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ORIGINAL ARTICLE

# Long noncoding RNA NEAT1 aggravates sorafenib-resistance in non-small cell lung cancer via regulating miRNA-335/c-Met

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## Summary

***Purpose:*** *The purpose of this study was to illustrate the role of long non-coding RNA (lncRNA) NEAT1 in inhibiting sorafenib sensitivity in non-small cell lung cancer (NSCLC) through targeting microRNA-335 (miR-335)/c-Met axis.*

***Methods:*** *Regulatory effects of NEAT1/miR-335/c-Met axis on proliferative ability of sorafenib-induced A549 and PC9 cells were assessed by cell counting kit-8 (CCK-8) and colony formation assay. Apoptosis changes influenced by nuclear paraspeckle assembly transcript 1 (NEAT1)/miR-335/c-Met axis after sorafenib treatment in lung cancer cells were examined by detecting apoptotic rate, as well as relative levels of Bcl-2 and Bax. The interaction among NEAT1/miR-335/c-Met was analyzed through dual-luciferase reporter gene assay.*

***Results:*** *Sorafenib treatment in A549 cells and PC9 cells attenuated the proliferation and induced apoptosis, which were more pronounced after silencing of NEAT1. MiR-335 was the downstream target of NEAT1, and its level was negatively regulated by NEAT1. Moreover, c-Met was the target gene of MiR -335. Rescue experiments verified the role of NEAT1/MiR-335/c-Met regulatory loop in reducing the proliferative ability and inducing apoptosis of sorafenib-treated lung cancer cells.*

***Conclusions:*** *LncRNA NEAT1 aggravates sorafenib resistance in NSCLC through inhibiting MiR-335 to upregulate c-Met level, manifesting as attenuated proliferation and accelerated apoptosis.*

***Key words:*** *NSCLC, sorafenib, NEAT1, miR-335 c-Met*

## Introduction

Multiple organ metastasis and lymph node metastasis of non-small cell lung cancer (NSCLC) lead to poor prognosis of affected people, especially in those with decreased immunity or complicated with cardiopulmonary dysfunction. The mortality rate of NSCLC is up to 25% [1]. Currently, drug therapy is a major approach for clinical treatment of NSCLC [2]. It is necessary to uncover the mechanism of chemotherapy resistance in NSCLC, thus improving the therapeutic efficacy.

Long non-coding RNAs (lncRNAs) are a type of non-coding RNAs with 200 nt long. They are functionally involved in epigenetic, transcriptional and post-transcriptional regulation. In addition, lncRNAs participate in the occurrence, progression and prognosis of human diseases [3]. NEAT1 (nuclear paraspeckle assembly transcript 1) is a newly discovered lncRNA with two monomeric forms, namely NEAT1-1 (3.7 kb) and NEAT1-2 (23 kb) [4]. Studies have shown that NEAT1 overexpression can remarkably promote the growth of lung cancer cells and trigger tumor metastasis [5].

MicroRNAs (miRs) are widely expressed and capable of regulating gene expressions at the post-transcriptional level [6]. They are able to mediate various cellular behaviors. In almost all types of tumors, differentially expressed miRs could be discovered, and exert a carcinogenic or

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anti-tumor effect [7,8]. Upregulation of miR-335 is discovered in colorectal cancer and astrocytoma [6,7]. Upregulated miR-335 is significantly increased in NSCLC tissues compared with that in para-tumor ones [8].

Cellular mesenchymal to epithelial transition factor (c-Met) is closely related to the tumorigenesis of NSCLC [9]. By binding to its unique ligand hepatocyte growth factor, c-Met triggers a series of downstream events and thus affects various biological effects [10]. As a vital therapeutic target, c-Met could sufficiently alleviate the progression of lung cancer [11].

Sorafenib is a novel type of anti-tumor drug, showing a high biological concentration *in vivo* (especially in lungs), long half-life and abundant anti-tumor activity. Sorafenib is able to suppress persistent proliferation of tumor cells and drug resistance by inactivating many tumor-related pathways [12]. In liver cancer, NEAT1 is able to inhibit sorafenib sensitivity by targeting miRNA-335/c-Met [13]. In this paper, we mainly explored the role of NEAT1/miRNA-335/c-Met regulatory loop in influencing sorafenib sensitivity in NSCLC.

