**LncRNA NNT-AS1 affect progesterone resistance by regulating miR-542-3p****/survivin axis in endometrial cancer**

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Running title: NNT-AS1 affect endometrial cancer resistance via miR-542-3p

Abstract:

Background: Progestin is commonly used for young patients suffering from endometrial cancer, but more than 30% of patients with progestin treatment have presented progestin resistance. The previous study showed that lncRNA NNT-AS1 could play an important role in cervical cancer. This study aimed to investigate the expression of NNT-AS1 in progesterone resistance endometrial cancer and assess its possible molecular mechanism.

Methods: Establishment of progesterone resistant Ishikawa (Ishikawa-PR) cell line that are resistant to the growth-inhibitory effects of progestin in vitro. Protein and mRNA expression were determined by western blot and qRT-PCR, respectively. MTT assay tested cell proliferation. Silence and overexpression of NNT-AS1 and survivin were performed by si-NNT-AS1/survivin and pcDNA-NNT-AS1/survivin, respectively. The effect of NNT-AS1 on Ishikawa-PR cell transplant tumor growth was measured by mice xenograft model. In addition, we evaluated the relationship between NNT-AS1 and miR-542-3p by RNA immunoprecipitation (RIP) and RNA pull down.

Results: The NNT-AS1 and survivin expression were both significantly increased, while miR-542-3p was decreased in Ishikawa-PR cell. Overexpression of NNT-AS1 was found to increase sensitive Ishikawa cells resistance, while inhibition of NNT-AS1 could reduce Ishikawa-PR cell resistance. NNT-AS1 functioned as a miR-542-3p decoy, and miR-542-3p could regulated Ishikawa cell resistance by survivin. In addition, NNT-AS1 was confirmed to regulate survivin expression and Ishikawa cell resistance by miR-542-3p. In vivo mice xenograft model revealed that silencing NNT-AS1 could improve endometrial cancer resistance.

Conclusion: LncRNA NNT-AS1 affect progesterone resistance by regulating miR-542-3p/survivin axis in endometrial cancer.

Keywords: LncRNA NNT-AS1; progesterone resistance; endometrial cancer; miR-524-3p; survivin;

Introduction:

Endometrial cancer is the most common gynaecological tumor in developed countries. Hormonal therapy, such as medroxyprogesterone acetate (MPA), has been applied in the conservative treatment of young patients who wish to preserve their fertility, as well as in the palliative treatment of advanced-state patients[[1](#_ENREF_1" \o "Park, 2015 #1575)]. But more than 30% of patients with progestin treatment have presented progestin resistance[[2](#_ENREF_2" \o "Gunderson, 2012 #1574)]. In recent years, there is some improvement has been demonstrated in endometrial cancer progesterone resistance. However, the mechanisms underlying acquired resistance to progestin is not entirely clear.

Long noncoding RNAs (lncRNAs) are over 200 nucleotides in length without protein-coding capacity. Guo et al[[3](#_ENREF_3" \o "Guo, 2015 #1577)] demonstrated that GAS5 acted as a tumor suppressor lncRNA in endometrial cancer, and could enhance PTEN expression to promote cancer cell apoptosis by inhibiting miR-103. Huang et al [[4](#_ENREF_4" \o "Huang, 2014 #1578)]reported that HOTAIR expression was increased in endometrial cancer cells and tissues, and the down-regulation of HOTAIR resulted in a significant inhibition of cell proliferation, migration, and invasion and in cell cycle arrest at the G0/G1 phase. Furthermore, HOTAIR depletion significantly suppressed the endometrial cancer tumorigenesis in vivo. On top of this, there are several lncRNA that play an important role in endometrial carcinoma development, such as ASLNC04080[[5](#_ENREF_5" \o "Zhai, 2015 #1579)], FER1L4[[6](#_ENREF_6" \o "Qiao, 2016 #1580)], BANCR[[7](#_ENREF_7" \o "Wang, 2016 #1581)], MEG3[[8](#_ENREF_8" \o "Guo, 2016 #1582)], etc. LncRNA Nicotinamide Nucleotide Transhydrogenase-antisense RNA1 (NNT-AS1), which located in 5p12 with 3 exons, has been mapped to chromosome 5 region 43573185–43603230 according to the NCBI (GRCh38.p2). Two recent articles by Wang[[9](#_ENREF_9" \o "Qian, 2017 #1583)] and Hua[[10](#_ENREF_10" \o "Hua, 2017 #1584)] have respectively reported that NNT-AS1 was increased in colorectal cancer and cervical cancer, and associated with advanced clinicopathological features and poor overall survival. However, NNT-AS1 have not been investigated in endometrial cancer, especially in progesterone resistance endometrial cancer.

