EZH2 Confers Sensitivity of Breast Cancer Cells to Taxol by Attenuating P21 Expression Epigenetically

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Abstract

Breast cancer isthe leading cause of death in women. Although numerous clinical regimens are employed to treat breast cancer and manifest satisfied efficacy, drug resistance is emerging as the major obstacle to their long-term use. It is critically necessary to decipher the molecular mechanism underlying this process to obtain improved and long-term use of each regimens. In the present study, we showed the negative relationship between EZH2 and chemoresistance to Taxol in breast cancer cells. EZH2 interference was capable of decreasing while overexpression increasing apoptosis of breast cancer cells challenged with Taxol. Meanwhile, p21, the inhibitor of cell cycle entry, interference up-regulated while overexpression down-regulated apoptosis induced by Taxol. Mechanistically, EZH2 was recruited to the promoter of p21 accompanied with H3K27me3 enrichment and transcription silencing. Collectively, EZH2 attenuates chemoresistance of breast cancer cells to Taxol by dampening p21 epigenetically.

Introduction

Breast cancer is the most prevalent cancer and the most common cause of death in women. Epidemiological studies demonstrated that there were around 1.68 million new cases and 0.53 million deaths of breast cancer in 2012 worldwide which was the leading malignancy in women (Torre et al., , 2015).The clinical treatment for breast cancer is surgery accompanied with ionizing

radiation, anti-cancer reagents as well as anti-estrogen regimens. However, satisfactory prognosis is hardly obtained due to chemo-resistance (Rivera and Gomez, 2010). The routine drugs applied for breast cancer treatment are anthracycline-based regimens and taxanes. Taxol, the derivative of taxane, is a widely used chemotherapy agent for various cancers including breast cancer(Weaver, 2014a). It functions through microtubules whose disruption obstacles cell division. However, it was reported that the concentration of intratumoral Taxol was too low to attain therapeutic effect which indicated other mechanisms were involved in the effect of Taxol upon breast cancer. Though Taxol is able to provide patients with 30%-70% response rates, much response will be attenuated progressively due to chemo-resistance(Rivera and Gomez, 2010). Therefore, it is necessary to decipher mechanisms of resistance of breast cancer to Taxol and thus prolongs its potency.

Cell cycle is co-regulated by CDKs and CDK inhibitors. As an important CDK inhibitor, p21 plays a crucial role in maintenance of normal cell cycle. Besides the inhibitory effect of p21 on complex of cyclin D1, CDK, and PCNA, p21 interacts and inhibits cyclin E/CDK2, cyclin A/CDK2, and cyclin B/CDK1 complexes likewise(Jung et al., , 2010). Of note, in addition to inhibit cell cycle progression, p21 exerts an anti-apoptotic effect within cancer cells(Jung et al.,, 2010). Ying Li et al reported that AKT/PKB was able to increase p21 through stabilizing p21 protein and thus promoted cell survival(Li et al., , 2002). Mojgan Mahyar-Roemer et al demonstrated that chemotherapeutic agents induced p21 expression and apoptosis independently of p53, but more apoptosis was observed after p21 interference in colon cancer(Mahyar-Roemer and Roemer, 2001). Moreover, p21 was overexpressed within breast cancer and Taxol prevented breast cancer through induction of p21(Abbas and Dutta, 2009, Choi and Yoo, 2012a). These clues made us a reason that p21 might be involved in Taxol-resistance in breast cancer.

EZH2, the histone methyltransferase, is a critical histone methylation enzyme which writes H3K27me3 marks into various genes and inhibits their expression. Numerous studies have shown that EZH2 was over-expressed in breast cancer(Bracken et al., , 2003, Kleer et al., , 2003).Also, EZH2 was reported to be involved in resistance of breast cancer to several chemotherapeutic agents(Jiang et al., , 2018, Huang et al., , 2018). Of note, an lncRNA was demonstrated to be involved in chemo-resistance to Taxol through EZH2(Si et al., , 2016). However, whether EZH2 was associated with Taxol-resistance in breast cancer was elusive.

In the present study, we studied the involvement of EZH2 in resistance of breast cancer to Taxol and its mechanism. We showed that EZH2 was negatively associated with resistance of breast cancer cells to Taxol. On the other hand, over-expressed p21 provided breast cancer cells with anti-apoptotic capacity and thus resistance. Mechanistically, EZH2 regulated resistance of breast cancer cells to Taxol by manipulating p21 through modifying H3K27me3 enrichment within its promoter.

