Taxotere suppresses breast cancer growth through inducing
lincRNA-p21 expression

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Submitted to the graduate degree program in Department of Molecular Biosciences and the
Graduate Faculty of the University of Kansas in partial fulfillment of the requirement for the
degree of Master of Arts

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Date defended: 31 August 2016
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Date Approved: 01/30/2017
Abstract

It has been reported that long non-coding RNA lincRNA-p21 is induced upon radiation and chemotherapy. This induction contributes to DNA-damage repair, cell death and cell cycle regulation. In this study, we focused on Taxotere (TXT) mediated chemotherapy of breast cancer cells and found that lincRNA-p21 is robotically induced upon TXT treatment. Secondly, we observed increased chemoresistance in three different breast cancer cell lines with lincRNA-p21 knockdown. Mechanistically, we found that lincRNA-p21 knockdown lead to decreased cell death during chemotherapy comparing to the negative control. In addition, our work showed that p21 is a downstream target of lincRNA-p21 in human breast cancer cells, and the loss of lincRNA-p21 caused p21 downregulation at both the RNA and protein levels.
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Introduction

More than 1.3 million patients are diagnosed with breast cancer every year all over the world. Even with advanced medical care and treatment, about 50,000 people will die from this disease each year[1]. Chemosresistance is one of the main reasons for failures in breast cancer treatments and tumor recurrence[2]. We now know that cell death deficiency and cell cycle dysregulation lead to tumor resistance[3-7]. Various protein-coding gene such as p53 and HER2 have been reported to influence chemoresponse of breast cancer cells[8, 9]. However, the role of long non-coding RNAs (lncRNAs) in regulating breast cancer chemoresistance remains to be characterized. More importantly, there is still no accurate prognosis guideline in clinical practice to differentiate breast cancer patients who would respond to chemo/radiotherapy from those who would not, based on our current knowledge about specific molecular or genetic markers.

Taxotere (TXT or docetaxel) is a chemotherapeutic drug that has been incorporated into first line adjuvant chemotherapy of breast cancer[9, 10]. Mechanistically, TXT promotes the assembly and stability of microtubules. As a consequence, TXT blocks cell cycle progression at M phase and induces cell death pathway by interrupting microtubules depolymerization[11, 12]. As reported before, TXT activates apoptosis pathway by upregulating p21, which is a validated downstream target of lincRNA-p21[13, 14]. However, a better understanding on the mechanism of TXT action in breast cancer is desired.

Long non-coding RNA (LncRNA) is a subset of RNA molecule classified based on RNA length (longer than 200bp in length) and translational potential (no protein product)[15]. The traditional understanding about human genome has focused on protein-coding genes, with the rest of genome considered ‘transcriptional noise’. However, advances in sequencing
technologies have shown that a mere 2% of the human genome was transcribed into mRNA, whereas 62.1% of genome was transcribed into non-coding RNAs that were processed. This finding indicates that the non-coding RNAs likely have the potential to affect biological functions in human cells[16-18]. For the past decades, lncRNAs were shown to have diverse regulatory functions on gene transcriptional process and post-transcriptional regulation, such as modulating RNA stability[19-22]. Investigating the biological functions of lncRNAs in regulating cell signaling pathways has largely advanced our understanding about multiple diseases, including cancer[15].

LincRNA-p21 is a ~3kb lncRNA that been identified in 2010 as p53 target and putative tumor suppressor[23]. Published data showed that the lincRNA-p21 induction in cells mediated by radiation or chemotherapy directly promoted apoptosis, senescence, and the G1/S cell-cycle checkpoint[14, 22-28]. Recently, our finding indicates that lincRNA-p21 is also an important mediator in taxotere chemotherapy of human breast cancer (Guo et al, manuscript in preparation). In brief, we found that TXT treatment rapidly activated lincRNA-p21 expression in human breast cancer cells. And the knockdown of lincRNA-p21 led breast cancer cells to become chemoresistant. At the molecular level, p21 is a downstream target of lincRNA-p21 and the loss of lincRNA-p21 attributed to a loss of p21 induction upon TXT treatment.
Results

1. LincRNA-p21 was induced upon chemotherapeutic drug Taxotere treatment in human breast cancer

To validate the suppressive effect of TXT on breast cancer cell growth, we treated human breast cancer cell line MDA-MB-231 with two different dosages of TXT and measured the cell growth. After treated for 24 hours, 0.5nM TXT treatment significantly suppressed MDA-MB-231 cells growth about 58% and 1nM TXT treatment inhibited cell over 90% comparing to control. This indicates that growth suppressive effect is dose-dependent (Fig. 1A).

