

RESEARCH ARTICLE

Influence of Ionizing Radiation on Ovarian Carcinoma SKOV-3 Xenografts in Nude Mice under Hypoxic Conditions

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Abstract

Purpose: We aimed to detect the expression of HIF-1 α , VEGF, HPSE-1 and CD31 in SKOV3 xenografts in nude mice treated with different doses of ionizing radiation, trying to explore the possible mechanism of hypoxia and radioresistance. **Methods:** Nude mice bearing SKOV3 xenografts were randomly divided into 4 groups: Group A (control group, no ionizing radiation), Group B (treated with low dose of ionizing radiation: 50cGy), Group C (treated with high dose of ionizing radiation: 300cGy), Group D (combined ionizing radiation, treated with ionizing radiation from low dose to high dose : 50cGy first and 300cGy after 6h interval). The mRNA levels of HIF-1 and VEGF in each group were detected by real time polymerase chain reaction, while HPSE-1 expression was measured by ELISA. The microvessel density (MVD) and hypoxic cells were determined through immunohistochemical (IHC) staining of CD31 and HIF-1 α . **Results:** Significant differences of HIF-1 α mRNA level could be found among the 4 groups ($F=74.164, P<0.001$): Group C>Group A>Group D> Group B. The mRNA level of VEGF in Group C was significantly higher than in the other three groups ($t=-5.267, P=0.000$), while no significant difference was observed among Group A, B and D ($t=1.528, 1.588; P=0.205, 0.222$). In addition, the MVD was shown to be the highest in Group C ($t=6.253, P=0.000$), whereas the HPSE-1 level in Group A was lower than in Group B ($t=14.066, P=0.000$) and higher than in Group C ($t=-21.919, P=0.000$), and similar with Group D ($t=-2.066, P=0.058$). Through IHC staining of HIF-1 α , the expression of hypoxic cells in Group A was (++) , Group B was (+), Group C was (+++) and Group D was (+). **Conclusion:** Ionizing radiation with lower-doses might improve tumor hypoxia through inhibiting the expression of HIF-1 and HPSE-1, whereas higher-doses worsen tumor hypoxic conditions by up-regulating HIF-1 α , HPSE-1, VEGF and CD31 levels. A protocol of low-dose ionizing radiation followed by a high-dose irradiation might at least partly improve tumor hypoxia and enhance radiosensitivity.

Keywords: Ionizing radiation - hypoxia inducible factor 1 - vascular endothelial growth factor - CD31 - HPSE-1

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Introduction

The response of tumor to ionizing radiation correlated with the existence of oxygen, which could facilitate permanent DNA damage by inducing free radicals. The hypoxic region in tumors required higher radiation doses to obtain similar biologic effects compared with normoxic regions (Lund et al., 2004). In hypoxic conditions, tumor cells would undergo gene mutation to adequate the circumstances they lived, which resulted in the radioresistance. Hypoxia may also enhance the aggressiveness of tumors and resulted to multidrug resistance (Seubwai et al., 2012; Li et al., 2013).

Hypoxia inducible factor 1 (HIF-1), consisting of HIF-1 α and HIF-1 β , is an important mediator in such process. As one of the subunits, HIF-1 β expressed constitutively, whereas HIF-1 α protein escaped from the immediate degradation under hypoxia. HIF-1 α is a transcription factor involved in the process of gene related hypoxic

adaptation of neoplasm. These genes also interacts with other transcriptional factors, such as the angiogenic growth factor, vascular endothelial growth factor (VEGF) and glucose transporter 1 (Glut-1).

