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Animal Cell Technology

Autophagy regulated by lncRNA HOTAIR contributes to the cisplatin-induced resistance in endometrial cancer cells

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Abstract

Objectives To identify whether the lncRNAs participate in the regulation of cisplatin-resistant induced autophagy in endometrial cancer cells.

Results Autophagy assay showed that autophagy activity was significantly boosted in cisplatin-resistant Ishikawa cells, a human endometrial cancer cell line, compared with that in parental Ishikawa cells. After analyzing the overall long noncoding RNA (lncRNA) profiling, a meaningful lncRNA, HOTAIR, was identified. It was down-regulated simultaneously in cisplatin-resistant Ishikawa cells and parental Ishikawa cells treated by cisplatin. RNA interference of HOTAIR reduced the proliferation of cisplatin-resistant Ishikawa cells and enhanced the autophagy activity of cisplatin-resistant Ishikawa cells with or without cisplatin treatment. And beclin-1, multidrug resistance (MDR), and P-glycoprotein (P-gp) were mediated by lncRNA HOTAIR.

Conclusions It is clear that lncRNAs, specifically HOTAIR, can regulate the cisplatin-resistance ability of human endometrial cancer cells through regulation of autophagy by influencing Beclin-1, MDR, and P-gp expression.

Keywords Autophagy, Cisplatin-resistance, Endometrial cancer, HOTAIR, Long noncoding RNA

Introduction

One of uterine cancers, endometrial cancer, is the common gynecologic malignancies worldwide. Currently, cisplatin, one of platinum compounds, is regarded as the first line drugs to treat endometrial cancer in either adjuvant or neo-adjuvant settings. Although cisplatin-based treatment prolongs the cancer progression-free intervals, the actual survival is unaltered, which dues to the development of chemoresistance. Therefore, to effectively control the endometrial cancer, the novel objective is focused on resolving the problem of chemoresistance.

Autophagy is a major cellular process that captures intracellular organelles and cytoplasmic materials in autophagosomes, and delivers them to the lysosomal compartment where they are degraded and recycled (Mizushima 2010). Recently, accumulating reports have revealed that autophagy was utilized to mediate the chemoresistance (Sui et al. 2013). Particularly, one of mechanisms of cisplatin-resistance in ovarian and endometrial cancer was demonstrated to correlate with autophagy (Fukuda et al. 2015). However, the detailed contributions of autophagy to cisplatin-resistant endometrial cancer remain extremely limited.

Some references suggest that many long noncoding RNA (lncRNA) have a role in mediating the chemoresistance of cancer cells (Liu et al. 2015; Liu et al. 2013). But the underlying mechanisms are less well documented, and whether lncRNA participates in the regulation of chemoresistance-induced autophagy in endometrial cancer remains unknown.

Herein, a meaningful lncRNA, HOTAIR, was identified and evidenced to be a tumor chemoresistant promoter. It’s mechanism of regulating autophagy contributing to the cisplatin-induced resistance in endometrial cancer cells was investigated.

Methods

Cell culture and treatment

The Ishikawa human endometrial cancer cell line and its cisplatin-resistant sub-clone were used in the in vitro studies. For cisplatin treatment, both cell lines were seeded into 96-well plates at a rate of 2×10^3 cells/well and incubated with DMEM containing various concentrations of cisplatin (0-80 μg/ml) for indicated times.

Autophagy assay using the DsRed-LC3-GFP reporter

To develop a dual-color autophagy reporter, we used *ptf*LC3 (Addgene plasmid 21074), which is a mRFP-green fluorescence protein (GFP) tandem fluorescent-tagged LC3 (also known as ATG8) plasmid that DsRed protein is fused with N terminus of LC3 protein and C terminus of the protein is connected to EGFP. To produce stable cell lines continuously reporting autophagy activity, recombinant retroviruses expressing the DsRed-LC3-GFP reporter were generated and used to infect target cells.

LncRNA profiling

To identify the involved lncRNAs, Human Disease-related LncRNA Profiler (System Biosciences) was utilized and overall lncRNAs were selected from the RNA database (http://research.imb.uq.edu.au/rnadb/Default.aspx) or lncRNA database (www.lncRNAdb.org). Total RNA was extracted from Ishikawa human endometrial cancer cell line and its cisplatin-resistant sub-clone pretreated with or without cisplatin. Reverse transcription was carried out by using RevertAid™ Reverse Transcriptase (Fermentas) and random primer mix (New England BioLabs). The values for the cells without treatment after normalization by the internal controls served as a basal level of expression of indicated lncRNAs; delta-delta Ct values (no treatment versus carboplatin plus docetaxel treatment) were used to determine their relative expression as fold changes.

