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Targeted inhibition of Notch1 gene enhances the killing effects of paclitaxel on triple negative breast cancer cells

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ABSTRACT

Objective: To study the influence of targeted inhibition of Notch1 gene on the killing effects of paclitaxel on triple negative breast cancer cells.

Methods: The triple negative [estrogen receptor (ER)/progesterone receptor (PR)/human epidermal growth factor receptor 2 (Her2)] breast cancer cell line MDA-MB-231 and ER/PR/HER-2-positive breast cancer cell line MCF-7 were cultured, transfected with Notch1-siRNA-overexpression plasmid and blank plasmid, and treated with different concentrations of paclitaxel, and then the cell proliferation activity and apoptosis rate as well as the mRNA expression of Caspase-3, Caspase-9 and Bcl-2 were determined.

Results: Paclitaxel could decrease the MDA-MB-231 and MCF-7 cell proliferation activity as well as Bcl-2 mRNA expression, and increase MDA-MB-231 and MCF-7 cell apoptosis rate as well as Caspase-3 and Caspase-9 mRNA expression in dose-dependent manners; with the same dose of paclitaxel treatment, the inhibitory effects on MDA-MB-231 cell proliferation activity and Bcl-2 mRNA expression as well as the promoting effects on MDA-MB-231 cell apoptosis and mRNA expression of Caspase-3 and Caspase-9 were weaker than those on MCF-7 cell; after 0.5 μM paclitaxel combined with Notch1-siRNA treatment, MDA-MB-231 cell proliferation activity and Bcl-2 mRNA expression were significantly lower than those after 0.5 μM paclitaxel combined with control plasmid treatment while cell apoptosis rate and mRNA expression of Caspase-3 and Caspase-9 were higher than those after 0.5 μM paclitaxel combined with control plasmid treatment.

Conclusions: Targeted inhibition of Notch1 gene may enhance the killing effects of paclitaxel on triple negative breast cancer cells by up-regulating the expression of Caspase-3 and Caspase-9 and inhibiting the expression of Bcl-2.

1. Introduction

Triple negative breast cancer (TNBC) is a special type of breast cancer that does not express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2), it is not sensitive to endocrine therapy and targeted therapy, and the overall prognosis is poor. Paclitaxel is first-line chemotherapeutic drug for clinical treatment of TNBC, and as the secondary metabolite of taxus plant, it can promote the microtubule polymerization and make the cell division arrest in mitosis, and thus inhibit cell proliferation. But in clinical practice, TNBC easily develops resistance to paclitaxel during chemotherapy, which influences the effect of chemotherapy [1,2]. Therefore, increasing the TNBC cell sensitivity to paclitaxel chemotherapy is the focus of clinical attention. Notch1 gene is an important member of the Notch family that has regulatory effect on the biological behaviors of lung cancer [3], gastric cancer [4], liver cancer [5] and other malignant tumor cells. Study about TNBC has confirmed that Notch1 genes are

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highly expressed in TNBC tissue [6], but it hasn't been reported whether Notch1 affects the chemosensitivity of TNBC cells. In the following study, the influence of targeted inhibition of Notch1 gene on the killing effects of paclitaxel on triple negative breast cancer cells was analyzed.

2. Materials and methods

2.1. Experimental materials

Triple negative breast cancer cell line MDA-MB-231 as well as ER/PR/HER-2-positive breast cancer cell line MCF-7 were from the ATCC cell bank in the United States, Notch1-siRNA-overexpression plasmid and blank plasmid were synthesized and provided by Shanghai Innovation Company, Lipofectamine™ 2000 liposomes (from Invitrogen Company), MTS kits (from Promega Company), Annexin V kits (from eBioscience Company) as well as RNA extraction kits, reverse transcription kits and fluorescence quantitative PCR kits (from Takara Company).

2.2. Experimental methods

2.2.1. Cell culture and treatment methods

MDA-MB-231 cell line MCF-7 cell line were recovered, cultured in DMEM medium containing 10% calf serum, digested and sub-cultured with trypsin, inoculated in culture plate and treated when the cell density reached about 90%. Paclitaxel treatment concentration was 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM and 0.5 μM, and the cells treated with no paclitaxel (0 μM) were used as control; Lipofectamine™ 2000 liposomes were used to transfect plasmids, and the transfected plasmids included Notch1-siRNA-overexpression plasmid and blank plasmid.

