LncRNA HOTTIP Participates in Cisplatin Resistance of Tumor Cells by Regulating miR-137 Expression in Pancreatic Cancer

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Aim: This study aimed to investigate the effect of HOTTIP and miR-137 on cisplatin resistance of pancreatic cancer cells, and study the mechanism of the effect of HOTTIP on the resistance to cisplatin in pancreatic cancer cells, so as to provide new targets for clinical treatment of pancreatic cancer.

Methods: Pancreatic cancer cells were induced to be resistant to cisplatin by gradually increasing cisplatin concentration at a low concentration gradient in vitro. The changes of HOTTIP and miR-137 were detected, and the effects of HOTTIP and miR-137 on cisplatin efficacy of pancreatic cancer cisplatin-resistant cells were analyzed to explore the mechanism of HOTTIP on cisplatin resistance of pancreatic cancer cells.

Results: After inducing cisplatin resistance in pancreatic cancer cells, the expression level of HOTTIP in pancreatic cancer cells further increased and miR-137 decreased. Silencing HOTTIP or over-expression of miR-137 can increase the sensitivity of pancreatic cancer cisplatin-resistant cells to cisplatin, inhibit the proliferation of pancreatic cancer cells, and promote apoptosis. And we found HOTTIP can target to inhibit miR-137 expression. Rescue experiments showed that regulating miR-137 cannot affect the expression of HOTTIP, miR-137 is a downstream target of HOTTIP, and down-regulation of miR-137 expression can obviously hinder the cisplatin sensitization effect of silencing HOTTIP on cisplatin-resistant pancreatic cancer cells.

Conclusion: Silencing HOTTIP reverses cisplatin resistance of pancreatic cancer cells by promoting miR-137 expression.

Keywords: IncRNA HOTTIP, miR-137, pancreatic cancer, cisplatin, drug resistance, targeted therapy

Core Tip
Pancreatic cancer is the third leading cause of death related to cancer death. Although great progress has been made in surgical techniques and chemotherapy schemes, the prognosis is still unsatisfactory. Chemotherapy resistance of tumor cells is one of the main reasons Cisplatin is the most widely used and effective chemotherapeutic drug in tumor therapy, which also almost leads to cisplatin resistance being the most common phenomenon in tumor therapy. This study explored the effect of HOTTIP and miR-137 on cisplatin resistance of pancreatic cancer cells, and the mechanism of HOTTIP affecting cisplatin resistance of pancreatic cancer cells, so as to provide new targets for clinical treatment of pancreatic cancer.
Introduction
Pancreatic cancer is the third leading cause of death related to cancer death, second only to lung cancer and colon cancer. Due to the late discovery of pancreatic cancer, the diagnosis is often in the advanced stage. Pancreatic cancer is highly invasive and the prognosis of patients is very poor, its 5-year survival rate is only 2–9%. The incidence of pancreatic cancer has regional differences. In 2015, the number of new cases of pancreatic cancer in the United States was 48,960, accounting for 3% of newly diagnosed cancer cases. The number of death was 40,560, accounting for 7% of cancer deaths. It is estimated that by 2030, the disease will become the second largest cancer-related death cause after lung cancer. Moreover, pancreatic cancer has a strong age dependence. There are also significant differences. In 2015, the number of new cases of pancreatic cancer in the United States was 48,960, accounting for 7% of cancer deaths. It is estimated that by 2030, the disease will become the second largest cancer-related death cause after lung cancer. With the increase of human life span and population aging, the incidence of pancreatic cancer has a further upward trend, which is a worldwide public health problem.