Material and methods

Cell culture

Human breast cancer cells MCF-7 and ZR-75-30 were purchased from FDCC (Shanghai, China). They were cultured in DMEM-H and DMEM/F12 respectively supplemented with 10% FBS and 1% penicillin and streptomycin. The Taxol-resistant cell lines of MCF-7 and ZR-75-30 were obtained by pulse selection using Taxol and designated as MCF-7R and ZR-75-30R respectively.

For the establishment of paclitaxel-resistant breast cancer cell lines, MCF-7R and ZR-75-30R was obtained by pulse selection from parental MCF-7S and ZR-75-30S. Here in our study, 1x106 cells were seeded in 10-cm dishes. Paclitaxel at dosage of IC50 was applied to challenge sensitive cells after 24 hours. Culture medium and paclitaxel was replaced weekly. As cell confluence attained 70-80%, cells were collected and re-seeded in new dishes followed by paclitaxel challenge at dosage of double IC50. The establishment process was ended as paclitaxel attained 10xIC50.

Cell number counting

$1x10^5$ cells were seeded to wells of the 6-well plates. After starvation with serum-free medium O/N, for dosage-dependent assay, cells were challenged with 0 µM, 5 µM, 10 µM, 15 µM, and 20 µM for 24 hours respectively. For time-dependent assay, cells were challenged with 15 µMTaxol for0 hours, 12 hours, 24 hours, 36 hours, and 48 hours respectively. Then cells were washed with PBS 3 times and trypsinized in incubator for 3 min. The complete culture medium was applied to stop digestion followed by gently pipetting to obtain single cell suspension. Cell number was counted using hematocytometer under inverted microscope.

MTS assay

Cells were digested and seeded in 96-well plates ($1x10^4$ per well) in complete medium. After starvation with serum-free medium O/N, cells were challenged with 15 µMTaxol for 0 hours, 12 hours, 24 hours, 36 hours, and 48 hours respectively. 20 µl MTS reagent (Biovision, Milpitas, USA) was applied to each well followed by incubation at 37°C for 2 hr. The plate was shaken for 30 sec and the absorbance was detected with plate reader.

Cell transfection with plasmid and siRNA

Cells were starved O/N for better transfection. 5µg plasmid and 10µl Lipofectamine 3000 (Thermo Fisher Scientific Corp, Waltham, USA) was added to 125µlOpti-MEM (Thermo Fisher Scientific Corp, Waltham, USA) respectively accompanied with co-incubation at RT for 5 min. The equal amount of empty vector was employed as Vector group. Opti-MEM containing plasmid and Lipofectamine 3000 was mixed for 20 min. Cells were washed with PBS and pre-incubated with 250µl Opti-MEM. Then, mix of Opti-MEM and Lipofectamine 3000 was added to cells and incubated in incubator for 6 hours followed by medium replacement with complete medium. For siRNA transfection, 5µl siRNA was applied as plasmid transfection procedure and negative control was designated as siNC group. The sequences of siEZH2 was5'-AGGTCAGTTACCATACTTATCTTATTGA-3' and its negative control (siNC) was 5'-ATTAATTCGAATTCCTAGTATCGTACTG-3'; thesequence of siP21 was 5'-GTCAGATTCTGTTACTTTACGTCATCCA-3' and its negative control was 5'-ACTCCTGATATTCGTCTACTTCGTAATG-3'.

Trypan blue staining

After treatment, culture medium containing suspending cells was collected into 15 ml tubes. Adherent cells were washed with PBS and detached with trypsin in incubator for 3 min. After digestion stop using complete medium, single cell suspension was obtained by gently pipetting. Cells were mixed and stained with 0.4% Trypan blue (Beyotime, Shanghai, China) for 5 min. Later, blue-staining cells number was counted using hematocytometer under inverted microscope.

Total RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted using total RNA extraction kit (BioTeke, Beijing, China)according to manufacturer’s instruction. RNA was reversely transcribed into cDNA using reverse transcription

kit (Thermo Fisher Scientific Corp, Waltham, USA) according to manufacturer's instruction. Relative mRNA expression level was assayed using SYBR Green PCR master mix (Transgene, Beijing, China) according to manufacturer's instruction. GAPDH was used as internal control. The relative expression level was normalized to control groups. The sequences of primers used in this study was: EZH2 GAGTTGGTGAATGCCCTTGG (forward) and TGCTGTGCCCTTATCTGGAA (reverse) ; p21 CCCAAGCTCTACCTTCCCAC (forward) and CTGAGAGTCTCCAGGTCCAC (reverse); GAPDH CCAGAACATCATCCCTGCCT (forward) and CCTGCTTCACCACCTTCTTG (reverse).