VEGF is an important angiogenic factor for endothelial cells, which has been mentioned elsewhere. It could protect endothelial cells from apoptosis by activating the Raf signal pathway. Tumor cells suffering ionizing radiation has shown to directly increase the expression of VEGF through the rapid MAPK pathway (Levy et al., 1995; Shweiki et al., 1995). In addition, the activation of VEGF expression can also be induced by HIF-1, just as above mentioned. Clinical studies have demonstrated that increased VEGF expression in tumor cells is associated with higher prevalence of metastasis and poorer prognosis, worsening the hypoxic conditions or oxidative stress. Gupta (Gupta et al., 2002) have reported that VEGF overexpressed xenografts became more resistant to the ionizing radiation compared with none VEGF-expressing

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ones. Increased VEGF expression was found to be negatively correlated with the efficacy of radiation therapy in advanced cervix carcinoma (Loncaster et al., 2000).

CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), is a 130-kd membranous glycoprotein of the immunoglobulin superfamily. CD31 level detection by IHC is proposed as a standard for microvessel density (MVD) studies.

Heparanase (HPSE-1), an endo-glucuronidase degrades the heparin sulfate (HS) side chains of HSPGs, is a critical mediator in the process of tumor angiogenesis and metastasis (Li et al., 2012). Thoughtfully, angiogenesis induced by hypoxia and pathological angiogenesis induced by tumor were regulated with the similar mechanisms, containing increased expression of VEGF and HPSE-1 (Bernaudin et al., 2002; Greenberg et al., 2005). In the early stage of angiogenesis, HPSE-1 is necessary for degradation of the physical barrier formed by the extracellular matrix (ECM) and basement membranes. Many investigations have revealed that the relationship between HIF-1, VEGF and radioresistance resulted from hypoxic condition (Harada 2011), providing that HIF-1, VEGF, HPSE-1 and CD31 could be regarded as indirect indicators of hypoxia and radioresistance.

This study aimed to detect the expression of HIF-1 α , VEGF, HPSE-1 and CD31 in SKOV3 xenograft in nude mice after treated with different doses of ionizing radiation. Furthermore, we explored the novel changes of hypoxic state in tumor tissues with ionizing radiation to provide preclinical data for overcoming radioresistance in hypoxic tumor cells.

Materials and Methods

Ethics Statement

All animals' experiments were carried out in accordance with protocols approved by the Animal Care Committee of the Affiliated Hospital of Qingdao University Medical College.

Cell Culture and Tumor Models

SKOV3 cells (purchased from the cell bank of Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640 (Invitrogen USA) and 10% FBS (Invitrogen USA) under the condition of 37°C temperature and 5% CO₂ concentration. The cell lines were harvested from exponential cultures at the concentration of 3 \times 10⁸/ml. To establish tumor xenografts, SKOV3 cells were injected into the right hind limb (6 \times 10⁷ cells in PBS) of BALB/c female nude mice (4~5 weeks of age, weighted 10-13 g, purchased from Vital River Laboratory Animal Technology Co. Ltd., qualified No. is SCXK (BJ) 2006-0010). The animals were housed under pathogen-free conditions. Tumor size was measured directly in millimeters by pathologists as the largest diameter of the tumor mass. Tumors were allowed to attain a maximal diameter of 20.0mm.

Group Division and Administration of IR

32 nude mice bearing SKOV3 xenografts were divided into four groups: Group A (control group, no

ionizing radiation); Group B (treated with low dose of ionizing radiation: 50cGy); Group C (treated with high dose of ionizing radiation: 300cGy); Group D (combined ionizing radiation, low dose first and high dose following: 50cGy/300cGy, 6h as interval). Tumor cells were irradiated with no fractions using a linear accelerator (Varian, USA) generating 6MV-X ray at a dose rate of 3Gy/min.

Tumor sample collection and disposal

Mice were disposed by cervical dislocation for 48hs after irradiation. The intact tumor masses were removed immediately. Samples of the tumors were fixed in formalin for 8 h, and then embedded with paraffin. Embedded tissues were cut 4 μ m thick and prepared for immunohistochemistry. The remaining tumor tissues were frozen in -80°C liquid nitrogen.