2.2.2. Cell proliferation activity detection methods

The cells digested and sub-cultured by trypsin were inoculated in 96-well cell plates (inoculation density 5×10^3/well), different concentrations of paclitaxel and two plasmids were used to transfect the cells after 12 h, 20 μL of testing liquid from MTS kit was added in each cell well after 24 h, the cells continued to be cultured in incubator for 4 h, the cell culture plates were taken out and shaken for 10 min, then the absorbance value (OD value) at 570 nm wavelength was read from the microplate reader, and the OD value was used as the cell proliferation activity. Three parallel wells were made for each bath of cells, and 4 batches were repeated.

2.2.3. Cell apoptosis detection methods

The cells digested and sub-cultured by trypsin were inoculated in 6-well cell plates (inoculation density 1×10^6/well) and transfected with different concentrations of paclitaxel and two different plasmids after the cell density reached 80%, the supernatant was abandoned after 24 h, the cells were kept, digested with trypsin and centrifuged, the obtained cells were added in 5 μL Annexin V and incubated for 15 min away from light, and the percentage of apoptotic cells was detected in flow cytometer.

2.2.4. Fluorescence quantitative PCR

The cells digested and sub-cultured by trypsin were inoculated in 12-well cell plates (inoculation density 0.5×10^5/well) and transfected with different concentrations of paclitaxel and two different plasmids after the cell density reached 80%, the supernatant was abandoned after 24 h, the cells were kept, RNA extraction kits were used to extract the total RNA in the cells, reverse transcription kits were used to reverse transcribe the total RNA into cDNA, finally fluorescence quantitative PCR kits were used to amplity Caspase-3, Caspase-9, Bcl-2 and β-actin, and β-actin was used as reference to calculate Caspase-3, Caspase-9 and Bcl-2 mRNA expression. Caspase-3, Caspase-9 and Bcl-2 primer sequences and annealing temperature were shown in Table 1.

2.2.5. Statistical methods

SPSS20.0 software was used to input and analyze data, the measurement data analysis between two groups was by t test, measurement data analysis among groups was by variance analysis and $P < 0.05$ indicated statistical significance in differences.

3. Results

3.1. Effect of paclitaxel on MDA-MB-231 and MCF-7 cell line proliferation and apoptosis

After different doses of paclitaxel treatment, MDA-MB-231 and MCF-7 cell line proliferation activity were significantly suppressed, and paclitaxel could reduce the MDA-MB-231 and MCF-7 cell line proliferation activity in dose-dependent manner; the inhibition of same dose of paclitaxel treatment on MDA-MB-231 cell proliferation activity was weaker than that on MCF-7 cell line.

After different doses of paclitaxel treatment, MDA-MB-231 and MCF-7 cell line apoptosis rate increased significantly, and paclitaxel could increase MDA-MB-231 and MCF-7 cell line apoptosis rate in dose-dependent manner; the increase of same dose of paclitaxel treatment on MDA-MB-231 cell apoptosis rate was weaker than that on MCF-7 cell lines (Table 2).

3.2. Effect of paclitaxel on Caspase-3, Caspase-9 and Bcl-2 expression in MDA-MB-231 and MCF-7 cell line

After different doses of paclitaxel treatment, Caspase-3 and Caspase-9 expression in MDA-MB-231 and MCF-7 cell lines

Table 1
PCR primer sequences and annealing temperature.

Genes	Primer sequences (5′→3′)	Annealing temperature (°C)	Product size (bp)
Caspase-3	Upstream: AGTCTTAGCTAAAGTCAGT Downstream: TTGACCGATCGTAGCTAAAG	59.5	294
Caspase-9	Upstream: GCGCATTAGCTATATGCATG Downstream: ATCGTAGCAAGCGCATGCTG	60.0	373
Bcl-2	Upstream: GCTAGCATGCTAGCTAAGCT Downstream: CAGTCCGATTATATAAGCGC	58.0	227

Table 2
Effect of paclitaxel on MDA-MB-231 and MCF-7 cell line proliferation and apoptosis and Caspase-3, Caspase-9 and Bcl-2 expression in MDA-MB-231 and MCF-7 cell line.

