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Original Research Article

LncRNA gas5 regulates granulosa cell apoptosis and viability following radiation by X-ray through sponging miR-205-5p and Wnt/β-catenin signaling pathway ingranulosa cell tumor of ovary

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Abstract

Purpose: *The study explored the role of lncRNA gas5 in ovarian granulosa cells exposed to X-ray in granulosa cell tumor of ovary(GCTO).*

Methods:*Exposed the KGN cell line (KALANG, Beijing, China) to X-ray to mimic the radiotherapy for GCSO patients in vitro, cell viability was checked by CCK8 assays. RT-qPCR detected the RNA expression of apoptosis-related genes while Western Blot for biomarkers in wnt/β-catenin signaling. Differential expressions of lncRNA gas5 were examined after cells exposed to X ray for 0,24,48hs. We over expressed gas5 and assessed resultant cell viabilities, apoptosis and signaling. The sponging between gas5 and miR-205-5p was verified through Luciferase Assay. CCK8, RT-qPCR and Western Blot were applied for investigations into the correlation between miR-205-5p and cell viability and apoptosis after miR-205-5p augmentation. Similarly, the interactions between the gas5 and miR-205-5p were assessed after co-transfection of miR-205-5p mimics and oe-gas5. Last, wnt inhibitor was used to study the role of signaling pathway in KGN cells.*

Results: *Exposure of KGN toX-ray reduced cell viabilities and increased apoptosis. Gas5 had reduced expression in cells while miR-205-5p increased. Gas5 upregulation could protect the cells from apoptosis and add to the cell viability and activation of wnt//β-catenin signaling. lncRNA gas5 targeted miR-205-5p and miR-205-5p mimics could counteract functions of up-regulated lncRNA gas5, regulating Wnt/β-catenin signaling pathway. Inactivation in Wnt/β-catenin could suppress cell viability.*

Conclusions: *lncRNA gas5 regulated the cell apoptosis and viability after cellular radiation, which might be a potential therapeutic target to combine into radiotherapy for GCTO patients in clinical stage.*

Keywords: *Ovary, proliferation, apoptosis, lncRNA gas5, x-ray*

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INTRODUCTION

Ovarian follicle is one of the most important structures among female organs which plays an important role in regulating progressions of proliferation, differentiation and apoptosis in granulosa cell tumor of ovary (GCTO). After primordial follicles are activated and grow into primary follicles and secondary follicles, granulosa cells begin to proliferate and form

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layers [1]. Granular cell layer is a composition of fast-changing ovarian follicles. Before oocyte and theca cells, granulosa cells are often observed in follicular initial cells that undergoapoptosis in atretic follicles, which indicate that they could be initiator of follicular atresia[2,3]. In addition, granulosa cells can secrete several factors such as gonadal steroids, endocrine hormones and growth factors, which are important for their growth and survival [4].

X-rays are types of electromagnetic radiation which are present in the outer space and are well known for their ability to penetrate human tissues [6]. They are frequently used in the diagnosis and treatment of diseases ,leading to pathological damage to tissues [5]. Among cell organelles, mitochondrion is the most sensitive to ionizing radiation [6]. Impaired mitochondrial function is an important index of oxidative damage [7]. With the advances in technology, damages from radiation, especially their effects on reproductive system, are receiving growing attention. Researches have also proved that oxidative stress is an essential pathological factor in the infertility of both males and females [8]. LncRNAs are non-coding RNAs which have more than 200 nucleotides in length [9]. These RNAs can regulate tumor growth through different mechanisms [10]. Researches have also proven that lncRNAs are important in the control of radio resistence of cancers [11]. When exposed to X-ray, expression of lncRNA-p21 increased, inhibiting β-catenin signaling and inducing apoptosis, leading to increase in sensitivity of CRC to radiation [12]. Up-regulated lncRNA ANRIL could increase resistance of cancers to radiation through suppressing apoptosis and inducing proliferation. Furthermore, its function in regulating tumors is mediated through negative controlling of miR-125a, which is a kind of tumor suppressor [13]. Though, noncoding RNAs played important roles in tumor cell growth and apoptosis as well as resistance to radiation. Currently, there are few research related to gas5 in GCTO.

In this work, functions of lncRNA gas5 and miR-205-5p would be measured to figure out mechanisms in regulating reproduction in female.