Although great progress has been made in surgical techniques and chemotherapy schemes, the prognosis is still unsatisfactory. Chemotherapy resistance of tumor cells is one of the main reasons. Cisplatin is the most widely used and effective chemotherapeutic drug in tumor therapy, which also almost leads to cisplatin drug resistance being the most common phenomenon in tumor therapy. Non-coding RNAs (IncRNAs) are a class of non-coding RNAs with a length of more than 200 nucleotides and play a vital role in translation, RNA splicing, and gene regulation. Recent studies have shown that IncRNAs is closely related to drug resistance of tumor cells. HOXA terminal transcript antisense RNA (HOTTIP) is a kind of IncRNAs. Mao et al reported that the expression level of HOTTIP in gastric cancer cisplatin-resistant cell lines increases and inhibition of HOTTIP expression can reduce epithelial-mesenchymal transformation ability of gastric cancer cisplatin-resistant cells to reverse drug resistance. Zhang et al reported that HOTTIP can promote the development of lung adenocarcinoma, and reduce the sensitivity of lung adenocarcinoma cells to paclitaxel through protein kinase B signaling pathway, thus promoting the formation of drug resistance. microRNAs (miRNAs) are a group of non-coding RNA with a length of about 19–24 nucleotides, which is also involved in the occurrence of drug resistance in tumor cells. Hu et al found that miR-137 could regulate the c-Myc-EZH2 axis to reverse cisplatin resistance in ovarian cancer cells. Shen et al reported in the study that miR-137 can also inhibit the growth of lung cancer tumors and increase the sensitivity of lung cancer cells to paclitaxel and cisplatin. These studies showed that HOTTIP and miR-137 play a role in the formation of cisplatin resistance in many tumors, but there are few reports on cisplatin resistance in pancreatic cancer.

This study explored the effect of HOTTIP and miR-137 on cisplatin resistance of pancreatic cancer cells, and the mechanism of HOTTIP affecting cisplatin resistance of pancreatic cancer cells, so as to provide new targets for clinical treatment of pancreatic cancer.

Data and Methods
Cell Culture
Human pancreatic cancer cell lines PANC-1, HS766T, AsPC-1 and human normal pancreatic duct epithelial cell lines HPDE6-C7 were all purchased from BeNa Culture Collection, with the cell numbers of BNCC338219, BNCC101687, BNCC100652 and BNCC338285. All cell culture media were composed of 90% DMEM high glucose culture fluid and 10% fetal bovine serum, and the culture conditions were at 37°C, with 5% CO2. The cells were passaged for 2–3 generations. DMEM high glucose medium and fetal bovine serum were all purchased from Thermofisher (China), with the item number of 10569044 and 10099141.

Construction of Drug-Resistant Cell Lines
The drug resistance of pancreatic cancer cells to cisplatin was induced by gradually increasing the concentration of cisplatin at a low concentration gradient in vitro. Pancreatic cancer cells in the logarithmic growth phase were taken, re-suspended, and conventionally inoculated into 96-well plates. The number of inoculated cells per well was about 3000. The cells were cultured in a incubator for 6 hrs at 37°C with 5% CO2, then added with a culture medium containing 0.01μg/mL of cisplatin and 48 hrs, and the culture medium was changed after the culture was completed. Cisplatin concentrations were increased in sequence, with cisplatin concentrations of 0.1, 1, and 10μg/mL, respectively. Five parallel wells were set at each concentration. MTT method was used to detect the cell growth inhibition rate and IC50 value was calculated. The cisplatin resistance of pancreatic cancer cells was induced by re-inoculation of pancreatic cancer cells at the initial concentration of 10% IC50. The drug concentration was increased after the cells grew steadily until the cancer cells finally survived at 1 μg/mL of cisplatin.
Construction and Transfection of Expression Vectors

All expression vectors were designed by ThermoFisher (China), including HOTTIP low expression vector (si-HOTTIP), HOTTIP over-expression vector (sh-HOTTIP), miR-137 low expression vector (mir-137-inhibitor), miR-137 over-expression vector (mir-137 mimics), blank vector miR-NC, blank vector si-NC, sh-NC, PMiR-miR-137-3′UTR wild type (Wt), pMiR-miR-137-3′UTR mutant (Mut), and blank vector pMiR-NC. Pancreatic cancer drug-resistant cell lines were digested with trypsin 24 hrs before transfection, and transfection of expression vector was carried out when the cells fuse to about 80%. The specific operation steps referred to the kit instructions, and the cells were cultured in an incubator at 37°C and with 5% CO₂ for 48 hrs, and the culture medium was changed every 6 hrs. qRT-PCR and Western blot were used to detect the transfection results. LipofectamineTM2000 transfection kit was purchased from Invitrogen Company of the United States, with the item number of 35050. Cells that did not receive any intervention were used as a control group. The interference sequence is shown in Table 1.

