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# Up-regulation of VEZT by small activating RNA inhibits the proliferation, invasion and migration of gastric cancer cells

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ABSTRACT

*Objective:* To identify an effective saRNA sequence that can specifically up-regulate VEZT expression and to determine the influence of saRNA had on gastric cancer cell growth, proliferation, invasion and migration.

*Methods:* Three various saRNAs, that target the VEZT gene promoter at different locations relative to the transcription start site were synthesized. A dsControl saRNA was synthesized as a negative control, and a specific shRNA was synthesized to knockdown VEZT and eliminate any off-target effects of the saRNA. Both SGC-7901 and M-28 cells were either transfected with the different saRNAs, or treated with Lipofectamine2000 alone. To determine the most effective saRNA, real-time PCR and Western blot were used to determine the VEZT mRNA and protein content, respectively, of each treatment group. After selection, both cell lines were treated with the chosen saRNA, dsControl or Lipofectamine2000. The saRNA treated cells were divided into two groups: the first group was used immediately in the experiments, and the second group was transfected with shRNA by using RNAi-Mate. The proliferation of cells transfected with saRNA, or saRNA and shRNA, as well as the other control cells, was detected by CCK-8. The invasive and migratory abilities were determined using the transwell chamber assay.

*Results:* We identified the most effective saRNA via real-time PCR and Western blot. The selected saRNA inhibited the growth, invasion and migration of GC cells by specially reactivating VEZT. The real-time PCR and Western blot results showed that treatment with saRNA caused a significant up-regulation of VEZT, and an obvious decrease in the proliferative, invasive and migratory abilities; compared with the control groups ($P < 0.01$); furthermore, there were no significant differences among the control groups ($P > 0.05$). This phenomenon provides a theoretical basis for saRNA design and gene therapy for gastric cancer.



## 1. Introduction

Gastric cancer (GC) is the fifth most commonly diagnosed cancer and the third most lethal malignancy worldwide; with about 952,000 new patients diagnosed in 2012 [1,2]. Although the global rate of GC has declined by nearly 2% every year, the total number of new cases has been rising due to the increasing population, and the incidence is rising because of the increasing elderly population, who are at higher relative risk [3]. Currently, surgery remains the only curative treatment; nonetheless, local relapse and/or distant metastasis have been leading cause of treatment failure and the death of GC patients [4]. Both oncogenes and tumour suppressor genes (TSGs) play vital roles in the proliferation, differentiation and apoptosis of cells; the aberrant activation of oncogenes and/or inactivation of TSGs are critical for GC progression [5]. Therefore, additional research on cancer genes is essential to explore new targets for cancer treatment, and can provide theoretical support to guard against cancer. Implementation of siRNA and saRNA is the most promising approach; in this study, we selected the TSG VEZT as the target of saRNA to explore the effectiveness of RNAa.

Early in the 1960s, Britten and others put forward the concept of activator RNA; however this idea was criticised. Soon afterwards, Li and Janowski both discovered saRNA and affirmed that [6,7]: RNA

*Abbreviations:* dsControl, double stranded control; dsRNA, double stranded RNA; GC, gastric cancer; VEZT, vezatin; RNAa, RNA activation; RNAi, RNA interference; saRNA, small activating RNA; siRNA, small interference RNA; TSG, tumour suppressor gene.

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can activate gene expression at the transcriptional level by small double stranded RNA (dsRNA), this phenomenon was called RNA activation (RNAa), and the small double stranded RNA was named small activating RNA (saRNA). Later, different models of RNAa have either been discovered or proposed, including transcriptional activation by targeting specific sequences of promoters [8–11] and/or gene antisense transcripts [12–14] that would lead to changes of chromatin structure at the targeted genes. Some studies have partially elucidated the mechanism of RNAa, which is that RNAa would strengthen post-transcriptional gene expression through saRNAs by either directly promoting translation [15] or antagonizing miRNA target recognition [16].

Vezatin (VEZT), a novel putative TSG located on chromosome 12q22, encodes a plasma membrane protein that plays a vital role in adhesion junctions [17,18]. Commonly, VEZT was studied in epithelial cells and contains two adjacent transmembrane domains that involved in its function. VEZT connects myosin VIIa and the E-cadherin catenin/actin complex through its intracellular and extracellular domains [19]. Currently, VEZT acts as a prognostic factor in gastric cancer.

In this study, we aimed to determine the effect of VEZT gene reactivation in the GC cell lines SGC-7901 and M-28 by using the RNAa technique, and how this reactivation influences the malignant behaviors of the cells, including proliferation, migration and invasion.

