

# Effect of modulation of epithelial-mesenchymal transition regulators Snail1 and Snail2 on cancer cell radiosensitivity by targeting of the cell cycle, cell apoptosis and cell migration/invasion (Review)

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**Abstract.** Cancer is one of the leading causes of cancer-associated mortality worldwide. Several strategies of treatment, including radiotherapy, have been developed and used to treat this disease. However, post-treatment metastasis and resistance to treatment are two major causes for the limited effectiveness of radiotherapy in cancer patients. Epithelial-mesenchymal transition (EMT) is regulated by SNAIL family transcription factors, including Snail1 and Snail2 (Slug), and serves important roles in progression and cancer resistance to treatment. Snail1 and Slug also have been shown to be implicated in cancer treatment resistance. For resolving the resistance to treatment problems, combining the modulation of gene expression with radiotherapy is a novel strategy to treat patients with cancer. The present review focuses on the effect of Snail1 and Slug on cancer radiosensitivity by targeting cell apoptosis, the cell cycle and cell migration/invasion.

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## 1. Introduction

Cancer is one of the leading causes of mortality in more and less economically developed countries (1). It was estimated that 14.9 million new cancer cases and 8.2 million cancer-associated mortalities occurred in 2012 globally, among which lung and breast cancer were the most frequently diagnosed cancer types in overall and less developed countries, respectively (2). It is predicted that 22 million new cancer cases will occur annually within two decades (3). Based on these data, cancer can be considered as a significant public health issue worldwide, and thus requires intense research into the improvement of prevention strategies and enhancing the effectiveness of treatment. Numerous types of curative therapy, including chemotherapy (4), gene therapy (5), radiotherapy and immunotherapy (6,7), are used to treat cancer. Radiotherapy is an effective and commonly employed treatment in the management of >50% of human malignancies and remains a standard therapeutic modality for cancer patients (8,9). However, intrinsic or acquired resistance, such as genetic abnormalities, which lead to the promotion of angiogenesis and tissue progression, limit the efficacy of radiotherapy (10,11). Cancer gene therapy, which represents one ideal therapeutic tool, can be combined with radiotherapy to enhance the radiotherapy effect (12). Epithelial-mesenchymal transition (EMT) is

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*Abbreviations:* EMT, epithelial-mesenchymal transition; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; shRNA, short hairpin RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TGF- $\beta$ , transforming growth factor- $\beta$ ; rAAV2, type 2 recombinant adeno-associated virus; MDCK, Madin-Darby canine kidney; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorting

*Key words:* cancer radiosensitivity, Snail1, Snail2, cell apoptosis, cell cycle, cell migration, cell invasion

involved in cancer cell invasion (13), migration (14), resistance to apoptosis (15), the cell cycle (16) and therapy resistance (17). Modulation of EMT could change the behaviors of cancer cells against therapies, particularly radiotherapy (18). The SNAIL transcription factor family has been associated with EMT (19). Snail1 and Slug are key SNAIL family transcription factors that regulate EMT, and are also involved in cancer progression (20) and resistance to treatment such as radiotherapy (21). The present study reviews the literature wherein the modulation of Snail1 and Snail2 (Slug) expression was shown to influence cancer cell apoptosis, the cell cycle and cell invasion/migration, and in which Snail1 and Slug modulation enhanced the efficacy of radiotherapy by targeting cancer cell apoptosis, the cell cycle and cell invasion/migration.