Chromatin immunoprecipitation (ChIP)

$1x10^7$cells were used for ChIP assay. Briefly, cells were cross-linked with 1% formaldehyde and chromatin was fragmented with high power sonication. 100-500bp DNA fragments were used by following analysis. DNA fragments were precipitated with anti-H3K27me3 antibody O/N and the immune complex was further precipitated with Protein A/G-coated magnetic beads for 2 hours according to manufacturer's instruction. Precipitated chromatin was reversely cross-linked by gently shaking at 65°C for 2 hours. The product was amplified usingSYBR Green PCR master mix (Transgene, Beijing, China) according to manufacturer's instruction and analyzed with quantitative PCR. The results were normalized to each control group. The sequences of primers used in this study was: p21 CAGTGGACCTCAATTTCCTCA (forward) and AAAACGATGCACCTCTCTGC(reverse).

Total protein extraction and immunoblotting

Total protein was extracted using RIPA tissue lysis buffer (Solarbio, Beijing, China). Samples were quantitated and de-natured and with SDS-loading buffer. Each samples were loaded to and analyzed by 10% SDS-PAGE gels at 120 V for 2 hours. Protein was transferred to PVDF membranes at 300 mA for 2 hours and membranes were blocked with 5% non-fat milk for 2 hours. Then, each membrane was incubated with the primary antibody against EZH2 (1:1000, Millipore, Burlington, USA), p21 (1:1000, Santa Cruz, Dallas, USA) and GAPDH (1:10000, Proteintech, Rosemont, USA) O/N and corresponding secondary antibody for 2 hours. The bands were visualized with ECL detection kit (Thermo Fisher Scientific Corp, Waltham, USA) and quantitated with Image J software. GAPDH was used as internal control. The results were normalized to each control group.

Cell apoptosis flow cytometry

Treated cells were collected and centrifuged at 1000g for 5min. The supernatant was discarded and the cell pellet was washed with cold PBS for 2 times. The obtained cells were treated and stained withAnnexin V-FITC cell apoptosis detection kit (Beyotime Biotechnology, Shanghai, China) in dark room according to manufacturer's instruction. Cell apoptosis was analyzed using flow cytometry.

Statistical analysis

Each experiment was repeated 3 times. Student's *t*-test was used to determine statistical significance. All results were expressed as means ± SEM. $P< 0.05$ was consideredas statistically significant.

All the uncropped original scans of membranes used for Western blot analysis are shown in Supplementary Figure 1. Meanwhile, the raw CHIP data included in this study is indicated in Supplementary Figure 2.

Results

EZH2 was altered markedly in breast cancer cell with various Taxol-resistance

EZH2 was significantly increased in breast cancer(Kim and Roberts, 2016). To manifest its contribution to Taxol-resistance in breast cancer, its expression in Taxol-sensitive and Taxol-resistant counterpart was determined. As showed in figure 1A, EZH2 showed significantly decreased expression of mRNA after induction of Taxol-resistance in different breast cancer cell lines. Of note, ZR-75-30, the breast cancer cells with intermediate Taxol-resistance, expressed higher EZH2 mRNA in comparison with MCF-7, the Taxol-resistant lines which was consistent with study reported by Xx Si et al(Si et al.,, 2016). Also, immunoblotting showed the same change at protein level. Next, the effect of Taxol on cell growth was determined. Figure 1C showed that cell growth was markedly decreased by Taxol challenge in sensitive breast cancer cells which were less decreased in resistant lines in time-dependent manner. In accordance with cell growth assay, Taxol-resistant breast cancer cell lines evidenced similar decrease in cell viability which was less decreased in resistant lines in time-dependent manner (Figure 1D). Moreover, the less Taxol-resistant breast cancer cells showed less cell growth and viability and vice versa (Figure1C and D). Then the dosage-dependency of Taxol upon cell growth and cell viability in different breast cancer cells was determined. It was showed that Taxol decreased growth and viability of breast cancer cells in dosage-dependent manner after 24 hour challenge. As 15μM decreased growth and viability remarkably and comparably compared with 10μM and 20μM respectively, it was used in subsequent analysis.

