

ORIGINAL ARTICLE

X-ray-induced epithelial-mesenchymal transition in SW480 colorectal cancer cells and its potential mechanisms

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Summary

Purpose: The purpose of this study was to investigate the relation between X-ray irradiation and epithelial-mesenchymal transition (EMT), as well as the potential mechanisms of X-ray-induced EMT in SW480 colorectal cancer (CRC) cells.

Methods: It is well known that EMT plays a critical role in invasive and metastatic of colorectal cancer progression. However, the possible role of X-ray irradiation on EMT in colorectal cancer is widely disputed and its potential mechanisms are unclear. SW480 CRC were irradiated (0, 2, 4, 6, 8 Gy) and cultured for 48 hrs, and then the cellular morphology was observed. Protein and mRNA expressions were examined by Western blot and QRT-PCR. Cell migratory and invasive capacity was evaluated by Transwell assay.

Results: In the 2, 4, 6, 8 Gy groups, SW480 CRC exhibited a classical mesenchymal phenotype compared with the 0

Gy group. The expression of E-cadherin was significantly decreased, while the expression of vimentin and Smad3 was significantly increased in the 2, 4, 6, 8 Gy groups ($p < 0.05$) compared with the 0 Gy group; still, the expression of K-ras decreased in the 4, 6, 8 Gy groups ($p < 0.05$) compared with the 0 and 2 Gy groups. Furthermore, the cell migration and invasion capacity was significantly enhanced in the 4 and 8 Gy groups compared with the 0 Gy group ($p < 0.05$).

Conclusion: These results support the fact that X-ray irradiation can induce EMT through promoting Vimentin and Smad3 expression in SW480 CRC cells.

Key words: epithelial-mesenchymal transition, K-ras, radiation, Smad3, SW480 human colorectal cancer cells

Introduction

CRC is a common cause of morbidity and mortality in humans [1]. It is the third most common cancer and the third leading cause of cancer related mortality in the United States [2]. Also, the morbidity and mortality of CRC present an upward trend in China [3], even above the world average [4]. With the development of modern medicine, treatments of malignant tumors trend towards diversification, but the survival rate of patients with CRC has not been obviously improved. After multi-modality therapy, almost half of patients with CRC will develop metastatic disease, with a quarter having distant metastatic lesions at diagnosis

[5]. The reason may be associated with the high metastasis characteristics of CRC.

Radiation therapy plays an important role in the treatment of CRC. It is reported that X-ray irradiation can treat cancer mainly through ionizing radiation which can induce double-strand DNA cleavage, single-strand break, kill cancer cells and change the biological characteristics of cancer cells [6]. However, some previous researches found that irradiation can enhance the invasion and migration capacity of cancer cells and promote distant metastasis [7,8]. Numerous studies have suggested that EMT is closely related to the

invasion and metastasis of CRC cells [9,10] and irradiation can lead to EMT in endometrial cancer, cervical cancer and breast cancer [11-13]. So, there may be an association between irradiation and EMT in poor prognosis CRC.

EMT means that epithelial cells transform into mesenchymal cells in physiological or pathological cases, which was proposed by Greenburg and Hay [14] in 1982. Decreased Cadherin expression, increased vimentin expression, intercellular separation, loss of polarity, spindle-cell shape and pseudopodia formation are its characteristics. Depending on these changes, cells obtain high migration and invasion capacities. EMT is currently considered as a key step to start metastasis of cancer, which has been observed in many cancer metastatic processes (e.g. pancreatic cancer, gastric cancer and CRC) [9,15,16].

It has been reported that irradiation can induce change of gene expression associated with cancer. There are many signaling pathways that can lead to EMT [17] such as TGF β ₁-Smads, EGF-ras, etc. In the present study, we investigated the effect of X-ray irradiation on EMT in CRC cells and the potential signaling pathway mechanisms of X-ray-induced EMT.

