Original Article
Reactivation of p21WAF1/CIP1 by saRNA inhibits proliferation, invasion and migration in gastric cancer cells

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Abstract: saRNA is a small molecular non-coding RNA, and it is proved to be effective in many cancer cells, such as prostate cancer cell, kidney tubular epithelial and breast cancer cell, but there has been no report on gastric cancer cells so far. dsP21-322, targeting p21WAF1/CIP1 (p21) gene promoter located at position-322 (dsP21-322) relative to the transcription start site, was synthesized to explore the RNAa-induced reactivation effects on gastric cancer cell lines SGC-7901 and M-28. Besides, a dsControl saRNA was synthesized as a negative control. SGC-7901 and M-28 cells both were transfected with the different saRNAs or treated with lipofectamine2000 alone. real-time PCR and Western blot were used to determine the p21 mRNA and protein content, respectively. The proliferation of transfected cells was assessed by CCK-8. The invasive and migratory abilities were determined by using Transwell assays. The results showed that dsP21-322 caused a significant up-regulation of p21 expression in a time- and dose-dependent manner, and an obvious decrease in proliferation, invasive and migratory abilities compared with the control groups (P<0.01). This phenomenon provides gene therapy for gastric cancer or other malignant tumours and theoretical basis for subsequent RNA activation mechanism research.

Keywords: Gastric cancer, saran, p21, invasion, migration

Introduction
Gastric cancer (GC) is the fifth most commonly diagnosed malignancy and the third lethal cancer around the world, with about 952,000 new cases diagnosed in 2012 [1, 2]. Although the rate of GC has been declining by nearly 2% every year, the total number of new patients has been rising owing to the increasing population, and the incidence is rising because of the increasing number of the elderly, who are at higher risk [3]. So far, surgery remains the only curative treatment option; however, local relapse and/or distant metastasis have been major causes of treatment failure and the death of GC patients [4]. Both the aberrant activation of oncogenes and inactivation of tumor suppressor genes (TSGs) play pivotal roles in the growth, differentiation and apoptosis of cells; and they are critical for GC occurrence and progression [5]. Therefore, the intensive study on cancer genes is essential to discover new targets for cancer treatment. siRNA and saRNA are among the most promising tools. In this study, we selected the TSG p21WAF1/CIP1 as the target of saRNA.

Early in the 1960s, Britten came up with the concept of activator RNA; nonetheless, this idea was controversial. Later, Li and Janowski both discovered and named RNAa [6, 7]: small double-stranded RNA (dsRNA) can activate gene expression at the transcriptional level; the dsRNA was named small activating RNA (saRNA), and this phenomenon was called RNA activation (RNAa). Various models of RNAa have been discovered or proposed including transcriptional activation and post-transcriptional activation. The transcriptional activation was achieved 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; The post-transcriptional activation was through...
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directly promoting translation [15] or antagonizing miRNA target recognition [16].

\( p21^{WAF1/CIP1} \) (p21), a tumor suppressor gene located on chromosome 6p, was marked by broad-acting cyclin-dependent kinase inhibitor and belonged to the Cip/Kip family of cyclin-dependent kinase inhibitor [17-19]. p21 is induced by wild-type p53 reacting to DNA damage and helped to cause G1 cell cycle arrest primarily through inhibiting the activity of cyclin/cdk2 complexes [17, 20, 21].

In this study, we aimed to explore the reactivation effect of p21 gene in GC cell lines SGC-7901 and M-28 through RNAa and how this up-regulation effects the cell proliferation, migration and invasion.

Materials and methods

Reagents


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 Uml\(^{-1}\)) and streptomycin (100 mgL\(^{-1}\)) and incubated at 37°C in a humidified atmosphere containing 5% CO\(_2\). After two passages, the cells were transfected. Li et al. [6] approved that RNAa was a time- and dose-dependent phenomenon. In order to achieve maximum efficiency, cells should be transfected with an indicated time and concentration. The cells were trypsinised, diluted with Opti-MEM without antibiotics, and seeded into six-well plates at a number of 2\(\times\)10\(^5\) per well for SGC-7901 and 2.5\(\times\)10\(^5\) per well for M-28. Both cell lines were cultured overnight until they reached 50%-70% confluence, and then transfected with either dsP21-322 and dsControl using Lipofectamine2000. Both were conducted with an indicated concentration and time. The mock groups were treated with Lipofectamine2000 alone.

