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Research paper

# *LncRNA GACAT3* acts as a competing endogenous RNA of *HMGA1* and alleviates cucurbitacin B-induced apoptosis of gastric cancer cells

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ABSTRACT

Long non-coding RNAs (lncRNAs) have been demonstrated to perform important roles in cancer development. Previously, we have shown that *lncRNA gastric cancer-associated transcript 3* (*GACAT3*) is overexpressed in gastric cancer and acts as a downstream target of interleukin 6/signal transducer and activator of transcription 3 (IL-6/STAT3) signaling. However, the role of *GACAT3* in regulating gastric cancer cell growth remains unclear. In this study, we demonstrate that *GACAT3* acts as a competing endogenous RNA of *high mobility group A1* (*HMGA1*), a typical oncogene that is overexpressed in most types of cancer, based on a search for common miRNA-binding sites and on experiments involving in vitro cell transfection with synthesized miRNA mimics. Furthermore, knockdown of *GACAT3* by its specific siRNA resulted in significantly decreased cell proliferation in gastric cancer cells, similar to the effect of an *HMGA1* knockdown. Moreover, *GACAT3* overexpression alleviated the apoptosis induced by cucurbitacin B, which is a widely used anticancer drug. Mechanistically, *GACAT3* amplified STAT3 expression and decreased the level of the apoptosis gene *bcl-2-associated X protein* (*BAX*). Thus, our study provides fundamental information regarding *GACAT3*, which could be a valuable target for gastric cancer therapy.

## 1. Introduction

Long non-coding RNAs (lncRNAs) are longer than 200 nucleotides and play multiple roles in fundamental biological processes (Kopp and Mendell, 2018; Mercer et al., 2009). An increasing number of studies have demonstrated that aberrant regulation of lncRNAs not only plays key roles in tumorigenesis but is also a potential diagnostic biomarker (Esteller, 2011; Schmitt and Chang, 2016; Wapinski and Chang, 2011). It has been reported that lncRNAs regulate gene expression at various levels, including transcription, post-transcription, and translation (Geisler and Coller, 2013; Mathy and Chen, 2017). One representative function of lncRNA is to act as a microRNA (miRNA) sponge (Bak and Mikkelsen, 2014). It is hypothesized that miRNAs, lncRNAs and other RNAs communicate with each other via common miRNAs. By sharing the same miRNA response elements, these different RNAs regulate the expression of each other and control intracellular miRNA function (Salmena et al., 2011). These RNAs, which affect each other's expression, are called competing endogenous RNAs (ceRNAs). Since lncRNAs are not actively translated, they are supposed to be highly effective ceRNAs. Recently, numerous studies have revealed that lncRNAs function as miRNA sponges in various tissues, such as *linc00152* in vascular endothelial cells (Teng et al., 2017), *lncRNA protein disulfide isomerase family A member 3 pseudogene 1* (*PDIA3P*) in oral squamous cell carcinoma (Sun et al., 2017), *long intergenic non-protein coding RNA 319*, *nuclear paraspeckle assembly transcript 1* (*NEAT1*) and *X-inactive specific transcript* (*XIST*) in lung adenocarcinoma (Fang et al., 2016; Sun et al., 2016a; Zhang et al., 2018), *urothelial carcinoma-associated* (*UCA1*) in bladder cancer (Luo et al., 2017), *HOX antisense intergenic RNA*

*Abbreviations:* lncRNAs, long non-coding RNAs; GACAT3, lncRNA gastric cancer-associated transcript 3; IL-6, interleukin 6; STAT3, signal transducer and activator of transcription 3; HMGA1, high mobility group A1; BAX, bcl-2-associated X protein; ceRNAs, competing endogenous RNAs; Bcl-2, B-cell lymphoma 2

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(*HOTAIR*) in gastric cancer (Liu et al., 2014), and *H19* in diverse physiological and pathological processes (Kallen et al., 2013).

In previous studies based on a lncRNA microarray of gastric cancer tissues, *gastric cancer-associated transcript 3* (*GACAT3*) was identified as a novel lncRNA (Chen et al., 2014; Song et al., 2013). The level of *GACAT3* has been suggested to be positively correlated with the tumor size, TNM stage, and distant metastasis, indicating that *GACAT3* could serve as a potential biomarker of GC (Xu et al., 2014). *GACAT3* is obviously overexpressed in GC tissues, and its expression is augmented through the signal transducer and activator of transcription 3 (STAT3) signaling pathway, which contributes to cell proliferation and inflammation and is classified as one of the major mediators of tumorigenesis (Shen et al., 2016). Constitutively activated STAT3 has been demonstrated to induce resistance to apoptosis by inducing the expression of anti-apoptotic *B-cell lymphoma 2* (*Bcl-2*) genes and inhibiting *bcl-2-associated X protein* (*BAX*) expression in various cancers (Aggarwal et al., 2009; Sun et al., 2016b). However, the functional mechanism of *GACAT3* in cancer cells remains unclear.

High mobility group A1 (HMGA1), a non-histone architectural protein, is detected to be highly expressed in various cancers (Fusco and Fedele, 2007). It was reported that HMGA1 could be downregulated by various microRNAs (Palmieri et al., 2012; Watanabe et al., 2009). Moreover, miRNA sponging role of HMGA1 was also demonstrated such as MALT1-miR-26-HMGA1 in lung cancer (Chen et al., 2016) and H19-miR-138-HMGA1 in colon cancer (Yang et al., 2017). During GC development, expression of HMGA1 was greatly increased (Akaboshi et al., 2009) and both HMGA1 and GACAT3 had a close association with STAT3 (Hillion et al., 2008; Shen et al., 2016). However, the link between GACAT3 and HMGA1 is unknown.

