Long non-coding RNA cartilage injury-related promotes malignancy in bladder cancer

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Abstract. Recent advances have highlighted the important roles of long non-coding RNAs (lncRNAs) in a number of biological processes, including oncogenesis. However, the function of lncRNA cartilage injury-related (lncRNA-CIR) in bladder cancer progression remains elusive. A novel function for lncRNA-CIR in bladder cancer was identified in the present study. Reverse transcription quantitative polymerase chain reaction, viability, invasion assay and in vivo implantation were used to evaluate the role of lncRNA-CIR. It was identified that the expression of lncRNA-CIR was frequently upregulated in 52 cancerous tissues and selected bladder cancer cell lines. Additionally, upregulating lncRNA-CIR was demonstrated to promote viability and invasion in T24 and SW780 cells, whereas siRNA-mediated lncRNA-CIR-knockdown consistently exhibited the opposite effects. High lncRNA-CIR levels also dictated poor overall survival among patients with bladder cancer. Furthermore, in vivo implantation experiments also supported a tumorigenic function for lncRNA-CIR, as decreasing lncRNA-CIR levels markedly attenuated Ki-67 staining and xenograft tumor growth. Overall, the present study identified a novel function of lncRNA-CIR and indicates that lncRNA-CIR may serve as a potential biomarker for bladder cancer treatment.

Introduction

Bladder cancer is among the most detrimental tumors of the urinary system, particularly in China (1). A total of ~70% of patients belong to the non muscle-invasive bladder cancer group and <50% of patients will suffer from recurrence; furthermore, ~20% will progress to the muscle-invasive bladder cancer type (2). The incidence and mortality rates of patients with bladder cancer have markedly increased in recent years (3). Numerous therapeutic methods for bladder cancer intervention have been investigated, including chemotherapy, radiotherapy and surgery; however, the overall survival (OS) rate remains poor (4). Therefore, identifying novel biomarkers for an early diagnosis and effective prognosis, as well as establishing the underlying mechanisms for bladder cancer progression, is critically important for increasing the survival rate among patients with bladder cancer.

The occurrence and development of bladder cancer has been ascribed to multiple mechanisms. For example, frequent inactivation of tumor suppressor pathways, including the p53 signaling pathway, contributes to bladder cancer progression (5). Similarly, aberrant activation of oncogenic pathways, including the Akt and mitogen-activated protein kinase pathways, has also been reported to increase motility and invasion in bladder cancer (6). Furthermore, epigenetic alterations, including loss of heterozygosity, hypermethylation and point mutations, have also been implicated in the development of multifocal bladder cancer (7,8). Notably, factors involved in chromosome abnormalities such as C14orf166 also function as high-risk biomarkers for bladder cancer progression (9). However, the intrinsic cellular mechanisms remain largely elusive and further studies are required to elucidate the mechanisms of bladder cancer development.

Although research regarding small non-coding NRAs (e.g., microRNAs) has been the focus in the field of molecular biology, numerous studies have indicated that long non-coding RNAs (lncRNAs) also exhibit essential roles in biological processes including proliferation, senescence and apoptosis (10). LncRNAs belong to a class of RNAs that are >200 nucleotides in length with no protein coding activities (11). Their inability to encode proteins, lncRNAs can regulate various biological pathways and are characterized by their complex mechanisms of activity (12). The aberrant expression of lincRNAs is critically involved in a variety of diseases, particularly cancer. Previously, Liu et al (13) identified that lncRNA cartilage injury-related (lncRNA-CIR) may increase the degradation of chondrocyte extracellular matrix in osteoarthritis (OA). However, the precise function of lncRNA-CIR especially in bladder cancer progression remains unknown.

In the present study, the role of lncRNA-CIR in the pathogenesis of bladder cancer was examined. It was identified that lncRNA-CIR was frequently upregulated in bladder cancer. Higher lncRNA-CIR expression promotes the viability and
invasion of bladder cancer cells. In addition, it was found that patients with higher lncRNA level were associated with poor overall survival. The lncRNA-CIR may consistently increase xenograft tumor growth in vivo. Overall, the results of the present study identified a novel and oncogenic role of lncRNA-CIR in bladder cancer and may provide important insights into potential therapeutic interventions targeting lncRNA-CIR.

