Research Article

NEAT1 siRNA Packed with Chitosan Nanoparticles Regulates the Development of Colon Cancer Cells via IncRNA NEAT1/miR-377-3p Axis

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This study was for verifying that transfecting colon cancer cells (CCCs) with lncRNA NEAT1 packed with siRNA chitosan nanoparticles (CNPs) can suppress lncRNA NEAT1 and biological behaviors of the cells. siRNA targeting lncRNA NEAT1 expression vector was constructed and then transfected into CCCs after being packed with CNPs. Subsequently, the impact of the transfection on biological behaviors of the cells was evaluated. As a result, with high expression in CCCs, NEAT1 was negatively bound up with miR-377-3p in cases with colon cancer (CC), and dual luciferase reporter assay confirmed the potential binding region. Additionally, after downregulating NEAT1 in CCCs, transfection of NEAT1 siRNA packed with CNPs brought a great inhibition on cell proliferation and a promotion on apoptosis, and inhibiting miR-377-3p was able to offset the role of silencing NEAT1 in CCCs. Therefore, in our opinion, NEAT1 siRNA packed with CNPs can hinder the growth and metastasis of CCGs by knocking down NEAT1 in CC, and its mechanism may be achieved by targeting miR-377-3p, which offers a novel direction for treating CC.

1. Introduction

Currently, with an increasingly high global prevalence and mortality, colon cancer (CC) poses a grave threat to human life and health [1, 2]. It is mainly treated via conventional means like surgery combined with chemoradiotherapy, but after such treatment, patients still show a high recurrence rate and incidence of adverse reactions and thus face unfavorable prognosis [3, 4]. Thus, searching for a novel treatment is critical for patients with CC.

For tumor eradication, targeted gene therapy has received more and more research attention [5]. Small interfering RNA (siRNA) is a short double-stranded RNA that can achieve sequence-specific gene silencing of complementary mRNA, induce mRNA degradation, and inhibit the production of target proteins [6, 7]. siRNA has shown potential as a molecular means to downregulate the expression of specific genes in cancer cells [8]. And siRNA has been adopted for various pathological conditions like viral infection, cancer, genetic diseases, and autoimmune diseases [9]. The siRNA-based therapies have emerged as a promising strategy for targeting a variety of diseases [10]. However, despite notable progress in design, chemical modification, and manufacture of siRNA, lacking an ideal delivery system still limits the development of siRNA-based drugs. Without the assistance of appropriate carriers, it is difficult to accomplish the task of targeted transport of tissues and cell uptake of therapeutic siRNA [11, 12]. Thus, it is imperative to develop a safe and effective delivery system for siRNA.

Various materials have been developed for the effective delivery of siRNA, including lipids, polymers, dendrimers, polymeric micelles, and metallic core nanoparticles [13, 14]. Nontoxic, biocompatible, and biodegradable chitosan nanoparticles (CNPs) serve as an effective platform vehicle that
can release regulation ability and lift bioavailability [15]. As a water-soluble polymer, they are able to solve the precipitation problem during preparation and transfection of gene vectors and can contribute to stronger targeting and cell penetration abilities of gene therapy [16]. Yu et al. demonstrated that CNPs-delivered CXCR4 siRNA reduced the CXCR4 expression and sensitizes breast cancer cells to cisplatin [17]. Nikkhoo et al. confirmed that carboxymethyl dextran-conjugated trimethyl chitosan (TMC-CMD) nanoparticles loaded with NIK/STAT3-specific siRNA and BV6 to synergistically induce apoptosis in the breast, colorectal, and melanoma cancer cell lines [18].

lncRNA NEAT1 has been proven to promote the progression of colorectal cancer [19], endometrial cancer [20], and gastric cancer [21]. Moreover, lncRNA NEAT1 can promote CC progression by regulating miR-495-3p/CDK6 axis [22]. Therefore, in the present study, we used CNPs to deliver lncRNA NEAT1 siRNA in the CC to counteract the progression of colorectal cancer [19], endometrial cancer [20], and gastric cancer [21]. Moreover, lncRNA NEAT1 can promote CC progression by regulating miR-495-3p/CDK6 axis [22]. Therefore, in the present study, we used CNPs to deliver lncRNA NEAT1 siRNA in the CC to confirm that CNPs-deliver siRNA is an effective method to target and inhibit the progress of CC.