Methods

Cell culture

SW480 human CRC cells purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in RPMI-1640 medium, supplemented with 10% v/v fetal bovine serum (FBS; Gibco, Grand Island, NY), streptomycin (100U/L), penicillin (100U/L), and incubated in humidified 5% CO₂ atmosphere at 37°C.

X-ray irradiation

CRC cells were irradiated with different 6MV X-ray doses (0, 2, 4, 6, 8 Gy), (Siemens, Germany) at room temperature. For a uniform X-ray distribution, we used culture dishes with 1.5 cm thick organic glass at the top and bottom.

Invasion & migration assay

The invasiveness capacity of cells was evaluated using 24-well matrigel invasion chambers (8 μ l pore size; Becton-Dickinson Biosciences, New Jersey, USA). Cells were irradiated by X-ray, after being cultured for 24 hrs and then cells were transferred into the upper chamber at a concentration of 10 \times 10⁴ in 200 μ l of RPMI-1640 containing 1% FBS and 600 μ l RPMI-1640 supplemented with 10% FBS were added to the lower chamber. After 24 hrs of incubation in humidified 5% CO₂ atmosphere at 37°C, cells remaining on the upper surface of the matrigel-coated polycarbonate membrane

were removed by scraping with a cotton swab, and the cells invading the lower surface were stained with 0.1% crystal violet. The number of cells was counted under microscope at a magnification of \times 200. Cell migration capacity was assessed using a similar approach but without matrigel coating.

RNA extraction and QRT-PCR

Total RNA was extracted from SW480 colorectal cancer cells 48 hrs post-irradiation by using TRIzol reagent as indicated by the manufacturer, then, treated by RNeasy kits with DNase 1. The purity and integrity of RNA in each sample was detected by spectrophotometer (Thermo, Shanghai, China). One μ g RNA was transformed into cDNA by using the M-MLV reverse transcriptase kit (TaKaRa, Dalian, China). The SYBR[®] Premix Ex Taq TM Kit (TaKaRa) was used in PCR analysis. Amplification reactions were performed in standard settings with 95°C for 10 min and 40 cycles of 95°C for 30 s and 60°C for 1 min. The sequences of the primers used are presented in Table 1. QRT-PCR assays were carried out in three independent experiments for each sample. The expression levels were analyzed by comparing the 2^{- $\Delta\Delta$ Ct} value.

Table 1. The sequences of the primers used

Gene	Primer sequences	Sizes (bp)
E-cadherin	F:5'-CCCCATACCAGAACCTCGAAC-3'	169
	R:5'-TTCTTGGGTTGGGTCGTTGTA-3'	
Vimentin	F:5'-GAGAAGCTTTGCCGTTGAAGC-3'	144
	R:5'-CTCAATGTCAAGGGCCATCT-3'	
GAPDH	F:5'-GGTCGGAGTCAACGGATTTG-3'	218
	R:5'-GGAAGATGGTGATGGGATTTC-3'	

Western blot analysis

SW480 cells were washed with PBS, and then homogenized in lysis buffer (Tris-buffered saline, PH 7.5, containing 1% Triton X-100). The supernatants were collected after centrifugation at 12000 rpm at 4°C for 5 min and frozen at -20°C until use. Protein concentration was determined by the BCA protein assay. Equal amounts of protein were separated by 10% SDS gel electrophoresis, and subsequently transferred onto nitrocellulose membranes. The membranes were blocked using 5% non-fat milk, supplemented with 0.05% Tween-20 (TBST) at room temperature for 1 hr. Then, the samples were incubated with primary antibody at 4°C overnight. The target signal bands were visualized by using the West Femto system (PIERCE, New Jersey, USA).

Statistics

All statistical analyses were performed using SPSS17.0. Results were expressed as the mean (\pm SD) of three independent experiments. Differences between groups were analyzed by using the one-way ANOVA multiple comparisons test. P<0.05(*) or P<0.01(**) were considered statistically significant.