Cell line	Paclitaxel dose (μM)	Proliferation activity	Apoptosis rate (%)	Expression level		
				Caspase-3	Caspase-9	Bcl-2
MDA-MB-231	0	1.05 ± 0.12	4.59 ± 0.52	1.04 ± 0.14	1.02 ± 0.14	1.06 ± 0.12
	0.1	0.92 ± 0.10^{a*}	5.88 ± 0.57^{a*}	1.13 ± 0.14	1.15 ± 0.12^{a*}	0.94 ± 0.11^{a*}
	0.2	0.87 ± 0.07^{ab*}	9.01 ± 1.17^{ab*}	1.27 ± 0.17	1.30 ± 0.15^{ab*}	0.87 ± 0.09^{ab*}
	0.3	0.83 ± 0.06^{abc*}	12.41 ± 1.39^{abc*}	1.40 ± 0.13	1.45 ± 0.17^{abc*}	0.82 ± 0.07^{abc*}
	0.4	0.79 ± 0.07^{abcd*}	17.83 ± 2.31^{abcd*}	1.66 ± 0.18	1.59 ± 0.14^{abcd*}	0.79 ± 0.05^{abcd*}
	0.5	0.77 ± 0.05^{abcde*}	31.42 ± 4.58^{abcde*}	1.72 ± 0.15	1.80 ± 0.22^{abcde*}	0.73 ± 0.06^{abcde*}
MCF-7	0	1.08 ± 0.14	4.62 ± 0.54	1.02 ± 0.15	1.05 ± 0.13	1.03 ± 0.14
	0.1	0.81 ± 0.09^{a}	8.41 ± 0.77^{a}	1.48 ± 0.18	1.56 ± 0.17^{a}	0.78 ± 0.06^{a}
	0.2	0.72 ± 0.08^{ab}	16.25 ± 2.02^{ab}	1.92 ± 0.24	2.03 ± 0.28^{ab}	0.64 ± 0.08^{ab}
	0.3	0.55 ± 0.04^{abc}	35.21 ± 4.64^{abc}	2.31 ± 0.33	2.42 ± 0.31^{abc}	0.55 ± 0.06^{abc}
	0.4	0.39 ± 0.05^{abcd}	61.25 ± 9.23^{abcd}	2.77 ± 0.39	2.79 ± 0.34^{abcd}	0.39 ± 0.05^{abcd}
	0.5	0.27 ± 0.04^{abcde}	78.65 ± 9.35^{abcde}	3.29 ± 0.41	3.41 ± 0.48^{abcde}	0.22 ± 0.04^{abcde}

$^{*}P < 0.05$ compared with MCF-7 cell line with same paclitaxel dose; $^{a}P < 0.05$ compared with same cell line with 0 μM paclitaxel; $^{b}P < 0.05$ compared with same cell line with 0.1 μM paclitaxel; $^{c}P < 0.05$ compared with same cell line with 0.2 μM paclitaxel; $^{d}P < 0.05$ compared with same cell line with 0.3 μM paclitaxel; $^{e}P < 0.05$ compared with same cell line with 0.4 μM paclitaxel.

significantly increased while Bcl-2 expression was significantly suppressed, and paclitaxel could increase Caspase-3 and Caspase-9 expression and inhibit Bcl-2 expression in dose-dependent manner; the promoting effect of same dose of paclitaxel treatment on Caspase-3 and Caspase-9 expression and the inhibiting effect on Bcl-2 expression in MDA-MB-231 cell lines were weaker than those in MCF-7 cell lines (Table 2).

3.3. Effect of paclitaxel combined with Notch1-siRNA on MDA-MB-231 cell line proliferation and apoptosis

The MDA-MB-231 cell line proliferation activity after 0.5 μM paclitaxel combined with blank plasmid transfection treatment was significantly lower than that after 0 μM paclitaxel combined with blank plasmid transfection treatment, and the cell apoptosis rate was higher; the MDA-MB-231 cell line proliferation activity after 0.5 μM paclitaxel combined with Notch1-siRNA-overexpression plasmid transfection treatment was significantly lower than that after 0.5 μM paclitaxel combined with blank plasmid transfection treatment, and the cell apoptosis rate was higher (Table 3).