qRT-PCR

The cells were collected. Total RNA was extracted from the cells by using TRIzol kit (Invitrogen, USA, 15596018). The purity, concentration, and integrity of total RNA were detected by ultraviolet spectrophotometer and agarose gel electrophoresis. A260/A280 value was required to be 1.8–2.1. HOTTIP and miR-137 were amplified by one-step method. EasyScript One-Step RT-PCR SuperMix kit was purchased from Beijing TransGen Biotech, with the item number of AE411-02. The reaction system was 1μg of RNA Template, 0.4μL of Forward GSP (10μM), 0.4μL of Reverse GSP(10μM), 10μL of 2 * One-Step Reaction Mix, 0.4μL of EasyScript One-Step Enzyme Mix, and 20μL of RNase-free Water supplementation reaction system value. Reaction conditions were as follows: 40°C for 30min, 94°C for 5min, 94°C for 30s, 60°C for 30s, 72°C2kb/min, 72°C for 10 min, for a total of 40 cycles. Each sample was given 3 repeat wells. GAPDH was used as the internal reference of HOTTIP, U6 as the internal reference of miR-137, and the results were analyzed by 2−ΔΔCT method. The primer sequences are shown in Table 2.

Double Luciferase Report

Human embryonic kidney cell 293T was purchased from BeNa Culture Collection, No. BNCC100530. After cell culture to the logarithmic growth phase, pmir-miR-137-3′UTR wild type (Wt), pmir-miR-137-3′UTR mutant (Mut), sh-HOTTIP, and sh-NC were transfected. After 48 hrs of transfection, fluorescence intensity was detected by double luciferase detection system (Beckman Cytoflow cytometer, USA). The sequence was designed by ThermoFisher (China).

MTT Cell Proliferation Experiment in vitro

Cells were harvested 24 hrs after transfection, and after routine digestion, the cell density was adjusted to 3*10⁴ cells/well. The cells were, respectively, inoculated on 96-well plates, and incubated at 37°C for 48 hrs. A 20μL of MTT solution (5 μ m g/mL) was added after incubation, and the cells were cultured at 37°C for 4 hrs. Then, 150μL of

| Table 1 primer |
|----------------|
| miR-137-inhibitor | UGGAUUUGUACCAUCUUCUGGAAGAAUGUACAAAUCCAAG |
| miR-137 mimic | CGGCGTGAACGTGCAGATCCACATCCGAGATGAGCTGAACAGTCACTTTTTT |
| si-HOTTIP#1 | UGGAUUUGUACCAUCUUCUGGAAGAAUGUACAAAUCCAAG |
| si-HOTTIP#2 | UUUGAGUGGGAUACCAACAGGGUGAUAACCCACUAAAAAG |
| si-HOTTIP#3 | AAGUUUUGUGUUAACCUGUUCAGGUGUAACAAAACUUCU |
| sh-HOTTIP | GTATATCCTGGGTGATAATA |

| Table 2 Primer Sequence |
|-------------------------|
| **Forward Primer** | **Reverse Primer** |
| HOTTIP | 5′-AGT GTG TCA TAG AGC TTC CTG TTT CAT CTC CCA GT-3′ | 5′-TGG AAC CAG GCC CCA GGG AAT CTT TCA GCT GCA TT-3′ |
| miR-137 | 5′-GAA ATC CGA CAG CCT AAG GAG GTT TGA-3′ | 5′-CAT TGC ACA CAG AGG ATT TGA TTT ACT-3′ |
| U6 | 5′-GGG CGT GTG GAA GGG TTC-3′ | 5′-GTG CAG GGT CCG AGG T-3′ |
| GAPDH | 5′-TGC ACC ACC AAC TGC TTA G-3′ | 5′-GAT GCA GGG ATG ATG TCC-3′ |
dimethyl sulfoxide was added to each well, and the OD value of each group of cells was measured under 490nm absorbance using an enzyme-labeled instrument (Shanghai Flash Spectrum Biotechnology Co., Ltd.). The MTT test kit was purchased from Thermofisher (China), NO. V13154.

Detection of Apoptosis
Cells were digested with 0.25% trypsin, rinsed twice with PBS after digestion, added with 100μL of binding buffer, prepared into 1*10^6/mL suspension, sequentially added with AnnexinV-FITC and PI, incubated at room temperature in dark for 5min, and detected with CytoFLE S flow cytometer system. The experiment was repeated for 3 times to get the average value. Annexin V-FITC/PI apoptosis detection kit was purchased from Invitrogen (USA), NO. V35113.