Cell proliferation assay

Cell proliferation was analyzed by using CCK-8 assay kit (Dojindo) according to manufacturer indications. In particular, cells were respectively incubated in the medium containing cisplatin in the absence or presence of small interference RNA (siRNA) against HOTAIR in 96-well plates for indicated times. After that, 5μl CCK-8 reagent was added to each well and incubated at 37 °C for 1 h. The cell numbers were assessed by measurement of absorbance at 450 nm. All the experiments were performed in triplicate.

RNA interference

The sequences of siRNA corresponding to lncRNA HOTAIR was synthesized (Qiagen). Ishikawa human endometrial cancer cell line and its cisplatin-resistant sub-clone were respectively transfected with three HOTAIR siRNAs (100 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Scrambled siRNA was used as negative control. Post-transfection later, cells were respectively collected for CCK-8 assay.

Western blotting

The expression levels of proteins were determined by western blotting using the enhanced chemiluminescent substrate (ECL, BioRad, Richmond, CA, USA) in accordance with the manufacturer's instruction.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) and subjected to analysis of variance (ANOVA) to assess the treatment effects by using SPSS 13.0 software. The Student's t-test was used to determine the statistically significant differences in numbers with two significant levels (0.05 and 0.01).

Results

Identification of cisplatin-resistant Ishikawa human endometrial cancer cell line

A CCK8-based proliferation assay was used to investigate the growth suppressive effect of cisplatin (CP) on Ishikawa and its resistant cell line (Ishikawa CP). Treatment with cisplatin inhibited cell proliferation in a dose-dependent manner, in which the cell viability of Ishikawa CP is obviously higher than its sensitive cell line (Fig. S1a). Moreover, the half-maximal inhibitory concentration (IC_{50}) values were approximately 20 and 60 μg/ml for Ishikawa and its resistant cell line (Fig. S1b). These findings suggest that the cisplatin-resistant Ishikawa cell sub-clone was selected.

Ishikawa CP shows higher autophagy

To examine whether Ishikawa CP possesses the increased autophagy, a dual color DsRed-LC3-GFP reporter was used, which is modified formation of the classical GFP-tagged LC3 reporter introducing two readouts for autophagy activity. The reporter has GFP separated from the C terminus of LC3 by a recognition site for the autophagic protease, ATG4, and fewer of GFP fluorescence means the increased autophagy. Ishikawa CP presented a significantly reduced GFP fluorescence in contrast to normal Ishikawa cells, indicating the abnormal autophagy activity in Ishikawa CP (Fig. 1a). Additionally, the more autophagosomes were detected in Ishikawa CP (Fig. 1b). These data suggest that higher autophagy is likely to positively involve the cisplatin resistance in Ishikawa CP.

Differential expression of lncRNAs between Ishikawa CP and normal Ishikawa cell

Next, whether lncRNAs involved in altered autophagy of Ishikawa CP was investigated. The expression of lncRNAs in normal Ishikawa and Ishikawa CP with or without CP treating was detected, Tumor-related lncRNAs were selected to analysis, and most of these lncRNAs expression had been changed in these four experimental groups (Fig. S2a). Among these differential lncRNAs, HOTAIR presents the less expression in Ishikawa CP, and Ishikawa and Ishikawa CP in presence of CP treatment in contrast to normal Ishikawa (Fig. S2b). Although the least expression of lncRNA HOTAIR was shown in Ishikawa CP in absence and presence of CP, the expression of lncRNA HOTAIR in these two groups

was identical (Fig. S2b), indicating that CP treatment impossibly changes the expression of HOTAIR in Ishikawa CP.