EXPERIMENTAL

Cell culture

Human ovarian granulosa cell line KGN was purchased (*KALANG, Beijing, China)*. The KGN cells were incubated in RPMI-1640 medium contained 20% fetal bovine serum (FBS), 100 umol/ml penicillin and streptomycin. After incubation, cells in log phase were collected and exposed to 10GY X-ray for 0h, 24h and 48h before harvest. The radiation was generated using Philips RT250 (Kimtron, USA)

Cell transfection

The pcDNA3.1 plasmid (4ul) (Invitrogen™, USA) was applied to clone the full-length sequence lncRNA gas5,constructing a pcDNA3.1-gas5 and an empty plasmid worked as a control. miR-205-5p mimics, mimics NC,miR-205-5p inhibitor and NC inhibitor were used for transfection adopting Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher, USA) . Hence, we achieved differential gas5 and miR-205-5p expressions in cells that had been exposed to radiation for 24h and 48h before.

RT-qPCR

Total RNAs were extracted from the cells using Trizol reagent (Beyotime, Shanghai, China) according to manufacturer's instructions. Then 20μl of TaqMan™ Reverse Transcription Reagents (Invitrogen™, USA) was applied to reverse the RNAs to cDNAs. Next, 20μl SYBR Green qPCR Mix(Beyotime, Shanghai, China) was used for PCR quantitation. Conditions of PCR: pre-denaturation, 95℃, 5min; denaturation, 95℃, 30s; annealing, 55℃, 30s; extension, 72℃, 30s, 40 cycles. T100™ Thermal Cycler (Bole, Shanghai, China) was used to analyze the results. The RNA primers for gas5, miR-205-5p, GAPDH, U6, Bcl-2, Bcl-xl and Caspas-3 were used. The relative expressions of these genes above were detected in cells with exposure to X-Ray for 0 (normal cells); 24h and 48h with or without transfection. 2-△△Ct methods were applied.

CCK-8

We collected the cells after 0,24 and 48h exposure of 10GY X-ray. Thencells were seeded into 96 well plate with 1×10^5 cells per well and incubated at 37℃, 5%CO_2. 10μl of CCK-8 solution was added into the plate at 24h, 48h and 72h and the optical density (OD) values of cells were quantified at 450nm using microplate reader (Thermo Fisher, USA). Similarly, the 24,48h groups after transfection were selected to undergo cell viability assays in order to research the correlations between the gene expression and viabilities.,

Luciferase report assay

A putative binding was predicted on Starbase(http://starbase.sysu.edu.cn/agoClipRN

A.php?source=lncRNA). KGN cells were co-transfected with 25ng lncRNA gas5 wt or lncRNA gas5 mut with 20ul mimics NC or miR-205-5p mimics through Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher, USA). After 48 hours, the luciferase reporter assay system (Promega, USA) was to analyze luciferase activity [14].

Western blot

Cells exposed to X-ray were washed with PBS twice then the cells were lysed with 250ul RIPA reagent (Beyotime, Shanghai, China) for 20min. After that, the supernatant liquid was collected after centrifugattion at 1000 rpm for 5min. Total proteins were quantified using BCA assay kit (Beyotime, Shanghai, China)[15]. Next, 40μg of total proteins were separated by SDS-PAGE and transferred into PVDF membranes after which the membranes were blocked with 8% non-fat milk powder at room temperature for at least 2h[16]. This was followed by the addition of primary antibodies and incubation of the membranes at 4℃ overnight. Primary antibodies adopted were as follows: Anti-Wnt3a antibody (1 μg/ml,ab28472), Anti-beta Catenin antibody (1:5000,ab32572) and anti-GAPDH (1:1500; ab181602). Then membranes were rinsed and Goat Anti-Mouse IgG H&L (HRP) (1:800; ab205719) and Goat Anti-Rabbit IgG H&L (HRP) (1:800; ab205718) were added and the mixture was incubated for 1h at room temperature. Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher, USA) was used for development and gray values of proteins were measured with GAPDH as the internal reference.

Statistical analysis

Data were displayed by mean±SD and analyzed through SPSS 19.0 (IBM, USA). All experiments were repeated three times. T-test was used to compare the two groups. $P<0.05$ was considered significant.

RESULTS

X-ray suppressed cell proliferation and promoted apoptosis

Compared to normal cells, cells that were radiated had lower level of cell viabilities (Figure 1A) which reduced gradually with time. The X-ray radiated cells had lower expressions of anti-apoptosis genes (Bcl-2 and Bcl-xL) and higher level of caspase-3, suggesting that irradiation promoted apoptosis (Figure 1B-D). Expressions of proteins examined by Western Blot indicated that X-ray could reduce expression level of both Wnt and β-catenin (Figure 1E).

