Effects of Huaier Regulating Oxaliplatin Resistance in Colorectal Cancer through PI3K/AKT Signaling Pathway

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Abstract [**Objectives**] To investigate whether Huaier (*Trametes robiniophila* Murr) can reverse oxaliplatin resistance of colorectal cancer and its molecular mechanism. [**Methods**] CCK8 assays were used to analyze the effect of Huaier on the survival rate of HCT8 and HCT8/L cells, Flow cytometry was used to verify the apoptosis rate and flow cytometry detected the cell cycle, gene expression lever of *P-glycoprotein* was detected by RT-qPCR assays, and the protein expression lever was detected by western blot. [**Results**] Huaier can significantly reduce the cell viability of HCT8 and HCT8/L cells, induce cell apoptosis and arrest the cell cycle at G_1/G_0 phase. Huaier could inhibit the expression lever of *MDR1* mRNA and inhibit the protein expression of P-glycoprotein, P85α, p-AKT, BCL-2, CDK4, CyclinD1, and promote the expression of BAX, Cleaved caspase-3 and cleaved caspase-9 in drug-resistant colorectal cancer cells. Through compared Huaier group with the 740Y-P group and the co-treated group, it shows that Huaier can reverse oxaliplatin resistance in colorectal cancer through PI3K/AKT signaling pathway. [**Conclusions**] This study shows that Huaier can reverse oxaliplatin resistance of colorectal cancer by inducing apoptosis and arresting cell cycle through PI3K/AKT signaling pathway.

Key words Huaier (*Trametes robiniophila* Murr), Drug resistance, Colorectal cancer, PI3K/AKT, Apoptosis

1 Introduction

Colorectal cancer (CRC) is the third most common tumor in the world, and its mortality rate ranks second in the world[1-2]. Surgery and chemotherapy are the keys to the treatment of CRC, among which oxaliplatin is the first-line chemotherapy drug commonly used in clinic[3], but chemotherapy resistance is often an important reason for the failure of treatment. There are many mechanisms about tumor drug resistance, but many have not been fully clarified. Abnormal expression of membrane transporters is one of the classic ways of drug resistance in CRC[4-5].

It has been found that the activation of PI3K/AKT signaling pathway is related to the regulation of cell proliferation, apoptosis and drug resistance of tumor. Inhibiting this signaling pathway can effectively reverse the drug resistance and induce apoptosis of tumor cells[6]. And the loss or decrease of BAX can also increase the drug-resistance of CRC to Oxa[7]. P-glycoprotein (P-gp) is an important member of ATP-binding cassette transportor superfamily, it is coded by *MDR1* gene[8-9]. Studies have shown that inhibiting the expression of P-gp can effectively reverse oxaliplatin resistance in CRC[10]. With the increasing in-depth research, more and more P-gp inhibitors have been found[11]. Although the new inhibitors have effectively reduced the toxic and side effects of drugs, the clinical therapeutic effect has not reached the expectations. Therefore, it is essential to develop natural drugs with high affinity and relatively innocuity as P-gp inhibitors.

Huaier (*Trametes robiniophila* Murr), a compound extracted from fungus, has major active substances including polysaccharides and proteins[12]. It has been proved that Huaier play an anticancer role in many kinds of tumors through many ways[13-16]. Many studies have shown that Huaier can enhance the sensitivity of tumor to oxaliplatin[17], but it is not clear whether it can reverse the resistance of CRC to oxaliplatin. Therefore, we used the drug-fast cells HCT8/L to study the potential effect of Huaier on the drug resistance of CRC. In this article, we aim to study the ability of Huaier in reversing drug resistance of CRC and its main mechanism.

2 Materials and methods

2.1 Cell culture and vectors HCT8 cells were purchased from Procell Biotech (CL-0098), and HCT8/L cells were resistant cells manufactured by Shanghai Aolu Biological Company (XB-4127, China), result of STR matching analysis in HCT8 and HCT8/L cells were detected by Gene Mapper v3.2 software (ABI), all of these are cultivated with dedicated 10% complete medium (Procell Company, China), and incubated in 37 ℃ and 5% CO_2 humid environment. Huaier granules were purchased from Qidong Gaitianli Pharmaceutical Company (Jiangsu, China). Preparation of Huaier stock solution: dissolved 2 g Huaier particles in 20 mL 1640 culture medium, shook with vortex oscillator for 10 min, fully mixed, and then filtered and sterilized with 0.22 μm filter (Millipore, USA). Finally, we obtained 100 mg/mL Huaier stock solution, packaged, sealed and stored in -40 ℃. P13K/AKT agonist 740Y-P was purchased from MCE Company (USA).