Protein analysis by western blotting

The transfected cells were washed three times with PBS. (pH 7.4) and re-suspended in lysis buffer (Beyotime, USA) on ice. The cell extracts were centrifuged for 30 minutes at 12000 rm\(^{-1}\) and the supernatants were collected. The protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Lnc., Rockford, IL, USA) following the manufacturer’s instruction. Equivalent amounts of protein were separated on 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes by voltage gradient-transfer. Then, the membranes were blocked in 5% non-fat 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. Anti-β-actin was used as a loading control.

mRNA analysis by real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from dsP21-322-, dsControl-, and mock-treated cells by using TRizol solution (TaKaRa RNAiso Plus) according to the manufacturer’s instruction. Reverse transcription was performed in a 20 µl reaction system following the manufacturer’s instruction (TaKaRa PrimeScript RT reagent Kit with gDNA Eraser). The cDNA was amplified using gene-specific primer sets in conjunction with the SYBR Premix Ex Taq (TaKaRa) following the manufacturer’s instruction. real-time PCR was performed in 20 µl reaction system which contained 10 µl of SYBR, 0.4 µl of forward primers,
0.4 µl of reverse primers, 7.2 µl of DEPC-treated water, and 2 µl of corresponding cDNA. The following primers were used for real-time PCR:
p21: forward, 5'-GTAACCCGTTGAACCCCATT-3', reverse, 5'-CCATCCAATCGGTAGTAGC-3'; and GAPDH: forward 5'-GGACCTGACCTGCCGTCTAG-3', reverse, 5'-GTAGCCCAGGATGCCCTTGA-3'.

**Cell proliferation assay**

The growth rate of the transfected GC cell lines SGC-7901 and M-28 was measured by CCK-8. SGC-7901 and M-28 cells were seeded in a 96-well plate at a number of 4x10³ and 5x10³ per well, respectively, for the proliferation

Figure 1. dsP21-322 induced the up-regulation of p21 in SGC-7901 and M-28 cells. The expression was assessed by Western blot. A. A schematic representation of p21WAF1/CIP1 promoter and the location of the dsP21-322 target. B, C. SGC-7901 and M-28 cells were treated with dsP21-322 at the indicated concentration for 72 h. The reactivation was a dose-dependent phenomenon that achieved maximum efficiency with 50 nM saRNA. D, E. SGC-7901 and M-28 cells were treated with 50 nM dsP21-322 at the indicated length of time. The reactivation was a time-dependent reactivation achieved maximum efficiency with 50 nM dsP21-322 for 72 h. *indicated P<0.05, and ** indicated P<0.01 compared to the control groups. The p21 protein expression levels were normalized to β-actin and the results were presented as means ± SD of three independent experiments.
Figure 2. dsP21-322 induced p21 protein and mRNA up-regulation and inhibited the proliferation in SGC-7901 and M-28 cells that were transfected with 50 nM dsP21-322 for 72 h. A, D. The expression of p21 protein was assessed by Western blot. dsP21-322 caused approximately 4.9-fold increase expression in SGC-7901 cells and 4.2-fold increase expression in M-28 cells. B, E. The expression of p21 mRNA was assessed by real-time PCR. dsP21-322 caused about 6.6-fold increase expression in SGC-7901 cells and 5.3-fold increase expression in M-28 cells. C, F. The proliferation of SGC-7901 and M-28 cells was detected by CCK-8. dsP21-322 inhibited the proliferation of SGC-7901 and M-28 cells. *indicated P<0.05, and **indicated P<0.01 compared to control groups. The p21 mRNA expression levels were normalized to β-actin and the results were presented as means ± SD of three independent experiments.
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After an overnight incubation, the cells were transfected with indicated concentration, 24 h, 48 h, 72 h, 96 h, 120 h after the transfection, 12 µl of CCK-8 was added to each well containing SGC-7901 cells, and 10 µl of CCK-8 for M-28 cells, then the cells were incubated at 37°C for an additional 4 h. The optical densities were determined on a microreader at 450 nm.