## Methods

*Cell culture and transfection*

A549 and PC9 were provided by Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 μg/mL penicillin and 0.1 mg/mL streptomycin, in a 37°C, 5%$CO_2$ incubator. Cells pre-seeded in 24-well plates were cultured at 70% confluence and transfected using Lipofactamine 2000 (Invitrogen, Carlsbad, CA, USA). Six hours later, the complete medium was replaced. Sequences of transfection vectors were as follows: siRNA-NEAT1: sense: 5'-GUGAGAAGUUGCUUAGAAACUUUCC-3' and antisense: 5'-GGAAAGUUUCUAAGCAACUUCUCACUU-3'; MiRNA-335 mimics: sense: 5'-UCAAGAGCAAUAACGAAAAAUGU-3' and antisense: 5'-AUUUUUUCGUUAUUGCUCUUUU-3'; MiRNA-335 inhibitor: sense: 5'-ACAUUUUUCGUUAUUGCUCUUGA-3' and antisense: 5'-CAGUACUUUUGUGUAGUACAA-3'.

*Quantitative real-time polymerase chain reaction (qRT-PCR)*

Extraction of total RNA in cells was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the cells were subjected to reverse transcription. The extracted complementary deoxyribose nucleic acid

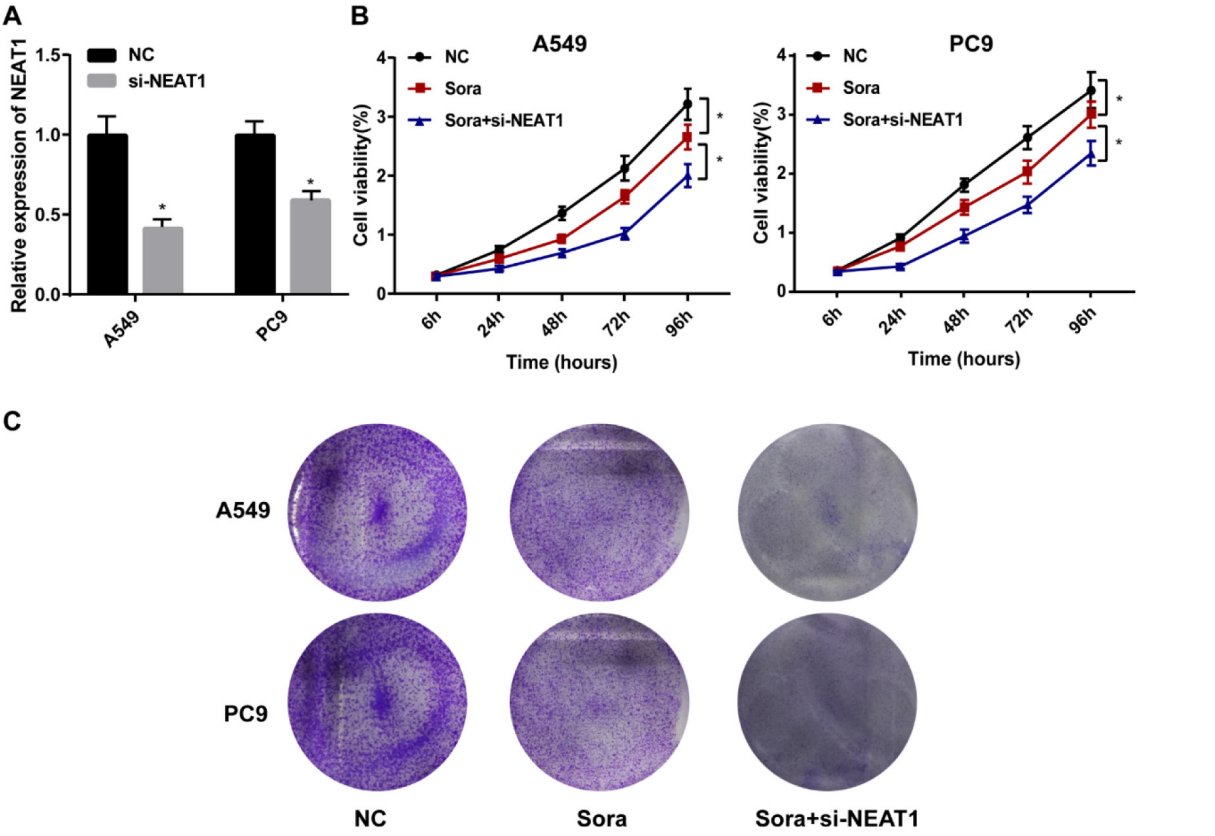


**Figure 1.** Knockdown of NEAT1 enhanced sorafenib-sensitivity in NSCLC. **A:** Transfection efficacy of si-NEAT1 in A549 and PC9 cells. **B:** Viability in A549 and PC9 cells with blank control, sorafenib treatment or sorafenib treatment + si-NEAT1 transfection. **C:** Clonality in A549 and PC9 cells with blank control, sorafenib treatment or sorafenib treatment + si-NEAT1 transfection (*p<0.05).

(cDNA) was applied for PCR using SYBR Green method (TaKaRa, Tokyo, Japan). Primer sequences were as follows: NEAT1: forward: 5'-GTGGCTGTTGGAGTCGGTAT-3' and reverse: 5'-TAACAAACCACGGTCCATGA-3' (antisense); MiRNA-335: forward: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACATTT-3' and reverse: 5'-GCGCTCAAGAGCAATAACGAA-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH: forward: 5'-AGCCCAAGATGCCCTTCAGT-3' and reverse: 5'-CCGTGTTCCTACCCCCAATG-3'.

*Cell counting kit-8 (CCK-8)*

Cells were seeded in 96-well plates with $3\times10^3$ cells per well and cultured overnight. Absorbance at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curves.

*Colony formation assay*

Cells were seeded in 6-well plates with 800 cells per well and were cultured for at least 10 days until the formation of visible colonies. These colonies were subjected to 15-min fixation in 4% paraformaldehyde and 5-min dyeing with violet crystal. After PBS wash and air dried, images of colonies were captured for counting.

*Apoptosis determination*

Cells were washed with PBS twice and centrifuged at 3000 r/min for 5 min. The precipitant was resuspended in 500 μL of binding buffer, incubated with 5 μL of Annexin V in the dark for 15 min, and 5 μL of propidium iodide (PI) at 4°C in the dark for 15 min. After 5-min centrifugation at 3000 r/min, the precipitant was dissolved in 300 μL of binding buffer and subjected to flow cytometry.

*Western blot*

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were loaded for electrophoresis and transferred on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skim milk for 2 h, the membranes were incubated with primary and secondary antibodies. Finally, bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

*Dual-luciferase reporter gene assay*

Wild type and mutant type vectors were constructed based on the binding sites in the promoter regions of