Materials and methods

Cell culture and specimen collection. The present study used bladder cancer T24, SW780, UBC-40, 5637 and UM-UC-3 cells lines, in addition to the transformed cell line, SV-HUC-1. The cell lines were all purchased from the Shanghai Institute of Cell Biology (Shanghai, China) and were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 200 U/ml penicillin and 50 mg/ml streptomycin (all Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and maintained in an incubator with 5% CO2 at 37°C. Matched fresh bladder cancer specimens and normal adjacent tissues were collected from 52 patients at The Affiliated Hospital of Guilin Medical College (Guilin, Guangxi, China) between February 2009 and March 2011. Following surgical resection, tissues were immediately stored at -80°C until total RNA was extracted. None of the patients had received preoperative chemotherapy or radiotherapy prior to surgery. All patients provided written informed consent, and the research on human specimens was reviewed and formally approved by the Ethics Committee of the Affiliated Hospital of Guilin Medical College.

lncRNA-CIR-knockdown and -overexpression. The sequences of specific siRNAs targeting lncRNA-CIR were designed and synthesized by Sigma-Aldrich (Merck KGaA). The lncRNA-CIR fragment obtained by RNA extraction and RT-qPCR was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s protocol. Briefly, cells were cultured with RPMI-1640 medium in a 24-well plate for 24 h at 37°C. Prior to transfection, the medium was replaced with 200 µl/well of medium without antibiotics and the cells were cultured for 24 h at 37°C. The DNA or RNA interference Lipofectamine® 2000 complex was prepared by mixing for 30 min, and then 100 µl of the complex was added to each well. The cells were cultured for 24 h at 37°C with normal RPMI-1640 medium and then subject to additional assays. The si-CIR sequences used were: si-CIR#1, sense: 5'-GGCCUGAACGAGCUGA-3', antisense: 5'-GACAGUCCGUACGC=3'; si-CIR#2, sense: 5'-CGUCCACAAAGGAGCA-3', anti-sense: 5'-AUGUCUCUUG-3', si-CIR#3: sense: 5'-CGAGAACUGCGGGACA-3', anti-sense: 5'-AUGCCACGAGUUC-3'.

RNA extraction and RT-qPCR. Total RNAs were isolated from T24 and SW780 cell lines and human specimens with TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following cell lysis, 0.5 ml chloroform (Sigma-Aldrich; Merck KGaA) was added and cells were maintained at 20°C for 5 min. Centrifugation was performed at 15,000 x g at 4°C for 10 min. The aqueous phase was then transferred to another tube and 0.2 ml isopropanol per 1 ml TRIzol was added. Following incubation for 15 min at 20°C, centrifugation was conducted at 10,000 x g at 4°C for 10 min. The RNA pellet was washed with 70% ethanol and the RNAs were dissolved using 0.05 ml fresh water and incubated for 15 min. Isolated RNA was reverse-transcribed with Improm-II reverse transcription kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. GAPDH was used as the control. Reactions were performed using the ABI PRISM® 7000 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The expression of lncRNA-CIR was calculated by the 2ΔΔCq method (14). The quantitative real-time PCR was performed using TaqMan Non-coding RNA Assays (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reactions were initially denatured at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 45 sec. The amplified products were extracted and purified using Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA), and then digested with Hind III and Xho I. Following this, the products were cloned into the pcDNA3.1 vector. The correct constructs were verified by DNA sequencing and were used to transfect T24 and SW780 cells. The constructs were sequenced using standard procedures of dye terminator chemistry on a 3700 Sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Sequences were compared to sequences in Gene Bank using the Blast program (version 2.5.0; http://blast.ncbi.nlm.nih.gov/Blast.cgi). All siRNAs against lncRNA-CIR were designed and synthesized by Sigma-Aldrich; Merck KGaA. All transfections were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Briefly, cells were cultured with RPMI-1640 medium in a 24-well plate for 24 h at 37°C. Prior to transfection, the medium was replaced with 200 µl/well of medium without antibiotics and the cells were cultured for 24 h at 37°C. The DNA or RNA interference Lipofectamine® 2000 complex was prepared by mixing for 30 min, and then 100 µl of the complex was added to each well. The cells were cultured for 24 h at 37°C with normal RPMI-1640 medium and then subject to additional assays. The si-CIR sequences used were: si-CIR#1, sense: 5'-GGCCUGAACGAGCUGA-3', antisense: 5'-GACAGUCCGUACGC=3'; si-CIR#2, sense: 5'-CGUCCACAAAGGAGCA-3', anti-sense: 5'-AUGUCUCUUG-3', si-CIR#3: sense: 5'-CGAGAACUGCGGGACA-3', anti-sense: 5'-AUGCCACGAGUUC-3'.