Previously, published reports showed that the induction of lincRNA-p21 mediated by radiation and doxorubicin played an important role in human colon cancer cell stress response. However, no evidence showed lincRNA-p21 was involved in TXT-mediated cell chemoresponse. To examine if lincRNA-p21 has potential functions in breast cancer TXT chemoresponse, we started to quantifying its induction level upon TXT treatment. By performing real-time qPCR, we observed that lincRNA-p21 expression level was rapidly upregulated upon TXT treatment and final expression level was increased by 15 times. We also found that the TXT-mediated induction of lincRNA-p21 appeared to be dose-dependent (Fig.1C).

Taken together, our data indicates that chemotherapeutic drug TXT efficiently suppressed the growth of human breast cancer cells. During this process, lincRNA-p21 transcriptional process was potently activated. Based on this observation, we hypothesized that lincRNA-p21 induction plays a significant role for breast cancer chemoresponse upon TXT treatment.
Fig 1: LincRNA-p21 was induced upon taxotere (TXT) treatment.

A. Treating MDA-MB-231 with 0.5nM TXT for 24 hours inhibited cell growth.

B. LincRNA-p21 was induced by 0.5nM TXT in breast cancer cell line MDA-MB-231 (0h – 48h).

C. LincRNA-p21 activation mediated by TXT in MDA-MB-231 was dose-dependent.
2. LincRNA-p21 knockdown promoted breast cancer cell chemoresistance

To test our hypothesis about the role of lincRNA-p21 in breast cancer cells, we designed and synthesized four lincRNA-p21 targeting antisense oligonucleotides (ASOs) for knockdown experiment. Since lincRNA-p21 localized in both the cytoplasm and nucleus[22], ASO/RNaseH complex is more efficient in knocking down lincRNA-p21 in both cell compartments comparing to RNA-induced silencing complex (RISC) [29, 30]. Before performing functional studies, we tested knockdown efficiency of all ASOs in MDA-MB-231 cells and picked the best two ASOs (ASO2 and ASO3) for further studies (Fig. 2A). In addition, we also validated that ASO2 and ASO3 efficiently blocked lincRNA-p21 in the presence of TXT (Fig. 2B).

To better understand its roles in breast cancer cells, we performed lincRNA-p21 knockdown assay in three different breast cancer cell lines: MDA-MB-231, BT-483 and HS-578T. We chose these three cell lines for two reasons: 1) lincRNA-p21 was expressed (data not shown); 2) they include ER+ (BT-483) and ER- (MDA-MB-231, HS-578T) breast cancer cells [31]. We used MTT-based cytotoxicity assay to evaluate cell chemoresistant level. IC-50 represented half-maximal inhibitory concentration. High IC-50 indicated high resistant level of the cell to the compound. With lincRNA-p21 knocked down, the IC-50 of MDA-MB-231 upon TXT treatment was almost doubled comparing to negative control (NC) group. This indicates that lincRNA-p21 promotes TXT resistance (Fig. 2C). This phenotype was also observed in two other breast cancer cell lines, BT-483 (ER+) and HS-578T (ER-) (Fig. 2C). However, the effect of lincRNA-p21 knockdown on chemoresistance was less significant in HS-578T and BT-483 cells. We deduced it could likely be attributed to the low transfection efficiency and high resistance to TXT.
Based on previous studies, lincRNA-p21 is a p53 target and its function is highly dependent on p53[23, 26, 28, 32]. Our finding showed that lincRNA-p21 also has function in p53-null (BT-483) and p53-mutant (HS-578T, MDA-MB-231) breast cancer cells. This demonstrates that the regulatory network of lincRNA-p21 in breast cancer potentially has p53-dependent and p53-independent mechanisms for gene expression. In conclusion, we found that TXT suppressed breast cancer growth through inducing lincRNA-p21 and the loss of lincRNA-p21 led to chemoresistance.
Fig 2: LincRNA-p21 knockdown promoted breast cancer cell chemoresistance.


B. ASO blocks lincRNA-p21 induction mediated by 1nM TXT in MDA-MB-231 cells.

C. LincRNA-p21 knockdown increases breast cancer cell chemoresistance of MDA-MB-231/ BT-483/ HS-578T.
3. LincRNA-p21 knockdown increased cell growth while blocking cell death

To date, no study has examined lincRNA-p21’s function in breast cancer. By knocking down lincRNA-p21 in three breast cancer cell lines (MDA-MB-231, BT-483 and HS-578T), we observed a consistent phenotype in all three cell lines that lincRNA-p21 knockdown significantly promoted cell growth as compared to negative control (Fig.3A). To quantify the influence of lincRNA-p21 knockdown on cell growth, we performed Acridine Orange and Propidium Iodide (AO/PI) staining assay with or without TXT treatment on MDA-MB-231 cells (Fig. 3B). As shown in Figure 3B, knockdown groups had significantly higher numbers of living cells than that of negative control for both conditions. This data confirm the potential tumor suppressive role of lincRNA-p21 in breast cancer cells. The lincRNA-p21 knockdown efficiency was lower in HS-578T and BT-483 cells (Fig. 3E, 3F), which might explain why lincRNA-p21 knockdown was less potent in altering chemoresistance and cell growth in these two cell lines.