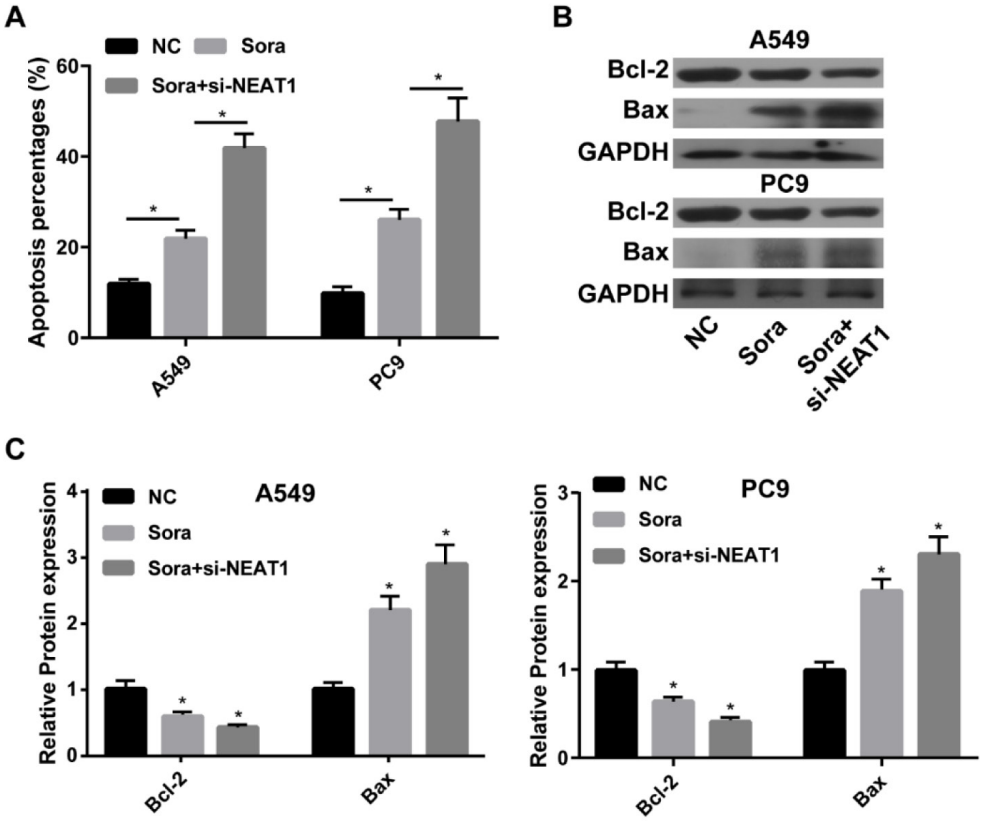


**Figure 2.** Knockdown of NEAT1 accelerated apoptotic sensitivity of NSCLC to sorafenib. **A:** Apoptosis percentage of A549 and PC9 cells with blank control, sorafenib treatment or sorafenib treatment+si-NEAT1 transfection. **B:** Western blot analyses of Bcl-2 and Bax in A549 and PC9 cells with blank control, sorafenib treatment or sorafenib treatment + si-NEAT1 transfection. **C:** Protein levels of Bcl-2 and Bax in A549 and PC9 cells with blank control, sorafenib treatment or sorafenib treatment+si-NEAT1 transfection (*p<0.05).

two genes. Cells were co-transfected with wild type/mutant type vectors and miR-335 mimic/control for 48 h. Afterwards, cells were lysed for determining luciferase activity (Promega, Madison, WI, USA).

*Statistics*

SPSS 22.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the t-test. P<0.05 was considered as statistically significant.

## Results

*Knockdown of NEAT1 enhanced sorafenib sensitivity in NSCLC*

First of all, we constructed si-NEAT1 and tested its transfection efficacy in A549 and PC9 cells (Figure 1A). Transfection of si-NEAT1 significantly downregulated the NEAET1 level. CCK-8 assay showed that sorafenib treatment markedly decreased the viability of lung cancer cells, which was further inhibited by transfection of si-NEAT1 (Figure 1B). Similarly, clonality was attenuated by sorafenib treatment, and silencing NEAT1 aggravated the clonality inhibition in NSCLC (Figure 1C). The above data demonstrated that NEAT1 influenced sorafenib sensitivity in NSCLC through attenuating the proliferative ability.

*Knockdown of NEAT1 accelerated apoptotic sensitivity of NSCLC to sorafenib*

Flow cytometry data revealed an elevation in the apoptotic rate after sorafenib treatment in lung cancer cells. Knockdown of NEAT1 further accelerated apoptosis in sorafenib-treated NSCLC (Figure 2A). Moreover, protein levels of apoptosis-related genes (Bcl-2 and Bax) were determined. As western blot analyses depicted, the protein level of Bcl-2 was downregulated and Bax was upregulated in sorafenib-treated lung cancer cells. Changes in Bcl-2 and Bax levels were much more obvious after transfection of si-NEAT1 (Figures 2B,2C).