Reverse transcriptase-polymerase chain reaction analysis

Total RNA was isolated using Trizol reagent (Gibco, USA). The whole sequences of target genes(HIF-1 α , VEGF) and reference gene (GAPDH) were obtained from NCBI Gene Bank. The corresponding primers were synthesized on the basis of Primer Express software: HIF-1 α (5'-CTTCTGGATGCTGGTGATTTG-3' and 5'-TATACGTGAATGTGGCCTGTG-3'), VEGF(5'-AGGAGGGCAGAATCATCACG-3' and 5'-TATGTGCTGGCCTTGGTGAG-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' and 5'-CATGTAGCCATGAGGTCCACCAC-3'). The primer pair amplified a 478 base pair (bp) fragment as HIF-1 α , a 256bp fragment as VEGF, two fragments with 258bp and 532bp as GAPDH.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with the isolated RNA and the synthesized primers as templates and primers, respectively. Total RNA(10 μ l) from the SKOV3 xenografts was transcribed into cDNA using LightCycler 480 (Roche Diagnostics, Germany) RT-PCR system (12.5 μ l reaction mixture, 10 μ l RNA, 0.8 μ l primer1 and 0.8 μ l primer2, 0.5 μ l TaKaRa AVM reverse transcriptase, 0.5 μ l AVM2Taq, 4.9 μ l purified water), with one cycle of denaturation for 30min at 60°C and annealing for 3min at 95°C. The cDNA was then amplified with 40 cycles of denaturation for 5 sec at 95°C, and extension for 20 sec at 60°C. After amplification, products were loaded onto a 2% agarose gel in Tris-borate-ethylene diamine tetraacetic acid (EDTA) buffer for agarose gel electrophoresis (AGE) at 120V for 20min. And the specific bands were visualized with ethidium bromide and photographed under ultraviolet light. Gel scanner system (EP0241904 B1 ISCO, Inc.) was used for absorptiometric analysis. For semi-quantitation, Absorbance of HIF-1 α and VEGF PCR products were normalized to that of GAPDH.

CD31 immunohistochemistry and microvessel density assessment

CD31 immunohistochemistry (IHC) for tumor blood vessels was performed on 4 μ m thick sections of formalin-fixed, paraffin-embedded tissues. CD31 was detected

with a rabbit anti-mouse monoclonal antibody(CD31, Abcam, HK, 200:1 dilution). After deparaffinisation and rehydration, tissue sections were treated with 3% H₂O₂ for 20 min to block endogeneous peroxidase activity. Sections were washed in PBS for 5min three times, and incubated for 20min at room temperature with a protein-blocking solution consisting of PBS with 10% goat serum. Excess blocking solution was drained. The primary antibody reaction was carried out at 4°C overnight. Sections were then washed with PBS for 5 min, three times. And later incubated with a biotin-labeled anti-rabbit IgG (Abcam, HK 50:1 dilution) for 30min at 37°C. Three times washing were repeated. Then HRP(horseradish peroxidase) conjugated Streptavidin was added onto the sections, baked at 370C for 30min and three times washing in PBS for 5min each time was repeated. 3,3,-diaminobenzidine (DAB) was used to detect antigen-antibody binding. Counterstaining was done with hematoxylin, and after dehydration, slides were mounted with glycerogelatin. Vessel density was interpreted in vessel profiles counts identified by CD31 staining. The average count of microvessel of 10 fields was calculated (×200 magnification).

HPSE-1 activity assays

The Elisa kit (Abnova, Taiwan) was used in this assay for the quantification of HPSE enzymatic activity. Equal quantities of tissues were cut off from each specimen. Total cell lysates were prepared using the extraction buffer supplied with the kit. The assay was then carried out according to the manufacture's protocol. The absorbance (OD value) was measured at 450 nm by enzyme linked immunoassay instrument (BIOBASE 2000 Italy). The HPSE concentrations were quantified by comparison with a series of HPSE standard samples included in the assay kit. All samples were done in duplicate independently, and readings were taken in triplicate.