Statistical Analysis
SPSS 19.0 (Asia Analytics Formerly SPSS China) was used. The measurement data were expressed as mean± standard deviation (mean±sd). One-way ANOVA was used for multi-group comparison and LSD was used for back testing. The P-value less than 0.05 was regarded as statistical significance.

Results
Expression Level Analysis of HOTTIP and miR-137 in Pancreatic Cancer Cells
qRT-PCR results showed that the expression of HOTTIP in pancreatic cancer cells PANC-1, HS766T and AsPC-1 was significantly higher than that in normal pancreatic duct epithelial cells HPDE-6-C7 (P<0.05), while the expression of miR-137 was significantly lower than that in HPDE-6-C7 cells (P<0.05). (Figure 1)

Determination of IC50 Value of Pancreatic Cancer Cell Line
A linear regression equation was established according to the inhibition results of cisplatin at different concentrations in pancreatic cancer cells, and IC50 was calculated. The IC50 of PANC-1, HS766T and AsPC-1 cells were 5.91μg/mL, 4.31μg/mL and 5.12μg/mL, respectively. (Figure 2)

Construction of Cisplatin-Resistant Pancreatic Cancer Cell Line
After the induction of drug resistance, the IC50 of pancreatic cancer cells was detected again. The results showed that the IC50 of PANC-1, HS766T, and AsPC-1 cells were 11.52μg/mL, 14.69μg/mL, and 11.27μg/mL, respectively. All of them survived stably under 1μg/mL cisplatin, which was considered successful modelling. The analysis results of the expression levels of HOTTIP and miR-137 in the re-detected cells showed that the expression levels of HOTTIP in PANC-1/CDDP, HS766T/CDDP, AsPC-1/CDDP were higher than those in PANC-1, HS766T, AsPC-1 cells (P<0.05), and the expression levels of miR-137 were lower than those in PANC-1, HS766T, AsPC-1 cells (P<0.05). (Figure 3)

Figure 1 Expression level analysis of HOTTIP and miR-137 in pancreatic cancer cells. (A) Expression of HOTTIP in pancreatic cancer cells. (B) Expression of miR-137 in pancreatic cancer cells. *Indicates that P<0.05.
Figure 2 Effects of different concentrations of cisplatin on the growth of pancreatic cancer cells. (A) Growth inhibition of PANC-1 cells by different concentrations of cisplatin. (B) Growth inhibition of HS766T cells by different concentrations of cisplatin. (C) Growth inhibition of AsPC-1 cells by different concentrations of cisplatin.

Figure 3 Construction results of cisplatin-resistant pancreatic cancer cell lines. (A) Growth inhibition of PANC-1/CDDP cells by different concentrations of cisplatin. (B) Growth inhibition of HS766T/CDDP cells by different concentrations of cisplatin. (C) Growth inhibition of AsPC-1/CDDP cells by different concentrations of cisplatin. (D) Expression changes of HOTTIP in pancreatic cancer drug-resistant cell lines. (E) Expression changes of miR-137 in pancreatic cancer drug-resistant cell lines. *Indicates that P<0.05.
Effect of HOTTIP on Cisplatin Sensitivity of Pancreatic Cancer Cisplatin-Resistant Cell Line

Among the three si-HOTTIP, si-HOTTIP#1 has the strongest inhibitory effect. The following related experiments were performed using si-HOTTIP # 1. After silencing HOTTIP, the expression level of HOTTIP in PANC-1/CDDP, HS766T/CDDP, AsPC-1/CDDP cells in si-HOTTIP group was significantly lower than that in control group and si-NC group (P<0.05), while there was no statistical difference between control group and si-NC group (P>0.05). Taking IC50 value of each drug-resistant cell as the addition concentration of cisplatin, the growth inhibition rate and apoptosis rate of cells in si-HOTTIP group were higher than those in control group and si-NC group (P<0.05), while there was no difference between control group and si-NC group (P>0.05). (Figure 4)

Effect of miR-137 on Cisplatin Sensitivity of Pancreatic Cancer Cisplatin-Resistant Cell Line