Transwell invasion assay. The invasion assay was performed using Transwell plates (pore size, 8 µm) with a Boyden chamber (Sigma-Aldrich; Merck KGaA). Transfected cells were washed twice with serum-containing RPMI-1640 medium (Sigma-Aldrich; Merck KGaA). A total of 1×105 T24 and SW780 cells were seeded onto the Transwell apparatus. Each insert was preloaded with Matrigel (50 µg; Sigma-Aldrich; Merck KGaA). Cells were suspended in 100 µl RPMI-1640 serum-free medium (Sigma-Aldrich; Merck KGaA) and placed in the top chambers. RPMI-1640 medium (100 µl; Sigma-Aldrich; Merck KGaA) containing 10% fetal calf serum (Sigma-Aldrich; Merck KGaA) was added to the bottom chambers. The chambers were incubated for 24 h at 37°C with 5% FBS in the lower chamber. Cells on the upper layer were removed by cotton buds and then washed with PBS. The cells that had invaded into the lower chambers were fixed with methanol for 15 min (Sigma-Aldrich; Merck KGaA) and stained with 5% crystal violet for 30 min at 20°C (Sigma-Aldrich; Merck KGaA). The results were visualized.
by light microscopy (DFC500; Leica Microsystems GmbH, Wetzlar, Germany) and the final values represent the mean from three fields on the membrane. The results were visualized at magnification, x100. Experiments were performed in triplicate.

Cell viability assay. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to evaluate cell viability. Following transfection with pcDNA, si-NC, pcDNA-CIR or si-CIR for 36 h, T24 and SW780 cells were re-suspended and seeded into a 96-well plate at a density of ~1x10^4 cell/ml. CCK-8 solution (25 ml/well) was then added and the plate was incubated for 3 h at 37°C. The viability was monitored once a day for 5 days. The optical density at 490 nm was used to determine cell viability with the Spectramax M5 microplate monitor (Molecular Devices, LLC, Sunnyvale, CA, USA) according to the manufacturer's protocol.

In vivo implantation assay. T24 cells transfected with pcDNA, si-NC, pcDNA-CIR or si-CIR for 36 h were continuously cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) for an additional 24 h at 20°C. The cells were then resuspended and ~1x10^6 cells were injected subcutaneously into nude mice. A total of 40 mice (BALB/C; female; age, 4-5 weeks; mean weight, 15.6 g) were used. Mice were housed at ~20°C, 55-60% humidity, with a light-dark cycle of 12 h. Ad libitum access to food and water was provided. The nude mice were obtained from the Model Animal Research Center (Nanjing, China). The Ethics Committee of Affiliated Hospital of Guilin Medical College approved the animal experiments. The volumes (length x width x height) of the tumors in vivo were recorded by external caliper every 3 days for 30 days. Animals were sacrificed when tumor volumes reached 2,000 mm³, or when animals lost >20% of initial body weight. After 30 days post-injection, all mice were sacrificed by an overdose of sodium pentobarbital (4%, 300 mg/kg with intraperitoneal injection; catalog no. 1507002; Sigma-Aldrich; Merck KGaA) and the implants were immunostained with Ki-67 (Sigma-Aldrich; Merck KGaA). Following antigen retrieval in the supplied solution, the primary antibody against Ki-67 (catalog no. P6834; dilution, 1:1,000; Sigma-Aldrich; Merck KGaA) was added and incubated for 30 min at 20°C. The image was visualized using a CX31-LV320 light microscope at magnification, x100 (Olympus Corporation, Tokyo, Japan).

Statistical analysis. All statistical results are shown as the mean ± standard deviation. Statistical significance was determined by Mann-Whitney test using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) and P<0.05 was used to indicate a statistically significant difference. One-way ANOVA was used to determine the statistical significance across multiple groups followed by Least Significant Difference post hoc comparison. Kaplan-Meier curves were evaluated by log-rank test. The contingency table for clinicopathological features was determined by Fisher's exact test.