Results

The morphological characteristics of SW480 CRC cells changed consistently with EMT after X-ray irradiation

The phenotypic changes were observed in both non-irradiated and irradiated cells. After 48 hrs of irradiation (2, 4, 6, 8 Gy), cells acquired the mesenchymal phenotype and lost the epithelial phenotype compared with non-irradiated cells, which was evidenced by intercellular separation, loss of polarity, spindle-cell shape and pseudopodia formation (Figure 1).

The invasion and migration capacity of SW480 CRC cells was enhanced after X-ray irradiation

Compared with non-irradiated cells, changes consistent with EMT were observed in irradiated cells. For further research, we evaluated the cell

invasion and migration capacity using Transwell chamber assays. In invasion assays, X-ray irradiation significantly increased cell invasion in the 4 Gy group ($p < 0.05$) and in the 8 Gy group ($p < 0.05$) compared with the control 0 Gy group, respectively. Invasion was also increased in the 8 Gy group compared with the 4 Gy group ($p < 0.05$) (Figures 2,4). X-ray irradiation also enhanced cell migration significantly in the 4 Gy ($p < 0.05$) and 8 Gy ($p < 0.01$) groups compared with the 0 Gy group, respectively. There was no significant difference between the 4 Gy and 8 Gy groups (Figures 3,4). X-ray irradiation significantly enhanced the invasion and migration capacity of CRC cells.

X-ray irradiated SW480 CRC exhibited changes in molecular expression associated with EMT

After X-ray irradiation, the phenotype and the invasion and migration capacity of cells were

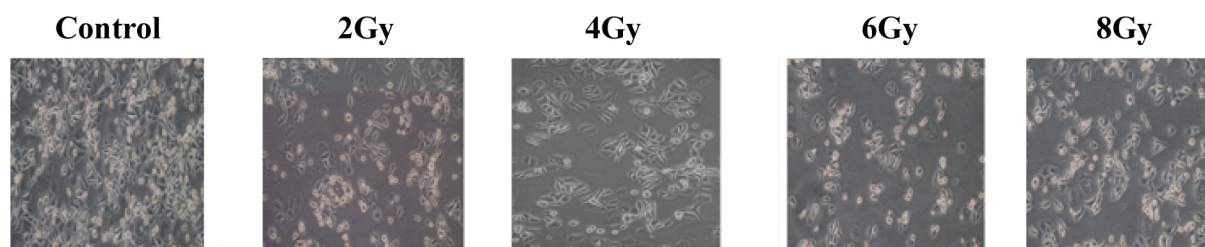


Figure 1. Compared with the control group (0Gy), X-ray irradiation (2, 4, 6, 8Gy) changed the morphology of SW480 colorectal cancer cells after 48hrs, which lost the epithelial phenotype and acquired the mesenchymal phenotype.

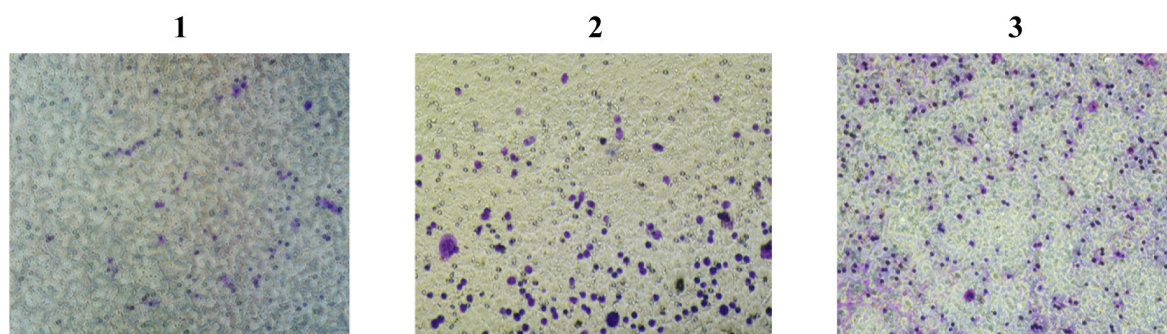


Figure 2. X-ray irradiation enhanced the invasion capacity of SW480 cells. **1:** 0Gy group, **2:** 4Gy group, **3:** 8Gy group. X-ray irradiation significantly increased cell invasion in the 4Gy and the 8Gy groups compared with the control 0Gy group. Also invasion was increased in the 8Gy group compared with the 4Gy group.