2. Materials and Methods

2.1. Collection of Clinical Specimens. Totally, 86 patients receiving radical resection against CC were enrolled, and 86 colon cancer tissue (CCT) specimens and 86 corresponding paracancerous tissue specimens were sampled from them during surgery with their permission. All enrolled participants were patients confirmed with CC via pathological examination who had not received associated therapy before specimen collection. Samples collected according to the approval of the Ethics Committees of our hospital, and all of them consented to take part in the study and signed informed consent forms.

2.2. Cell Culture and Passage. Colon cancer cell lines (HCT116, LoVo, and SW480) and normal colon epithelial cells (NCM460) from American Type Culture Collection (ATCC) were cultured in high-glucose DMEM (HyClone) with 10 fetal bovine sera (FBS) (Gibco) and 1% penicillin/streptomycin (HyClone) under 95% humid atmosphere (37°C, 5% CO₂).

2.3. Preparation Method of NEAT1 siRNA-Encapsulated Nanoparticles. Acetone was gradually slowly dropped into the prepared chitosan-acetic acid solution via magnetic stirring, and then, CNP suspension was obtained via homogenization, followed by 2 h centrifugation (20000 × g) for purifying nanoparticles that were saved after vacuum freeze-drying. CNPs were resuspended in aqueous solution with NEAT1 siRNA plasmid or its si-NC (the weight ratio of chitosan to DNA: 1 : 1) for obtaining gene-carrying CNPs (nano-NEAT1 siRNA) and empty sequences-carrying CNPs (nano-NC). The size and Zeta potential of the former were determined via a Zetasizer 3000 (Malvern Instruments Ltd, Worcestershire, England), and their morphology was evaluated under a scanning electron microscope. Additionally, an ultraviolet spectrophotometer was adopted for determining the entrapment efficiency and the gel electrophoresis for determining the gene protection on enzyme degradation.

2.4. Cell Transfection. LoVo and SW480 cells seeded into a 96-well plate were transfected with miR-377-3p-mimics, miR-377-3p-Inhibitor, miR-NC, nano-NEAT1 siRNA, nano-NC, si-NEAT1 sequence, or its si-NC via a Lipofectamine™ 2000 kit (Invitrogen) under strict kit guidelines.

2.5. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA (10 ng) acquired from cells via TRI-ZOL reagent (Tiangen) was synthesized into cDNA via RevertAid reverse transcriptase for the qRT-PCR on miRNA. Under the manufacturer’s guidelines, a SYBR Green PCR kit was adopted for PCR (with internal reference of U6) via 95°C/5 min, followed by 40 cycles of 95°C/12 s and 62°C/40 s. Calculation about miRNA was conducted based on 2^ΔΔCt method.

2.6. MTT Assay. Totally, 20 μL MTT solution (5 mg/mL) was put into a 96-well plate seed with LoVo and SW480 cells in log-growth stage at 3 × 10⁴ cells/mL at 0, 24, 48, and 72 h after cell seeding, respectively. After 4 h incubation, 150 μL DMSO was added into each well, followed by determination of optical density via a microplate reader at 490 nm.

2.7. Apoptosis Assay. The transfected cells were subjected to digestion via 0.25% trypsin before two times of washing via PBS and then prepared into 1 × 10⁶ cells/mL in 100 μL binding buffer. Subsequently, the suspension was subjected to 5 min incubation with dark surroundings after being successively added with Annexin-V-FITC and PI. Finally, cell determination was performed via a FACSVerse flow cytometer.