3.4. Effect of paclitaxel combined with Notch1-siRNA on Caspase-3, Caspase-9 and Bcl-2 expression in MDA-MB-231 cell lines

Caspase-3 and Caspase-9 expression in MDA-MB-231 cell lines after 0.5 μM paclitaxel combined with blank plasmid transfection treatment were significantly higher than those after 0 μM paclitaxel combined with blank plasmid transfection treatment, and the Bcl-2 expression was significantly lower; Caspase-3 and Caspase-9 expression in MDA-MB-231 cell lines after 0.5 μM paclitaxel combined with Notch1-siRNA-overexpression plasmid transfection treatment were significantly higher than those after 0.5 μM paclitaxel combined with blank plasmid transfection treatment, and the Bcl-2 expression was significantly lower (Table 3).

4. Discussion

Paclitaxel is the first-line chemotherapeutic drug for clinical treatment of triple negative breast cancer, but the triple negative breast cancer cell sensitivity to paclitaxel is poor, and drug resistance easily appears in the process of chemotherapy [7,8]. In order to define the differences in the ER/PR/HER-2 positive breast cancer cell and triple negative breast cancer cell sensitivity to paclitaxel, the ER/PR/HER-2 triple negative breast cancer cell line MDA-MB-231 and ER/PR/HER-2 positive cell line MCF-7 apoptosis and proliferation were compared in the study after different doses of paclitaxel treatment. It was found in the study that paclitaxel treatment could inhibit the proliferation activity and promote the apoptosis of the two type of cell lines in dose-dependent manners. Further comparison of the differences in MDA-MB-231 and MCF-7 cell proliferation activity and apoptosis rate after paclitaxel treatment showed that the inhibiting effect of same dose of paclitaxel on MDA-MB-231 cell line proliferation activity and the promoting effect on apoptosis were weaker than those on MCF-7 cell lines. It means that triple negative breast cancer cell line MDA-MB-231 has obvious resistance to paclitaxel, and the killing effect of

Table 3
Effect of paclitaxel combined with Notch1-siRNA on MDA-MB-231 cell line proliferation and apoptosis and Caspase-3, Caspase-9 and Bcl-2 expression in MDA-MB-231 cell lines.

Treatment conditions	Proliferation activity	Apoptosis rate (%)	Expression level		
			Caspase-3	Caspase-9	Bcl-2
Paclitaxel 0 μM + blank plasmid	1.01 ± 0.13	5.03 ± 0.66	1.05 ± 0.16	1.07 ± 0.13	1.05 ± 0.12
Paclitaxel 0.5 μM + blank plasmid	0.73 ± 0.08	35.14 ± 5.25	1.69 ± 0.22	1.74 ± 0.18	0.76 ± 0.08
Paclitaxel 0.5 μM + Notch1-siRNA	0.31 ± 0.05	74.52 ± 9.25	3.35 ± 0.41	3.51 ± 0.47	0.25 ± 0.05

paclitaxel on triple negative breast cancer cells is weaker than that on ER/PR/HER-2 positive breast cancer cells.

Excessive activation of PI3K/Akt signaling pathway is related to the breast cancer cell tolerance to chemotherapeutic drugs [9–12], PI3K/Akt of cascade phosphorylation activation can make transcription factor FoxO3 activate, combine with the DNA promoter regions, and then adjust the expression of a variety of proliferation- and apoptosis-related genes [13–15]. Bcl-2 gene is an important target gene regulated by FoxO3, and the activated FoxO3 is combined with Bcl-2 gene promoter regions and then increases its expression [16,17]. The protein encoded by Bcl-2 gene can regulate the opening and closing state of mitochondrial membrane transition pore, reduce the release of cytochrome C, inhibit the Caspase cascade apoptosis pathway mediated by cytochrome C and antagonize apoptosis process [18,19]. In the study, analysis of Caspase-3, Caspase-9 and Bcl-2 expression in two breast cancer cell lines after paclitaxel treatment showed that paclitaxel treatment could restrain the Bcl-2 gene expression and increase the Caspase-3 and Caspase-9 gene expression in the two cell lines in dose-dependent manner. Further analysis of the differences in above gene expression in MDA-MB-231 and MCF-7 cells showed that the promoting effect of same dose of paclitaxel treatment on Caspase-3 and Caspase-9 expression and the inhibiting effect on Bcl-2 gene expression in MDA-MB-231 cell lines were weaker than those in MCF-7 cell lines. It illustrates that the low expression of Caspase-3 and Caspase-9 as well as the high expression of Bcl-2 in triple negative breast cancer cell line MDA-MB-231 may be closely related to the breast cancer cell insensitivity to the effects of paclitaxel.