LncRNA gas5 expressed lower and promoted proliferation in X-ray exposed cells and inhibited apoptosis

Expressions of lncRNA gas5 were detected in cells exposed to X-ray respectively for 0h,24h and 48 hr. Compared to normal cells(0h), lncRNA gas5 was expressed lower in X-ray treated cells (Figure 2A). The expression of lncRNA gas5 decreased significantly as the exposure time increased (Figure 2B, C). We upregulated the lncRNA gas5 in cells with 24h and 48h exposure to X-Ray and CCK8 results showed that increased lncRNA gas5 could promote viabilities (Figure 2D). Moreover, cell viabilities were higher in cells that were pretreated with radiation for 24h compared to those for 48h (Figure 2E). On the other hand, overexpression of lncRNA gas5 could up regulate expressions of Bcl-2 and Bcl-xL but inhibited expression of caspase-3 (Figure 2F, G). Expressions of wnt3a and beta-catenin were increased by overexpressed lncRNA gas5 (Figure 2H).

MiR-205-5p is the target of lncRNA gas5 and promoted viability in cells with pre-exposure to radiation

Starbase v2.0 predicted that miR-205-5p targeted lncRNA gas5 (Figure 3A). Therefore, luciferase reporter assay was applied to make sure of the binding sites between lncRNA gas5 and miR-205-5p, showing that the luciferase activity only decreased significantly in the group that was co-transfected with wild-type of gas5 and miR-205-5p mimics, which indicated that wild type of miR-205-5p could directly bind lncRNA gas5 (Figure 3B). miR-205-5p expression was higher in the group with X-ray exposure than untreated cells (Figure 3C). Thereafter, we knocked down miR-205-5p by transfecting miR-205-5p inhibitor into the cells with pre-exposure to radiation for 24h and 48h.

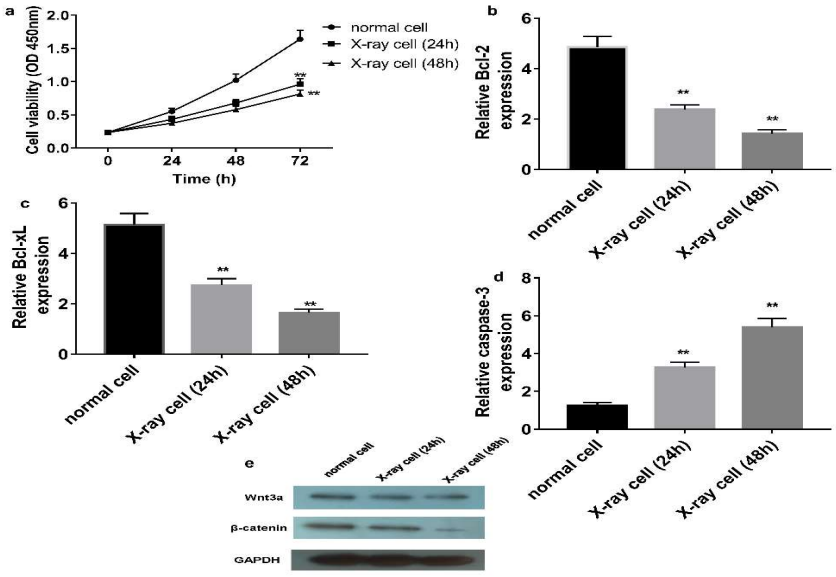

Figure 1: X-ray suppressed proliferation and accelerated apoptosis of KGN cells. A. Cell viabilities were detected through CCK-8, p<0.05. B, C, D. RT-qPCR was applied to measure expressions of factors related to apoptosis, p<0.05. E. western blot was used to evaluated expressions of proteins in Wnt/β-catenin signaling pathway, p<0.05.