2.2 Cell viability determination Cell viability was measured by Cell Counting Kit-8 (CCK8, MCE, USA). The cells in logarithmic growth period were planted in 96-well plate with about 5 000 cells per well, and cultured with 10% complete medium un-

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til the cells adhere to the wall. There were six groups of cells (0, 6, 9, 12, 15 and 18 mg/mL, respectively), and each group was provided with three holes. The supernatant was discarded, added with the corresponding concentration of Huaier, and then cultured in an incubator. After 24 h, discarded the supernatant and added the driving fluid of CCK8: 10 μL CCK8 and 100 μL 1640 medium per well, placed in an incubator for 2 h, and then detected the absorbance at 460 nm with a microplate reader. And then the same concentration of the Huaier was accompanied, it was cultured in an incubator for 24 h, and the absorbance was measured again in the same way as before. Cell viability = $[(As - Ab)/(Ac - Ab)] \times 100\%$, where *As* is absorbance of assays group; *Ac* is absorbance of control group; *Ab* is absorbance of blank group. All assays were performed three times.

2.3 Cell cycle detection by flow cytometry Cell cycle detection kit (BD, USA) was used to detect cell cycle. Set up a group of HCT8 cells, and four groups of HCT8/L cells were planted in a six-well plate with the density of 50%, and cultured in an incubator until the cells were adhere to the wall, discarded the supernatant, washed it with PBS (Gibco, USA), and added complete medium containing with the concentration of Huaier (0, 6, 9, 12 mg/mL, respectively) in HCT8/L cells, the cells were digested after 24 h, washed with PBS twice and centrifuged. After discarding the supernatant, re-suspended the cells with 1 mL PBS and dropwised them into 3 mL precooled absolute alcohol. Fixed at −20 ℃ overnight. Took out the fixed cells, centrifuged at 1 500 rpm for 10 min, discarded supernatant, used PBS wash the cells twice, added 3 mL PBS for hydration with 15 min, centrifuging and discarding the supernatant. Added 1 mL BD dye solution, mixed well, keeped out of light for 30 min, sieved with 300 mesh screen panel. And the cell cycle was analyzed by flow cytometry machine and analysed by the by Modfit.

2.4 Cell apoptosis detection by flow cytometry Apoptosis was detected by apoptosis detection kit (Sizhengbai, China). Set the seven groups on six-well plates, four holes as the non-drugs group, and the other three holes were dosed according to the above concentrations of Huaier. After 24 h, the cells were digested with trypsin without EDTA, and the cells were dispersed by light blowing at 1 500 rpm for centrifugation. After 10 min, discarded the supernatant, re-suspended the cells in PBS, sieved with a 300 mesh screen panel, and used PBS clean and centrifuged for twice, and then discarded the supernatant. Each group was added with 500 μL of buffer: 100 μL of 5 × Binding Buffer and 400 μL of ultrapure water. No solution was added to the cells in the first tube, 10 μL PI staining solution was added to the second tube, and 5 μL Annexin V was added to the third tube. The remaining cells in each group were mixed with 10 μL PI dye solution and 5 μL Annexin V, incubated in the dark at room temperature for 5 – 10 min, and detected on the flow cytometry machine and analyzed by the Flow Jo.

2.5 Quantitative real-time PCR (qRT-PCR) The gene expression lever was analyzed by RT-qPCR. The groups of Huaier were treated with different concentrations of Huaier for 24 h, then taken out, washed with PBS and dried. Total RNA was extracted from feach well of 1 mL trizol (Biosharp, China). After measuring the RNA concentration, the cDNA was synthesized according to the instructions of cDNA synthesis kit (Abclonal, USA) and retrovirus was carried out according to the instructions of PCR kit (Abclonal, USA). Quantitative reverse transcription polymerase chain reaction. The primer sequences used in RT-qPCR assays were: *MDR1* forward primer (5'-TCTATGGTTGGCAACTAACACT-3'), *MDR1* reverse primer (5'-CTCCTGAGTCAAAGAAACAACG-3'), *GAPDH* forward primer (5'-GCACCGTCAAGGCTGAGAAC-3'), GAPDH reverse primer (5'-TGGTGAAGACGCCAGTGGA-3'). The results were analyzed by $2^{-\triangle\triangle Ct}$ method.