Results

lncRNA-CIR is upregulated in bladder cancer tissues and cells. RT-qPCR was used to evaluate the expression of lncRNA-CIR in bladder cancer tissues and corresponding normal adjacent tissues. The relative expression of lncRNA-CIR was quantified by dividing the value in bladder cancer tissues by the associated value in normal adjacent tissues. The results demonstrated that lncRNA-CIR was significantly upregulated in bladder cancer tissues (Fig. 1A). Notably, >80% of tissues were overexpressed in lncRNA-CIR by >2-fold increase (Fig. 1B). The lncRNA-CIR expression was markedly associated with metastasis and tumor size (P=0.005 and P=0.004 respectively; Table I). However, lncRNA-CIR expression was not significantly associated with age, sex or pathological grade (Table I). The samples were classified into two groups based on the median value of expression (4.0489; Fig. 1C). The expression of lncRNA-CIR was further examined in bladder cancer cell lines and the level of lncRNA-CIR was demonstrated to be consistently significantly increased in cancer cell lines compared with that in normal cells (Fig. 1D). These results suggested lncRNA-CIR was overexpressed in bladder cancer cells and specimens. The T24 and SW780 cell lines were selected for further analysis as they displayed the highest lncRNA-CIR expression among the bladder cancer cell lines examined.

Table I. Association between CIR and clinicopathological features.

| Clinicopathological factor | CIR expression |
|----------------------------|----------------|
|                            | Low, n (%)     | High, n (%) |
| **Age, years**             |                |             |
| <60                        | 30 (46.7)      | 16 (53.3)   | 0.779 |
| ≥60                        | 22 (54.5)      | 10 (45.5)   |       |
| **Sex**                    |                |             |
| Male                       | 24 (45.8)      | 13 (54.2)   | 0.391 |
| Female                     | 28 (53.6)      | 13 (46.4)   |       |
| **Metastasis**             |                |             |
| Absent                     | 23 (73.9)      | 6 (26.1)    | 0.005*|
| Present                    | 29 (31.0)      | 20 (69.0)   |       |
| **Tumor size, cm**         |                |             |
| <3                         | 21 (76.2)      | 5 (23.8)    | 0.004*|
| ≥3                         | 31 (32.3)      | 21 (67.7)   |       |
| **Pathological grade**     |                |             |
| Low                        | 27 (40.7)      | 16 (59.3)   | 0.267 |
| High                       | 25 (60.0)      | 10 (40.0)   |       |

*P<0.05. The median value (4.0489) was used as the cutoff. CIR, cartilage injury-related. Fisher exact test was used to determine significance.
lncRNA-CIR expression was significantly associated with improved survival (P<0.01; Fig. 2). Furthermore, the difference in OS rate between the two groups generally increased during the follow-up period (Fig. 2). These results suggested that higher lncRNA-CIR levels were associated with poor overall survival among patients with bladder cancer.

lncRNA-CIR promotes malignant phenotypes in bladder cancer cell lines. To further explore the potential oncogenic effect of lncRNA-CIR in vitro, a series of in vitro experiments were performed in the T24 and SW780 bladder cancer cell lines and lncRNA-CIR was demonstrated to be either knocked down or overexpressed in T24 and SW780 cells. The knockdown and overexpression efficiency were verified and the si-RNA-mediated knockdown and pcDNA-mediated transfection was demonstrated to significantly alter the expression of lncRNA-CIR compared with that in the corresponding control group (Fig. 3A). si-CIR#1 displayed the most significant effect to knock down CIR expression in the two cell lines (Fig. 3A). Therefore, si-CIR#1 was used for further analysis (abbreviated as si-CIR thereafter). CCK-8 assay was used to evaluate the viability of T24 and SW780 cells. Overexpressing lncRNA-CIR was demonstrated to significantly increase the viability of T24 cells (Fig. 3B). Decreasing lncRNA-CIR by si-RNA instead markedly inhibited the T24 cell viability compared with that in corresponding controls (Fig. 3B). Similar results were obtained in SW780 cells (Fig. 3C).

The invasion was further investigated and the present study identified that overexpression of lncRNA-CIR could significantly upregulate the invasive cell numbers of bladder cancer cells, while decreasing lncRNA-CIR expression displayed the opposite effect compared with that in the corresponding control group (Fig. 3D and E). These results indicated that...
LncRNA-CIR promotes the viability and invasion of bladder cancer cell lines in vitro.

LncRNA-CIR increases xenograft tumor proliferation and growth. To further ascertain whether lncRNA-CIR exhibited a tumorigenic effect in vivo, tumor implantation assays were performed. Approximately 1x10⁶ T24 cells were subcutaneously injected into nude mice and the tumor volume was monitored every 3 days. It was observed that lncRNA-CIR overexpression substantially promoted tumor growth (Fig. 4A). However, reducing lncRNA-CIR expression markedly inhibited tumor growth compared with that in the control group (Fig. 4B). Reduction in lncRNA-CIR expression by si-RNA-mediated knockdown was demonstrated to consistently decrease tumor weight (Fig. 4B). The Ki-67 staining indicative of cell proliferation also exhibited enhanced staining in the pcDNA-CIR transfection group, while the proliferation was significantly weakened with si-CIR-mediated knockdown compared with that in the control group (Fig. 4C). These results demonstrated that lncRNA-CIR can support tumor growth and further implied an oncogenic role of lncRNA-CIR in vivo.

