Tropical Journal of Pharmaceutical Research June 2020; 19 (6): 1153-1159
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i6.5

Original Research Article

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

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Sent for review: 23 Feb 2020 *Revised accepted: 21 Mar 2020*

Abstract

Purpose: *To investigate the role of lncRNA gas5 in ovarian granulosa cells exposed to x-ray in granulosa cell tumor of ovary (GCTO).*
Methods: *KGN cell line was exposed to X-ray to mimic the radiotherapy for GCSO patients in vitro, cell viability was checked by CCK8 assays. RNA expression of apoptosis-related genes was determined by quantitative reverse transcriptase-polymerase reaction (qRT-PCR) while Western Blot for biomarkers in wnt/β-catenin signaling. Differential expressions of lncRNA gas5 were examined after cells were exposed to a ray for 0,24,48hs. We over expressed gas5 and assessed resultant cell viability, apoptosis and signaling. The sponging between gas5 and miR-205-5p was verified by luciferase assay. CCK8, qRT-PCR and Western blot were applied to investigate the correlation between miR-205-5p, cell viability, and apoptosis after miR-205-5p augmentation. Similarly, interaction between gas5 and miR-205-5p was assessed after co-transfection of miR-205-5p mimics and oe-gas5. Finally, wnt inhibitor was used to study the role of signaling pathway in KGN cells.*
Results: *Exposure of KGN to x-ray reduced cell viability and increased apoptosis. Gas5showed reduced expression in the cells, while miR-205-5p expression increased. Gas5 upregulation protected the cells against apoptosis and contributed to cell viability and activation of wnt//β-catenin signaling. lncRNA gas5 targeted miR-205-5p and miR-205-5p mimics counteracted the functions of up-regulated lncRNA gas5, regulating Wnt/β-catenin signaling pathway. Inactivation of Wnt/β-catenin suppressed cell viability.*
Conclusions: *lncRNA gas5 regulates cell apoptosis and viability following cellular radiation, thus presenting a potential therapeutic target for the application radiotherapy in GCTO patients.*

Keywords: *Ovary, Proliferation, Apoptosis, lncRNA gas5, Radiotherapy, β-catenin signaling*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Ovarian follicle is one of the most dynamically evolving 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 layers [1]. Granular cell layer is a composition of fast-changing ovarian follicles. Granulosa cells are often observed in follicular initial cells undergoing apoptosis in atretic follicles before oocyte and theca cells, which indicate that they could be initiator of follicular atresia [2,3]. In addition, granulosa cells can secrete several factors including 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 resistance 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. There are few research related to gas5 in GCTO.

The purpose of this study was to evaluate the role of lncRNA gas5 and miR-205-5p in ovarian granulosa cells exposed to X-ray in granulosa cell tumor of ovary in order to determine the 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 was applied.

CCK-8

After 0, 24 and 48h exposure of 10GY X-ray, the cells were seeded into 96 well plate with 1×10^5

cells per well and incubated at 37℃, 5%CO_2. CCK-8 (10μl solution) was added into the plate at 24h, 48h and 72h and the optical density (OD) values of cells were determined at 450nm using microplate reader (Thermo Fisher, USA). The cells exposed to the X-ray for 24, 48 h after transfection were selected to undergo cell viability assays in order to determine the correlations between the gene expression and cell viability.

Luciferase report assay

A putative binding was predicted on Starbase (http://starbase.sysu.edu.cn/agoClipRNA.php?source=lncRNA). KGN cells were co-transfected with 25ng lncRNA gas5 wt or lncRNA gas5 mut with 20 μl 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 and then lysed with 250 μl RIPA reagent (Beyotime, Shanghai, China) for 20 min. Then the supernatant liquid was centrifuged at 1000 rpm for 5 min and the total proteins were quantified using BCA assay kit (Beyotime, Shanghai, China) [15]. This was followed by the separation of the total proteins (40μg) using SDS-PAGE and transfer into PVDF membranes. THe the membranes were blocked with 8% non-fat milk powder at room temperature for at least 2 h [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

All experiments were carried out three times as appropriate. Data are presented as mean ± SD and analyzed with SPSS 19.0 (IBM, USA) using Student's. t-test. $P < 0.05$ was considered statistically 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 1 A) 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 1 B - D). Expressions of proteins examined by Western Blot indicated that x-ray can reduce expression level of both Wnt and β-catenin (Figure 1 E).