Cucurbitacin B is a triterpenoid compound extracted from the medicinal herb *Trichosanthes kirilowii* Maximowicz (Cucurbitaceae family), which has been used for a long time as an oriental medicine owing to its anti-inflammatory effects (Chen et al., 2005). Cucurbitacin B has been reported to play an antiproliferative role by inhibiting the constitutively activated STAT3 signaling pathway, resulting in cell cycle arrest and apoptotic cell death (Garg et al., 2018). Recently, the antiproliferative effect of cucurbitacin B has been demonstrated in several types of cancer, such as ovarian carcinoma (Qu et al., 2017), pancreatic cancer (Thoennissen et al., 2009), neuroblastoma (Shang et al., 2014), and breast cancer (Wakimoto et al., 2008).

In the present study, we demonstrate that *GACAT3* plays a role in regulating GC cell growth by acting as a miRNA sponge of *HMGA1*, which is an oncogene in most types of cancer. Moreover, *GACAT3* overexpression alleviated apoptosis induced by cucurbitacin B, a widely used clinical anticancer drug component.

## 2. Materials and methods

### 2.1. Cell culture and treatments

Human BGC-823 and SGC-7901 gastric cancer cells were cultured in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich, USA) and 100 U/ml penicillin-streptomycin (Sigma-Aldrich, USA). Cucurbitacin B was purchased from Lianke Biotechnology (Hangzhou, China) and dissolved in DMSO (Sigma-Aldrich, USA). BGC-823 and SGC-7901 cells were treated with various concentrations of cucurbitacin B, as indicated in the figures.

### 2.2. Plasmids, miRNA mimics, and transfection

To prepare *HMGA1* expression constructs, a 291-bp CDS (protein coding region) fragment and 1347-bp 3′-UTR fragment of the *HMGA1* gene (Gene ID: 3159) were synthesized in vitro and cloned into a pcDNA3 vector at Shanghai Novobio Biotechnology (Shanghai, China). The clones were named pcDNA3-HMGA1 and pcDNA3-HMGA1 3′-UTR. The *GACAT3* expression construct was prepared as described previously (Shen et al., 2016). The mimics of miR-7, miR-128, and miR-138 as well as the negative control were synthesized by Shanghai GenePharma (Shanghai, China). Transfection was performed using Lipofectamine 3000 according to the manufacturer's protocol (Invitrogen, Germany).

### 2.3. siRNAs and knockdown experiments

The *GACAT3*, *HMGA1*, and GL3 control siRNAs were prepared as described previously (Shen et al., 2016), and the siRNAs were transfected into the cells using RNAiMAX according to the manufacturer's instructions (Invitrogen, Germany).

### 2.4. Quantitative RT-PCR

RNA was extracted using TRIzol (Invitrogen, Germany) and reverse-transcribed using the GoScript Reverse Transcription System (Promega, USA). Quantitative PCR was performed using LightCycler 480 SYBR Green I Master Mix in a LightCycler 480 II instrument (Roche, USA). The relative fold change was quantified relative to β-actin.

### 2.5. Cell proliferation assay and flow cytometry analysis

Cell proliferation was assessed after transfection. Cells were counted using a hemocytometer. The apoptotic or dead cells were excluded by using 0.2% trypan blue (Sigma-Aldrich, USA) staining. Flow cytometry was used for cell cycle analysis and apoptosis detection. Stained cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, USA).

### 2.6. Western blot analysis

Proteins were extracted using a standard RIPA buffer and separated by SDS-PAGE as described previously (Shen et al., 2016). Primary antibodies against HMGA1 (#39615, ActiveMotif), p21 (#AF6290, Affinity), p27 (#AF6324, Affinity), BAX (#2772S, Cell Signaling Technology), STAT3 (#4904S, Cell Signaling Technology), and β-actin (#A1978, Sigma-Aldrich) were used.

### 2.7. Dual luciferase assay

The 3′ UTR fragment of the *HMGA1* and *GACAT3* sequences were cloned into pGL3 vector at Shanghai Novobio Biotechnology (Shanghai, China) and named as pGL3-HMGA1-3UTR and pGL3-GACAT3. Dural luciferase assay was performed according to the manufacturer's protocol (Promega, USA). Briefly, the constructed reporter vector and the reference vector pRL-SV40 were co-transfected into 293T cells by X-tremeGENE™ HP DNA transfection reagent (Roche, USA). After 48 h post-transfection, the mimics of miRNAs were applied as previously described in present research. For luciferase measurement, firefly luciferase activities were normalized to renilla luciferase activities.

### 2.8. Statistical analysis

The graphic images were created by GraphPad software Prism 6 (San Diego, CA, USA). The SPSS software 18.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data are presented as the mean ± S.D. A two-tailed Student's *t*-test was applied, and statistical significance was considered at $p < 0.05$.

## 3. Results

### 3.1. GACAT3 and HMGA1 are regulated by the same miRNAs

The entire mRNA sequence of *GACAT3* and the 3′-UTR sequence of *HMGA1* were obtained from the NCBI database (National Center for