Effects of Decitabine on the proliferation of K562 cells and the expression of DR4 gene

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Abstract

Objective: To investigate the role of DR4 gene in the occurrence, development and prognosis of acute myeloid leukemia (AML), find a new regulatory gene of Decitabine for the treatment of AML, namely DR4 gene, and explore the molecular mechanism of AML in the treatment of AML.

Methods: The methylation level and the mRNA expression level of DR4 gene promoters of bone marrow mononuclear cells in 122 patients with newly diagnosed AML and 24 patients with iron deficiency anemia (IDA) were detected using Methylation specific PCR (MS-PCR) and Q-RT-PCR, respectively, and a correlation analysis of them was conducted. The effects of Decitabine on the proliferation of K562 cells were detected using CCK-8 assay. Then, the effects of Decitabine on the methylation level and the mRNA expression level of DR4 genes of K562 cells treated with Decitabine were detected using MS-PCR and Q-RT-PCR, respectively. The effects of Decitabine on the cell cycle and apoptosis of K562 cells were detected using flow cytometry.

Results: Compared with the control group, the methylation level (P = .002) of DR4 genes of bone marrow mononuclear cells in patients with newly diagnosed AML was high. The methylation level (P = .01) of DR4 genes of bone marrow mononuclear cells in patients of the positive group of enlargement of liver, spleen and lymph node was lower than that of the negative group, and the methylation level (P = .006) of DR4 genes in patients of the high risk group of clinical stage was lower than that of the low risk group, and the methylation level (P = .03) of DR4 genes in patients of the group where patients did not achieve complete remission (CR1) after a course of induction chemotherapy was lower than that of the group where patients achieved complete remission (CR1) after a course of induction chemotherapy. There was a significant negative correlation (P < .01) between the methylation level and the mRNA expression level of DR4 genes of bone marrow mononuclear cells in 122 patients with newly diagnosed AML. After the K562 cells were treated with Decitabine for 48 h, the methylation level of DR4 gene promoters gradually decreased, while the mRNA expression level of DR4 genes gradually increased, both of which showed a concentration-dependent relationship. After the K562 cells were treated with 5 \textmu mol/L Decitabine for 48 h, the K562 cells in G0/G1 phase and G2/M phase increased significantly, and the K562 cells in S phase decreased significantly.

Conclusion: DR4 gene played an important role in the occurrence and development of AML. Decitabine can effectively inhibit the proliferation of K562 cells, which probably partly because it can terminate the methylation effect of DR4 gene promoters and restore the mRNA expression of DR4 genes.

1. Background

Recent studies found that abnormal epigenetic modification is an early event of tumorigenesis, and gene methylation change is one of the key steps for the occurrence of tumors of hemic and lymphatic systems (Oakes et al., 2016). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) superfamily, induces the apoptosis of tumor cells by binding with death receptor 4 (DR4), but has no killing
effect on normal cells, which thus captures scholars’ attention to TRAIL and its DR4 in the treatment of tumors (Yagita et al., 2004). Studies have reported that the aberrant methylation of DR4 gene promoter region in a variety of tumors leads to the decreased mRNA expression of DR4 genes, and then the occurrence, development and invasion of tumors (Horak et al., 2005; Shin et al., 2001; Lee et al., 2009). In this study, the methylation level of DR4 genes of bone marrow mononuclear cells in patients with newly diagnosed AML was detected, and the correlation between the methylation level and the mRNA expression level of DR4 genes was analyzed, and then the role of DR4 genes in the occurrence, development and prognosis of AML was investigated so as to find a new regulatory gene of Decitabine for the treatment of AML.

2. Materials and methods

2.1. Main reagents and apparatuses

The K562 cells came from Central Laboratory of Henan Province People’s Hospital, and purchased from American Type Culture Collection (ATCC); Decitabine purchased from Chia Tai Tianqing Pharmaceutical Group (CTTQ); CCK-8 purchased from Vazyme Biotech Co., Ltd.; DNA Extraction Kit purchased from Sangon Biotech (Shanghai) Co., Ltd.; EZ RNA Methylation™ Kit and ZymoTaq™ DNA Polymerase purchased from Beijing Tianmo SciTech Development Co., Ltd.; Q-RT-PCR Kit purchased from TaKaRa Bio; primers and probe sequences synthesized by Sangon Biotech (Shanghai) Co., Ltd.; PCR Amplification System adopted the Step one Plus RQ-RT-PCR System (Applied Biosystems); flow cytometry produced by Beckman Coulter, Inc.