Survivin, the smallest member of IAP (inhibitor of apoptosis) family, is a dual functional protein acting as a critical apoptosis inhibitor and key cell cycle regulator. Survivin is reported to highly express in most human cancers, such as lung, pancreatic and breast cancers[[11](#_ENREF_11" \o "Chen, 2016 #1590)]. Also, survivin plays an essential role in the drug resistant phenotype of multiple human cancers[[12](#_ENREF_12" \o "Kar, 2015 #1593),[13](#_ENREF_13" \o "Berinstein, 2015 #1594)]. Especially, survivin was involved in progesterone resistance endometrial cancer/hyperplasia[[14](#_ENREF_14" \o "Chen, 2009 #1591),[15](#_ENREF_15" \o "Fan, 2017 #1597)]. Overexpression of survivin was found in progestin-resistant endometrial samples, and down regulation of survivin increased the progestin sensitivity in endometrial cancer. A number of studies have shown that many miRNAs regulated cancer cell function and drug resistance by targeting survivin, for example miR-218[[16](#_ENREF_16" \o "Hu, 2015 #1601)], miR-214-3p [[17](#_ENREF_17" \o "Phatak, 2016 #1602)], miR-138-5p[[18](#_ENREF_18" \o "Rong, 2016 #1603)], etc. Althoff et al [[19](#_ENREF_19" \o "Althoff, 2015 #1599)]using reporter assays confirmed miR-542-3p directly targeted survivin in neuroblastoma. In addition, miR-542-3p played an important role in endometrial decidualization by regulating IGFBP1[[20](#_ENREF_20" \o "Tochigi, 2017 #1596)], and was increased in paired ectopic/eutopic endometrium as compared with normal endometrium[[21](#_ENREF_21" \o "Toloubeydokhti, 2008 #1595)]. Therefore, we hypothesized that miR-542-3p may regulate endometrial cancer cell resistance by targeting survivin.

Base on the result of predictive analytics software, NNT-AS1 may function as a miR-542-3p decoy. Thus, this study aimed to investigate the expression of NNT-AS1 and miR-542-3p and evaluate whether NNT-AS1 affect progesterone resistance by regulating miR-542-3p/survivin axis in progesterone resistance endometrial cancer.

Material and methods:

Routine cell culture and establishment of progestin‑resistant cell line.

Ishikawa cells were grown in DMEM Medium (Invitrogen) containing 10% FBS (Gibco), 50 mg/mL gentamycin. Progestin-resistant Ishikawa cell lines were induced as previous study [[22](#_ENREF_22" \o "Zhao, 2013 #1571)]. The progestin-resistant Ishikawa cell were grown in DMEM Medium (Invitrogen) containing 10% FBS (Gibco), 50 mg/mL gentamycin and maintained with a kind of synthetic progestin medoxyprogesterone acetate (MPA, 10μM, Sigma).

MTT assay

Seven day growth curves of the parent Ishikawa cells and the progestin-resistant Ishikawa cells were tested by MTT assay. Cells were plated in a 96 well ﬂat bottomed microplate with 100 µl cell suspension (1~2\*104 cells/ml) per well. The parent Ishikawa cells were grown in the complete medium with absence of MPA. The progestin-resistant Ishikawa cells were routinely maintained in the complete medium containing 10 µM MPA. Culture media were changed every other day.

After transfection, Ishikawa cell were stimulated with 1μM MPA, while progestin-resistant Ishikawa cell were stimulated with 5 μM MPA. And then cell proliferation was detected by MTT assay. A total of 10 µl MTT (5 mg/ml) was added into each well. After incubation for 4h, the medium was poured off, and formazan crystals were dissolved with 150 µl DMSO by shaking. The absorbance was measured at 490 nm with a microplate reader.