## 2. EMT and Snail1/Slug of the SNAIL family

EMT is recognized as a phenotypic conversion that occurs during embryo development, as gastrulation, and during neural crest formation in nervous system development (22). EMT has also been described in the process of re-epithelialization during wound healing and in the generation of tissue fibroblasts during the process of organ fibrosis. EMT is a key step in the metastasis and invasion of tumors (23), in the development of tumor resistance to apoptosis and in cancer radiotherapy resistance (24). A central group of EMT regulators is the SNAIL superfamily of transcription factors, which includes Snail1 and Slug (Fig. 1), the most highly studied members of this family (25-28). SNAIL family members are highly expressed in a variety of cancer types and have been implicated in the regulation of tumor invasion, metastasis, cell survival and cell proliferation (29-36). Proteins of the SNAIL family have a similar structural organization. The carboxyl terminus contains (4 to 6 motifs in the terminus) C2H2 zinc-finger motifs that facilitate the direct binding of the protein to DNA. The CAGGTG sequence is the consensus DNA binding site for SNAIL family proteins and this motif is a subset of the E-box sequence to which a number of basic helix-loop-helix transcription factors bind (37,38).

The SNAIL gene (Snail1/Snail2) is implicated in EMT via the suppression of epithelial markers (E-cadherin, vascular endothelial cadherin, claudin, occludin, desmoplakin, cytokeratin and mucin) associated with an epithelial phenotype (39-42) and via upregulation of mesenchymal markers (fibronectin and vitronectin) associated with the mesenchymal phenotype (31,39). Snail-mediated EMT (Snail1/Snail2) associated with the suppression of E-cadherin causes inhibition of cancer cell adhesion and promotes the migratory capacity (43). At the molecular level, EMT regulation by Slug is often associated with its ability to transcriptionally repress the expression of epithelial gene E-cadherin (26). In epithelial cells, Snail transcription is low and E-cadherin expression is high, which prevents the stimulation of NK- $\kappa$ B and other signaling pathways. Moreover, external stimuli, such as transforming growth factor- $\beta$  (TGF- $\beta$ ) expression, can induce Snail1/Snail2 protein activation (44), which then binds to the SNAIL gene (Snail1 protein binds to Snail1 gene and Snail2 protein binds to Snail2 gene) (45,46). When E-cadherin expression is inhibited, SNAIL (SNAIL1/SNAI2) expression is amplified by a self-stimulation loop due to the suppression of nuclear factor- $\kappa$ B (NF- $\kappa$ B).

Thus, the activity of the self-stimulation loop is enhanced by the downregulation of E-cadherin via SNAIL (Snail1/Snail2). Additionally, the induction of mesenchymal genes and other suppressors, including zinc finger E-box-binding homeobox 1 (Zeb1), by NF- $\kappa$ B activation, leads to SNAIL inhibition by Zeb1 without a phenotype reversion. This could explain why the SNAIL (Snail1/Snail2) gene is required for triggering EMT (47).

## 3. Snail1, Slug and cancer cell apoptosis

Snail1- or Slug-mediated EMT in cancer cells generates a phenotype closely associated with the resistance of cancer cells to apoptosis (48,49). However, little research has been performed on cancer cells with regard to the link between the direct or indirect modulation of Snail1/Slug and cancer cell apoptosis.

*Modulation of Snail1 and cancer cell apoptosis.* It has been reported that the direct or indirect modulation of Snail1 expression affects cancer cell apoptosis. Through use of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays to assess apoptosis, Aletaha *et al* (50) showed that knockdown of Snail1 enhanced breast cancer cell apoptosis. In another study, Kajita *et al* (51) reported that, following induction of DNA damage by exposing breast cancer cells to topoisomerase inhibitor Adriamycin (ADR), the relative apoptotic activity of parental breast cancer cells was substantially increased relative to that of adeno-Snail1 MCF-7 cells (overexpressing Snail1), suggesting that Snail1 acts to prevent ADR-induced cell death in breast cancer cell lines. In a prostate cancer cell line, following the evaluation of caspase 3 and caspase 7 activities by fluorescence detection as a marker of apoptosis, Osorio *et al* (48) showed that Snail1 overexpression decreased the rate of cell apoptosis and that prostate cancer cells with Snail1 silencing (shRNA-Snail1) exhibited increased apoptosis (48). Franco *et al* (52) also found that Snail1 downregulation enhanced the apoptosis in murine hepatic cells, and that activation of its expression blocked the apoptotic effect of TGF- $\beta$  in adult hepatocytes. Wan *et al* (53) reported that the inhibition of Snail1 enhanced TRAIL-induced apoptosis by upregulating cellular tumor antigen p53 expression following combined hepatocarcinoma cell transfection with lentiviral short hairpin (sh)Snail1 and adenovirus type 5-TRAIL. However, in contrast to the aforementioned reports, the study by Olmeda *et al* (54) showed that there was no significant difference in the apoptotic index of the tumors caused by sh-Snail1-derived cells and their corresponding controls, and that there was also no change in the apoptotic response to serum deprivation in HaCa4 shSnail1 and CarB-ShSnail1 cells compared with that in their corresponding parental or control cells. Taken together, these data suggest that Snail1 acts as an inhibitor of apoptosis and that this function is dependent on the type of cell line or tissue.

