regulate the expressions of cyclin D1, CDK2 and CDK6 proteins

The Western-blot results showed that the expression of Cyclin D1, CDK2 and CDK6 protein in the inhibitor group were significantly lower than the control group (*P<0.01*) (**Figure 7**). It was suggested that low expression of

AURKA down-regulate the expressions of Cyclin D1, CDK2 and CDK6 proteins.

Discussion

Cervical cancer is one of malignant tumors harm to women health, which has a increased incidenceamong which more than 80% cases occurred in the developing country [10, 11]. In China, the prevalence of cervical cancer in Uighur women has been very high (526/100,000), and 3 to 4 times higher than the average level in China (138/100,000) [12].

For a long time, surgery, radiotherapy and chemotherapyare the main treatment for cervical cancer. Radiotherapy is the major method for the uterus cervical carcinoma, which has a wide scope of application, especially the tumor recurrence and metastasis,according to the NCCN Clinical Practice Guidelines for Cervical Cancer (version 2014). However, the difference of individual physique and tumor heterogeneityare different in the radiosensitivity. The radiation resistance is one of the most important reasons for treatment failure, relapse and metastasis [13].

The cell cycle is a successive process between two mitosis divisions, there is much difference between different cell cycle phases in cell radiosensitivity. For instance, G2 and M phase cells are the most sensitive, G1 phase cells are moderately sensitive, S phase cells are non sensitiveto radiation. And cell cycle specific proteins and Cyclin-dependent-kinase are directly involved in cell cycle progression regulation.

Cyclin D1, CDK2, CDK6 are important cell cycle specific proteins. Cyclin D1 affected G1-S checkpoints, and the complexes which are made of acombination of Cyclin D1 and CDK4/6, could regulate Rb protein's phosphorylation state [14], relieve the inhibitory effects of Rb to E2F [15], acceleratethe synthesis of DNA, lead the advance of G1 to S phase in cell cycle. That CDK2 binding to Cyclin A and Cyclin B, could promote cells transform to G2/M phase and promote cell mitotic [16]. Moreover, the combination of CDK2 of Cyclin E could promote cells transform to G1/S phase[17].

The Aurora kinases family, belonging to serine/threonine protein kinases, and Aurora-A has a controlling role in cell mitosis. Aurora-A kinase plays an important role in centrosome separation, maturation and bipolar spindle assembly and chromosome segregation processes during mitotic phase, it's the criticalfactor in regulating of the cell cycle progression [18]. Aurora-A is also involved in the regulation of the G_2/M transition and checkpoint [19, 20]. Aurora-A is closely related to cell cycle protein expression, but the mechanism of cell cycle progression remains to be further studied.

In normal cells, the expression level of Aurora-A was relatively high inthymus, whereas low levels of expression were detected insmall intestine, testicles, colon, spleen and brain. Based on series of researches, high expression of Aurora-A mRNA and its protein have been observed in human malignancies from lots of organs, such as neck, ovary, prostate, Oral squamous cell, throat, etc. And have also suggest aggressive potential and poor prognosis. Studies also showed that reducing Aurora-A expressionhas great significance in inhibiting the proliferation of tumor cell from small cell lung cancers, osteosarcoma, breast cancer, and promoting its apoptosis, and inducing cell cycle arrest in G2/M phase, as a consequence, to increase tumor cell sensitivity to radiation therapy. The abnormal expression of Aurora-A, which has identified as proto-oncogene, can cause unequal distribution, and finally leads to cancers [21, 22].

Interest in Aurora-A inhibitor is rapidly increasing. MLN8237, known as the second-generation selective Inhibitors, which IC50 is 1.2 nM, has no significantinhibition effect on the activity of other 205 kinds of protein kinase [23-25]. The MLN8237 could induce cell cycle arrest in G2/M phase, and significant reduce radiation resistance. Thus, targeting Aurora-Amay not only inhibit the proliferation of tumor cell, increase the radiation sensitivity, but also provide new ideas and theoretical basis for future clinical antitumor. At present, MLN8237 is an effective strategy in oncotherapy [26]. Phase I [27] and Phase II [28] clinical trial of MLN8237 have obtained gratifying result and have a good safety profile, and now MLN8237 has already been applied to phase III clinical trial [29, 30].

According to this study, Aurora-A was overexpression in CSCC, it may be related to cancer cell invasion and metastasis, and it was an independent prognostic factor affecting disease free survival and overall survival. Cell pro-

life ration and cell apoptosis of MLN8237 treated group wereincreased after X-irradiation. And the expression of Cyclin D1, CDK2 and CDK6 were down-regulated to induce cell cycle arrest at G2/M phases.

In conclusion, Aurora-A inhibitor can blocks cell proliferation and cell cycle progression, promote cell apoptosis, and increase the sensitivity of cervical cancercells to radiotherapy by regulating the expression of cell cycle related protein. Thus, Aurora-A may be used as one of therapeutic targets and an independent prognostic factor to increase the sensitivity of CSCC radiotherapy. Whether the Aurora-A inhibitor directly lead this, or through inhibiting cyclin expression of Cyclin D1, CDK2 and CDK6, the exact mechanism remains to be further studied.

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Disclosure of conflict of interest

None.

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