Cell transfection

MiR-542-3p mimic/inhibitor or negative control mimic/inhibitor were purchased from Genepharma, and the NNT-AS1 and survivin expression plasmids were also constructed by Genepharma (Shanghai, China). Si-NNT-AS1, si-survivin and their respective negative control were chemically synthesized by RiboBio (Shanghai, China). Ishikawa cells (with progestin-resistant) were transfected with NNT-AS1 and survivin siRNA or expression plasmids using Lipofectamine 2000 (Invitrogen). To overexpress or knockdown miR-542-3p in Ishikawa cells (progestin-resistant), the cells were transfected with miR-542-3p mimics or inhibitor using Lipofectamine 2000 (Invitrogen). After transfection, Ishikawa cell were stimulated with 1μM MPA, while progestin-resistant Ishikawa cell were stimulated with 5 μM MPA.

Western blot

The total protein was extracted from tissues and cells. Equal amounts of proteins were fractioned with SDS-PAGE followed by electrophoretic transfer of proteins onto nitrocellulose membranes. The blots were probed with antibodies against MDR-1 and survivin (Abcam), and followed by incubation with secondary antibodies conjugated with horseradish peroxidase (ThermoFisher). The immune complexes were detected with a WesternBright™ ECL Western Blotting HRP Substrate kit and analyzed with image lab software (BioRad, USA).

RNA extraction and qRT-PCR analysis

Total RNA from tissues and cells were extracted using Trizol reagent (TAKARA). RNA was reverse transcribed into cDNAs using the Primer-Script one step RT-PCR kit (TAKARA, Dalian, China). The cDNA template was amplified by real-time RT-PCR using the SYBR Premix Dimmer Eraser kit (TAKARA). Gene expression in each sample was normalized to β-actin expression. Real-time PCR reactions were performed using the ABI7500 system (Applied Biosystems, USA). The real-time PCRs were performed in triplicate. Relative expression fold change of mRNAs was calculated by the 2−ΔΔCt method.

RNA pull down

To determine whether NNT-AS1 is associated with the RISC complex, we performed an RNA precipitation assay by synthesized NNT-AS1 as a probe and then detected AGO2 from the pellet by western blot and miR-542-3p by qRT-PCR. The biotin-labeled full length lncRNA NNT-AS1 was transcribed in vitro with the Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Roche), treated with RNase-free DNase I (Roche), recycled with QIA quick Nucleotide Removal Kit (Qiagen) and purified with the RNeasy Mini Kit (Qiagen). Loc285194 was also cloned as a control and used in precipitation experiments for comparison. Ishikawa cells proteins were mixed with biotin-labeled lncRNA-NNT-AS1 incubated at 4℃ for 1 hour. The streptavidin agarose beads (Invitrogen) were added to each binding reaction and incubated at room temperature for 1 hour. Western blot was performed to detect AGO2, and the three group of precipitates were used for detecting miR-542-3p expression according to the standard procedures.

RNA immunoprecipitation

RIP assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s protocol. Briefly, Ishikawa cells were lysed by RIP lysis buffer. Then, 100-μL cell extract was incubated with RIP buffer containing magnetic beads conjugated with human anti-Ago2 antibody (Millipore) or negative control (normal mouse IgG, Millipore). After the antibody was recovered by protein A/G beads, the AGO2 was detected by immunoprecipitation-western, and qRT-PCR was performed to detect NNT-AS1 and miR-542-3p level in the precipitates.

Reversed progestin resistance effects of siRNA-NNT-AS1 in vivo.

BALB/c mice used in the present study were treated according to the protocols approved by the ethical committee of Affiliated Hospital of Inner Mongolia University Nationalities. Mice at the age of 4–6 weeks (n =6) were injected subcutaneously with siRNA-NNT-AS1 transfected- Ishikawa-PR cells (5\*106 in 100 ul PBS) in the right and left hind limbs. The control mice (n =6) were injected with the same volume of vector only. Seven days after injection, the mice received MPA (100 mg/kg bodyweight), which was injected three times/week (nine times in total). The mice were killed at day 28 after tumor cell transplantation. Tumor size was measured with calipers weekly, and tumor volume was calculated using the following formula: tumor volume (mm3) = (tumor length \* tumor width 2)/2. All experimental procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Statistical analysis

Data analysis was performed using SPSS 17.0. Data were represented as mean ± SD based on at least three repeats. Group difference was assessed using Student’s t test, and P<0.05 was considered as statistically significant.