*MiRNA-335 was the downstream target of NEAT1*

Cumulative evidence has uncovered the role of lncRNAs to negatively regulate miR levels [14]. Here, bioinformatics analysis pointed out binding sites in the promoter regions of NEAT1 and miR-

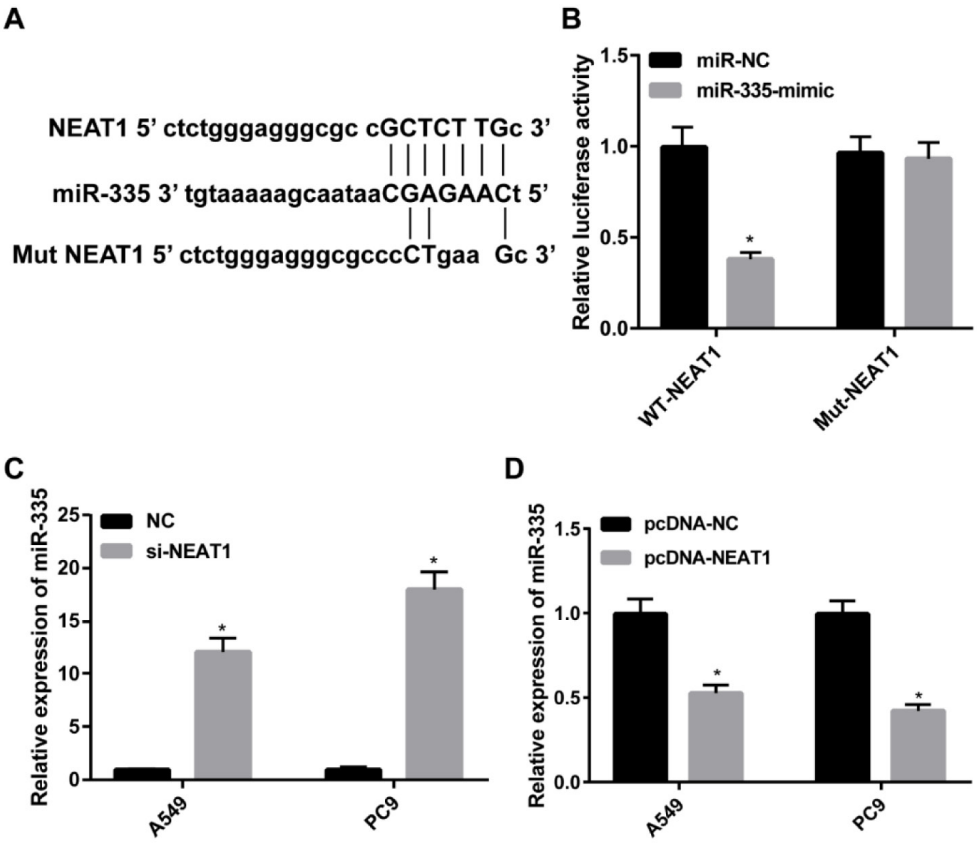


**Figure 3.** MiR-335 was the downstream target of NEAT1. **A:** Binding sites in the promoter regions of NEAT1 and miR-335. **B:** Luciferase activity in A549 cells co-transfected with miR-NC/miR-335 mimic and WT-NEAT1/Mut-NEAT1. **C:** MiR-335 level in A549 and PC9 cells transfected with NC or si-NEAT1. D: MiR-335 level in A549 and PC9 cells transfected with pcDNA-NC or pcDNA-NEAT1 (*p<0.05).

335 (Figure 3A). Transfection of wild type NEAT1 vector was able to quench the luciferase activity of miR-335, supporting the fact that miR-335 was the downstream target of NEAT1 (Figure 3B). Moreover, transfection of si-NEAT1 upregulated miR-335 level, and conversely, transfection of pcDNA-NEAT1 downregulated its level (Figures 3C,3D). It was proved that NEAT1 targeted and negatively regulated miR-335.

*Knockdown of miRNA-335 alleviated sorafenib sensitivity in NSCLC*

To explore the potential function of miR-335 in regulating sorafenib sensitive NSCLC, cells were transfected with miR-335 inhibitor. Silencing NEAT1 in sorafenib-treated A549 and PC9 cells inhibited the viability and clonality, which were partially reversed by co-transfection of miR-335 inhibitor (Figures 4A,4B). Apoptotic rate was elevated after knockdown of NEAT1 in sorafenib-treated cells, which was reversed by knockdown of miR-335 (Figure 4C).