EZH2 attenuated Taxol-resistance of breast cancer cells

As EZH2 was altered in breast cancer cells with different resistance to Taxol significantly, the effects of its overexpression or interference on Taxol-resistance and cell apoptosis were determined. The efficiency of overexpression or interference of EZH2 in MCF-7 and its resistant lines was showed in Figure 2A and 2B. Cell apoptosis flow cytometry demonstrated that Taxol challenge remarkably induced apoptosis in Taxol-sensitive MCF-7 cells. However, this induction was obviously mitigated by EZH2 interference. In contrast, apoptosis was induced by Taxol challenge in less extent compared with MCF-7 in Taxol-resistant MCF-7R cells which was significantly aggravated by EZH2 overexpression (Figure 2C and 2D). In accordance, apoptosis staining showed decrease in apoptosis after EZH2 interference in MCF-7 cells while increase in apoptosis after EZH2 overexpression in MCF-7R cells (Figure 2D). Notably, apoptosis of breast cancer cells with different resistance to Taxol showed corresponding alterations (Figure 2C and 2D). These results demonstrated that EZH2 was positively associated with sensitivity of breast cancer cells to Taxol.

p21 potentiated Taxol-resistance of breast cancer cells

Recently, it was reported that p21, a vital cell cycle inhibitor, was involved in breast cancer cell survival(Braun et al., , 2011). Accordingly, p21 expression was remarkably induce by Taxol challenge which indicated that p21 might be involved in Taxol-resistance. Therefore, the implication of p21 within this process was determined. It was showed that both mRNA and protein of p21 was significantly elevated in Taxol-resistant breast cancer cells in comparison with Taxol-sensitive breast cancer cells (Figure 3A and 3B). Again, breast cancer cells with intermediate resistance to Taxol manifested lower p21 level (Figure 3A and 3B). Subsequently, interference and overexpression of p21 was employed to explore the effects of p21 on apoptosis induced by Taxol

(Figure 3C and 3D). Flow cytometry showed that p21 interference in MCF-7R has increased the cell apoptosis while overexpression in MCF-7 has decreased the cell apoptosis remarkably (Figure 3E and 3F). Apoptosis staining showed similar alteration in apoptosis of breast cancer cells challenged with Taxol (Figure 3G). These data demonstrated that p21 was capable of potentiating Taxol-resistance of breast cancer cells.

EZH2 down-regulated p21 expression through enrichment of H3K27me3 within its promoter

As a critical component of polycomb repressive complex 2 (PRC2), EZH2 functions as the methyltransferase which methylate's histone H3 at lysine 27 and thus inhibits gene expression. Tao Fan et al have showed that EZH2 was able to inhibit p21 expression in melanoma(Fan et al., , 2011), however, whether EZH2 was able to inhibit p21 expression via its methyltransferase activity was not certified. ChIP assay manifested that breast cancer cells with Taxol-resistance showed lower H3K27me3 enrichment within promoter of p21 (Figure 4A). Although breast cancer cells with less Taxol-resistance evidenced higher H3K27me3 enrichment, there was no statistical significance (Figure 4A). Thereafter, the effects of EZH2 on expression of p21 were detected. As showed in figure 4B and 4D, EZH2 recovery in MCF-7R inhibited p21 at both mRNA and protein levels significantly. On the contrary, EZH2 interference in MCF-7 increased p21 correspondingly. These results showed that EZH2 regulated p21 expression by directly modifying H3K27me3 enrichment within its promoter.

p21 rescued Taxol-resistance attenuated by EZH2 in breast cancer cells

Eventually, whether modifications upon p21 could mediate Taxol-resistance attenuated by EZH2 in breast cancer cells was explored. In accordance with figure 2C, EZH2 interference within MCF-7 cells decreased cell apoptosis induced by Taxol. However, this effect was remarkably reversed by p21 interference (Figure 5A, upper panel). In contrast, EZH2 overexpression increased apoptosis induced by Taxol in MCF-7R cells which was significantly rescued by p21 complement (Figure 5A and 5B, lower panel). Apoptosis staining showed similar results (Figure 5C). These results demonstrated that p21 positively regulated the Taxol-resistance attenuated by EZH2 in breast cancer cells.

Discussion

Breast cancer has been the leading cause of death in women worldwide. Though various chemo-therapeutic agents have been developed to obtain better prognosis, drug-resistance is the major obstacle of their long-term use and efficacy. Cellular microtubule destabilizer Taxol was first discovered in 1964 and approved by FDA for breast cancer treatment in 1994(Weaver, 2014b). However, clinical use of Taxol gets into the same situation as other anti-cancer drugs resulting from resistance of breast cancer cells(Weaver, 2014b, Li et al., , 2018). Therefore it is increasingly necessary to unveil the mechanism of the resistance of breast cancer cells to Taxol and to define novel therapeutic targets to counteract this effect. Here, in our study, we found that EZH2, an epigenetics modifier, decreased theTaxol-resistance in breast cancer cells by down-regulating p21 expression through increasing H3K27me3 enrichment in its promoter.