After over-expression of miR-137, the expression level of miR-137 in PANC-1/CDDP, HS766T/CDDP, AsPC-1/CDDP cells in mimic group were significantly lower than that in control group and miR-NC group (P<0.05), while there was no statistical difference between control group and si-NC group (P>0.05). Taking IC50 value of each drug-resistant cell as cisplatin concentration, the growth inhibition rate and apoptosis rate of cells in si-HOTTIP group were higher than those in mimic group and miR-NC group (P<0.05), while there was no difference between control group and miR-NC group (P>0.05). (Figure 5)

Effect of HOTTIP on Expression of miR-137 in Cisplatin-Resistant Pancreatic Cancer Cell Line

After silencing HOTTIP, the expression level of miR-137 in PANC-1/CDDP, HS766T/CDDP, AsPC-1/CDDP cells in si-HOTTIP group was significantly higher than that in control group and si-NC group (P<0.05), while there was no statistical difference between control group and si-NC group (P>0.05). Double luciferase report results showed that HOTTIP can target inhibit miR-137 expression (P<0.05). (Figure 6)

Effect of Co-Transfection of Si-HOTTIP and miR-137 Inhibitor on Cisplatin Sensitivity of Pancreatic Cancer Cisplatin-Resistant Cell Line

After co-transfection of si-HOTTIP and miR-137 inhibitor (co-transfection group), the expression level of HOTTIP in PANC-1/CDDP, HS766T/CDDP, AsPC-1/CDDP cells in co-transfection group was not significantly different from that in si-HOTTIP group (P>0.05), but miR-137 was significantly lower than that in si-HOTTIP group (P<0.05). Taking IC50 value of each drug-resistant cell as cisplatin concentration, the growth inhibition rate and apoptosis rate of cells in co-transfection group were lower than those in si-HOTTIP group (P<0.05). (Figure 7)

Discussion

HOTTIP and miR-137 are two non-coding RNA closely related to the occurrence and development of pancreatic cancer. In many studies, HOTTIP and miR-137 can regulate the growth of pancreatic cancer cells, increase the expression level of HOTTIP in pancreatic cancer, increase the proliferation, survival and migration of pancreatic cancer cells. However, the expression level of miR-137 in pancreatic cancer is reduced, which can induce pancreatic cancer cell senescence and trigger cancer suppression network.

This study first analyzed the differential expression of HOTTIP and miR-137 in three pancreatic cancer cells and one pancreatic ductal epithelial cell. The expression level of HOTTIP in the three pancreatic cancer cells was higher than that in pancreatic ductal epithelial cells, while miR-137 expression significantly decreased in pancreatic cancer cells. Moreover, we found that the expression level of HOTTIP in pancreatic cancer cells further increased after inducing cisplatin resistance in pancreatic cancer cells. The expression of miR-137 also further reduced. We speculated that HOTTIP and miR-137 are not only related to the occurrence and development of pancreatic cancer, but also may be closely related to the occurrence of cisplatin resistance in pancreatic cancer cells. HOTTIP and miR-137 have been reported to be associated with drug resistance in pancreatic cancer cells. Li et al. reported in the study that inhibition of HOTTIP can enhance the toxic effect of gemcitabine on pancreatic cancer cells, block the cell cycle process, inhibit epithelial-mesenchymal transition of pancreatic cancer cells, and reduce the cell invasion ability. Xiao et al. found that over-expression of miR-137...
Figure 4 Effect of HOTTIP on cisplatin sensitivity of pancreatic cancer cisplatin-resistant cell line. (A) Effect of different si-HOTTIP on drug-resistant cell line HOTTIP. (B) Silencing HOTTIP result. (C) Effect of cisplatin on growth inhibition of pancreatic cancer drug-resistant cells. (D) Effect of cisplatin on apoptosis of pancreatic cancer drug-resistant cells. (E) Cell flow apoptosis image of PANC-1/CDDP. (F) Cell flow apoptosis image of HS766T/CDDP. (G) Cell flow apoptosis image of ASPC-1/CDDP. *Indicates that P<0.05.
Figure 5 Effect of miR-137 on cisplatin sensitivity of pancreatic cancer cisplatin-resistant cell line. (A) Over-expression of miR-137 results. (B) Effect of cisplatin on growth inhibition of pancreatic cancer drug-resistant cells. (C) Effect of cisplatin on apoptosis of pancreatic cancer drug-resistant cells. (D) Cell flow apoptosis image of PANC-1/CDDP. (E) Cell flow apoptosis image of HS766T/CDDP. (F) Cell flow apoptosis image of ASPC-1/CDDP. *Indicates that P<0.05.
can increase the sensitivity of pancreatic cancer cells to 5-fluorouracil and inhibit tumor growth.