Results:

The increased lncRNA NNT-AS1 expression in progesterone-resistant Ishikawa cells

To establish progestin resistant endometrial cell line (Ishikawa-PR), we used MPA, a kind of synthetic progestin medoxyprogesterone acetate to induce Ishikawa cell as described in the literature[[22](#_ENREF_22" \o "Zhao, 2013 #1571)]. Cells were plated in 96‑well ﬂat‑bottomed microplates as described in Materials and methods. The 7 days growth curve of Ishikawa cells and the progestin-resistant Ishikawa cells shown in Fig1A, which suggested a successfully established experimental progesterone-resistant Ishikawa cell. And then the expression levels of lncRNA NNT-AS1, miR-542-3p and survivin in the two cells were analyzed by RT-qPCR or western blot. The RT-qPCR results showed that lncRNA NNT-AS1 expression was significantly increased, while the miR-542-3p expression was obviously decreased in Ishikawa-PR cells (Fig1B and C). The western blot results showed that survivin expression was also significantly increased in Ishikawa-PR cells (Fig1D). These data revealed that the expression of lncRNA NNT-AS1, miR-542-3p and survivin were different in sensitive Ishikawa cell line and Ishikawa-PR cells, and the lncRNA NNT-AS1 and survivin expression were significantly increased while miR-542-3p was decreased in progesterone-resistant Ishikawa cells.

The effect of lncRNA NNT-AS1 on Ishikawa cell resistance

To investigate the effect of overexpressing exogenous and RNA-interfered endogenous lncRNA NNT-AS1 on Ishikawa cell resistance, the sensitive Ishikawa cell transfection of pcDNA-NNT-AS1 was stimulated by MAP (0, 0.01, 0.1, 1, 2 μM), and the Ishikawa-PR cells transfection of si-NNT-AS1 was stimulated by MAP (0, 0.5, 1.0, 5.0, 10 μM). The MTT assay showed that when the MAP concentration was greater than 0.1μM, the cell proliferation in pcDNA-NNT-AS1-transfected cell was significantly higher than that in pcDNA-transfected cell, suggesting overexpression NNT-AS1 promoted cell resistance in sensitive Ishikawa cells (Fig2A). Moreover, when the MAP concentration was greater than 5μM, the cell proliferation in si-NNT-AS1-transfected cell was significantly lower than in si-control-transfected cell, suggesting knockdown of NNT-AS1 reduced cell resistance in Ishikawa-PR cells (Fig2B). These results indicated that lncRNA NNT-AS1 played an important role in Ishikawa cell resistance.

LncRNA NNT-AS1 functioned as a miR-542-3p decoy in Ishikawa cells

As NNT-AS1 expression was opposite to miR-542-3p expression, we suspected that NNT-AS1 may function as a miR-542-3p decoy. The Fig 3A displayed the predicted positions of miR-542-3p binding sites on NNT-AS1 transcript, and then their relationship were confirmed by RNA immunoprecipitation and RNA pull-down assay. The AGO2 antibody was used into RNA immunoprecipitation, and the RT-PCR results showed that, comparing to IgG complex, the NNT-AS1 and miR-542-3p were both existed and obviously increased in AGO2 complex (Fig 3B). The RNA pull-down result showed that the AGO2 existed in NNT-AS1 drop-down compound, which confirmed a direct interaction of NNT-AS1 with miR-542-3p (Fig 3C). The miR-542-3p expression was significantly increased in NNT-AS1 drop-down compound, which further confirmed NNT-AS1 functioned as a miR-542-3p decoy (Fig 3D). These data suggested that NNT-AS1 could function as a miR-542-3p decoy in Ishikawa cells.

The miR-542-3p could regulate Ishikawa cell resistance by survivin

Previous study have reported that miR-542-3p exerted tumor suppressive functions by targeting survivin in neuroblastoma[[23](#_ENREF_23" \o "Althoff, 2015 #1572)], and survivin expression was opposite to miR-542-3p expression in our study. So we speculated that miR-542-3p could regulate Ishikawa cell resistance by targeting survivin. In sensitive Ishikawa cells, transfection of miR-542-3p inhibitor increased cell resistance and MDR-1 expression, however, co-transfection with si-survivin reversed the increased cell resistance and MDR-1 expression (Fig4A). In Ishikawa-PR cells, transfection of miR-542-3p mimic reduced cell resistance and MDR-1 expression, however, co-transfection with pcDNA-survivin reversed the reduced cell resistance and MDR-1 expression (Fig4B). These data implied that miR-542-3p could regulate Ishikawa cell resistance by regulating survivin.