*Modulation of Slug and cancer cell apoptosis.* It has also been reported that the modulation of Slug can affect cancer cell apoptosis. By analyzing the expression levels of the B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X (Bax) apoptosis markers, Wu *et al* (49) revealed that silencing of Slug using Slug-shRNA or microRNA-497 (miR-497) in a non-metastatic breast cancer cell line (MCF-7) enhanced the apoptotic index.

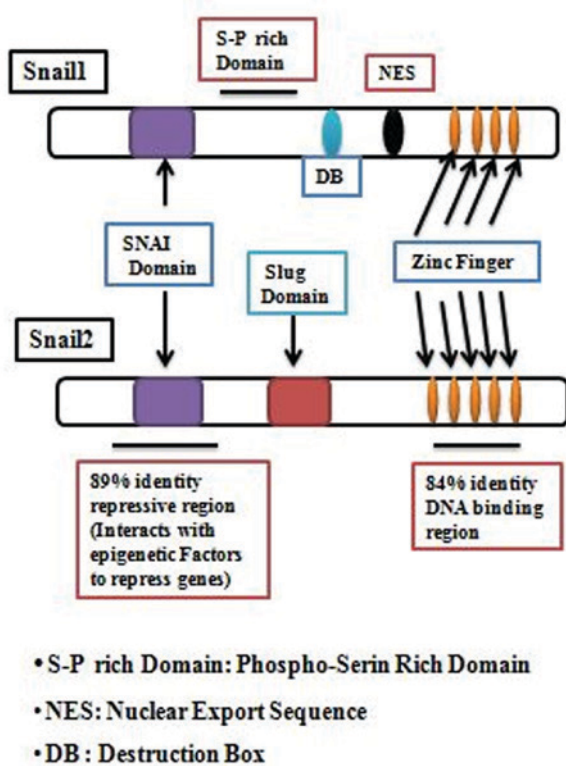


Figure 1. Schematic diagram of the function domain of Snail1 and Snail2 zinc finger transcription factors.

Kajita *et al* (51) also reported that following transfection of MCF-7 cells with Slug adenovirus to induce slug overexpression (MCF-7adSlug) and treatment with ADR as a cell apoptosis inducer, there was a notable reduction in the apoptotic abilities of treated cells (MCF-7adSlug) relative to that of untreated cells, suggesting that Slug acted as an apoptosis inhibitor in the breast cancer cell line. In another cancer cell line (PyMT-N-cad), Kim *et al* (55) showed that Slug attenuation by shRNA or fibroblast growth factor receptor inhibitor in a mammary tumor cell line increased caspase3 activity and poly(adenosine diphosphate ribose) polymerase levels, which are markers of apoptosis. It was previously shown that Slug silencing in human alveolar epithelial A549 cells and treatment with apoptosis-inducer tumor necrosis factor- $\alpha$  increased the apoptotic index in Slug-silenced cells (56). Mancini *et al* (57) also demonstrated that Slug overexpression contributes to apoptosis resistance in leukemic progenitors. In contrast to this study, and in confirmation of other aforementioned studies, Zhang *et al* (58) assessed apoptosis by measuring caspase 3 activity, TUNEL assay and Hoechst 33258 staining, and showed that slug overexpression does not have a significant effect on the apoptotic index in the TE-7 cell line, but that the inhibition of Slug expression in the esophageal cancer OE33 cell line leads to a marked increase in apoptosis *in vitro* and *in vivo*. This suggests that Slug silencing can effectively inhibit tumor growth *in vitro* and *in vivo* through the induction of apoptosis. According to these data, Snail1 and Slug modulation could have a significant role in cancer therapy and improve cancer therapy effectiveness when the modulation is combined with another cancer therapeutic strategy such as