HIF1- α immunohistochemistry and hypoxic tumor cell assessment

HIF1- α immunohistochemistry for hypoxic tumor cells was performed on 4 μ m thick sections of formalin-fixed, paraffin-embedded tissues. HIF1- α was detected with a rabbit anti-mouse monoclonal antibody (HIF1- α , Abcam, HK, 300:1 dilution). After deparaffinisation and rehydration, tissue sections were treated with 3% H₂O₂ for 20min to block endogeneous peroxidase activity. Sections were washed in PBS for 5min three times, and incubated for 20min at room temperature with a protein-blocking solution consisting of PBS with 10% goat serum. Excess blocking solution was drained. The primary antibody reaction was carried out at 4°C overnight. Sections were then washed with PBS for 5 min, three times. And later incubated with a biotin-labeled anti-rabbit IgG (Abcam, HK 50:1 dilution) for 30min at 37°C. Three times washing were repeated. Then HRP(horseradish peroxidase) conjugated Streptavidin was added onto the sections, baked at 370C for 30min and three times washing in PBS for 5min each time was repeated. 3,3,-diaminobenzidine(DAB) was used to detect antigen-antibody binding. Counterstaining was done with

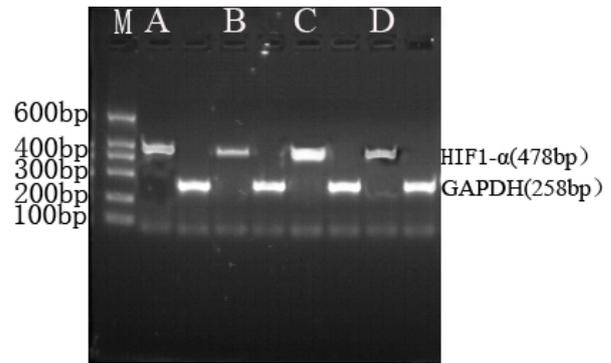


Figure 1. Expressions of HIF1- α mRNA Detected by RT-PCR in Different Groups

hematoxylin, and after dehydration, slides were mounted with glycerogelatin.

Semiquantitative criteria: slides were first scanned by×100 magnification: ten cellular fields were randomly chosen and a total of 200 cells and the number of HIF1- α positive cancer cells were counted (×400 magnification) and interpreted by two investigators without knowledge of the corresponding clinic pathological data. Immunohistochemical staining was assessed semi-quantitatively by measuring both the intensity of the staining (0 for nonstaining, 1 for yellow staining, 2 for brown yellow staining, and 3 for brown staining) and the extent of the staining (0, 0-5%; 1, 6-25%; 2, 26-50%; 3, 51-75%;4,76-100%). The scores for the intensity and extent of staining were multiplied to give a weighted score for each case. For the statistical analysis, the weighted scores were grouped in four categories where scores of 0 to 3 (-), 4 to 6 (+),7 to 9 (++) and 10 to 12 (+++) respectively.

Statistics Analysis

SPSS17.0 (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Level of HIF-1 α and VEGF transcription and expression of CD31, HPSE-1 and HIF1- α were presented as mean \pm standard deviation (SD). One-way ANOVA was performed for comparison among group A, B, C, and D on the above parameters and subsequently student's t-test was applied for statistic analysis between groups. *P*-values were 2 sided and *P*<0.05 was considered statistically significant.

Results

HIF1- α mRNA transcription of different groups

All groups showed certain expressions of HIF1- α mRNA. Semiquantitative results were as follows: GroupA:0.51 \pm 0.015, GroupB:0.46 \pm 0.014, GroupC:0.74 \pm 0.056 and GroupD:0.45 \pm 0.039 (F=70.096, *P*=0.000). HIF1- α mRNA levels of Group B or Group D were obviously lower compared with Group A(*t*=2.495, 3.087; *P*=0.03, 0.09). HIF1- α mRNA level of Group C was significantly higher than Group A(*t*=12.053,*P*=0.000). There was no significant difference between Group B and D (*t*=0.569, *P*=0.619) (Figure 1 and Figure 2).