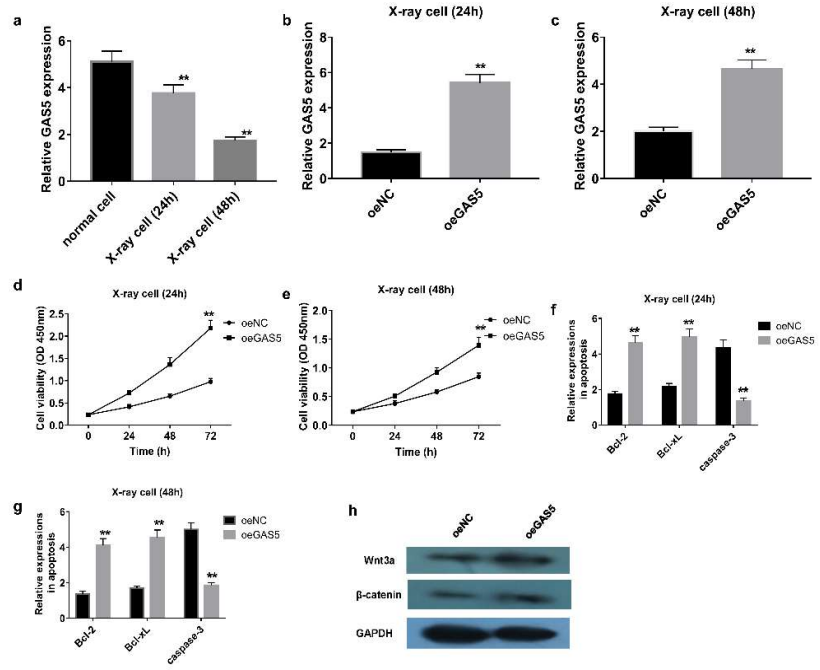

Figure 2: LncRNA gas5 expressed lower in X-ray treated cells with promoting proliferation and repressing apoptosis. A. Expressions of gas5 were analyzed through RT-qPCR, p<0.05. B, C. overexpressed gas5 expressions were detected by RT-qPCR, P<0.05. D, E. CCK-8 was applied to measure cell viabilities, p<0.05. F, G. Apoptosis was validated through RT-qPCR, p<0.05. H. Expressions of proteins were evaluated by western blot, p<0.05

Lower expression of miR-205-5p was detected (Figure 3D, E). Correspondingly, cell viabilities were measured, which disclosed that in cells with pre-exposure to radiation for both 24h and 48h groups, inhibited miR-205-5p could promote viabilities of cells (Figure 3F, G). As for apoptosis, inhibition of miR-205-5p could activate Bcl-2 and Bcl-xL and silence caspase-3,signifying deterred cellular apoptosis (Figure 3H, I).

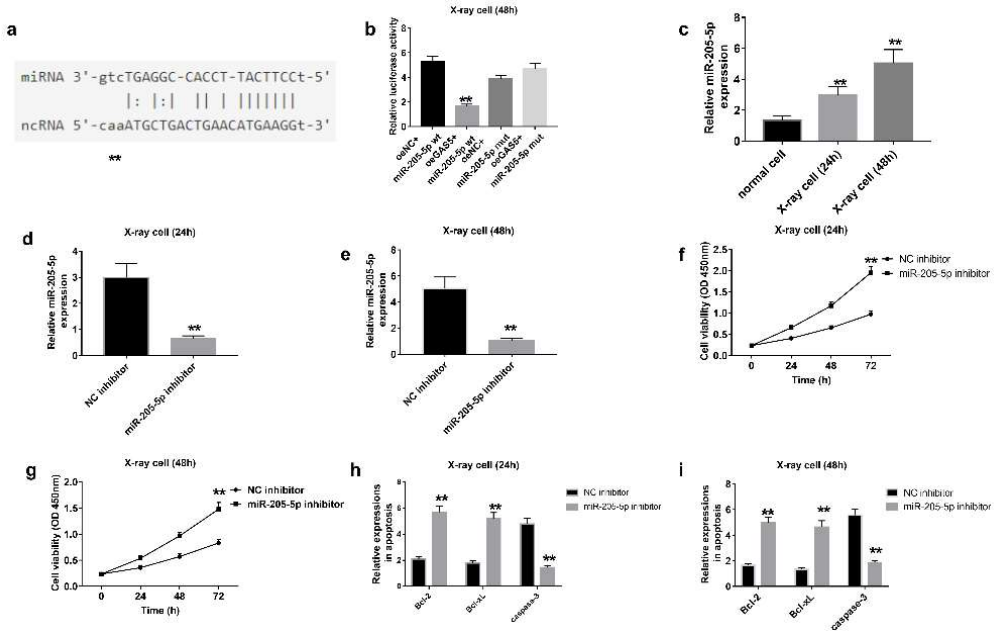

Figure 3: MiR-205-5p was the direct target of gas 5 and promoted proliferation A. Starbsase v2.0 was for finding predicted binding sites of gas5 and miRNAs. B. Luciferase report assay was used for determine binding conditions between gas5 and miR-205-5p, p<0.05. C. Expressions of mi-205-5p was detected by RT-qPCR, p<0.05. D, E. RT-qPCR was used to measure expressions of transfected miR-205-5p, p<0.05. F, G. Cell viabilities with inhibited miR-205-5p and NC was validated by CCK-8, P<0.05. H, I. RT-qPCR was for evaluating expressions of factors in apoptosis, p<0.05