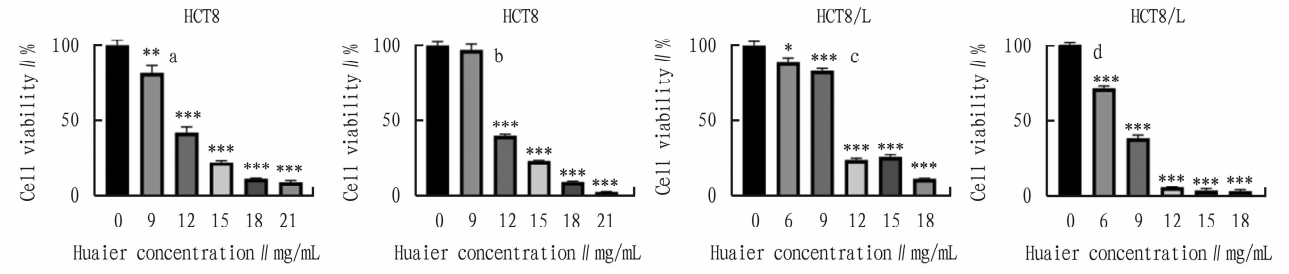
2.6 Western blot The expression lever of protein was analyzed by western blot. After the cells were treated with different concentrations of Huaier for 24 h. Total protein was extracted by RIPA lysate (Solarbio, China), and confected as 40 μg/15 μL system. Separated the protein gradient by gel electrophoresis. Protein has been transferred to PVDF membrane (Millipore, USA), 5% BCA fixed for 2 h, and then the first antibody was incubated for 4 ℃ overnight. The primary antibody includes β-actin (Affinity Biosciences Cat# AF7018, RRID:AB_2839420), P-gp (Bioss Cat# bs-1468R, RRID:AB_10854437), AKT (Affinity Biosciences Cat# AF0836, RRID:AB_2834120), p-AKT (Affinity Biosciences Cat # AF0016-pBP, RRID:AB_2847798), BAX (ABclonal Cat # A0207, RRID:AB_2757021), BCL-2 (ABclonal Cat# A0208, RRID:AB_2757022), P85α (Proteintech Cat # 60225-1-Ig, RRID:AB_11042594), cleaved caspase-3 (Proteintech Cat # 19677-1-AP, RRID:AB_10733244), Cleaved caspase-9 (Proteintech Cat# 10380-1-AP, RRID:AB_2068632), Cyclin D1 (Proteintech Cat# 60186-1-Ig, RRID:AB_10793718), CDK4 (Proteintech Cat# 66950-1-Ig, RRID:AB_2882273). TBST washed for 5min × 4times in a shaking table, and the corresponding HRP Goat anti-mouse IgG(H + L) (ABclonal Cat# AS003, RRID:AB_2769851), HRP Goat anti-rabbit IgG(H + L) (ABclonal Cat# AS014, RRID:AB_2769854) was incubated at room temperature for 1 h, and TBST washed for 5 min × 4 times. Immuno complexes were detected by enhanced chemiluminescence kit (Biosharp, China), and developed by Image Lab. Image J analyzed the protein gray value.

2.7 Statistical analysis Repeat each assays at least three times. Using SPSS 26.0 software program (New York, USA) to make statistical analysis of the data. Results were shown by the mean ± SD. The *t* test was used for comparison between the two groups, and one-way ANOVA was used for comparison among multiple groups, and multiple comparisons were made. Graphics were generated by GraphPad 8 (California, USA). $P < 0.05$ is considered to be statistically significant.

3 Results and analysis

3.1 Cytotoxicity and reverse drug resistance of Huaier Different concentrations of Huaier were used to treat cells, as shown