2.2. Isolation of bone marrow mononuclear cells and detection of methylation level of its DR4 genes

After obtaining informed consent from patients, the bone marrow specimens of 122 patients with newly diagnosed AML in Hematology Department of Henan Province People’s Hospital from February 2015 to January 2017 were collected, and the bone marrow specimens of 24 patients with IDA that were excluded from tumor factors were taken as controls. 2 ml bone marrow fluid was added to an EDTA anticoagulant tube to mix, then, the bone marrow mononuclear cells were separated with lymphocyte separating solution (Tianjin), and the RNA was extracted using TRIZOL reagent (Invitrogen) one-step method, and the A260/A280 value was determined by UV spectrophotometer, and stored at −80 °C. The extraction of genomic DNA referred to the Instructions of Small Amount Column Genomic DNA Extraction Kit of Sangon Biotech (Shanghai) Co., Ltd. The methylation of DNA referred to the operation steps of EZ DNA Methylation-Lighting Kit, and the DNA was recovered and dissolved in 10 μL M-Elution buffer, and stored at −20 °C. The methylation primer (M) and non-methylation primer (U) of DR4 genes were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The sense strand of the methylation primer (M) of DR4 genes was: 5’-TTCGAATTTCGGGAGCGTAGC-3’; the antisense strand of the non-methylation primer (U) of DR4 genes was: 5’-GGAACACAGCATGCTAGCGAA-3’. Reverse primer, 5’-TGCATCTCAGGGCGTACAT3’; Probe, 5’-(FAM)-CGAGCTGACAGCGATGCAG CACAAGTAC-(TAMRA)-3’. The GAPDH housekeeping gene was used to normalize DR4 expression. Real-time amplification protocol: Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA concentration and purity were determined by spectrophotometry. The reverse transcription was carried out with a Prime Script RT-PCR Kit. The standard reaction volume of reverse transcription was 10 μL and contained 2 μL 5 × RT buffer, 300 ng template mRNA, 0.5 μL Oligo dT Primer (50 μM), 0.5 μL RT Enzyme mix, 2 μL Random 6 mers (100 μM), 2 μL RNase Free dH2O. This was incubated at room temperature for 15 min and then maintained in a heat block at 85 °C for 5sec. For DR4 and GAPDH transcripts, 2 μL of target cDNA was added to 20 μL PCR mix containing 10 μL Universal PCR Master Mix, 400 nM of each primer and 800 nM of the probe, according to the manufacturer’s protocol. The GAPDH housekeeping gene and the genes of interest were amplified in parallel. The reaction was initiated at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 33 s. Data were recorded as cycle threshold (Ct) on a ABI Step one plus System using analytical software from the same manufacturer. The amount of DR4 mRNA, normalized to GAPDH, was given by the relation 2−ΔΔCt (Livak and Schmittgen, 2001). A bone marrow sample was considered positive for DR4 expression if the expression level was 1-fold higher than the mean expression in control samples.

2.3. Detection of the cell viability of K562 cells by CCK-8 assay

The K562 cells in logarithmic growth phase were inoculated in a 96-well plate, with cell density of 8 × 10^4/mL, 100 μL per well. 3 experimental groups of 2, 5, 10 μmol/L Decitabine and 1 control group were set up, 4 complex holes in each group, and 4 plates were inoculated. After being cultured for 0 h, 24 h, 48 h and 72 h, the absorbance value (A) of each complex hole under 450 nm wavelength was determined with a Microplate Reader. 2 h before each detection, 10 μL CCK-8 reagents were added into each complex hole respectively to continue culture for 2 h. Among which, the 0 h plate was immediately added with 10 μL CCK-8 reagents after the medium plate, and placed in the incubator to detect the A after being cultured for 2 h in the incubator, and the A of each complex hole was averaged. Each experiment was repeated 3 times, and the A of each complex hole was averaged. The cell viability of K562 cells was calculated according to the following formula: cell viability of K562 cells (%) = (A experimental group-A control group)/(A control group-A blank group) × 100%. The point-and-figure charts of the cell viability of K562 cells treated with different concentrations of Decitabine for different time were drawn.