LncRNA NNT-AS1 regulated survivin expression by miR-542-3p

To explore whether the survivin expression could be regulate by NNT-AS1 through miR-542-3p, the two cells were cotransfected with overexpressing exogenous and RNA-interfered endogenous NNT-AS1 and miR-542-3p mimic and inhibitor, respectively. As shown in Fig5A, sensitive Ishikawa cells transfected with pcDNA-NNT-AS1 upregulated survivin mRNA and protein expression, while co-transfection with miR-542-3p mimic reversed the increased survivin expression. As Fig 5B showed that Ishikawa-PR cells transfected with siRNA-NNT-AS1 downregulated survivin mRNA and protein expression, while co-transfection with miR-542-3p inhibitor reversed the decreased survivin expression. These results suggested that lncRNA NNT-AS1 could regulate survivin expression by miR-532-3p in Ishikawa cells.

LncRNA NNT-AS1 regulated Ishikawa cell resistance by miR-542-3p

Next, to demonstrate miR-542-3p was involved in the regulation mechanism of Ishikawa cell resistance by lncRNA NNT-AS1, the two cells were cotransfected with overexpressing exogenous and RNA-interfered endogenous NNT-AS1 and miR-542-3p mimic and inhibitor, respectively. As shown in Fig 6A, sensitive Ishikawa cells transfected with pcDNA-NNT-AS1 increased cell resistance and upregulated MDR-1 expression, while co-transfection with miR-542-3p mimic reversed the effect of pcDNA-NNT-AS1. As Fig 6B showed that Ishikawa-PR cells transfected with siRNA-NNT-AS1 reduced cell resistance and downregulated MDR-1 expression, while co-transfection with miR-542-3p inhibitor reversed the effect of siRNA-NNT-AS1. These results suggested that lncRNA NNT-AS1 could regulate Ishikawa cell resistance by miR-542-3p.

Knockdown of NNT-AS1 improved endometrial cancer resistance in mice.

To further study the role of RNA-interfered endogenous NNT-AS1 in improvement of endometrial cancer resistance in vivo, siRNA-NNT-AS1 transfected-Ishikawa-PR cells were injected subcutaneously into the right and left hind limbs of mice, and 7 days after injection, the mice received MPA. According to the measured tumor size, siRNA-NNT-AS1 group had a smaller tumor than that in si-control group, which indicated that siRNA-NNT-AS1 minimized endometrial cancer resistance in mice (Fig7A). As Fig7B showed that, the NNT-AS1 expression in tumor tissues that isolated from siRNA-NNT-AS1 transfected-progestin resistance Ishikawa cells transplantation mice was significantly lower than that from si-control transfected-progestin resistance Ishikawa cells transplantation mice. The miR-542-3p expression was obviously increased in siRNA-NNT-AS1 transfected-progestin resistance Ishikawa cells transplantation mice (Fig7C). In addition, the survivin and MDR-1 protein expression were both decreased in siRNA-NNT-AS1 transfected-progestin resistance Ishikawa cells transplantation mice, which further suggested that siRNA-NNT-AS1 could minimize endometrial cancer resistance in mice(Fig7D). These results demonstrated that RNA-interfered endogenous NNT-AS1 could improve endometrial cancer resistance in mice.

Discussion:

Progestin resistance is the main obstacle to successful conservative therapy in young endometrial cancer patients. To investigate the molecular events that lead to progestin resistance and to find a possible way to reverse progestin resistance in endometrial cancer, we established an Ishikawa-PR cell line that was resistant to the growth-inhibitory effects of progestin in vitro. The 7 days growth curve of Ishikawa cells and the Ishikawa-PR cell (Fig1A) indicated a successfully established experimental progesterone-resistant Ishikawa cell line. Survivin was involved in progesterone resistance endometrial cancer/hyperplasia[[14](#_ENREF_14" \o "Chen, 2009 #1591)]. In the present study, we found survivin protein expression was increased in Ishikawa-PR (Fig1D), and inhibition of survivin increased the progestin sensitivity and decreased MDR-1 expression (Fig4A), which was in agreement with previously reported results[[15](#_ENREF_15" \o "Fan, 2017 #1597)].