Invasion and migration assays

After the indicated time, the cells were harvested following treatment with the dsP21-322, dsControl or mock. The transfected cells and control groups were suspended in medium. For SGC-7901 cells, the density for invasion and migration was $1 \times 10^5$ ml$^{-1}$; and for M-28 cells, it was $1.5 \times 10^5$ ml$^{-1}$. Then, 0.2 ml of each suspension was added to the top of a transwell chamber PET membranes (24-well insert, 8 µm pore size; Millipore, Bedford, MA) that was either uncoated (for migration assays) or coated with diluted Matrigel (for invasion assays). Medium (0.6 ml) supplemented with 10% foetal bovine serum was added to the lower chamber of each well to act as a chemo-attractant. Cells were incubated for 24 h, and those that did not migrate through the pores were removed by scraping the upper surface of the membrane with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed for 10 minutes in 100% methanol and stained with 0.1% crystal violet for 5 min. The cells that invaded through the insert were counted. All of these experiments were conducted in triplicate and were performed in a minimum of three times.

Statistical analysis

All results are expressed as the means ± standard deviation (SD). Statistical analyses were performed using SPSS statistical software. Student’s t-test and one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests were adopted. Values of $P<0.05$ were considered statistically significant, and $P<0.01$ was considered highly statistically significant.

Results

$dsP21-322$ induced the reactivation of $p21$ gene in GC cells, and the reactivation were time- and dose-dependent

$dsP21-322$ targeted the $p21$ gene promoter at position-322 to initiate RNAa (Figure 1A). The up-regulated expression of $p21$ gene was determined by using Western blot and real-time PCR. The reactivation were dose-dependent (Figure 3A). dsP21-322 inhibits the invasion of SGC-7901 cells. B. The number of invading cells was significantly reduced in the dsP21-322 transfection group: 23.4 vs. 113.6 and 109.5 for the mock- and dsControl-treated groups, respectively ($P<0.01$). C. dsP21-322 inhibits the migration of SGC-7901 cells. D. The number of migratory cells was significantly reduced in the dsP21-322 transfection group: 49.3 vs. 215.7 and 210.6 for the mock- and dsControl-treated groups, respectively. *indicated $P<0.05$, and **indicated $P<0.01$. 

![Figure 3](image-url)
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1B, 1C) and time-dependent (Figure 1D, 1E). dsP21-322-treated groups were highly reactivated compared with the control groups (P<0.01), and achieved the maximum efficiency with 50 nM saRNA for 72 h. dsP21-322 caused over 5-fold expression of p21 in SGC-7901 cells (Figure 2A, 2B), and 4-fold in M-28 cells (Figure 2D, 2E). There was no obvious difference among the mock- and dsControl-treated groups (P>0.05).

dsP21-322 effectively inhibited proliferation and growth of GC cells

To determine the inhibitory effect of dsP21-322 on SGC-7901 and M-28 cell growth, we used the CCK-8 assay. The optical density showed a significant difference between the dsP21-322-treated groups and the control groups (P<0.01); and the most pronounced effect was observed on the third day (Figure 2C, 2F). There was no obvious difference between the mock- and the dsControl-treated groups (P>0.05).

dsP21-322 effectively inhibited migration and invasion of GC cells

Many reports have revealed that loss of p21 expression or function leads to a more invasive phenotype, and restoration by traditional means can inhibit migration and invasion in many types of tumours [17-21]. The migration and invasion abilities were analysed by using a transwell chamber (Figure 3). For SGC-7901 cells, the dsP21-322-treated groups exhibited weaker migratory and invasive abilities compared with those of the control groups (P<0.01). Conversely, there was no significant difference among the control groups (P>0.05) (Figure 4). Similar phenomena were observed in the M-28 cells.