2.4. Quantification by real-time RT-PCR

Primers and probes were designed using Primer-Express software (PE Biosystems). Each probe was labeled with a fluorescent 5’-reporter dye (FAM: 6-carboxy-fluorescein) and a 3’-quencher (TAMRA: 6-carboxy-tetramethyl-rhodamine). The DR4-specific primers and probe were designed as follows: forward primer, 5’-GGGAACACAGCATGCTAGCGAA-3’, Reverse primer, 5’-TGCATCTCAGGGCGTACAT3’; Probe, 5’-(FAM)-CGAGCTGACAGCGATGCAGCACAAGTAC-(TAMRA)-3’. The GAPDH housekeeping gene was used to normalize DR4 expression. Real-time amplification protocol: Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA concentration and purity were determined by spectrophotometry. The reverse transcription was carried out with a Prime Script RT-PCR Kit. The standard reaction volume of reverse transcription was 10 μL and contained 2 μL 5 × RT buffer, 300 ng template mRNA, 0.5 μL Oligo dT Primer (50 μM), 0.5 μL RT Enzyme mix, 2 μL Random 6 mers (100 μM), 2 μL RNase Free dH2O. This was incubated at room temperature for 15 min and then maintained in a heat block at 85 °C for 5sec. For DR4 and GAPDH transcripts, 2 μL of target cDNA was added to 20 μL PCR mix containing 10 μL Universal PCR Master Mix, 400 nM of each primer and 800 nM of the probe, according to the manufacturer’s protocol. The GAPDH housekeeping gene and the genes of interest were amplified in parallel. The reaction was initiated at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 33 s. Data were recorded as cycle threshold (Ct) on a ABI Step one plus System using analytical software from the same manufacturer. The amount of DR4 mRNA, normalized to GAPDH, was given by the relation 2−ΔΔCt (Livak and Schmittgen, 2001). A bone marrow sample was considered positive for DR4 expression if the expression level was 1-fold higher than the mean expression in control samples.
2.5. Detection of the cell cycle and apoptosis of K562 cells by flow cytometry

The K562 cells in the experimental group where the K562 cells were treated with 5 μmol/L Decitabine for 48 h and the control group were collected, washed with PBS 2 times, and the cell concentration was adjusted, and 5 μL RNAse A was added to incubate at 37 °C for 30 min; then, 125 μL PI dyeing solution was added to incubate away from light at 4 °C for 30 min and detect the cell cycle parameters of K562 cells by computer. Also, 100 μL binding buffer resuspension cells, 5 μL Annexin V-FITC and 5 μL PI dyeing solution were added to react away from light at room temperature for 10 min. Then, 400 μL binding buffer was added to mix and detect the apoptosis of K562 cells by computer.

2.6. Statistical treatment

Each experiment was repeated 3 times, and data analysis was carried out using SPSS17.0 software. The comparison of measurement data was performed using Mann-Whitney U test and Kruskal-Wallis H test, and the comparison of rates was performed using Fourfold Table Chi-square test, and correlation analysis was carried out using Spearman test. P < .05 indicated that the difference was statistically significant.

3. Results

1. The methylation of DR4 gene promoters of bone marrow mononuclear cells in 122 patients with newly diagnosed AML: The methylation of DR4 gene promoters of bone marrow mononuclear cells in 122 patients with newly diagnosed AML was detected using MS-PCR, and the results showed that the methylation of DR4 gene promoter region of bone marrow mononuclear cells in 111 patients (90.9%) with newly diagnosed AML was positive (Fig. 1), and the methylation of DR4 gene promoter region of bone marrow mononuclear cells in 3 patients (12.5%) with IDA was positive. There was significant difference between the two groups (P = .002).

2. The relationship between the methylation level of DR4 genes and the clinical characteristics and prognosis of AML in patients with newly diagnosed AML: The study found that the age and gender of patients with newly diagnosed AML had no effect on the methylation level of DR4 genes, while the methylation level (P = .01) of DR4 genes of bone marrow mononuclear cells in patients of the positive group of enlargement of liver, spleen and lymph node was lower than that of the negative group, and the methylation level (P = .005) of DR4 genes of bone marrow mononuclear cells in patients of the high risk group of clinical stage was lower than that of the low risk group, and the methylation level (P = .03) of DR4 genes of bone marrow mononuclear cells in patients of the group where patients did not achieve complete remission (CR1) after a course of induction chemotherapy was lower than that of the group where patients achieved complete remission (CR1) after a course of induction chemotherapy (Table 1). The results showed that the lower the methylation level of DR4 genes, the worse the clinical prognosis and chemotherapy effect.