MiRNAs are small RNA molecules (22 nt) that interact with their target mRNAs inhibiting translation or/and cleavaging the target mRNA. Studies by Yoon [[24](#_ENREF_24" \o "Yoon, 2010 #1600)]and Althoff [[19](#_ENREF_19" \o "Althoff, 2015 #1599)]both demonstrated that miR-542-3p could directly target survivin in A549 cells and neuroblastoma cell lines, respectively. Meanwhile, miR-542-3p was increased in paired ectopic/eutopic endometrium as compared with normal endometrium, and played an important role in endometrial decidualization [[20](#_ENREF_20" \o "Tochigi, 2017 #1596),[21](#_ENREF_21" \o "Toloubeydokhti, 2008 #1595)]. In our study, miR-542-3p was significantly reduced in Ishikawa-PR (Fig1C). MiR-542-3p inhibitor was found to increase sensitive Ishikawa cell resistance and MDR-1 expression, and miR-542-3p mimic reduced Ishikawa-PR cell resistance and MDR-1 expression. Moreover, in Ishikawa cell, si-survivin reversed the miR-542-3p inhibitor-increased cell resistance and MDR-1 expression, and in Ishikawa-PR cell, pcDNA-survivin reversed the miR-542-3p mimic-reduced cell resistance and MDR-1 expression (Fig4). The data presented here suggested that miR-542-3p could regulate Ishikawa cell resistance by survivin.

Increasing lines of evidence show that lncRNAs are important factors in cancer and confirmed that lncRNAs might function as a competing endogenous RNA or a molecular sponge in modulating miRNA[[25](#_ENREF_25" \o "Karreth, 2013 #2512)]. Li et al [[26](#_ENREF_26" \o "Li, 2016 #1585)] using luciferase reporter and qRT-PCR assays confirmed that miR-200c bound directly to MALAT1, and disrupting MALAT1/miR-200c sponge decreased invasion and migration in endometrioid endometrial carcinoma. This study using RIP and RNA pull down assay provided the first evidence that NNT-AS1 functioned as a miR-542-3p decoy in Ishikawa cells (Fig3). Furthermore, overexpression NNT-AS1 was found in colorectal cancer and cervical cancer by literature, and NNT-AS1 inhibition could suppress cancer cells proliferation and invasion ability in vitro[[9](#_ENREF_9" \o "Qian, 2017 #1583),[10](#_ENREF_10" \o "Hua, 2017 #1584)]. In this study, NNT-AS1 was obviously higher in Ishikawa-PR than in sensitive Ishikawa cell (Fig1B). Further studies have revealed that overexpression NNT-AS1 promoted cell resistance in sensitive Ishikawa cells, but knockdown of NNT-AS1 reduced cell resistance in Ishikawa-PR (Fig2).

In addition, we found that pcDNA-NNT-AS1 upregulated survivin expression which could be reversed by miR-542-3p, and siRNA-NNT-AS1 downregulated survivin expression which could be reversed by miR-542-3p inhibitor, suggesting that NNT-AS1 regulated survivin expression by miR-542-3p (Fig5). Further, we also found that pcDNA-NNT-AS1 increased cell resistance and upregulated MDR-1 expression which could be reversed by miR-542-3p in sensitive Ishikawa cells, and siRNA-NNT-AS1 reduced cell resistance and downregulated MDR-1 expression which could be reversed by miR-542-3p inhibitor in Ishikawa-PR cell, suggesting that NNT-AS1 regulated survivin expression by miR-542-3p (Fig6). In consideration of the above-described biological behaviors of NNT-AS1, to further investigated the role of NNT-AS1 in improvement of endometrial cancer resistance in vivo, we utilized siRNA-mediated knockdown of NNT-AS1 and evaluated that the resultant effects on tumor growth. We found that silencing NNT-AS1 obviously improved the sensitivity of Ishikawa-PR cell transplant tumors to MAP, and the expression level changes of miR-542-3p, survivin and MDR-1 were in accordance with in vitro (Fig7). All these data implied that NNT-AS1 affected progesterone resistance by miR-542-3p/survivin axis in vitro and in vivo.