HOTAIR ensures higher proliferation of Ishikawa CP in CP treatment

To examine the mechanism that links HOTAIR to the CP-resistant activity of Ishikawa CP, HOTAIR was knocked down by using RNA interference technique. Primarily, three HOTAIR siRNA were selected, and endogenous HOTAIR mRNA level was analyzed by RT-PCR and detected to be lower in HOTAIR siRNA2-transfected cells as compared to cells transfected with scrambled siRNA control, demonstrating the effectiveness of HOTAIR siRNA2 (Fig. 2a). Since treating with CP suppressed the expression of HOTAIR and Ishikawa CP showed the fewer expression of HOTAIR, the effect of HOTAIR inhibition on anti-CP resistant action was analyzed. Interestingly, the HOTAIR siRNA-transfected cells (including normal Ishikawa and Ishikawa CP) have a specific reduction in cell proliferation as compared to the control-transfected cells through CCK-8 assay (Fig. 2b).

Higher autophagy of Ishikawa CP is dependent on LncRNA HOTAIR

Using the decrease of GFP fluorescence intensity as readout for enhanced autophagy, we detected that HOTAIR siRNA treatment did not impact the autophagy activity in Ishikawa, but improved the autophagy action in Ishikawa in presence of CP incubation. In contrast, HOTAIR siRNA markedly induced the autophagy activity in Ishikawa CP in absence or presence of CP treatment (Fig. 3). These observations indicate that a CP resistance-related lncRNA, HOTAIR, play a significantly positive role in autophagy activity in endometrial cancer.

LncRNA HOTAIR regulates the expression of autophagy regulator, Beclin-1

Given the positive inter-relationship between autophagy activity and HOTAIR expression, the expression of autophagy regulator, Beclin-1, was measured by using HOTAIR siRNA. In normal, Ishikawa CP

represented higher expression of Beclin-1 than Ishikawa cells, and HOTAIR siRNA dramatically down-regulated the expression of Beclin-1 in both cell lines. Upon the CP treatment, significantly more expression of Beclin-1 was observed in Ishikawa CP cells compared with that in Ishikawa cells. Moreover, HOTAIR siRNA treatment resulted in decreases in Beclin-1 expression in Ishikawa CP cells (Fig. 4). These results demonstrate that HOTAIR may control the autophagy activity of endometrial cancer by mediating the expression of Beclin-1.

LncRNA HOTAIR regulates the expression of MDR and P-gp

To assess the potential interaction between HOTAIR and resistance-related proteins, the expression level of MDR and P-gp in Ishikawa and Ishikawa CP cells with or without transfection with HOTAIR siRNA was examined by Western blot analysis (Fig. 4). Originally, Ishikawa CP cells highly expressed MDR and P-gp. After incubating with CP only, the expressions of these two proteins were up-regulated in both Ishikawa cell lines. Upon the HOTAIR siRNA transfection only, the protein levels of MDR and P-gp were significantly reduced in Ishikawa CP cells. When integrated administration of CP and HOTAIR siRNA, MDR, and P-gp presented the declined protein level in both Ishikawa cell lines in contrast to that in only CP treatment, thereby eliciting a possible that transfection with HOTAIR siRNA in Ishikawa CP cells has made it partially sensitive to cisplatin because of decreased MDR and P-gp expression.

Discussion

Cisplatin is a commonly used chemotherapy drug and is the first chemotherapeutic choice for endometrial cancer. However, acquired cisplatin resistance is the greatest obstacle to clinical chemotherapy. In this study, we found that the HOTAIR expression level was significantly decreased in cisplatin-resistant Ishikawa CP cell compared with parental Ishikawa cells. The HOTAIR inhibition appeared to reverse the chemoresistance of Ishikawa CP cells to cisplatin. HOTAIR appeared to function as a tumor chemoresistant promoter by regulating the expression of Beclin-1, MDR, and P-glycoprotein.

HOTAIR, with a length of 2158 bp, is one of the few biologically well-defined lncRNAs and functions on the regulation of trans-silencing (Woo and Kingston 2007). HOTAIR has been found to be highly expressed in many kinds of human cancers and identified to be a negative prognostic indicator in colon, liver, breast, and pancreatic cancer patient survival, thereby evidencing a close association with increase in cancer cell metastasis (Kim et al. 2013; Kogo et al. 2011). Additional new report is now providing evidence to indicate that HOTAIR is important in regulating the expression of genes involved in cisplatin resistance of human lung adenocarcinoma cells (Liu et al. 2013). Similarly, our present study found that HOTAIR contributes to the resistance of endometrial cancer to cisplatin, and HOTAIR inhibition increases the autophagy in vitro. Previous studies did not reveal how any lncRNA might be responsible for autophagy-related chemoresistance. In current, we show that HOTAIR contributes to the cisplatin resistance of Ishikawa CP cells through autophagy pathway, demonstrating a mechanistic link among HOTAIR, autophagy, and cisplatin resistance in endometrial cancer. The cisplatin resistance regulated by autophagy and autophagy regulated by lncRNA have been extensively documented in the literature (Liu et al. 2015; Wang and Wu 2014). However, the ability of lncRNA to reverse chemoresistance through regulation of autophagy activity and in turn inhibit proliferation in endometrial cancer was previously unrecognized.