Discussion

Nowadays, GC is one of the most commonly diagnosed malignancies worldwide. Every year it causes about 700,000 deaths globally, which composes up to 10% of the world’s cancer-related deaths [2, 22]. The malignant behavior is primarily because of the complexity of its occurrence and progression, including the genetic and epigenetic factors [23]. Thus it can be seen, GC is a more heterogeneous disease than originally thought, and more curative treatment options are difficult to implement through conventional means [24]. Therefore, it is essential to develop a new approach to achieve the goals, and this leads to the suggestion of RNAa and saRNA.

saRNA, a novel type of gene regulatory molecule, is different from siRNA. Despite both of
them being double-stranded RNA that comprise 21-23 nucleotides in each strand with two overhanging nucleotides on both 3'-ends, they are different in the target sequence, functional dynamics and regulatory mechanism [6, 7, 25-28]. For example, siRNA can play an important role in cancer treatment through blocking the expression of genes [25, 26], while saRNA can achieve the up-regulation of targeted genes through transcriptional and post-transcriptional activation [8-16]. Given that both activated oncogenes and inactivated TSGs are related to GC progression, for cancer treatment, saRNA appears to be at least as consequential as siRNA. In addition, saRNA has many benefits compared with the other traditional therapeutic tools, including low toxicity, high specificity and efficacy; and what is more, some small molecules such as proteins and specific hormones could act in the role of saRNA [6, 7, 29], this will broaden the source or usage of RNAa. Currently, the restrictions of RNAa development have been illustrating the accurate mechanism of RNAa and designing saRNA, but recent studies appear to gradually eliminate the limitations. Li discovered how E-cadherin and VEGF was reactivated in several types of cells and loss of Histone 3 Methylation at lysine-9 was associated with RNAa [6]. Subsequent studies demonstrated that saRNA-guided Ago2 targeted the RITA complex to promoters to stimulate transcription initiation and elongation [30]; further studies showed that RNAa could achieve activation by interacting with the heterogeneous nuclear ribonucleoprotein A2/B1 [31]. In addition, more effective saRNAs were reported, including dsP53-285, dsPAR4-510, WT1-319 and VEZT [12, 32, 33]. All these experiments help to illustrate the mechanism. Above all, Ren and Kang both discovered that formulating saRNA into lipid nanoparticles could inhibit tumorigenicity through animal experiments independently [34, 35].

In this study, we reactivated p21 expression in GC cell lines SGC-7901 and M-28 by dsP21-322, which had been approved to be effective in RNAa [32, 36, 37]. The results of the Western blot showed more details about time- and dose-dependent phenomenon that achieved maximum efficiency with 50 nM dsP21-322 for 72 h; the most appropriate concentration was around 50 nM rather than the higher the better. real-time PCR explain the reactivation effect on the other hand. Furthermore, dsP21-322 could lead to the phenotypic changes in SGC-7901 and M-28 cells. The results of CCK-8 and Transwell chamber assays showed that dsP21-322 could inhibit the growth, migration and invasion of SGC-7901 and M-28 cells. The whole experiment provided evidences that reactivation of p21 by dsP21-322 might be efficient in cancer treatment; saRNA could be a novel therapeutic tool with the potential to improve the effectiveness of cancer treatment.

All in all, though the mechanism of RNAa and saRNA design is unclear, more relative details and effective saRNAs have been discovered [12, 32, 33]; besides, we could predict the effectiveness of saRNAs by using bioinformatics software and design corresponding experiments to identify these molecules. Therefore, the current limitations would not prevent RNAa from becoming a promising therapeutic tool. Certainly, more studies need to be performed to explore the mechanism of RNAa and saRNA design.

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Disclosure of conflict of interest

None.
References