2.8. Western Blot (WB). Total protein was acquired from cells via RIPA lystate, followed by determination of protein concentration via the BCA means. Then, with a concentration adjusted to 4 μg/μL, the protein was subjected to SDS-PAGE, followed by transfer to a PVDF membrane that was then subjected to 2 h immersion in 5% nonfat milk. The membranes were then incubated with primary antibodies Bax (1 : 500), Bcl-2 (1 : 500), and β-actin (1 : 1000) overnight at 4°C. The membrane was washed for eliminating the primary antibody, followed by 1 h incubation (37°C) with goat anti-rabbit secondary antibody (1 : 1000) and three times of washing via PBS (5 min/time). Finally, electrochemiluminescence (ECL) and development were carried out.

2.9. Dual Luciferase Reporter (DLR) Assay. Candidate miR-NAs able to bind to NEAT1 were searched in bioinformatics database (Starbase v2.0), and NEAT1 target sequence-contained oligonucleotides were treated via amplification and cloning into pmirGLO plasmid (WT). The established wild-type mirGLO-NEAT1-3’TUTR (Wt) and mutant mirGLO-NEAT1-3’TUTR (mut) were transferred to the downstream of luciferase reporter gene for sequencing and identifying the constructed plasmids. Then, Lipofectamine 2000 was adopted for cotransfecting luciferase reporter
plasmid and miR-377-3-mimic or miR-NC into CCCs. After 48 h, the luciferase activity was determined in a DLR system.

2.10. Statistical Analysis. This study adopted SPS819.0 for statistical analyses of obtained data and GraphPad 7 for drawing of required figures. In terms of comparison, intergroup and multigroup comparisons were conducted via the independent t-test and one-way ANOVA, respectively, and post hoc pairwise comparison and comparison of data at various time points were performed via the LSD-t and repeated measure analysis of variance, respectively. Additionally, Bonferroni post hoc test was applied. P < 0.05 denoted a notable difference.

3. Results and Discussion

3.1. NEAT1 Was Upregulated in CC Tissues and Cell Lines. As shown in Figure 1(a), the NEAT1 expression in CCTs was significantly increased compared to those in the paracancerous tissues (P < 0.05). In addition, the expression of NEAT1 further confirmed in cc cell lines HCT116, LoVo, SW480, and normal colon epithelial cells NCM460. The NEAT1 expression was significantly upregulated 1.77 ± 0.21 in HCT116, 1.84 ± 0.25 in LoVo, and 1.86 ± 0.26 in SW480 cell lines compared with NCM460 cell (Figure 1(b)) (P < 0.05). These results suggested that the NEAT1 expression might play the role in the development of CC.

3.2. Characterization of Nano-NEAT1 siRNA and Its Impact on the Viability of CCCs. As the characterization of nano-NEAT1 siRNA under TEM showed, it was analogously spherical, with a size of 120-150 nm, and the nanoparticles were evenly dispersed and encapsulated and dispersed well (Figure 2(a)). After transfection, NEAT1 was downregulated in CCCs in the ano-NEAT1 siRNA and si-NEAT1 groups, but its inhibition in the former was stronger (P < 0.05). Moreover, before and after transfection, the NEAT1 expression in LoVo cells was 0.41 ± 0.03 and 0.51 ± 0.06, respectively, and that in SW480 cells was 0.42 ± 0.04 and 0.58 ± 0.05, respectively (all P < 0.05, Figure 2(b)). In contrast to the nano-NC and si-NC groups, the nano-NEAT1 siRNA and si-NEAT1 groups showed a notable decrease in cell viability, and the decrease in the former group was more notable (P < 0.05) (Figure 2(c)).