Previous study reported that HOTAIR worked together with the PRC2 and LSD1/CoREST/REST, which causes the modifications of DNA-binding proteins and eventually regulates overall gene expressions (Chang and Hung 2012; Cao et al. 2012; Semaan et al. 2011; Murati et al. 2012). In HOTAIR-induced cisplatin resistance of lung adenocarcinoma cells, p21 might be an important mediator (Liu et al. 2013). In the present study, transfection of HOTAIR1 siRNA could significantly decline the increased expression of Beclin-1, MDR, and P-gp in Ishikawa CP cells, indicating that Beclin-1, MDR, and P-gp were the downstream targets of HOTAIR. Certainly, the molecular mechanisms involved in HOTAIR-induced Beclin-1, MDR, and P-gp down-regulation need to be further elucidated in future.

In summary, we have clearly lucubrated that lncRNAs, specifically HOTAIR, can regulate the cisplatin-resistance ability of human endometrial cancer cells through regulation of autophagy by influencing Beclin-1, MDR, and P-gp expression. Our study provides the new clues and valuable information towards development of a complete understanding of the pathogenesis and development of the cisplatin resistance in human endometrial cancer. This raises the possibility that anti-HOTAIR may have potential therapeutic value for those cisplatin-resistant endometrial cancer patients.

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Conflict of interest

We declare that we have no conflict of interest in relation to this article exists.

References

Cao W, Rde Ribeiro O, Liu D, Saintigny P, Xia R, Xue Y, Lin R, Mao L, Ren H (2012) EZH2 promotes malignant behaviors via cell cycle dysregulation and its mRNA level associates with prognosis of patient with non-small cell lung cancer. PLoS One 7:e52984.

Chang CJ, Hung MC (2012) The role of EZH2 in tumour progression. Br J Cancer 106:243-247.

Fukuda T, Oda K, Wada-Hiraike O, Sone K et al (2015) The anti-malarial chloroquine suppresses

proliferation and overcomes cisplatin resistance of endometrial cancer cells via autophagy inhibition Gynecol Oncol 137:538-545.

Kim K, Jutooru I, Chadalapaka G, Johnson G, Frank J, Burghardt R, Kim S, Safe S (2013) HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene 32:1616-1625.

Kogo R, Shimamura T, Mimori K, Kawahara K et al (2011) Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. Cancer Res 71:6320-6326.

Liu J, Wan L, Lu K, Sun M, Pan X, Zhang P, Lu B, Liu G, Wang Z (2015) The Long Noncoding RNA MEG3 Contributes to Cisplatin Resistance of Human Lung Adenocarcinoma PLoS One 10:e0114586.

Liu Z, Sun M, Lu K, Liu J, Zhang M, Wu W, De W, Wang Z, Wang R (2013) The long noncoding RNA HOTAIR contributes to cisplatin resistance of human lung adenocarcinoma cells via downregualtion of p21(WAF1/CIP1) expression. PLoS One 8:e77293.

malignancies: mutations, models and management. BMC Cancer 12:304.

Mizushima N (2010) The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol 22:32-139.

Murati A, Brecqueville M, Devillier R, Mozziconacci MJ, Gelsi-Boyer V, Birnbaum D (2012) Myeloid

Semaan A, Qazi AM, Seward S, Chamala S et al (2011) MicroRNA-101 inhibits growth of epithelial ovarian cancer by relieving chromatin-mediated transcriptional repression of p21(waf(1)/cip(1)). Pharm Res 28:3079-3090.

Sui X, Chen R, Wang Z, Huang Z et al (2013) Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment Cell Death Dis 4:e838.

Wang J, Wu GS (2014) Role of autophagy in cisplatin resistance in ovarian cancer cells. J Biol Chem

289:17163-17173.

Woo CJ, Kingston RE (2007) HOTAIR lifts noncoding RNAs to new levels. Cell 129:1257-1259.