At present, there are still no targeted drugs for clinical treatment of triple negative breast cancer, and the key molecules that can be targeted to adjust triple negative breast caner cell proliferation and apoptosis remains uncertain. Notch1 in Notch family is a newly discovered transmembrane receptor protein with promoting effect on tumor, and research has proved that the Notch1 expression significantly increases in triple negative breast cancer tissue and is closely related to the TNM staging of tumor [6]. It shows that high Notch1 gene expression is associated with the occurrence and development of triple negative breast cancer and it is predicted that Notch1 is a possible target to inhibit triple negative breast cancer cell proliferation and increase triple negative breast cancer cell sensitivity to chemotherapeutic drugs. In order to test this theory, transfection of Notch1-siRNA-overexpression plasmid was used in the study to continuously target and inhibit the Notch1 expression in triple negative breast cancer cells, Notch1-siRNA-overexpression plasmid and paclitaxel were used together to treat triple negative breast cancer cell line MDA-MB-231, and it was found that the inhibiting effect of 0.5 μM paclitaxel combined with Notch1-siRNA treatment on cell proliferation activity and the promoting effect on cell apoptosis were more significant than those of 0.5 μM paclitaxel combined with blank plasmid treatment. It indicates that targeted inhibition of Notch1 gene expression can improve the triple negative breast cancer cell sensitivity to paclitaxel, and after the Notch1 gene expression is inhibited, the killing effect of paclitaxel on triple negative breast cancer cells is enhanced.

In the occurrence and development of malignant tumor, the excessive expression and activation of Notch1 genes will increase its intracellular fragment hydrolysis, and after the transmembrane fragment NIC from intracellular fragment hydrolysis transfers from the cytoplasm into the nucleus, it can regulate the expression of a variety of tumor-related genes [20]. As mentioned above, the low expression of Caspase-3 and Caspase-9 as well as the high expression of Bcl-2 in triple negative breast cancer cell line may be closely related to the cancer cell insensitivity to the effects of paclitaxel, and in order to define the effect of targeted inhibition of Notch1 gene on the expression of gene related to paclitaxel sensitivity in triple negative breast cancer cells, the anti-apoptotic gene Bcl-2 and the pro-apoptotic genes Caspase-3 and Caspase-9 expression were analyzed in the study, and the results showed that the inhibiting effect of 0.5 μM paclitaxel combined with Notch1-siRNA treatment on the Bcl-2 expression and the promoting effect on Caspase-3 and Caspase-9 expression in cells were more significant than those of 0.5 μM paclitaxel combined with blank plasmid treatment. It indicates that targeted inhibition of Notch1 gene combined with paclitaxel intervention can significantly inhibit the expression of anti-apoptotic gene Bcl-2 and increase the expression of pro-apoptotic genes Caspase-3 and Caspase-9, and then target to increase the triple negative breast cancer cell line sensitivity to paclitaxel through the change of Bcl-2, Caspase-3 and Caspase-9 expression.

Based on above discussion, the killing effects of paclitaxel on ER/PR/Her-2 triple negative breast cancer cells is weaker than that on ER/PR/Her-2 positive breast cancer cells, and targeted inhibition of Notch1 gene may up-regulate the expression of Caspase-3 and Caspase-9 and inhibit the expression of Bcl-2 to enhance the killing effects of paclitaxel on triple negative breast cancer cells.

Conflict of interest statement

We declare that we have no conflict of interest.

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