3. The mRNA expression of DR4 genes in patients with newly diagnosed AML: The mRNA expression of DR4 genes of bone marrow mononuclear cells in 122 patients with newly diagnosed AML was detected using Q-RT-PCR. The results showed that the mRNA expression level of DR4 genes in the experimental group of 2, 5, 10 μmol/L Decitabine and the control group at 24 h, 48 h and 72 h also had statistical significance (P < .001), and the difference between the cell viability of K562 cells of the experimental groups of 2, 5, 10 μmol/L Decitabine and the control group at 24 h, 48 h and 72 h also had statistical significance (P < .001).

4. The effects of Decitabine on the proliferation of K562 cells: The cell viability of K562 cells decreased with the increase of the concentration of Decitabine (Fig. 2). The difference between the experimental groups of 2, 5, 10 μmol/L Decitabine and the control group at 24 h, 48 h and 72 h had statistical significance (P < .001), and the difference between the cell viability of K562 cells of the experimental groups of 2, 5, 10 μmol/L Decitabine and the control group at 24 h, 48 h and 72 h also had statistical significance (P < .001).

5. The effects of Decitabine on the methylation level of DR4 genes after the K562 cells were treated with Decitabine: The detection using MS-PCR found that the DR4 genes of K562 cells showed methylation status, after the K562 cells were treated with 2, 5, 10 μmol/L Decitabine for 48 h, the methylation level of DR4 gene promoters of K562 cells gradually decreased, and showed a concentration-dependent relationship. At high concentration (10 μmol/L), the MS-PCR did not detect the methylated amplified fragments of DR4 genes (Fig. 3).

6. The effects of Decitabine on the mRNA expression level of DR4 genes after the K562 cells were treated with Decitabine: After the K562 cells were treated with 2, 5, 10 μmol/L Decitabine, the mRNA expression level of DR4 genes was detected using Q-RT-PCR. The results showed that the mRNA expression level of DR4 genes in the control group was low, namely 0.57, while the mRNA expression level of DR4 genes in the experimental groups of 2, 5, 10 μmol/L Decitabine increased with the increase of the concentration of Decitabine, namely 8.09, 27.32 and 90.51, respectively.
8. The effects of Decitabine on the cell cycle and apoptosis of K562 cells: Compared with the control group, after the K562 cells were treated with 2, 5, 10 μmol/L Decitabine for 48 h, the K562 cells in G0/G1 phase and G2/M phase increased to 77.24% from 47.08%, and the K562 cells in G2/M phase increased to 18% from 3.41%, and the K562 cells in S phase decreased to 4.75% from 43.72%, suggesting that the cell cycle of K562 cells was arrested in G0/G1 and G1/M phases, thereby inhibiting the proliferation of K562 cells (Fig. 4).