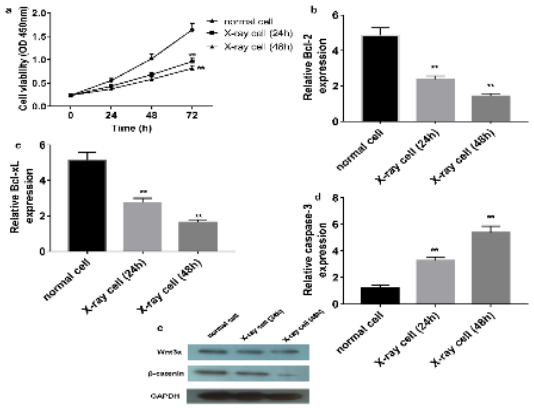

Figure 1: X-ray suppressed proliferation and accelerated apoptosis of KGN cells. A. Cell viabilities were detected through CCK-8, P<0.05. B, C, D. RT-qPCR was applied to measure expressions of factors related to apoptosis, P<0.05. E. western blot was used to evaluated expressions of proteins in Wnt/β-catenin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

LncRNA gas5 expressed lower protein levels and promoted proliferation in x-ray exposed cells and inhibited apoptosis

Expression of lncRNA gas5 was detected in cells exposed to x-ray for 0 h, 24 h and 48 h. Compared to normal cells (0 h), the expression of lncRNA gas5 was lower in X-ray treated cells (Figure 2A). The expression of lncRNA gas5 decreased significantly as the exposure time increased (Figure2B, C). When lncRNA gas5 in cells was upregulated with 24h and 48h exposure to X-Ray, CCK8 results showed that increased lncRNA gas5 promoted viability (Figure 2D). Moreover, cell viability was 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 up regulated the expressions of Bcl-2 and Bcl-xL but inhibited expression of caspase-3 (Figure 2F, G). Expression of wnt3a and beta-catenin was

increased by overexpressed lncRNA gas5 (Figure 2H).

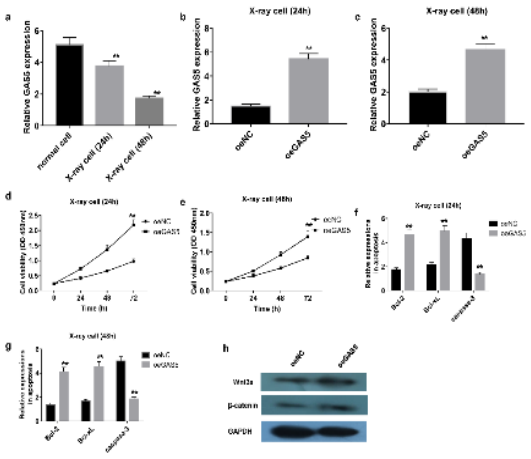

Figure 2: LncRNA gas5 expressed lower in X-ray treated cells with promoting proliferation and repressing apoptosis. A. Expressions of gas5 were analyzed through RT-qPCR, P<0.05. B, C. overexpressed gas5 expressions were detected by RT-qPCR, P<0.05. D, E. CCK-8 was applied to measure cell viabilities,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

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 3 A). Therefore, luciferase reporter assay was applied to determine the binding sites between lncRNA gas5 and miR-205-5p. 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 can directly bind lncRNA gas5 (Figure 3 B). MiR-205-5p expression was higher in the group with X-ray exposure than untreated cells (Figure 3 C). Thereafter, miR-205-5p was knocked down by transfecting miR-205-5p inhibitor into the cells with pre-exposure to radiation for 24h and 48h. Lower expression of miR-205-5p was detected (Figure 3 D and E). Correspondingly, cell viability was measured, which disclosed that in cells with pre-exposure to radiation for both 24 and 48 h groups, inhibited miR-205-5p could promote viability of cells (Figure 3 F and 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 3 H and I).