LncRNA gas5 regulated cell progression by targeting miR-205-5p through Wnt/β-catenin signaling pathway

After assessment of the functions of lncRNA gas5 and miR-205-5p individually, interplays in between were further studied. First, when miR-205-5p was up-regulated, expression of lncRNA gas5 decreased (Figure 4A). lncRNA gas5 increased viabilities of cells while overexpressed miR-205-5p could partly reverse the promotive effect generated by upregulated gas5 (Figure 4B). After that, apoptosis was detected through expressions of Bcl-2, Bcl-xL and caspase-3. Up-regulated miR-205-5p could restore Bcl-2 and Bcl-xL and reverse the suppression of caspase-3 induced by lncRNA gas5 upregulation (Figure 4C). Moreover, expressions of Wnt 3a and β-catenin were both increased by upregulated lncRNA gas5 while miR-205-5p mimics could offset the boost (Figure 4D). In order to make sure that Wnt/β-catenin signaling pathway played a part in the cellular functions, IWP-4, a Wnt/β-catenin signaling pathway inhibitor was introduced, which could silence Wnt 3a and β-catenin so as to investigate the changes in cell viabilities brought by the inactivation of Wnt/β-catenin signaling (Figure 4E, F). The CCK8 results unveiled the reduced cell viabilities after the IWP-4 treatment, supporting the previous hypothesis that gas5 might regulate cell progression by targeting miR-205-5p through Wnt/β-catenin signaling pathway

DISCUSSION

The present research mainly dedicated to the exploration of the role of lncRNA gas5 in granulosa cell tumor of ovary after X-ray treatment in vitro. At first, the GCTO-like cell line KGN was acquired and treated with X-ray for 0,24 and 48 hours to mimic the radiotherapy for GCTO patients in vitro. Cell viability decreased and apoptosis increased when the treated time increased. We found gas5 was silenced when the cells were radiated and upregulation of gas5 could promote the cell viability and inhibit apoptsosi, suggesting that gas5 might deter the pro-apoptosis effect of X-ray. In addition, bioinformatics and Luciferase assays confirmed the targeted gene of lncRNA gas5, miR-205-5p. Therefore, we studied the role of miR-205-5p in cellular functions singly first and discovered that miR-205-5p expression increased as radiation was pre-treated; miR-205-5p inhibitor promoted the cellular viabilities and deterred apoptosis. Then we went further into interactions between gas5 and miR-205-5p in modulations of the radiation-exposedcell functionsandcame to afinding that miR-205-5p mimics could partially reverse the cellular changes in viabilities and apoptosis induced by gas5 upregulation.