In conclusion, our data showed an abnormal expression of lncRNA NNT-AS1 in progesterone resistance Ishikawa cell, and demonstrated that inhibition of NNT-AS1 reduced progesterone resistance by regulating miR-542-3p/survivin axis. Therefore, NNT-AS1 is a potential therapeutic target for the treatment of progesterone resistance endometrial cancer.

Competing interest:

The authors declared that they have no competing interest

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Figure legends:

Figure 1. The increased NNT-AS1 expression in progesterone-resistant Ishikawa cells.

(A) The growth of the two cells were tested by MTT assay and analyzed by plotting cell growth curve. (B) The NNT-AS1 expression was significantly increased in Ishikawa-PR. (C) The miR-542-3p expression was significantly decreased in Ishikawa-PR. (D) The survivin expression was increased in Ishikawa-PR. \*P<0.05 vs Ishikawa.



Figure 2. The effect of NNT-AS1 on Ishikawa cell resistance.

(A) Overexpression NNT-AS1 promoted cell resistance in sensitive Ishikawa cells. (B) Knockdown of NNT-AS1 reduced cell resistance in progesterone-resistant Ishikawa cells. \*P<0.05 vs si-control or pcDNA.



Figure 3. NNT-AS1 functions as a miR-542-3p decoy in Ishikawa cells.

(A) The predicted positions of miR-542-3p binding sites on the NNT-AS1 transcript. (B) The AGO2 antibody used into RIP-western, and the RT-PCR results showed that the NNT-AS1 and miR-542-3p were both existed in AGO2 complex. (C) Western blot showed that the AGO2 existed in NNT-AS1 drop-down compound, the NC as control. (D) The RNA pull-down assay revealed a direct interaction of NNT-AS1 with miR-542-3p. The RT-PCR showed that miR-542-3p expression was significantly higher in NNT-AS1 drop-down compound than in NC. \*P<0.05 vs IgG, #P <0.05 vs NC.



Figure4. The miR-542-3p regulated Ishikawa cell resistance by survivin.

(A) miR-542-3p inhibitor increased sensitive Ishikawa cell resistance and upregulated MDR-1 expression, while si-survivin reversed the effect of miR-542-3p inhibitor. (B) miR-542-3p mimic reduced Ishikawa-PR cell resistance and MDR-1 expression, pcDNA-survivin reversed the effect of miR-542-3p mimic. \*P<0.05 vs NC or pre-NC, #P <0.05 vs miR-542-3p inhibitor +si-control or miR-542-3p mimic + pcDNA.



Figure 5. NNT-AS1 regulated survivin expression by miR-542-3p.

(A) pcDNA-NNT-AS1 increased survivin mRNA and protein expression, while miR-542-3p mimic reversed the effect of pcDNA-NNT-AS1 in sensitive Ishikawa cell. (B) siRNA-NNT-AS1 suppressed survivin mRNA and protein expression, while miR-542-3p inhibitor reversed the effect of si-NNT-AS1 in Ishikawa-PR cell. \*P<0.05 vs pcDNA or si-control, #P <0.05 vs pcDNA-NNT-AS1+pre-NC or siRNA-NNT-AS1+NC.



Figure 6. NNT-AS1 regulated Ishikawa cell resistance by miR-542-3p.

(A) pcDNA-NNT-AS1 increased cell resistance and MDR-1 expression, while miR-542-3p mimic reversed the effect of pcDNA-NNT-AS1 in sensitive Ishikawa cell. (B) siRNA-NNT-AS1 reduced cell resistance and MDR-1 expression, while miR-542-3p inhibitor reversed the effect of siRNA-NNT-AS1 in Ishikawa-PR cell. \*P<0.05 vs pcDNA or si-control, #P <0.05 vs pcDNA-NNT-AS1+pre-NC or siRNA-NNT-AS1+NC.



Figure 7. RNA interference of NNT-AS1 improved endometrial cancer resistance in mice.

(A) siRNA-NNT-AS1 minimized endometrial cancer resistance in mice. In siRNA-NNT-AS1 transfected-Ishikawa-PR cell transplantation mice: (B) The NNT-AS1 expression was significantly decreased. (C) The miR-542-3p expression was significantly increased. (D) The MDR-1 and survivin expression were both decreased. \*P<0.05 vs si-control.