In this study, HOTTIP can reverse the cisplatin resistance of pancreatic cancer cells by regulating miR-137. For the first time, we found that silencing HOTTIP or over-expressing of miR-137 can increase the sensitivity of pancreatic cancer cisplatin-resistant cells to cisplatin, inhibit the proliferation of pancreatic cancer cells and promote apoptosis. Furthermore, we found that HOTTIP was negatively correlated with miR-137 expression. Double luciferase reports showed that HOTTIP can target inhibit miR-137 expression. It was speculated that this might be the mechanism of HOTTIP affecting cisplatin resistance of pancreatic cancer cells. Therefore, we have co-transfected two vectors, si-HOTTIP and miR-137 inhibitor. The transfection results showed that miR-137 was significantly inhibited compared with si-HOTTIP, the expression of HOTTIP was unchanged, and the growth inhibition rate and apoptosis rate of pancreatic cancer cells in the co-transfected group were significantly reduced. These analyses results showed that regulation of miR-137 cannot affect HOTTIP expression, miR-137 is a downstream target of HOTTIP, and down-regulation of miR-137 expression can significantly hinder the cisplatin sensitization effect of silencing HOTTIP on cisplatin-resistant pancreatic cancer cells. Therefore, we have preliminarily verified from in vitro cell experiments that silencing HOTTIP reverses cisplatin resistance of pancreatic cancer cells by promoting miR-137 expression.

HOTTIP has many complicated mechanisms in regulating cisplatin resistance in tumor cells. Li et al\textsuperscript{23} reported in the study that over-expression of HOTTIP can activate Wnt/β-catenin signaling pathway to induce cisplatin resistance in osteosarcoma cells. Sun et al\textsuperscript{24} found that HOTTIP can also induce cisplatin resistance of small cell lung cancer cells by inhibiting miR-216a. In addition, miR-137 also has a downstream mechanism to induce cisplatin resistance of tumor cells and plays a role in promoting cisplatin resistance of tumor cells in lung cancer. Su et al\textsuperscript{25} showed that miR-137 can induce cisplatin resistance of non-small cell lung cancer cells by inhibiting apoptosis regulator caspase-3. Lu et al\textsuperscript{26} also reported that miR-137 over-expression reduces the sensitivity of non-small cell lung cancer to cisplatin by inhibiting serine/threonine kinase 2. This suggested that although miR-137 is related to cisplatin resistance of multi-tumor cells, the role miR-137 plays in it may be completely opposite, which may be related to tumor micro-environment. The biological effect of the gene depends to a large extent on the cell environment.\textsuperscript{27} Therefore, the results of this study need to be verified by in vivo experiments and further clinical experiments. In addition, whether there is a sponge effect between HOTTIP and miR-137 still needs to be verified by RNA immunoprecipitation experiment.

To sum up, we have preliminarily verified from in vitro cell experiments that silencing HOTTIP reverses cisplatin resistance of pancreatic cancer cells by promoting...
Figure 7 Effect of co-transfection of si-HOTTIP and miR-137 inhibitor on cisplatin sensitivity of pancreatic cancer cisplatin-resistant cell line. (A) Expression changes of HOTTIP in pancreatic cancer drug-resistant cells after co-transfection. (B) Expression changes of miR-137 in pancreatic cancer drug-resistant cells after co-transfection. (C) Effect of cisplatin on growth inhibition of pancreatic cancer drug-resistant cells. (D) Effect of cisplatin on apoptosis of pancreatic cancer drug-resistant cells. (E) Cell flow apoptosis image of PANC-1/CDDP. (F) Cell flow apoptosis image of HS766T/CDDP. (G) Cell flow apoptosis image of ASPC-1/CDDP. *Indicates that P<0.05.
miR-137 expression, which may be a potential therapeutic strategy of cisplatin in the treatment of pancreatic cancer.

Disclosure
The authors report no conflicts of interest in this work.

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