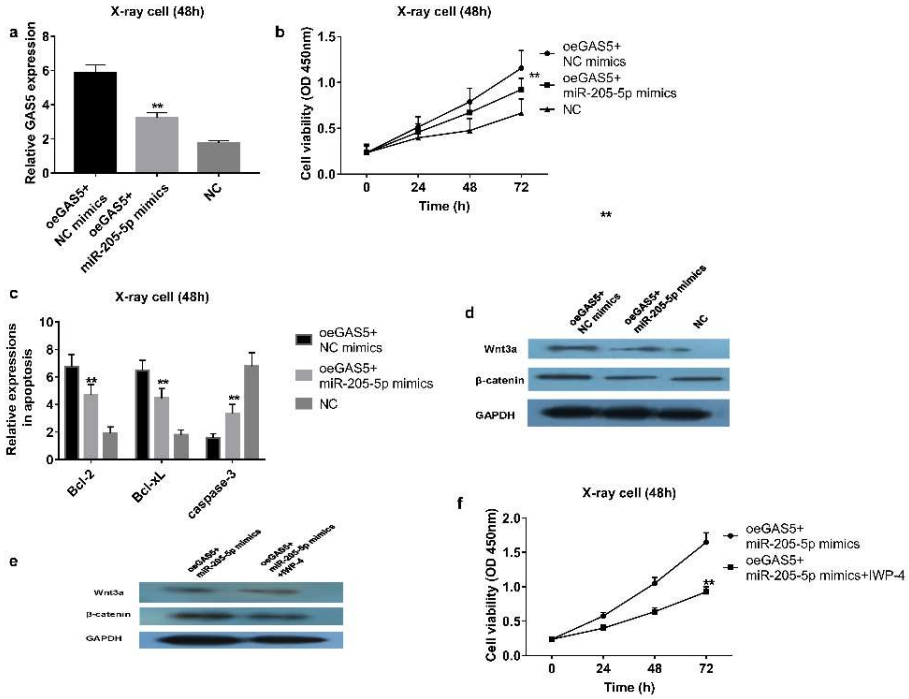

Figure 4: LncRNA gas5 regulated proliferation and apoptosis of X-ray treated cells through Wnt/β-catenin signaling pathway A. Relative expressions of gas5 was measured through RT-qPCR, p<0.05. B. Cell viabilities were detected through CCK-8, P<0.05. C. Apoptosis were evaluated by RT-qPCR, p<0.05. D, E. Western blot was for analyzing expressions of proteins, P<0.05. F: CCK-8 was used to measure cell viability, p<0.05

Granulosa cell tumors of ovary (GCTO) was a type of gynecological oncology which occurs rare [17]. The treatments for GCTO included radio-therapy[18]. Recent years, researches discovered that RNAs were involved with the GCTO progression. For instance, the presence of TRET C228T mutation tended to appear in recurrent tumors instead of primary ones in GCTO[19]. Silencing FOXO/PTEN in Granulosa cells were corelated with the progression of GCTO[20]. There have been some previous researches concerning lncRNAs and GCTO. It was disclosed that lnc LET could induce cell apoptosis and inhibit viabilities in GCTO-like cells; lncRNA SRA might promote cell growth and suppress viabilities in GCTO mice and lncRNA MEG3 promoted the cell viability and inhibit viabilities in GCTO mouse cells via p53/p66 signaling pathway[21-23].

LncRNA gas5 was widely reported to participate in the regulation of ovarian disorders[24, 25]. Yet, there is no literature relating to gas5 and GCTO. Therefore, we surveyed the role of gas5 in GCTO.

lncRNAs could be regulated by radiation and showed radioresistance in many kinds of cancer cells[26, 27]. Researches have also discovered that lncRNAs transcripts were changed because of ultraviolet rays or ionizing radiation in peripheral blood mononuclear cell (PBMC)[28], thymocyte[29] and melanocyte[30]. LncRNA gas5 was discovered in various cancers to play a regulator in radiotherapy efficacy[31-35]. Therefore, we researched the role of gas5 in GCTO regarding radiation *in vitro*.

Wnt signaling pathway was first verified in 1982 and Wnt1 was the first gene of Wnt family[36], which is an important signaling pathway in regulating cell progression. Previous researches have proved that Wnt/β-catenin signaling pathway could activate expression of survivin, an anti-apoptosis gene, to increase radioresistance of progenitor cells of mammary glands[37]. We found in this research that wnt/beta-catenin signaling changed as X-ray exposure, gas5 or miR-205-5p adjusted, giving a hint that the signaling pathway might be involved with the regulatory mechanism beneath the lncRNA gas5. Hence, we introduced the signaling pathway inhibitor, IWP-4, to silence the signaling and observed the corresponding changes in cell viability and results showed that resultant cellular viabilities were inhibited by IWP-4, indicating that

the lncRNA gas5/miR-205-5p axis might regulate the cell viability and apoptosis via wnt/beta-catenin signaling in GCTO-radiation cellular model.

CONCLUSION

LncRNA gas5 was expressed lower in GCTO-radiation cellular model and upregulation inhibited cell apoptosis and promoted cell proliferation by suppressing miR-205-5p and activating Wnt/β-catenin signaling, indicating that it could be a potential target to be considered combining with radiotherapy in GCTO. However, the present study requires more substantial animal and clinical studies for further validation.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Zaixue Jiang performed most experiments and took part in drafting the manuscript. Xingui Tian designed the work, analysed the data and participated in drafting the manuscript. Xiaomei Lu helped analyse the data. Baimao Zhong designed and supervised the work, and edited the final version of the manuscript. All authors read and approved the final version of the manuscript.

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