## 2. Materials and methods

### 2.1. Reagents

The sequences of saRNA targeted the promoter at various positions as follows: saRNA-1 (dsVEZT-2126): S, 5'-GAACUUGUGUCCAGAGUAUTT-3', AS, 5'-AUACUCUGGACACAAGUUCTT-3'; saRNA-2 (dsVEZT-1328): S, 5'-CCAGCCUAUACUUUCUUAUTT-3', AS, 5'-AUAAGAAAGUAUAGGUUGGTT-3'; saRNA-3 (dsVEZT-94): S, 5'-CUGUCCUAAAUACAACCCATT-3', AS, 5'-UGGGUUGUAUUUAGGAUAGTT-3'; dsControl: S, 5'-UUCUCCGAACGUGUCACGUTT-3', AS, 5'-ACGUGACACGUUCGGAGAATT-3'; and pGPU6/GFP/Neo-shRNA: S, 5'-CACCGCTTCCCACTTGGTGGATTGTTTCAAGAGAACAATCCACCAAGTGGGAAGCTTTTTTG-3', AS, 5'-GATCCAAAAAAGCTTCCCACTTGGTGGATTGTTCTCTTGAAACAATCCACCAAGTGGGAAGC-3'. All RNAs were synthesized by GenePharma (Shanghai, China). For immunoblotting, the anti-VEZT, and anti-β-actin (Santa-Cruz Biotechnology, Santa Cruz, CA) were used. Culture medium comprised: Opti-MEM and RPMI-1640 (Gibco). Transfections were performed by Lipofectamine2000 and RNAi-Mate as appropriate.

### 2.2. Cell culture and transfection

The GC cell lines SGC-7901 and M-28 were obtained from our lab. Both cell lines were cultured in RPMI-1640 supplemented with 10% heat-inactivated foetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/L) and incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. After two passages, the cells were transfected. Li et al. [6] proved that RNAa was a time and dose dependant phenomenon that achieved maximum efficiency with 50 nM saRNA for 72 h. The cells were trypsinised, diluted with Opti-MEM without antibiotics, and seeded into six-well plates at a density of $2 \times 10^5$ well$^{-1}$ for SGC-7901 and $2.5 \times 10^5$ for M-28 cells. Both cell lines were cultured overnight until they reached 50%–70% confluence, and then transfected with either saRNA and dsControl using Lipofectamine2000 as a transfectant. The mock groups were treated with Lipofectamine2000 alone. After the cells were cultured for 72 h, the saRNA-transfected groups were subdivided into one group that was used immediately for experiments and a second group that was transfected with shRNA by using RNAi-Mate and subjected to the CCK-8 and transwell chamber assays.

### 2.3. mRNA analysis by real-time polymerase chain reaction

Total RNA was extracted from the saRNA-, dsControl-, and mock-treated cells by using a TRIzol solution (TaKaRa RNAiso Plus) according to the manufacturer's instruction. Reverse transcription was performed in a 20 μl reaction system using the TaKaRa PrimeScript RT reagent Kit with gDNA Eraser following the manufacturer's instruction. The cDNA was amplified using gene-specific primer sets in conjunction with the SYBR Premix Ex Taq (TaKaRa). Real-time PCR was performed in 20 μl reaction system containing 10 μl of SYBR mix, 0.4 μl each of forward and reverse primer, 7.2 μl of DEPC-treated water, and 2 μl of corresponding cDNA. The following primers were used for real-time PCR: VEZT: forward, 5'-AGATGAACCACAAGCAGATGGA-3', reverse, 5'-TCTTCCTCCTCACCACCAAAA-3'; and GAPDH: forward, 5'-GGACCTGACCTGCCGTCTAG-3', and reverse, 5'-GTAGCCCAGGATGCCCTTGA-3'.

### 2.4. Protein analysis by western blotting

The transfected cells were harvested and washed three times with PBS. (pH 7.4) and re-suspended in lysis buffer (1 mM dithiothreitol, 0.125 mM EDTA, 5% glycerol, 1 mM phenylmethylsulfonylfluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 1% Triton X-100 in 12.5 mM Tris-HCl buffer, pH 7.0) on ice. The cell extracts were centrifuged for 30 min at 12,000 r/min, and the supernatants were collected. The protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) following the manufacturer's instruction. Equivalent amounts of protein were separated on an 8% SDS-polyacrylamidegel and transferred to polyvinylidene difluoride membranes by voltage gradient-transfer. Then, the membranes were blocked in 5% nonfat dry milk for 2 h at room temperature and subsequently washed three times with TBST. Later, the membranes were incubated overnight with the appropriate primary antibody at a dilution specified by the manufacturer. After the primary antibodies were removed and the blots were extensively washed three times with TBST, the blots were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at a proper dilution ratio for 2 h at room temperature. The blots were subsequently washed three times with TBST, developed using an Enhanced Chemiluminescence kit (NENTM Life Science Product Inc., Boston MA, USA), and exposed to X-ray film. Detection of β-actin was used as a loading control. The most effective saRNA was chosen for further experiments.

### 2.5. Cell proliferation assay

The growth rate of the GC cell lines SGC-7901 and M-28 that were transfected by either the selected saRNA or saRNA and shRNA was measured by CCK-8. SGC-7901 and M-28 cells were seeded in a 96-well plate at a density of $4 \times 10^3$ well$^{-1}$ and $5 \times 10^3$ well$^{-1}$, respectively, for the proliferation assay. After an overnight incubation, the cells were transfected with the selected saRNA. At 24 h, 48 h, 72 h, 96 h, 120 h after transfection, 12 μl of CCK-8 was added to each well containing SGC-7901 cells, and 10 μl of CCK-8 for M-28, they were incubated at 37 °C for an additional 4 h. The optical densities were determined on a microreader at 450 nm.

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