*MiRNA-335 directly targeted c-Met*

Subsequently, the target gene of miR-335 was predicted by bioinformatics method (Figure 5A). Luciferase activity declined after co-transfection of WT-c-Met and miR-335 mimic confirmed the binding relationship between miR-335 and c-Met (Figure 5B). It was found that c-Met level was downregulated after transfection of miR-335 mimic, whereas it was upregulated by transfection of miR-335 inhibitor (Figure 5C).

*NEAT1 induced sorafenib resistance in NSCLC via targeting miR-335/c-Met axis*

To clarify the potential role of NEAT1/miR-335/c-Met in sorafenib-resistant NSCLC, a series of rescue experiments were conducted. Silencing NEAT1 decreased the viability of sorafenib-treated A549 cells and PC9 cells, which was partially reversed by silencing miR-335 or overexpressing c-Met (Figure 6A). Additionally, the increased apoptotic rate in sorafenib-treated lung cancer cells

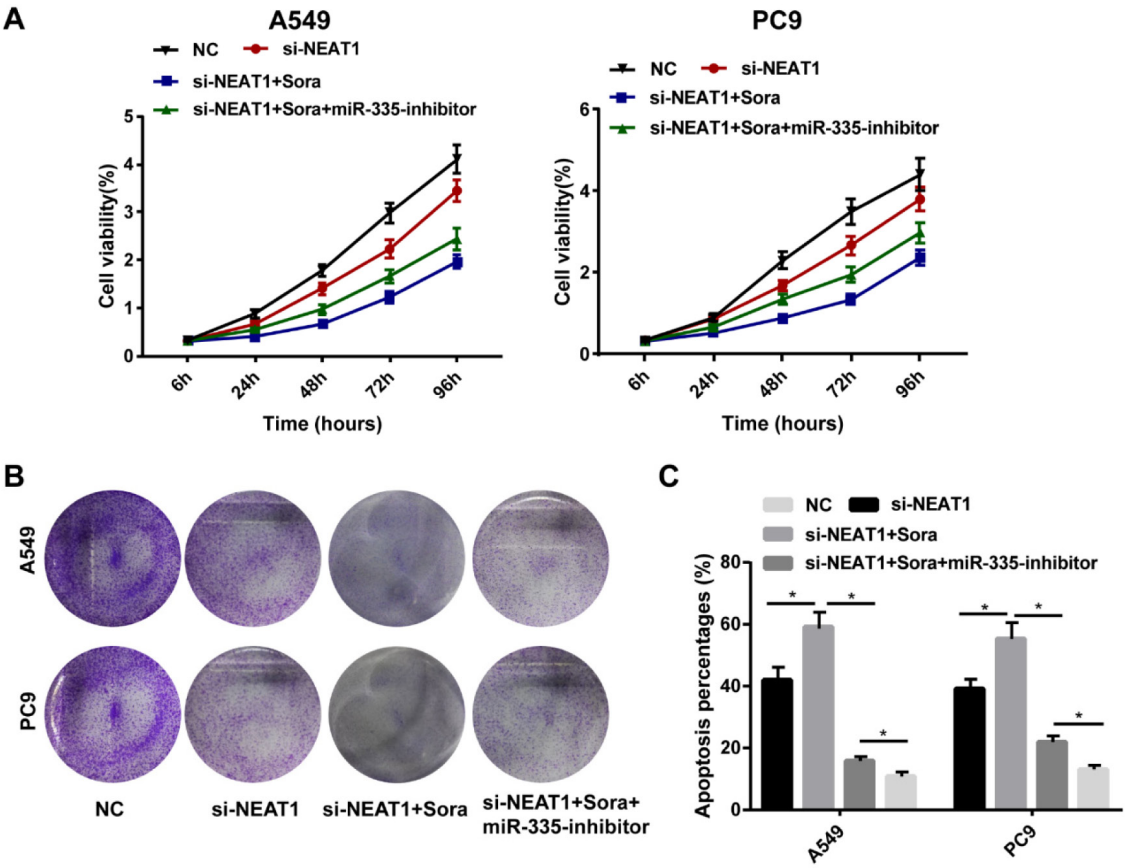