Furthermore, AO/PI staining results also showed that lincRNA-p21 knockdown increased breast cancer cell survival (Fig. 3G, 3H). More relevant to chemoresistance, we tested this effect of lincRNA-p21 knockdown upon TXT treatment. Consistent with above data, the loss of lincRNA-p21 directly led to robotic cell growth increase with decreased cell death after TXT treatment (Fig. 3G).
Fig 3: LincRNA-p21 knockdown promoted cell growth while blocking cell death.

A. Cells with lincRNA-p21 knocked down grew faster than NC (MDA-MB-231, BT-483, HS-578T).

B. Living MDA-MB-231 cell number with (lower) or without (upper) TXT treatment tested by AO/PI staining.

C. Percentage of dead MDA-MB-231 cells with (lower) or without (upper) TXT treatment.

D. LincRNA-p21 knockdown efficiency in BT-483 (upper) and HS-578T (lower).
4. **p21 is a downstream target of lincRNA-p21**

To identify downstream targets of lincRNA-p21 in breast cancer, we performed qPCR and Western blot with lincRNA-p21 knockdown samples. Previously, p21 was demonstrated to be the direct target of lincRNA-p21. Specifically, lincRNA-p21 activated P21 transcription in *cis* by recruiting hnRNPK and transcriptional activators to its promoter[14]. p21 acts as cell cycle and cell death regulator[3]. We hypothesize that lincRNA-p21 regulates breast cancer cell cycle distribution and cell death by activating p21. Our data showed that lincRNA-p21 knockdown led to decrease in p21 expression at both RNA and protein levels in MDA-MB-231 cells (Fig. 4A left, 4B). This regulatory relationship was also confirmed upon TXT treatment (Fig. 4A right). Currently, we are working on optimizing the PI staining flow-cytometry protocol to validate this hypothesis. Besides P21 gene, the expression of BRCA1 was also downregulated while knocking down lincRNA-p21 in breast cancer cells. Based on literature, BRCA1/p21 pathway is involved in cell cycle arrest upon stresses such as chemotherapy[5, 33, 34]. It is reasonable to deduce that lincRNA-p21 enhances breast cancer chemoresponse through activating BRCA1/p21 pathway. However, further experiments are still needed to examine this possibility.
Fig 4: p21 is downstream target of lincRNA-p21 in breast cancer.

A. Real-time qPCR quantification of P21 in MDA-MB-231 cells with (right) or without (left) taxotere treatment.

B. Western Blot of p21 protein in MDA-MB-231 cells.

5. dCas9-CRISPR mediated lincRNA-p21 activation

When we started this project, only a partial sequence of human lincRNA-p21 had been published and the remaining sequence was unknown[22]. Hence, all human lincRNA-p21 overexpression experiments used partial human lincRNA-p21 expressing vector[22, 26, 27, 35]. Since long non-coding RNAs function as RNA molecules instead of protein molecules, using a partial RNA sequence likely alters lncRNA functional domains and its activities. To better understand the functions and downstream targets of lincRNA-p21, we applied dCas9-CRISPR mediated gene activation (CRISPRa) system to achieve endogenous human lincRNA-p21 ‘overexpression’[36].

CRISPR technology is a new and efficient gene editing method recently established(ref). Mechanistically, Cas9 nuclease is guided by specific 20 base single guide RNA (sgRNA) to the targeting genome locus through nucleic acid base-paring and achieves gene editing [37, 38]. After being established in 2012, CRISPR technology is widely used in numerous areas. Different from traditional Cas9-CRISPR, CRISPRa uses the catalytically inactivated dCas9-sgRNA complex to target gene promoter and activate gene transcriptional process [39]. In brief, dCas9 protein is fused to the transcriptional activator, VP64. The modified sgRNA bears MS2 stemloops, which brings two extra activators (P65, HSF1) through MS2 aptamer-mediated recruitment. During activation process, sgRNA-P65-HSF1 complex associates with dCas9-VP64 fusion protein and then binds to its targeting gene promoter. This system induces endogenous gene expression in cells that captures the complexity of transcript isoform variance.