radiotherapy. However, further studies are required regarding the link between Snail1/Slug inhibition or overexpression and the apoptosis in different cancer cell lines.

#### 4. Snail1, Slug, cell apoptosis and radiosensitivity

Apoptosis, also known as programmed cell death, serves an important role in cancer cell radiation sensitivity. To date, there have been few studies concerning the roles of EMT transcription factors Snail1 and Slug in cancer radiosensitivity, specifically by targeting cell apoptosis. According to the aforementioned description of the association between Snail1 and cancer apoptosis, the modulation of Snail1 could impair cancer radiosensitivity by targeting cell apoptosis. Mezencev *et al* (59) found that MCF-7 cells with ectopic expression of Snail1 displayed increased radiosensitivity, but the association with apoptosis has yet to be studied. This study does not correlate with the study by Escriva *et al* (60), which showed that ectopic expression of Snail1 in the MDCK cell line induced cancer cell radioresistance by diminishing the apoptosis caused by irradiation where only 8-10% of cells that ectopically expressed Snail1 underwent apoptosis 48 h after  $\gamma$ -irradiation (60). However, cells with decreased Snail1 expression displayed a higher sensitivity to irradiation-induced apoptosis, as described in the study by Zhang *et al* (61), which stated that the combination of  $\gamma$ -irradiation (6 Gy) and type 2 recombinant adeno-associated virus (rAAV2)-small interfering (si)RNA-Snail lead to a markedly enhanced apoptotic response and radiosensitivity in pancreatic PANC-1 cells and to decreased radiosensitivity in MDCK cells via the targeting of apoptosis due to Snail1 overexpression (60). The data suggest that the modulatory role served by Snail1 in cancer cell radiosensitivity via the targeting of apoptosis is dependent on the type on cells. As with Snail1, it has been reported that Slug modulation also impairs cancer cell radiosensitivity by targeting cell apoptosis. Zhang *et al* (62) used the TUNEL assay to show that transfection with Slug siRNA and adenovirus rAAV2 for Slug silencing combined with 6 Gy irradiation significantly increased the apoptosis in a human cholangiocarcinoma cell line compared with that found with Slug silencing or irradiation alone. In the oral squamous carcinoma HSC3 and HSC6 cell lines, Jiang *et al* (63) used caspase 3, Bax and Bcl-2 expression levels as apoptosis markers and showed that X-ray irradiation improved Slug expression, and that the inhibition of Slug associated with X-ray irradiation enhanced the apoptosis induced (63). Inoue *et al* (64) also reported that Slug (-/-) mice are more radiosensitive compared to the Wild-type mice, and used TUNEL assays to demonstrate that hematopoietic progenitor of Slug (-/-) mice exhibited increased apoptosis 6 h after irradiation (6 Gy). Arienti *et al* (65) used western blotting to analyze the expression level of certain apoptotic marker proteins (caspase 3, p53 upregulated modulation of apoptosis and p21) and found that the inhibition of slug expression in melanoma cells enhanced their radiosensitivity by increasing the expression of these proteins. Therefore, Slug inhibition was shown to improve radiosensitivity by targeting apoptosis in the melanoma cell line. Thus, Snail1 and Slug modulation is suggested to modulate cancer cell radiosensitivity. However, this suggestion should be confirmed in various types of cancer cell lines.