**Figure 4.** Knockdown of miR-335 alleviated sorafenib-sensitivity in NSCLC. **A:** Viability of A549 and PC9 cells with blank control, si-NEAT1 transfection, sorafenib treatment+si-NEAT1 transfection or sorafenib treatment + co-transfection of si-NEAT1 and miR-335 inhibitor. **B:** Clonality of A549 and PC9 cells with blank control, si-NEAT1 transfection, sorafenib treatment + si-NEAT1 transfection or sorafenib treatment+co-transfection of si-NEAT1 and miR-335 inhibitor. **C:** Apoptosis percentage of A549 and PC9 cells with blank control, si-NEAT1 transfection, sorafenib treatment + si-NEAT1 transfection or sorafenib treatment + co-transfection of si-NEAT1 and miR-335 inhibitor (*p<0.05).

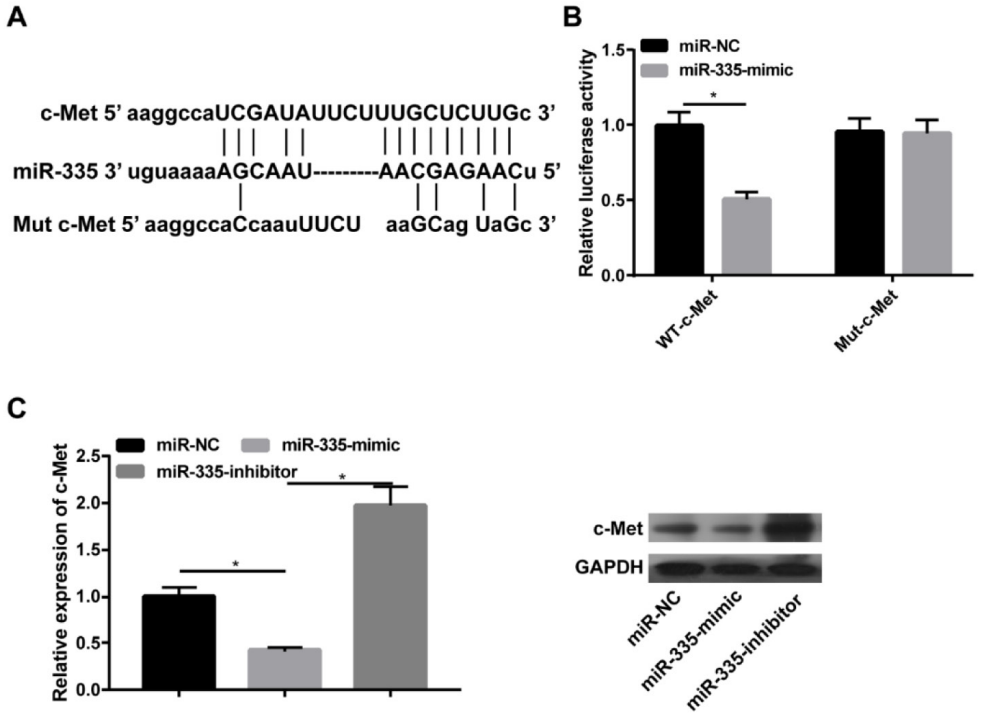


**Figure 5.** MiR-335 directly targeted c-Met. **A:** Binding sites in the promoter regions of miR-335 and c-Met. **B:** Luciferase activity in A549 cells co-transfected with miR-NC/miR-335 mimic and WT-c-Met/Mut-c-Met. **C:** Relative level of c-Met in A549 cells transfected with miR-NC, miR-335 mimic or miR-335 inhibitor (*p<0.05).