Based on literature, sgRNAs that target the first 200bp upstream from transcriptional start site (TSS) have the best activation efficiency[36]. Since we only had a partial lincRNA-p21 sequence available, we started with predicting the locations of lincRNA-p21 TSS and promoter
in human genome. We reached the epigenetic signatures of human lincRNA-p21 using sequencing data from *Roadmap Epigenomic Project* [40, 41]. H3K27ac and H3K4me1 are two markers that frequently appear at promoter region [42]. The promoter region is also marked with enriched DNaseI hypersensitive sites and transcriptional factor binding sites. As a consequence, the sequencing data indicated that the 1000bp upstream of the partial lincRNA-p21 sequence likely contains the promoter region and the +1 site TSS (Fig. 5A). Recently, the full-length sequence of human lincRNA-p21 has been published [43]. Our prediction is consistent with this new finding.

With this information, we designed four sgRNAs for CRISPRa assay using online tools (Fig. 5B). Considering the low transfection efficiency of breast cancer cells, we initially tested the CRISPRa system in HEK-293FT cells. As shown in Fig. 5C, sgRNA1/2/3 all activated lincRNA-p21 expression with different efficiencies. This result was consistent with original publication [36], that the closer the sgRNA is to TSS, the more efficient activation it achieves.

To make sure the activation was mediated by CRISPRa system and not by non-specific effects, we designed and developed lincRNA-p21 promoter luciferase assay (Fig. 5D upper). The luciferase assay was conducted in HEK-293FT cells for 24 hours and 48 hours after transfection. As shown in Figure 5D (lower panel), comparing to two negative controls, four lincRNA-p21 sgRNAs significantly activated the transcriptional process from lincRNA-p21 promoter.

To improve the activation efficiency, we established the HEK-293FT dCas9-complex lentiviral stable cell line and performed CRISPRa using this cell line. Consistent with our previous finding, the p21 gene was highly upregulated after lincRNA-p21 activation by
CRISPRa. To examine tissue specificity, the activation assay will be tested on breast cancer cells in the future studies.
Fig 5: LincRNA-P21 CRISPR activation system.

A. Epigenetic modification and lincRNA-p21 promoter prediction.
B. Designing of lincRNA-p21 CRISPR activation system.
C. CRISPRa efficiency in HEK-293ft lenti-dCas9 cells.
D. Validation of CRISPRa specificity in HEK-293ft cells: luciferase assay.
E. Downstream target of lincRNA-p21 in HEK-293ft cells.
Discussion

Breast cancer is one of the most common solid tumors, which affect about 12% women in US. Failure to respond to chemotherapy represents a critical problem in breast cancer treatment[44]. After decades of exploration, the mechanism of chemoresistance still remains not totally clear. Incorporating a new component promoting chemosensitivity into current cancer chemotherapy would provide immense clinical relevance that would benefit cancer patients. Long non-coding RNAs (lncRNAs) have emerged as important regulators of many cellular processes including chemoresponse. Among lncRNAs, lincRNA-p21 has been shown as a crucial tumor suppressor in many cancer types[14, 23, 26-28, 45]. However, the significance of lincRNA-p21 in breast cancer chemoresistance remains to be characterized.

Our results indicate that lincRNA-p21 is a phenotypic regulator of breast cancer chemosensitivity upon TXT treatment. LincRNA-p21 is highly upregulated during chemotherapy and suppress tumor growth. To our knowledge, this is the first study examining the role of lincRNA-p21 in human breast cancer and TXT chemotherapy. Taxotere is a first line chemotherapeutic drug that has been used in breast cancer treatment for decades. The p21 pathway contributes to TXT-mediated tumor suppression. Our finding provided a new layer of understanding of p21 regulation in TXT treatment and chemoresistance.

By now, we only used lincRNA-p21 knockdown to exam the role of lincRNA-p21 in breast cancer. For the future study, experiments on validating lincRNA-p21 functions and downstream targets are needed to have a better understand the mechanism of action. Specifically, it will be valuable to test and optimize our CRISPR activation system which achieves endogenous human lincRNA-p21 ‘overexpression’ on breast cancer cells. First, though the human lincRNA-p21 full-length sequence is now published, the CRISPRa system has the advantage of achieving
endogenous activation and covering all transcript isoforms. Secondly, our current lincRNA-p21 CRISPR activation system hasn’t achieve significant effect on any breast cancer cells. This could possibly due to: 1) low transfection efficiency; 2) histone modification on sgRNA binding sites. To solve this potential problem, we will establish lenti-viral CRISPRa system and test more sgRNAs. As double validation, we will overexpress human lincRNA-p21 using expression plasmid and compare the phenotype with the one of CRISPRa to conclude lincRNA-p21 functions in breast cancer.