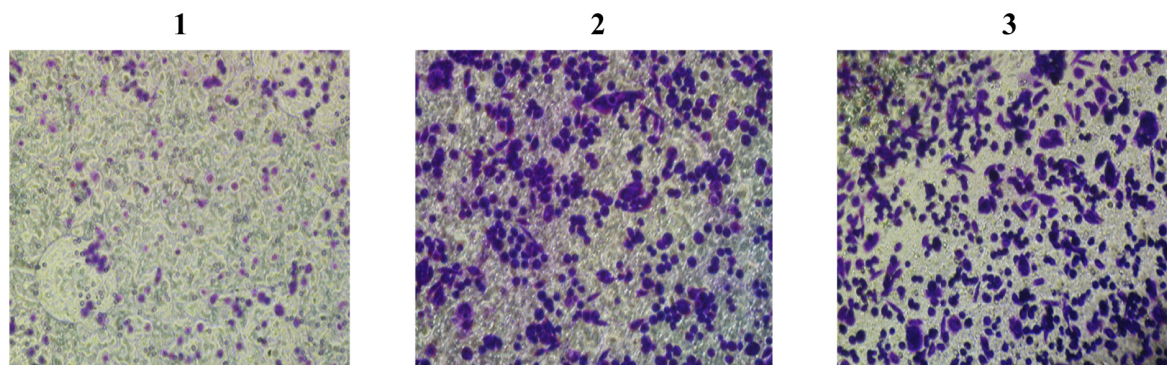


Figure 3. X-ray irradiation enhanced the migration capacity of SW480 cells. 1, 2 and 3 groups are the same as in Figure 2. X-ray irradiation enhanced cell migration significantly in the 4Gy and 8Gy groups compared with the 0Gy group, but no significant difference was noted between the 4Gy and 8Gy groups.

changed consistently with EMT. To detect the association of irradiation and EMT, we performed SYBR Green quantitative real-time PCR (Figure 5). Compared with non-irradiated cells, the expression of E-cadherin associated with epithelium was significantly decreased ($p < 0.05$ or $p < 0.01$) in X-ray irradiated cells (2, 4, 6, 8 Gy). However, the expression of the mesenchymal marker vimentin was significantly increased in irradiated cells (2, 4, 6, 8 Gy) compared with non-irradiated cells ($p < 0.05$ or $p < 0.01$). Furthermore, the decrease of E-cadherin in the 8 Gy group was more significant than in

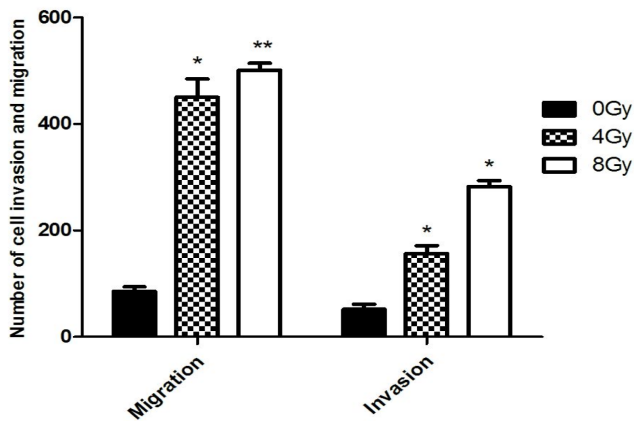


Figure 4. Compared with non-irradiated cells, the invasion and migration capacities in different dose of X-ray dose groups were increased. X-ray irradiation enhanced significantly cell invasion and migration in the 4Gy and 8Gy groups compared with the 0Gy group. * $p < 0.05$, ** $p < 0.01$.