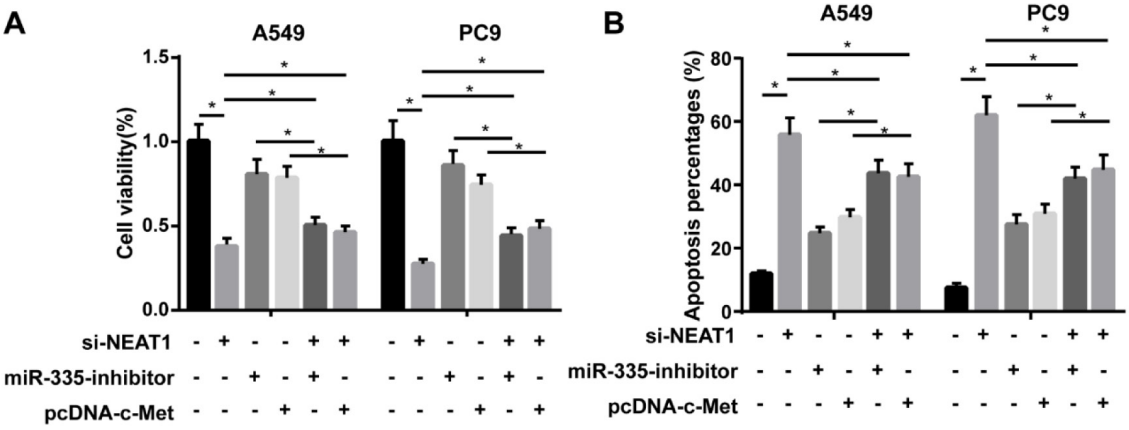


**Figure 6.** NEAT1 induced sorafenib-resistance in NSCLC via targeting miR-335/c-Met axis. Viability **(A)** and apoptosis percentage **(B)** of A549 and PC9 cells with different treatments (*p<0.05).

with NEAT1 knockdown was reversed by miR-335 knockdown or c-Met overexpression as well (Figure 6B). Collectively, NEAT1 alleviated sorafenib sensitivity in NSCLC via targeting miRNA-335/c-Met.

## Discussion

NSCLC is a malignant tumor that seriously threatens human lives and health. The morbidity and mortality of NSCLC are in the first place, no matter in China or worldwide [15]. Most NSCLC patients lose their optimal chance for surgical resection owing to the atypical signs and symptoms in the early disease stage [16]. Sorafenib is a small-molecular, multi-biotargeted anti-tumor drug. It is the first discovered oral polykinase inhibitor, presenting a promising application [17]. Explorations on factors affecting sorafenib sensitivity in NSCLC are of clinical value.

LncRNAs are closely related to tumor progression and metastasis by regulating gene expressions at the transcriptional or post-transcriptional lev-

els [18]. MiRs are extensively involved in almost all developmental and pathological processes in animals. Dysregulation of miRs is associated with many human diseases, especially tumors [2]. With in-depth research on these non-coding RNAs, lncRNAs and miRs exhibit their therapeutic potentials in malignant diseases. In recent years, a novel theory proposed that lncRNA could competitively bind to a target miR, thus affecting expressions and functions of downstream genes, that is, ceRNA hypothesis [10]. NEAT1 is reported to upregulate the oncogene E2F3 by absorbing miR-337-3p, therefore aggravating the progression of NSCLC [5]. CTR-1 upregulation remarkably enhances cisplatin concentration in tumor cells and chemotherapy efficacy [20]. Jiang et al. [21] demonstrated that NEAT1 serves as a sponge for miR-98-5p, which further upregulated CTR-1 level in NSCLC. In this paper, our findings uncovered the interaction among NEAT1/miR-335/c-Met in NSCLC. Rescue experiments confirmed that regulatory effects of NEAT1 in sorafenib-treated lung cancer cells could be partially reversed by miR-335 knockdown or c-Met overexpression. Collectively, NEAT1 aggravated sorafenib resistance in NSCLC through absorbing miR-335 to upregulate c-Met level.

## Conclusions

LncRNA NEAT1 suppresses sorafenib sensitivity in NSCLC by inhibiting miR-335 to upregulate c-Met level, manifested as attenuated proliferation and accelerated apoptosis.

## Conflict of interests

The authors declare no conflict of interests.

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