VEGF mRNA transcription of different groups

All groups showed certain expressions of

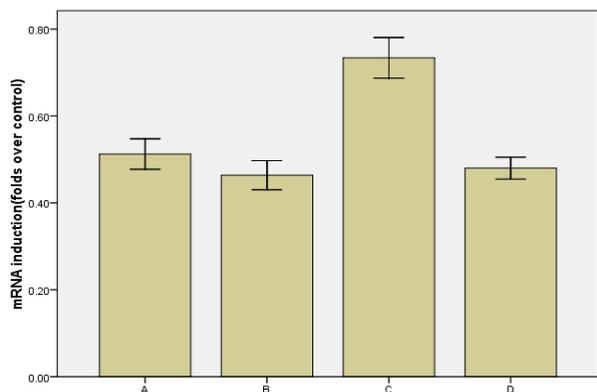


Figure 2. The variation of HIF1-α mRNA induction in different Groups

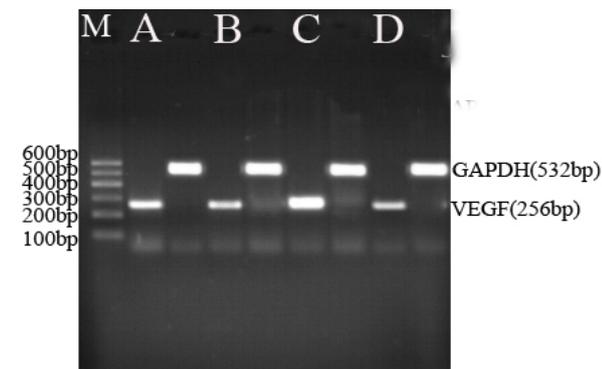


Figure 3. Expressions of VEGF mRNA Detected by RT-PCR in Different Groups

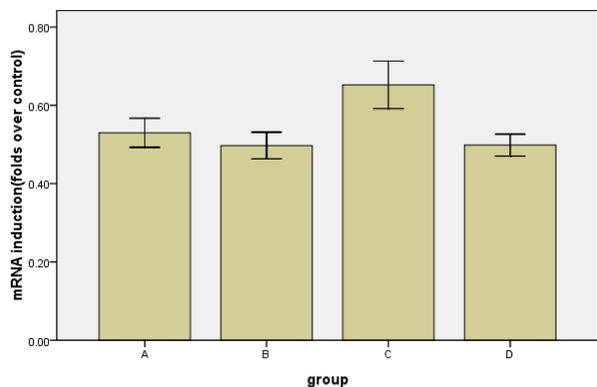


Figure 4. The Variation of VEGF mRNA Induction in Different Groups

VEGF mRNA. Semiquantitative results were as follows: GroupA:0.53±0.044, GroupB:0.49±0.046, GroupC:0.65±0.073 and GroupD:0.50±0.034 (F=17.208, P=0.000). There were no significant differences of VEGF mRNA levels in Group B or Group D compared with Group A (t=1.528, 1.588; P=0.205, 0.222). VEGF mRNA level of Group C was significantly higher than Group A (t=-5.267, P=0.000). There was no significant difference between Group B and Group D (t=-0.067, P=0.961) (Figure 3 and Figure 4).