## 5. Snail1, Slug and the cell cycle

*Modulation of Snail1 and the cell cycle.* The cell cycle is one of the important steps to cancer cell progression and the response to radiotherapy. Moreover, Snail1 has a major role in certain steps of cancer development, including cell cycle progression. To date, few studies have been conducted on the link between Snail1 and the cancer cell cycle. Using a fluorescence-activated cell sorting (FACS) assay to assess the cell cycle, Aletaha *et al* (50) showed that Snail1 inhibition in MDA-MB-468 cells regulated G<sub>1</sub> phase transition (early and late) and the G<sub>1</sub>/S checkpoint, which resulted in cell cycle arrest at the sub-G<sub>1</sub> and S phases. Moreover, Vega *et al* (66) reported that in MDCK-Snail1 cells stably overexpressing Snail1 following transfection, the majority (93%) were in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle under basal conditions after 72 h in culture. These data suggested that Snail1 modulation can impair cell cycle progression by causing cell cycle arrest and that the phase of the cell cycle in which the cells are arrested is dependent on the type of cancer cell.

*Modulation of Slug and the cell cycle.* As with Snail1, Slug is also involved in the control of cell cycle progression. Mittal *et al* (67) showed that Slug is positively correlated with cyclin D1, which serves a pivotal role in cell cycle control in normal and cancer cells, particularly in the G<sub>1</sub> phase of the cell cycle (68). Downregulation of Slug could lead to the inhibition of cyclin D1 expression and cell cycle arrest in the G<sub>1</sub> phase (67). This hypothesis does not correlate with the findings of the study by Liu *et al* (69), in which Slug expression was negatively correlated with cyclin D1 expression in a prostate cancer cell line. According to this study, the induced expression of Slug can lead to the inhibition of cyclin D1 and cell cycle arrest in the G<sub>0</sub>/G<sub>1</sub> phase; therefore, the effect of Slug modulation on the cancer cell cycle is also dependent on the type of cancer cell line. However, Essmann *et al* (70), through use of FACS assays, reported that the treatment of the PC3-16 cells line with Slug siRNA to downregulate its expression resulted in G<sub>0</sub>/G<sub>1</sub> cell cycle phase arrest in the majority (84.2±2.6 vs. 69.6±0.62%) of cells at 72 h post-treatment, meaning that Slug modulation has an impact on G<sub>1</sub> phase transition.

## 6. Snail1, Slug, the cell cycle and cancer radiosensitivity

Since radiosensitivity has previously been shown to be dependent on the phase of the cell cycle (71,72) it is hypothesized that targeting the cell cycle by modulating Snail1 or Slug could enhance the effect of radiotherapy. Mezencev *et al* (59) used a FACS assay to show that the ectopic expression of Snail1 increased the proportion of MCF-7 cells in the G<sub>2</sub>/M phase. This suggested that this proportion of G<sub>2</sub>/M phase cells could be increased following irradiation, which can be implicated in high radiosensitivity, as cells are more sensitive to irradiation during the M and G<sub>2</sub> phases (72). In the MDCK cell line, it was reported that MDCK-Snail1 clones presented with a high proportion of cells in the G<sub>1</sub> phase relative to the control (40 vs. 20%), during and at 48 h post-irradiation. Additionally, an increase in G<sub>2</sub>/M MDCK-Snail1 cell cycle arrest (56% of MDCK-Snail1 cells in the G<sub>2</sub>/M phase) was noted (66). As aforementioned, Slug also can modulate the

radiosensitivity of cancer cells by targeting the cell cycle. In melanoma cancer cell lines, Arienti *et al* (65) showed that Slug silencing with or without irradiation impaired cell cycle progression. Radiation treatment enhanced the percentage of G<sub>2</sub>/M phase cells in the M14 and M19 cell lines, an effect that was greater with 5 Gy than with 2.4 Gy of treatment (65). Slug silencing further increased the proportion of G<sub>2</sub>/M phase M14 cells following irradiation with 5 Gy, confirming the results of the study by Mezencev *et al* (59), and increased the percentage of M19 cells in the S phase. Concordant with the results by Arienti *et al* (65) using M19 cells, the study by Jiang *et al* (63) found that X-ray treatment combined with Slug silencing increased the proportion of cells arrested in the S phase, as compared with Slug silencing or X-ray alone, in the HSC3 and HSC6 cell lines. Therefore, the modulation of Snail1 and Slug expression can impair cell cycle arrest and modulate cancer cell radiosensitivity by targeting the cell cycle. However, the efficacy of this approach is dependent on the type of cancer.