3.3. Enhancement of Nano-NEAT1 siRNA on Apoptosis of CCCs. According to the flow cytometry, the apoptosis of LoVo cells in the si-NEAT1, Si-NC, nano-NEAT1 siRNA, and si-NEAT1 groups was 28.13 ± 2.05, 16.87 ± 1.21, 41.34 ± 3.52, and 17.16 ± 1.27, respectively, and that of SW480 cells in these groups was 29.03 ± 2.02, 15.86 ± 1.16, 42.09 ± 3.64, and 16.63 ± 1.11, respectively. Therefore, in contrast to the nano-NC and si-NC groups, the other three groups presented notably increased apoptosis, and the increase in the nano-NEAT1 siRNA group was more notable (P < 0.05) (Figure 3).

3.4. Bax and Bcl-2 in Cells. According to the WB assay, the expression of Bax and Bcl-2 in LoVo cells in the si-NEAT1 group was 0.89 ± 0.09 and 0.51 ± 0.06, respectively; that in the nano-NEAT1 siRNA group was 1.15 ± 0.10 and 0.42 ± 0.04, respectively; that in the si-NC group was 0.51 ± 0.05 and 0.62 ± 0.06, respectively; and that in the nano-NC group was 0.53 ± 0.06 and 0.61 ± 0.07, respectively (Figure 4(a)). Additionally, the expression of Bax and Bcl-2 in SW480 cells in the si-NEAT1 group was 0.83 ± 0.08 and 0.52 ± 0.05, respectively; that in the nano-NEAT1 siRNA group was 1.16 ± 0.14 and 0.41 ± 0.05, respectively; that in the si-NC group was 0.51 ± 0.05 and 0.62 ± 0.06, respectively; and that in the nano-NC group was 0.52 ± 0.05 and 0.63 ± 0.06, respectively (Figure 4(b)). Therefore, si-NEAT1 and nano-NEAT1 siRNA groups presented notably higher Bax and notably lower Bcl-2 than the miR-NC and nano-NC groups, and the nano-NEAT1 siRNA group presented notably higher Bax and notably lower Bcl-2 than the si-NEAT1 group (all P < 0.05).
3.5. DLR Assay. The starbase-based online prediction of positional miR of NEAT1 demonstrated a potential binding target between NEAT1 and miR-377-3p (Figure 5(a)), so we conducted a DLR assay further and found notable inhibition of miR-377-3p-mimics on the fluorescence activity of NEAT1-WT (Figure 5(b)), notable upregulation of knocking down NEAT1 on miR-377-3p in LoVo and SW480 cells, and more remarkable upregulation of nano-NEAT1 siRNA on miR-377-3p in them (Figure 5(c)).

3.6. Reversion of Inhibiting miR-377-3p on the Inhibition of NEAT1 to the Growth of CCCs. For further verifying whether NEAT1 works by targeting miR-377-3p in CCCs, nano-NEAT1 siRNA and miR-377-3p-inhibitor were cotransfected into SW480 cells for functional analyses. The MTT assay revealed the successful reversion of cotransfection with miR-377-3p-inhibitor on the upregulation of nano-NEAT1 siRNA in CCCs (Figure 6(a)), and the MTT assay revealed the inhibition of nano-NEAT1 siRNA on the proliferation of SW480 cells and reversion by miR-377-3p-inhibitor on the inhibition (Figure 6(b)). Additionally, the flow cytometry revealed the reversion of miR-377-3p-inhibitor on the promotion of nano-NEAT1 siRNA in cell apoptosis (Figure 6(c)). These data demonstrate that NEAT1 works in CC by regulating miR-377-3p.