Reduced apoptosis was thought as the main alteration of resistance of breast cancers to chemotherapeutic agents. P21 plays various roles in different conditions, such as cell cycle progression, cell death, DNA repair, senescence, and reprogramming pluripotent stem cell. Nonetheless, its best known function is disrupting association between PCNA and cyclin/CDK

complex and thus arresting cell mitosis(Jung et al.,, 2010). However, recently, it was reported that p21 acted as an anti-apoptosis protein under various conditions(Jung et al.,, 2010, Lin et al., , 2007). We therefore speculated that p21 was involved in resistance of breast cancer cells. Indeed, J Wendt et al demonstrated that p21 induction by ionizing radiation attenuated caspase-3-mediated apoptosis in breast cancer cells(Wendt et al., , 2006). As the first-line routine drug for breast cancer, extensive spotlight has been shed on Taxol-resistance. Numerous studies have explored the application of combination of Taxol with other drugs in breast cancer, such as trial of ganetespib in combination with Taxol and trastuzuab by Komal Jhaveri et al (Jhaveri et al., , 2017). However, it would make better sense to comprehensively get insight into the mechanism of resistance. Taxol could induce p21 which was thought as the mechanism of Taxol inhibition on proliferation of breast cancer cells(Choi and Yoo, 2012b). Consistently, Taxol-resistant breast cancer cells induced by Taxol showed higher expression of p21 (Figure 3A and 3B), and down-regulating p21 was able to recover cell sensitivity to Taxol (Figure 3E, 3F and 3G). This suggested that p21 conferred Taxol-resistance to breast cancer cells.

EZH2 is the methyltransferase of PRC2 which mainly provides histone H3 with H3K27me3 mark and silences their expression. This mark predominantly regulates expression of genes characterizing various cell lineages, such as HOX, BMP, WNT, and NANOG(Conway et al., , 2015). However, a large number of studies suggested that EZH2 was overexpressed in various cancers including breast cancer(Kim and Roberts, 2016).The meta-analysis suggested that overexpression of EZH2 was considered as a poor prognosis indicator of breast cancer(Wang et al., , 2015).Bachmann IM et al showed that high EZH2 was associated high proliferation rate as well as aggressive phenotype(Bachmann et al., , 2006). On the other hand, EZH2 was associated with metastasis of breast cancer through different signalings(Huang et al.,, 2018, Du et al., , 2012). Jiang B et al reported that an lncRNA increased the doxorubicin-resistant of breast cancer through interacting with EZH2(Jiang et al.,, 2018). Study reported by Wang W et al demonstrated that EZH2 attenuated resistance of breast cancer to PI3K/AKT inhibitor through suppressing IGFBP5(Wang et al., , 2018). This study was in accordance with our observations that EZH2 was down-regulated within Taxol-resistant breast cancer cells (Figure 1A and 1B). Also, Xinxin Si et al showed that EZH2 was able to repress the expression of pro-apoptotic gene BIK which was consistent with resistance-dampening effect of EZH2 reported in our study(Si et al.,, 2016). However, further study was required to manifest how Taxol down-regulated EZH2 after long-term administration clinically. As both mRNA and protein levels fluctuated, we reasoned that the transcriptional mechanism played some role during this process. Nevertheless, this could not rule out the possibility of post-translational regulation of EZH2 which was reported to be regulated in this manner largely(Kuserabali et al., , 2018, Wan et al., , 2015).

As an epigenetic modifier, we investigated the possibility of p21 regulation by EZH2 through promoter methylation. Surprisingly, H3K27me3 enrichment within promoter of p21 was significantly decreased in Taxol-resistance breast cancer cells (Figure 4A). Since H3K27me3 enrichment was able to decrease expression of target genes, p21 expression at both mRNA and protein levels was determined. Apparently, p21 was decreased after EZH2 overexpression in Taxol-resistant breast cancer cells at both mRNA and protein levels and *vice versa* (Figure 4B, 4C and 4D). These results were consistent with studies reported by YUNG HYUN CHOI et al (Choi and Yoo, 2012b).

In sum, the present study concluded that EZH2 sensitized Taxol-resistant breast cancer cells

through repressing p21 expression by enhancing H3K27me3 enrichment within its promoter. Therefore, it was the potential target for attenuating Taxol-resistance and thus prolonging use of Taxol clinically (Figure 6).

Conclusion

In sum, the present study concluded that EZH2 sensitized taxol-resistant breast cancer cells through repressing p21 expression by enhancing H3K27me3 enrichment within its promoter. Therefore, it was the potential target for attenuating taxol-resistance and thus prolonging use of taxol clinically (Figure 6).

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Disclosure

No competing financial interests exist.

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