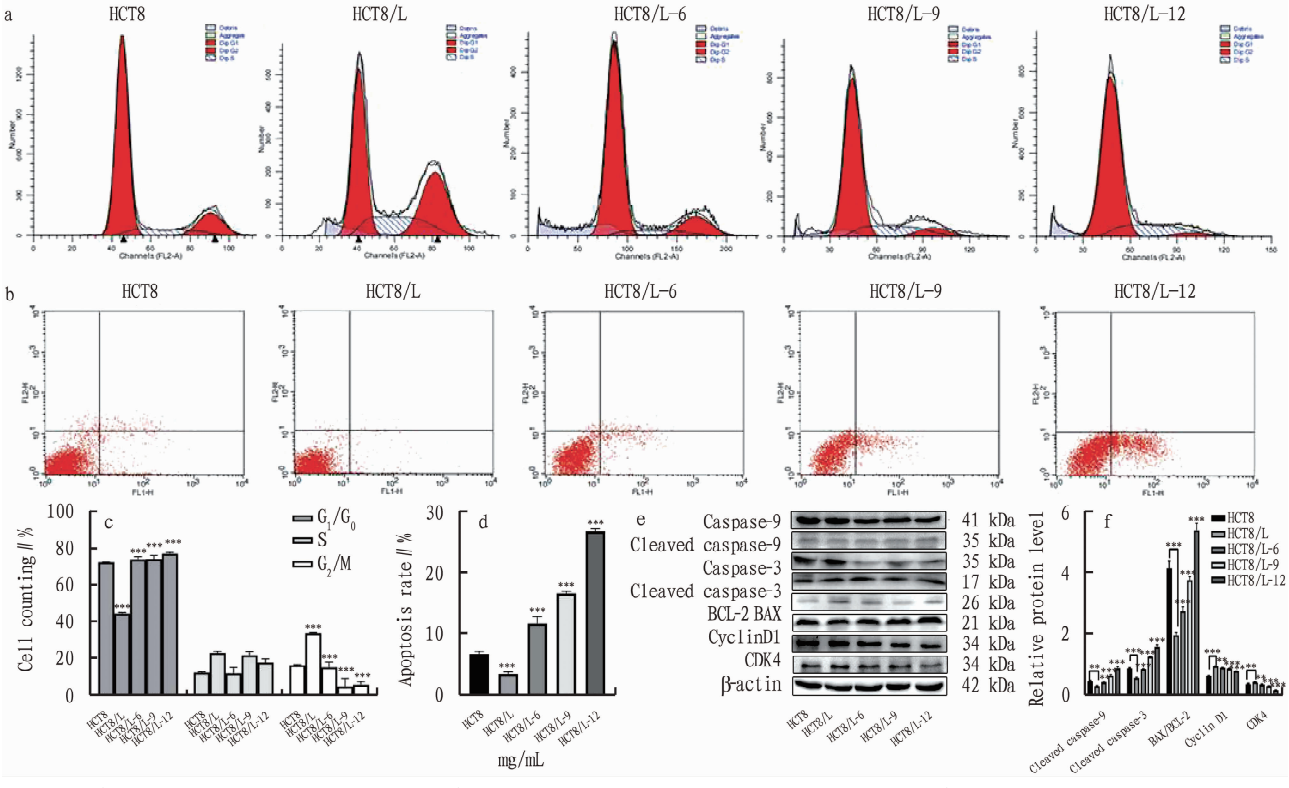
in Fig. 1, Huaier inhibited the growth of HCT8 and HCT8/L cells in a concentration-dependent and time-dependent manner. When took Huaier for 24 h on HCT8/L cells, IC_{50} value was 10.7 mg/mL, and the inhibitory rate of 6 mg/mL Huaier was 8.36% ±2.42%, the inhibitory rate of 9 mg/mL was 20.20% ±3.31%, and the inhibitory rate of 12 mg/mL was 78.14% ±1.97%. Therefore, the concentrations (6, 9 and 12 mg/mL) were selected to study the reverse oxaliplatin resistance in CRC.



NOTE The picture shows three independent assays of concentration-viability diagram ($n=3$ each group); a and b are effects of Huaier on HCT8 cells for 24 and 48 h; c and d are the effect of Huaier on HCT8/L for 24 and 48 h. $P<0.05$ is considered statistically significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. The same below.

Fig. 1 Effects of Huaier on the cell survival rate of HCT8 and HCT8/L

3.2 Reducing cell proliferation and inducing apoptosis Our assays showed that Huaier can arrest the cell cycle of HCT8/L in G_0/G_1 phase. The number of drug-fast cells in G_0/G_1 phase was significantly less than sensitive cells (Fig. 2a). However, the number of drug-resistant cells in G_0/G_1 phase in the drug-dosing group was significantly higher than the non-dosing group, and the number of cells increased with the increased concentration of Huaier. It was proved that Huaier can arrest the cell cycle of HCT8/L in G_0/G_1 phase to reduce cell proliferation. The apoptosis rate of drug-resistant cells decreased significantly, while the apoptosis rate of drug-resistant cells in the drug-added groups was significantly higher than that in the non-drug group, and the apoptosis rate of drug-resistant cells was significantly increased with the incremental concentration of Huaier (Fig. 2b). Through detecting