4. Discussion

Epigenetic dysregulation is one of the pathogeneses of many tumors, including AML, and the inactivation of tumor suppressor genes mediated by the methylation of the CpG island loci of gene promoter region plays an important role in the occurrence and development of AML (Geonmic, 2013). DR4, also known as TRAIL receptor 1 (Yagita et al., 2004), is a type I membrane protein containing 468 amino acids. DR4 combines with TRAIL specificity to form a “receptor trimer”, which activates and conducts apoptotic signals through death domain, and activates the caspase cascade reaction, leading to cell death, and is therefore called death receptor. However, not all tumor cells are sensitive to TRAIL, and the nonfunctional DR4 expression may be one of the reasons for cells’ resistance to the apoptosis mediated by TRAIL, which can increase cells’ sensitivity to TRAIL by up-regulating the mRNA expression of DR4 genes (Drosopoulos et al., 2005; Van Geelen et al., 2004). It has been proved that the mRNA expression of DR4 genes shows expression deficiency or down regulation in many tumor tissues, such as gastric cancer, colon cancer, breast...
cancer and uterine cancer. Its inactivation is mainly related to promoter methylation, and can be restored after demethylation (Horak et al., 2005; Shin et al., 2001; Lee et al., 2009). Horak et al. (2005) found in their study that the loss of the mRNA expression of DR4 genes in uterine cancer due to epigenetic effect is one of the mechanisms for uterine cancer’s resistance to TRAIL. The methylation of DR4 genes exists in breast cancer, which leads to a decrease in the mRNA expression of DR4 genes and is a cause for breast cancer cells’ resistance to the apoptosis mediated by TRAIL (Shin et al., 2001). A number of studies have shown that only a small proportion of patients with acute leukemia (AL) are sensitive to TRAIL: the study by Clodi et al. (2000) showed in their study that the pre-B cell ALL specimens of 38% (11/29) patients were sensitive to TRAIL. The study by Wuchter et al. (2001) also proved the above conclusion, namely, 27% pre-B cell ALL specimens were sensitive to TRAIL, and the pre-B cell ALL specimens of only 9% patients with AML were sensitive to TRAIL. The use of TRAIL combined with chemotherapeutic agent increases leukemic cells’ sensitivity to TRAIL, which is a mechanism that increases the antitumor effect of TRAIL by up-regulating the mRNA expression of DR4 genes in leukemic cells. The results of this study showed that the methylation rate of DR4 gene promoters in patients with newly diagnosed AML was 90.9%, which was slightly lower than that in patients with gastric cancer and breast cancer. The methylation level of DR4 genes in patients with newly diagnosed AML was related with the clinical characteristics and prognosis of AML in patients with newly diagnosed AML, in other words, the lower the methylation level of DR4 genes, the worse the clinical prognosis and chemotherapy effect. In addition, the study found that the mRNA expression level of DR4 genes of bone marrow mononuclear cells in 122 patients with newly diagnosed AML significantly decreased, and there was a significant negative correlation between the methylation level and the mRNA expression level of DR4 genes of bone marrow mononuclear cells in 122 patients with newly diagnosed AML.

Decitabine is a DNA methyltransferase inhibitor that can restore the mRNA expression of tumor suppressor genes by removing aberrant methylation, thereby achieving antitumor effects. Clinically it has been used in the treatment of myelodysplastic syndromes (MDS), and also shows significant efficacy in the preliminary clinical trials of AML, but the molecular mechanism of its target molecule and anti leukemia is still not clear (Kim et al., 2015). The experimental results showed that 10 μmol/L Decitabine had the highest demethylation efficiency for DR4 gene promoters of K562 cells, compared with 5 μmol/L Decitabine for gastric cancer and breast cancer, it might be due to different reaction of different cell lines to Decitabine. The high methylation status of DR4 gene promoters was gradually relieved, and the demethylation was enhanced with the increase of the concentration of Decitabine and the extension of treatment time. The expression of DR4 genes could be significantly increased by treating cells with Decitabine, and such up-regulation occurred at the mRNA level. Decitabine can significantly inhibit the proliferation of K562 cells, which became more significantly with the increase of the Concentration of Decitabine and the extension of treatment time. Compared with the control group, the cell division cycle of K562 cells was blocked and the apoptotic cells increased slightly, suggesting that the inhibition of the proliferation of K562 cells by Decitabine may be achieved mainly by regulating the cell cycle of K562 cells. Weeks et al. (2016) found in their study that in childhood acute B lymphocyte leukemia, Decitabine regulates the growth of B lymphocyte leukemia cells by blocking the cell cycle of leukemic cells. Benigno and Valdez (2010) found in their study that Decitabine inhibits the proliferation of leukemia cells by inducing the apoptosis of leukemia cells and arresting the cell cycle of leukemia cells in G0/G1 phase.

DR4 gene plays an important role in the occurrence and development of AML. Decitabine can effectively inhibit the proliferation of K562 cells, which probably partly because it can terminate the methylation effect of DR4 gene promoters and restore the mRNA expression of DR4 genes. Therefore, the results of this study can provide new experimental data for the exploration of the molecular mechanism of AML in the treatment of AML as well as a theoretical foundation for the reversion of AML’s resistance to TRAIL by up-regulating the expression of DR4 gene with chemotherapy drugs.

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