HPSE-1 expression of different groups

All groups showed certain expressions of HPSE-1. Semiquantitative results were as follows:

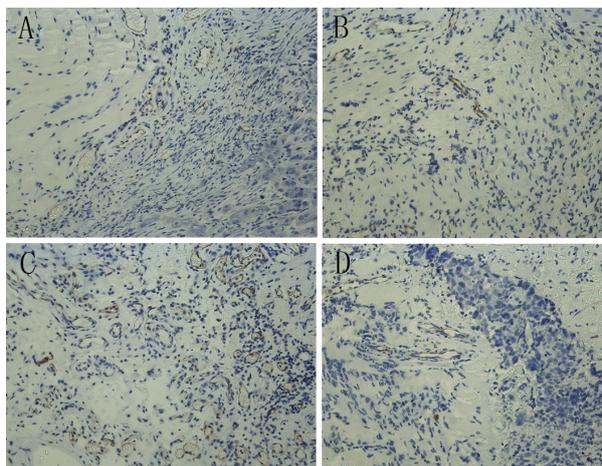


Figure 5. Different Expression Levels of CD31 by Immunohistochemistry in Each Group

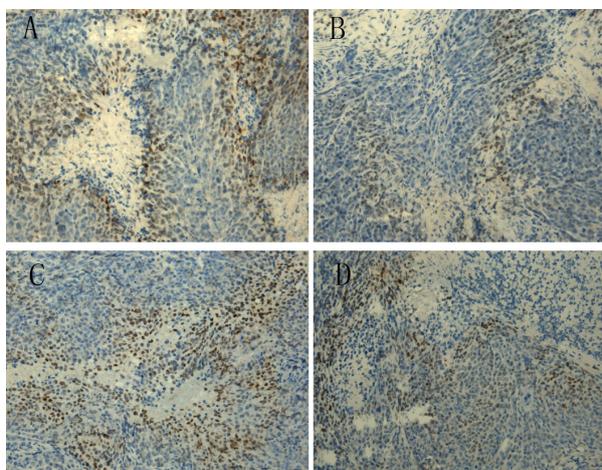


Figure 6. Different Expression Levels of HIF1-α by Immunohistochemistry in Each Group

GroupA:10.45±0.51ng/ml, GroupB:6.97±0.48ng/ml, GroupC:15.09±0.32 ng/ml and GroupD:10.89±0.32ng/ml (F=508.73, P=0.000). The level of HPSE-1 in Group B was lower than Group A (t=14.066, P=0.000) while that in Group D was higher compared with Group A (t=-21.919, P=0.000). There was no significant difference between Group A and Group D (t=-2.066, P=0.058).

CD31 immunohistochemistry and microvessel density

The MVD counts for each group was as follows: Group A 15.7±1.4, Group B 15.4±0.9, Group C 20.3±1.5 and Group D 16.4±0.9. MVD levels were significantly different among groups (F=22.66, P=0.000). Obvious escalation of MVD was observed in group C compared with group A (t=-6.253, P=0.000) while no significant differences were observed between group B or group D against the control group (t=0.638, -1.063; P=0.534, 0.306 respectively) (Figure 5).

HIF1-α immunohistochemistry assessment

Immunohistochemistry assessment showed various expression levels of HIF1-α (Figure 6). Group A was (++), Group B was (+), Group C was (+++) and Group D was (+).

Discussion

The most remarkable character of tumor cells is uncontrolled proliferation, leading to escalated oxygen consumption and hypoxic condition surrounding tumor cells. Hypoxia, significantly related to the radioresistance and failure of radiation therapy, is a common phenomenon in solid tumors. Overcoming radioresistance of hypoxia would effectively improve the efficacy of cancer radiation therapy (Brown, 2007). As a significant transcription factor closely correlated with hypoxia, HIF-1 α has been focused for a long time (Liang et al., 2013; Ping et al., 2013; Zhang et al., 2013). Many investigations have revealed that the relationships between HIF-1 α , VEGF and radioresistance resulted from hypoxic condition (Harada 2011) regarding HIF-1 α , VEGF, HPSE-1 and CD31 as indirect indicators of tumor hypoxia and radioresistance.