3.7. Discussion. Over the past few years, siRNA-based cancer treatment technology has captured much attention. Many earlier studies adopted plasmid vectors for expressing siRNA...
in cells, which can not only escape from the treatment of RNA and lower the cost but also prolong the action time of siRNA and obtain long-run stable siRNA effect, but the transfection efficiency of plasmid was blindingly low [23, 24]. Thus, development of new suitable siRNA delivery vectors is still an urgent. During the last couple of years, nanoparticles constructed by polymers, as transfection vectors, have delivered favorable outcomes [25]. Chitosan demonstrates remarkable strengthens as a drug or genetic carrier for its strong adsorption ability to organisms, few side effects, abundance, and low cost [26]. Moreover, it is a simple means requiring no strict preparation conditions, and it will not break the structure and function of plasmid genes with no use of organic solvent [27]. Our study constructed nano-NEAT1-siRNA based on the design principle of siRNA and packed it with CNPs for analyzing its impact on CCCs.

**Figure 5:** DLR assay. (a) Possible binding targets between NEAT1 and miR-377-3p. (b) DLR enzyme activity. (c) Impact of NEAT1 on miR-377-3p in CCCs. ** denotes $P < 0.05$; * and # denote $P < 0.05$ vs. si-NEAT1 group; $P < 0.05$ in terms of * vs. #.

![Figure 5](image-url)
experimental requirement, with a size of 120-150 nm and high entrapment efficiency, and it successfully transfected CCCs and thus downregulated NEAT1 in them and showed notably higher transfection efficiency than conventional plasmid transfection. The results indicate the effective transfection of nano-NEAT1-siRNA packed with chitosan nano-carrier for CC. Afterwards, we further elaborated its antitumor ability in CCCs. Firstly, we discovered the effective inhibition of transfecting nano-NEAT1-siRNA on the viability of CCCs and also found the remarkable promotion effect of it on apoptosis of CCCs through flow cytometry. The results denote the role of NEAT1 as an oncogene in CC, the ability of transfecting nano-NEAT1-siRNA on the viability of CCCs and also found the remarkable promotion effect of it on apoptosis of CCCs through flow cytometry. The results denote the role of NEAT1 as an oncogene in CC, the ability of transfecting nano-NEAT1-siRNA in effectively causing weaker cell proliferation and stronger apoptosis, and its stronger inhibition effect on CC than conventional transfection of NEAT1 siRNA. The obtained data further verify the stability of CNPs in delivering siRNA, which is helpful for further in vivo drug delivery experiments.

As we all know, lncRNA, a competitive endogenous RNA, regulates protein by binding to miRNA. For instance, NEAT1 regulates SOX2 by targeting miR-132, thereby suppressing the migration and invasion of glioma cells [30]. In addition, NEAT1 has been found to regulate miR-1224-5p in cases with gastric cancer and to be bound up with the imbalance of NEAT1 and unfavorable prognosis [31]. Our study provided further evidence for the participation of lncRNA in the role of mRNA posttranscriptional regulation in the pathogenesis of CC as CERNA. miR-377-3p can bind to NEAT1 according to biological analysis, and their targeting association was verified via the DLR assay. For further confirming that NEAT1 works in cases with CC by regulating miR-377-3p, we also conducted a rescue experiment and found the reversion of miR-377-3p-inhibition on the inhibition of nano-NEAT1 siRNA to the development of CCCs and its promotion on their apoptosis. This is the first time that we have discovered the anticancer role of nano-NEAT1 siRNA via upregulating miR-377-3p. One earlier study has also reported the role miR-377-3p in CC and found its anticancer influence in CC through directly targeting WEB/β-catenin [32]. However, we have not explored the follow-up mechanism of miR-377-3p.

4. Conclusion

In our study, prepared CNPs can efficaciously deliver siRNA, with a high safety in vivo, and nano-NEAT1 siRNA targeting NEAT13 siRNA has demonstrated a strong inhibition on the growth of CCCs after transfection and can strongly accelerate
the apoptosis of CCCs. As these results show, CNPs are potential candidate vectors for siRNA-based cancer gene therapy.

**Data Availability**

All data generated or analyzed during this study are included in this article.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Authors’ Contributions**

Tianyu Li, Nenghui Deng, and Ruimei Xu contributed equally to this work.

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