In addition, extra experiments such as rescue study can be conducted to validate p21 as the direct target of lincRNA-p21 upon TXT treatment. With the activation and overexpression tools available, we will be able to perform additional functional studies with human lincRNA-p21. In addition, our data show that BRCA1 mRNA abundance was altered in lincRNA-p21 knockdown samples. This demonstrates that BRCA1 might be a novel and relevant target of lincRNA-p21 in human breast cancer. Based on literature, BRCA1 regulates p21 expression, which is highly involved in breast cancer chemoresponse. Further studies related to the regulatory relationship between lincRNA-p21 and BRCA1 could be conducted in the future.

Lastly but not the least, we found that tumor suppressor lincRNA-p21 was not significantly lost from breast cancer cells comparing to normal cells. LincRNA-p21 is a highly dynamic lncRNA whose half-life is less than 2 hours. We hypothesized that the induction of lincRNA-p21 upon taxotere treatment was correlated with breast cancer chemosensitivity. As a consequence, further assays of determining the expression of lincRNA-p21 in taxotere-treated breast cancer cells and correlating lincRNA-p21 response with the cells’ chemosensitivity should be performed. These findings will contribute to identifying new biomarkers and incorporating new lncRNA components into breast cancer chemotherapy prognosis.
Materials and Methods

Cell Culture, Transfection and Antisense Oligonucleotide
Human breast cancer cell lines MDA-MB-231, BT-483 and HS-578T were cultured in DMEM (Invitrogen) supplemented with 10% FBS and antibiotics in a 37° and 5% CO₂–humidified incubator. All Antisense Oligonucleotides (2’OMe-ASOs) including NC ASO were purchased from IDT (Integrated DNA Technologies) diluted with RNase/DNase free water into 20mM. ASOs are listed in Table1. Cells (300,000 cells/well MDA-MB-231, 100,000 cells/well HS-578T, 300,000 cells/well BT-483 were seeded into 6-well-plate 24 hours before transfection. Cells were transfected with ASO at 20nM (MDA-MB-231, BT-483) or 30nM (HS-578T) final concentration using lipofectamine 2000 (Invitrogen). Culture media was changed 5 hours after transfection.

Western Blot Analysis
After 48 hours of transfection with ASOs, cells were washed twice with cold PBS and collected. Whole-cell lysates prepared in RIPA buffer were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto PVDF membranes. Primary antibodies were used: anti-P21 (Cell Signaling) and anti-tubulin (Sigma).

CRISPR activation
CRISPR activation was performed as previously described [36]. sgRNA sequence were listed in Table2. All plasmids used were obtained from Addgene. In brief, lenti-dCas9 HEK293ft cells were maintained in high-glucose DMEM with GlutaMAX. 450,000cells/well lenti-dCas9 HEK293ft were plated in 6-well-plate 24 hours before transfection. For lincRNA-p21 activation, 1µg sgRNA plasmid were transfected using lipofectamine2000 (1:2.5 ratio). Media were changed 5h post-transfection.

Promoter Luciferase Reporter Assay
The promoter region of lincRNA-p21 (1kb) was cloned into pGL4.20 [luc/Puro] vector (Promega). HEK293ft cells seeded in 96-well-plate were transfected with sgRNA, dCas9-VP64, MS2-p65-HSF1,
pGL4-lincp21 and Renilla. 48h or 72h after transfection, cells were harvested and assayed using the Dual-Luciferase Reporter Assay System (Promega).

**AOPI staining**

AOPI staining was performed using VisStain™ AOPI Staining Solution in PBS and Cellometer from Nexcelom following manufacturer's instructions.

**MTT-based Cytotoxicity Assay**

5,000 cells/well were plated in 96-well-plate and treated with serially diluted TXT in triplicates. After 4 to 6 days, WST-8 (Sigma) and plate reader were used to measure the result as described previously [46].

**RNA extraction, cDNA synthesis and real-time PCR analysis.**

Total RNA was extracted using Direct-zol® MiniPrep kit (Zymo Research) following manufacturer’s instructions. The cDNA synthesis was performed using random primer and SuperScript™ III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed using gene specific primers (listed in Table3), SYBR® Selected Master Mix (Applied Biosystems, Life Technologies) and normalized to GAPDH.

**Table 1. LincRNA-p21 ASOs**

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**Table 2. LincRNA-p21 CRISPRa sgRNAs**

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### Table 3. Primers

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Human lincRNA-p21 promoter clone primers:

Real-time PCR primers:

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