Discussion
Due to the increasing progress in sequencing technology, lncRNAs have been demonstrated to be associated with
the pathology of various diseases, including cancer (15). Significant advances in IncRNA research have greatly enriched the level of understanding regarding IncRNA profiles. An increasing number of studies have implied that IncRNAs can exert a diverse range of functions in various types of tumors (16,17). For example, IncRNAs may assist in classifying subtypes in glioma, colon and breast cancer (17). BRAF-activated LncRNA may suppress tumor development in papillary thyroid cancer (18). The IncRNA Gm15290 may instead promote invasion and proliferation of lung cancer via interactions with miR-615-5p (19). However, the mechanisms by which lncRNAs are involved in bladder cancer progression have not been fully identified. In the present study, an unexpected function of lncRNA-CIR in the development of bladder cancer was identified. lncRNA-CIR expression was demonstrated to be frequently upregulated in bladder cancer tissues and cell lines when compared with that in normal tissues and cell lines. Meanwhile, increasing lncRNA-CIR expression was also associated with poor survival and the malignant phenotypes of bladder cancer cells, including advanced proliferation, migration and invasion. The in vivo oncogenic effect for lncRNA-CIR was also confirmed, suggesting that lncRNA-CIR may exhibit a tumorigenic role in the present study.

A limited number of studies have focused on lncRNA-CIR, particularly in cancer-associated research. A study by Liu et al (13) previously identified that lncRNA-CIR can specifically promote the degradation of chondrocyte extracellular matrix in osteoarthritis (OA). In OA cartilage, the expression of lncRNA-CIR is highly expressed among the 152 differentially expressed lncRNAs and regulates the expression of matrix metalloproteinase 13 and a disintegrin and metalloproteinase with thrombospondin motifs 5 (13). Silencing lncRNA-CIR may elevate the induction of collagen and aggrecan, while overexpressing lncRNA-CIR consistently raises the level of matrix-degrading enzymes (13). However, in the field of tumor biology, the implications of lncRNA-CIR have never been revealed. Hence, the results of the present study may broaden the understanding of lncRNAs in cancer research.

A number of the functions of IncRNAs have not yet been comprehensively identified. It has been argued that the RNA products from pseudogenes can induce mRNA degradation via the coding copies, which leads to the production of small RNAs (24). The long antisense transcripts generated from the pseudogenes can interact with the spliced counterparts and form double-stranded RNAs, which will be cleaved by Dicer (24). Therefore, the mRNAs that can encode proteins may instead promote the formation of RNA-induced silencing complex to consume additional copies of mRNAs and decrease the expression of protein-coding genes (25).
The IncRNA-CIR is, however, a pseudogene product of vimentin (13) and may possibly serve as an siRNA to downregulate vimentin expression. Vimentin is known as an important factor in cell stiffness, the disruption of which may induce weakened cellular integrity and promote cell migration or invasion (26). Hence, we hypothesize that IncRNA-CIR may inhibit the expression of vimentin through the aforementioned mechanism and extracellular matrix degradation to promote the invasion of bladder cancer cells. Whether IncRNA-CIR also presents oncogenic roles in other types of tumor requires an in-depth investigation in the future.

There are also certain limitations in the present study. The clinical materials assessed were from Chinese patients only; IncRNA-CIR must be verified as a diagnostic and prognostic biomarker in different populations prior clinical usage. Additional multicenter studies that include diverse ethnic populations are required. In addition, the detailed molecular mechanism of IncRNA-CIR in bladder cancer was not identified. Additional studies are required to unravel the underlying mechanisms of IncRNA-CIR-mediated bladder cancer progression. Whether IncRNA-CIR serves a role in other types of cancer remains a focus for future studies.

In summary, the results of the present study suggest a tumorigenic role for IncRNA-CIR in bladder cancer. The involvement of IncRNA-CIR in bladder cancer progression may further extend the notion that many ncRNAs are critical in inhibiting cell migration and invasion of bladder cancer cells. Whether IncRNA-CIR serves a role in other types of cancer remains a focus for future studies.

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