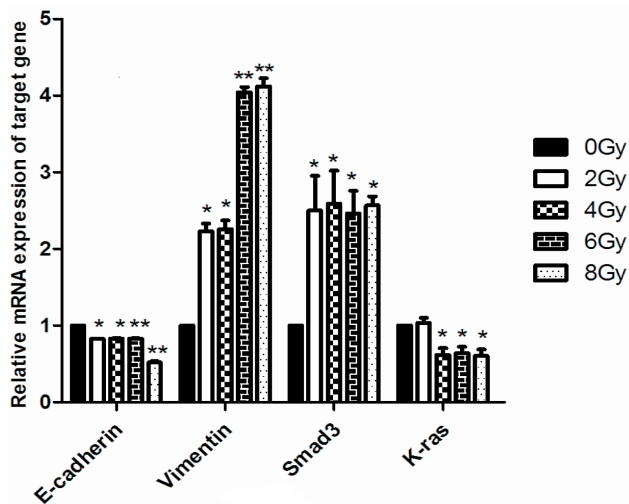


Figure 5. The mRNA expression of the target genes in different X-ray dose groups. Compared with non-irradiated cells, the expression of E-cadherin was significantly decreased in X-ray irradiated cells (2, 4, 6, 8Gy), but the expression of vimentin was significantly increased. The decrease of E-cadherin in the 8Gy group was more significant than in the 2, 4 and 6 Gy groups and there was no significant change between the 2, 4 and 6 Gy groups. The increase of vimentin was more significant in the 6 and 8Gy groups than in the 2 and 4Gy groups. * $p < 0.05$, ** $p < 0.01$.

the 2, 4, 6 Gy groups, and there was no significant change between the 2, 4, 6 Gy groups. Nevertheless, the increase of vimentin was more significant in the 6, 8 Gy groups than in the 2 and 4 Gy groups. No significant change was found between the 2 Gy and 4 Gy groups or between the 6 Gy and 8 Gy groups.

To determine the potential mechanisms of X-ray-induced EMT in SW480 CRC cells, we examined the expression of Smad3 and K-ras. They were associated with TGF- β_1 and EGF signaling pathways, respectively, and, both of them can induce EMT. QRT-PCR revealed that the expression of Smad3 was enhanced in irradiated cells (2, 4, 6, 8 Gy) compared with non-irradiated cells ($p < 0.05$); however, the expression of K-ras was decreased in the 4, 6, 8 Gy groups compared with the 0 Gy group. There were no significant changes between different X-ray dose groups (Figure 5).

The expression of EMT-associated protein in X-ray irradiated SW480 colorectal cancer cells

To determine whether X-ray irradiation can induce changes consistent with EMT and through which signaling pathway X-ray induces EMT in SW480 CRC cells, we performed Western blot (Figure 6). Compared with non-irradiated cells, the expression of E-cadherin in irradiated cells (2, 4, 6, 8 Gy) was significantly decreased, and the higher the X-ray dose, the lower its expression level. However, the expression of vimentin was increased in irradiated cells (2, 4, 6, 8 Gy) compared with non-irradiated cells, and the higher the X-ray dose, the