Our study was aimed to explore the influence of radiation on hypoxia and microvessel formations within tumor microenvironment. We observed the expressions of HIF1- α , HPSE-1 and VEGF, which could reveal various hypoxic conditions under different ionizing radiation doses in SKOV-3 ovarian carcinoma xenograft in nude mice and discussed the functions of HIF1- α and associated factors. Our data demonstrated the existence of hypoxia within tumor tissues and that high-dose ionizing radiation would exacerbate tumor hypoxia, exhibiting elevated expressions of HIF1- α , HPSE-1 and VEGF ($P < 0.05$). High-dose ionizing radiation might aggravate local hypoxic condition by inducing expression of HIF1- α , which substantially initiates the transcription of VEGF, HPSE-1 and CD31, leading to aberrant angiogenesis. Abnormal angiogenesis results in escalation of immature vessel density within tumor tissues which could not display equivalent function as normal vessels. The functional defect could not make for the density augmentation so that vascular permeability and interstitial fluid pressure would be increased. In this condition, the actual oxygen supplication is far less enough for continuously mounting oxygen consumption as tumor proliferation and hypoxia take a turn for the worse. In Group B with low-dose ionizing radiation, the expressions of HIF1- α and HPSE-1 were down-regulated with VEGF expression insignificantly changed. Parallel with Yu's (Yu et al., 2013) statements, we supposed that low-dose ionizing radiation would lead to an adaptive response for vascular dilation and intravascular pressures reduction, relieving the hypoxic conditions and down-regulating HIF1- α and HPSE-1 reactively. It seems that low-dose ionizing radiation might be a promising modulation in revolving clinical radioresistance.

In Group D, low-dose inducing and high-dose following, expression level of HIF1- α decreased and that of HPSE-1, VEGF and CD31 were insignificantly changed. We surmised that low-dose ionizing radiation before high-dose might relieve the exacerbated hypoxic condition resulted from the latter, improving radioresistance and radiosensitivity. It was in coincidence with the molecular changes in low-dose ionizing radiation group, which excellently interprets our suppose that adaptive response occur after low dose ionizing radiation. More research work should be concerned on this as it would be quite

valuable for optimization of neoplasm treatment protocols for clinicians.

Many studies showed that HIF-1 was a vital point for regulation of VEGF signal pathway under hypoxia (Cascio et al., 2010). It promotes the activity of VEGF transcription and enhances the stability of mRNA for VEGF as well, thus inducing expressions of VEGF. The specific binding of VEGF with Flt-1 and KDR on vascular endothelial cells membrane would facilitate neoplasm angiogenesis including generation of new endothelial cells and increased vascular permeability (Takahashi 2011). An investigation from Dukes (Shweiki et al., 1995) stated that expressions of HIF-1 α were remarkably up-regulated in tumor cells after ionizing radiation and the stability of this molecular was also enhanced attributed to ionizing radiation as the nitrosylation took place between HIF-1 α protein and nitric oxide. The latter was released from tumor related macrophages. Several scholars held that ionizing radiation would enhance local invading capacity of tumor cells. HPSE-1, the degrading enzymes of which participated completely in angiogenesis, was one of the significant influential factors in pre-angiogenesis process (Vlodavsky et al., 2011). Degradation of basal membrane following the migration after endogenous or exogenous stimulation in vascular endothelial cells played a critical role on the early stage of angiogenesis. Aberrant angiogenesis in tumor cells was as similar as the above. HPSE-1 might advance the generation and invasion of vascular endothelial cells in tumor tissues. High expression of HPSE was observed in capillaries of tumor tissues while none in mature vascular of normal tissues (Gupta et al., 2002).

In conclusion, our research found that several molecular markers closely correlated with tumor hypoxic condition and angiogenesis changed variously corresponding to different dose of ionizing radiation, upon which we supposed that low-dose ionizing radiation would inhibit expressions of HIF-1 α and HPSE-1 to improve tumor hypoxia while high-dose ionizing radiation induces high expressions of HIF-1 α , HPSE-1, VEGF and CD31, worsening tumor hypoxic condition and that low-dose inducing combined with high-dose following ionizing radiation could improve tumor hypoxic state and enhance radiosensitivity.

Acknowledgements

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