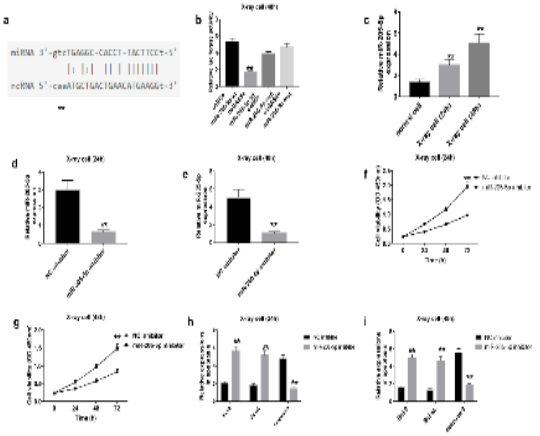

Figure 3: MiR-205-5p was the direct target of gas 5 and promoted proliferation A. Starbsase v2.0 was for finding predicted binding sites of gas5 and miRNAs. B. Luciferase report assay was used for determine binding conditions between gas5 and miR-205-5p, $p < 0.05$. C. Expressions of mi-205-5p were determined by RT-qPCR, $p < 0.05$. D and 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 via 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 viability of cells while overexpressed miR-205-5p partly reversed 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 restored Bcl-2 and Bcl-xL and reversed 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 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. This could silence Wnt 3a and β-catenin to be able 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.

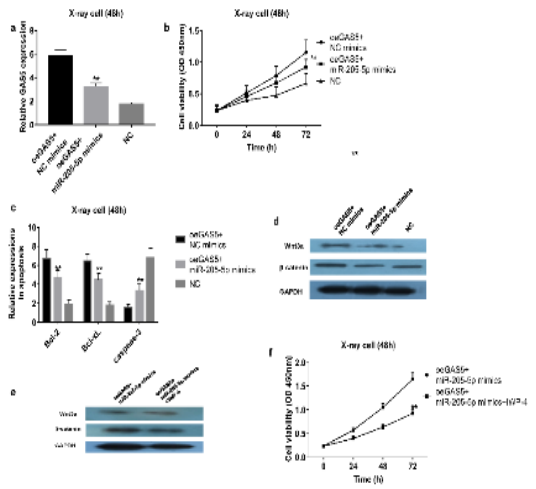

Figure 4 LncRNA gas5 regulated proliferation and apoptosis of X-ray treated cells through Wnt/β-catenin signaling pathway A. Relative expressions of gas5 was measured through RT-qPCR, $p < 0.05$. B. Cell viability was evaluated by CCK-8, P<0.05. C. Apoptosis were evaluated by RT-qPCR, $p < 0.05$. D and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

DISCUSSION

The present study mainly explored 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 period of treatment increased. Gas5 was silenced when the cells were radiated. The upregulation of gas5 can promote the cell viability and inhibit apoptosis, 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, the role of miR-205-5p in cellular functions was studied and it was discovered that miR-205-5p expression increased as radiation was pre-treated; miR-205-5p inhibitor promoted the cellular viabilities and deterred apoptosis. On the investigation of interactions between gas5 and miR-205-5p in modulations of the radiation-exposed cell functions, it was observed that miR-205-5p mimics can partially reverse the cellular changes in cell viability and apoptosis induced by gas5 upregulation.

GCTO was a type of gynecological oncology which occurs rarely [17]. The treatments for GCTO include radio-therapy [18]. In recent years, studies indicate that RNAs are involved in 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 corelated with the progression of GCTO [20]. There have been some previous studies concerning lncRNAs and GCTO. It was disclosed that lnc LET can induce cell apoptosis and inhibit cell viability in GCTO-like cells; lncRNA SRA might promote cell growth and suppress cell viability in GCTO mice and lncRNA MEG3 promoted the cell viability and inhibit viabilities in GCTO mouse cells via p53/p66 signaling pathway [21-23].

Although LncRNA gas5 was widely reported to participate in the regulation of ovarian disorders [24,25], there is no literature relating to gas5 and GCTO. lncRNAs can be regulated by radiation as it showed radio-resistance 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 regulatory role in radiotherapy efficacy [31-35]. This explains why the role of gas5 in GCTO regarding radiation *in vitro* was investigated.

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]. In this study, it was found that wnt/beta-catenin signaling changed as X-ray exposure, gas5 or miR-205-5p adjusted, suggesting that the signaling pathway might be involved with the regulatory mechanism beneath the lncRNA gas5. As a result, the signaling pathway inhibitor, IWP-4, was introduced to silence the signaling. The corresponding changes in cell viability showed that resultant cellular viabilities was inhibited by IWP-4. This is an indication 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

Acknowledgement

This research is funded by Key Specialty Construction Project of Pudong Health and Family Planning Commission of Shanghai (No. PWZzk2017-21) and also Science and Technology Development Fund of Shanghai Pudong New Area (no. PKJ2017-Y13).

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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