## 7. Snail1, Slug and cell migration/invasion

Several studies have implicated the modulation of Snail1 and Slug expression in cancer cell migration and invasion (48,73-75). A study using a scratch-wound assay to assess the directional migration of the breast cancer MDA-MB-468 cell line reported that silencing Snail1 significantly reduced the cell migration and invasion at 24 and 48 h post-transfection (50). Zhang *et al* (76) showed that parental MDA-MB-231 cells (overexpressing Snail1) exhibited high mobility compared with MDCK cells (used as a good cell line for invasion/migration studies and with no Snail1 expression). Following transfection with AdvSnail1, MDCK-Snail1 cells started to migrate faster into the wound region relative to the control, and Adv-antisense-Snail1-transfected MDA-MB-231 cells colonized ~50% of the wound region at 24 h post-wounding, while the mock infected cells occupied almost 95% of the wound region. Following assessment of invasion ability, the percentage of invasive Adv-Snail1-transfected MDCK cells increased (0.236±0.022%), whereas the percentage of invasive non-transfected parental cells was 0.126±0.015%. The invasive ability of the MDA-MB-231 cells transfected with Adv-antisense-Snail1 was markedly decreased compared with that of the non-transfected parental cells (0.215±0.0140 vs. 0.392±0.021%) (76). Smith *et al* (77), using migration and invasion assays performed on collagen I and fibronectin matrices, reported that MCF-7-Snail1 displayed decreased cell adhesion and increased cell migration compared with mock MCF-7-Neo cells (77). These data suggested that Snail1 expression is positively correlated with cell migration and invasion abilities.

As with Snail1, the modulation of Slug expression has also been implicated in the impairment of cell invasion and migration capacities (78-84). Bai *et al* (85) used Transwell and wound-healing assays to show that the relative migrated distance at 24 h post-transfection compared with the corresponding control was ~38.5 and 23.1% in the control and MDA-MB-231 siRNA-Slug groups, respectively; similar results were found at 60 h post-transfection. The study by Chen *et al* (86) indicated that the inhibition of slug in MDA-MB-231 and MDA-MB-436 cells can lead to the inhibition of migration

to ~40% of the rate observed in control cells. This result corroborates that of the study by Liang *et al* (87), in which Slug silencing using miR-124 reduced the migration and invasion abilities of the MDA-MB-231 cell line, whereas activation of Slug by overexpression of Slug in MDA-MB-231 miR-124 cells abrogated this reduction of migration and invasion capacities. Contrary to the hypothesis that Slug-knockdown reduces cell migration/invasion, Kim *et al* (55) showed that Slug-knockdown did not inhibit cell migration and invasion in the PyMT-N-cad metastatic cell line, meaning that Snail1 and Slug inhibition-mediated reduction of cell migration and/or invasion depends on the type of the cancer cell line.