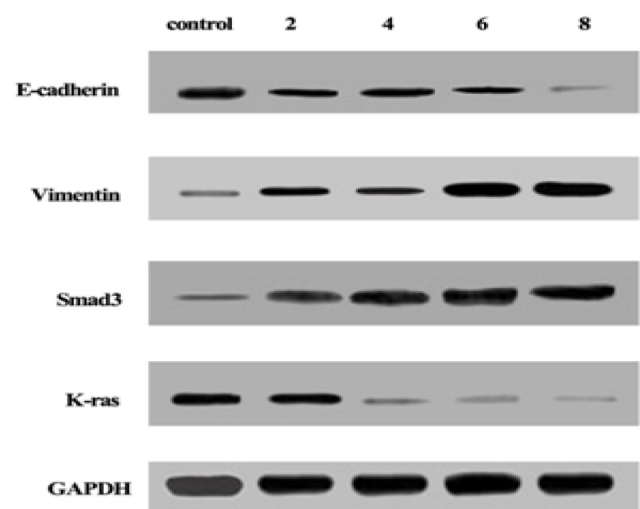


Figure 6. Western blot for the protein expression of the target genes in different X-ray dose groups. Compared with non-irradiated cells, the expression of E-cadherin and K-ras in irradiated cells (2, 4, 6 and 8Gy) was significantly decreased and the expression of vimentin and Smad3 was increased in irradiated cells (2, 4, 6 and 8Gy) compared with non-irradiated cells.

higher was its expression level. To investigate the potential mechanisms of these changes, we also observed the protein expression of Smad3 and K-ras (Figure 6). Compared with non-irradiated cells, the expression of Smad3 was increased in irradiated cells (2, 4, 6, 8 Gy), but that of K-ras was decreased.

These results indicate that X-ray irradiation can induce EMT in SW480 CRC cells, through the Smad signaling pathway.

Discussion

Radiotherapy plays an important role in the adjuvant therapy of CRC. Compared with the common complications induced by irradiation, the changes of biological characteristics in cancer cells are more serious. There were some reports that irradiation can promote the invasion and migration of CRC cells [18,19], and these characteristics were similar to those of EMT; EMT in CRC cells can also enhance the invasion and migration capacity [9,10].

In 1982, Greenburg and Hay showed that EMT means that epithelial cells transform into mesenchymal cells in physiological or pathological cases [14]. Intercellular separation, loss of polarity, spindle-cell shape and pseudopodia formation are characteristics of EMT, and these characteristics can promote invasion and migration of cancer cells. It has been reported that irradiation could induce EMT in endometrial cancer [11], cervical cancer [12], breast cancer [13], nasopharyngeal cancer [20], etc. However, studies about irradiation-induced EMT in CRC were very few and those about its potential mechanisms even fewer. X-ray irradiation can induce EMT through many signaling pathways [17], such as TGF β ₁-Smads, EGF-ras, etc. In the TGF β ₁ signaling pathway, Smad3 is a representative marker; Smad3 has 92% homology to Smad2, and together they compose P-smad2/3 which plays an important role in the TGF β ₁ signaling pathway. Nevertheless, ras also is a repre-

sentative marker in the EGF signaling pathway, and K-ras has a high expression in CRC.

In our study, the SW480 CRC cell phenotype and the markers associated with epithelial or mesenchymal cells were observed before and after X-ray irradiation. The changes of cell phenotype consistent with EMT, the enhancement of invasion and migration capacities, the increase of E-cadherin and the decrease of vimentin indicate that X-ray irradiation can induce EMT in SW480 CRC cells. Furthermore, we evaluated the expression of Smad3 and K-ras by using QRT-PCR and Western blot to determine through which signaling pathway X-ray irradiation induces EMT in CRC cells. Our studies indicate that X-ray irradiation may induce EMT in SW480 CRC cells through the Smad signaling pathway, which is evidenced by the increased mRNA and protein expression of Smad, and the decreased expression of K-ras. This observation suggests that further studies are necessary to determine the potential mechanisms and affecting factors of irradiation-induced EMT in CRC cells.

In conclusion, X-ray irradiation can enhance the invasion and migration capacities of CRC cells. X-ray irradiation may induce EMT in SW480 CRC cells through the Smad signaling pathway. Suppressing the expression of Smads may be a strategy to improve the survival of CRC patients, nevertheless further studies are needed.

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Conflict of interests

The authors declare no conflict of interests.

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