### 8. Snail1 and Slug affect radiosensitivity by targeting cell migration and invasion

The malignant progression of cancer depends on various cell properties, including mobility, invasiveness and metastatic potential, among others. It has been demonstrated that radiation can enhance the invasiveness and migratory capacity in a number of cancer cell lines (88). Young *et al* (89) also reported that a 2.3 Gy dose of irradiation was sufficient to increase the migration of metastatic MDA-MB-231 cells. Conversely, Rodman *et al* (90) reported that 2 Gy of irradiation can reduce the migratory ability of MDA-MB-231 cells. Based on these reports, the effect of radiation on cell migration and invasion is dependent on the cell type. Combining gene therapy techniques (modulating Snail1 or Slug expression) with radiotherapy could enhance the radiotherapy efficacy by reducing the migration and invasion potential caused by irradiation or by enhancing the reduction of the migration and invasion effect of radiotherapy or of the modulation of Snail1 and/or Slug. In the study by Du *et al* (91), it was demonstrated that heat shock protein 70 silencing significantly inhibited the cell invasion prior to and following irradiation in the human endometrial cancer ISK cell line. Moreover, activation of caspase 9 combined with irradiation enhanced the human glioma SNB19 cell invasion ability compared with the use of caspase 9 activation or irradiation alone (92). Taken together, radiotherapy and gene therapy may have a greater benefit on cell invasion or migration compared with irradiation or gene therapy alone. However, more studies should be performed in future confirming the aforementioned data and hypotheses, particularly that regarding Snail1 or Slug inhibition enhancing cancer radiosensitivity by targeting cell migration and invasion, in various types of cancer cell line.

Based on these data, Snail1 and Slug inhibition can be considered to have an ability to improve cancer radiosensitivity by increasing cancer cell apoptosis, acting by arresting the cancer cell cycle, while the association between Snail1 and Slug inhibition and cancer radiosensitivity by targeting cell migration or invasion remains to be elucidated (Fig. 2).

### 9. Conclusions

Snail1 and Slug are SNAIL family transcription factors that have been studied in a number of cancer cell types. As EMT regulators, Snail1 and Slug are highly expressed in numerous cancer cell types and are implicated in cancer cell cycle progression, cell apoptosis and cell migration/invasion.

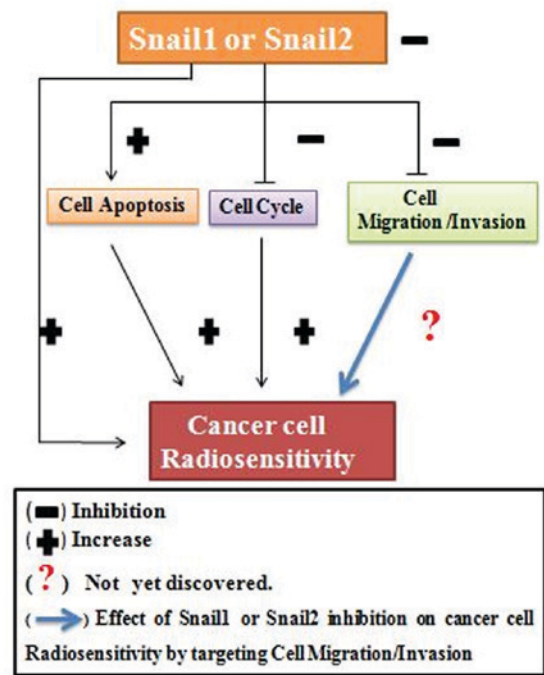


Figure 2. Schematic diagram of the link between Snail1 and Snail2 inhibition, cell apoptosis, the cell cycle, cell migration/invasion and cancer cell radiosensitivity.

Modulation of the expression of these genes is implicated in the impairment of cancer progression and cancer cell radiosensitivity by targeting of cell apoptosis, the cell cycle and migration/invasion. Due to the limitations of radiotherapy or gene therapy alone, the combined use of gene therapy, for inhibiting the expression of Snail1 or Slug, and radiotherapy, enhances the cancer cell radiosensitivity. However, more research is required concerning the effect of the modulation (inhibition or overexpression) of expression of Snail1 and Slug on cancer radiosensitivity, particularly in different types of cancer cell line. This could aid radio-oncologists in mastering how to manipulate these genes prior to or following radiotherapy for the enhancement of radiotherapeutic efficacy.

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### Availability of data and materials

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### Authors' contributions

GA was in charge of designing and writing the manuscript. YZ was involved in drafting the manuscript. Both authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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