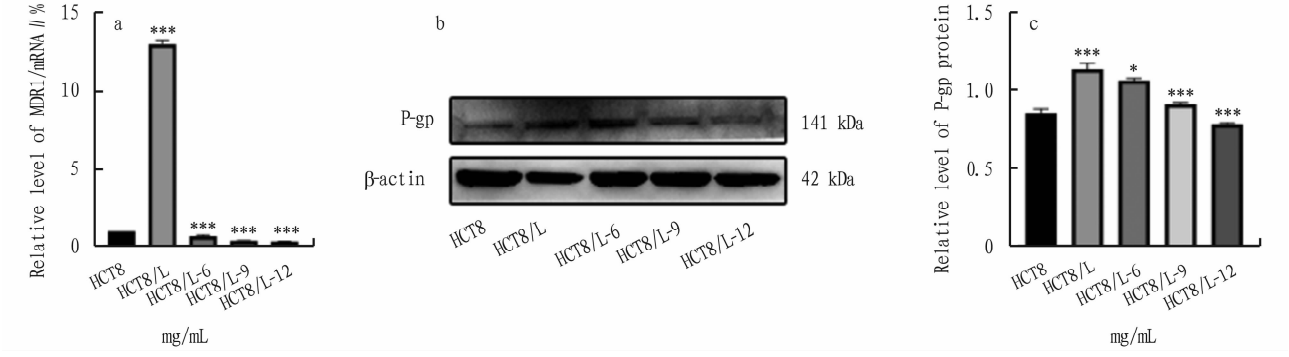


NOTE (a) cell cycle was detected by PI assays; (b) cell apoptosis was detected by Annexin-V/PI assays; (c) the proportion of different groups of cells in different cell cycles obtained from three independent assays; (d) the proportion of apoptotic cells in different groups obtained from three independent assays; (e) the expression levels of proteins related to proliferation and apoptosis were detected by western blot; (f) the expression levels of different proteins in different groups obtained from three independent assays.

Fig. 2 Effects of Huaier on proliferation and apoptosis of HCT8/L cells

proliferation-related and apoptosis-related proteins by western blot, we further confirmed that Huaier can reduce cell proliferation and increase cell apoptosis in HCT8/L cells. BCL-2, CDK4 and CyclinDl of HCT8/L were significantly higher than HCT8, and BAX, cleaved caspase-3 and cleaved caspase-9 were significantly lower than HCT8. In addition, Huaier could significantly inhibit the expression of CyclinDl and CDK4, and induce the expression of apoptosis-related proteins BAX, cleaved caspase-3 and cleaved caspase-9 (Fig. 2e).

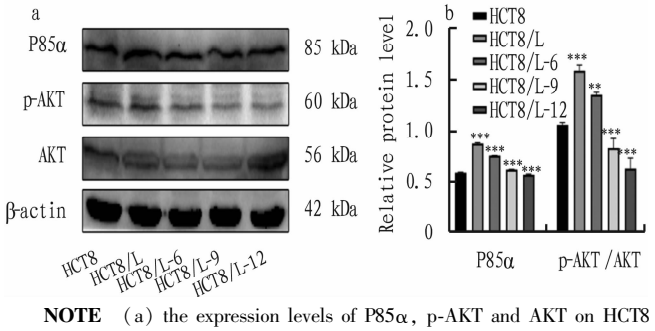
3.3 Reversing drug resistance by inhibiting the expression lever of MDR1 gene RT-qPCR and western blot assays were used to detect the expression level of *P-gp*, and we concluded that Huaier reverse drug resistance by inhibiting of *MDR1* gene and protein expression lever. RT-qPCR assays results showed the expression level of *MDR1* mRNA in HCT8/L cells was significantly higher than HCT8, while the groups with Huaier was significantly lower (Fig. 3a). Western blot showed the protein expression level of P-gp in HCT8/L was higher than HCT8 cells, and the drug-treated groups were lower than the non-drug group, and its expression level decreased with the increased of concentration (Fig. 3b).



NOTE (a) comparison of *MDR1* mRNA expression levels between HCT8 and HCT8/L, and different drug concentrations in HCT8/L cells compared with no-drug; (b) the protein expression level of P-gp of HCT8 and HCT8/L and different drug concentrations of HCT8/L cells were compared with no-drug, (c) the protein expression level of P-gp in different groups obtained from three independent assays.

Fig. 3 Effects of Huaier on MDR1 gene expression level

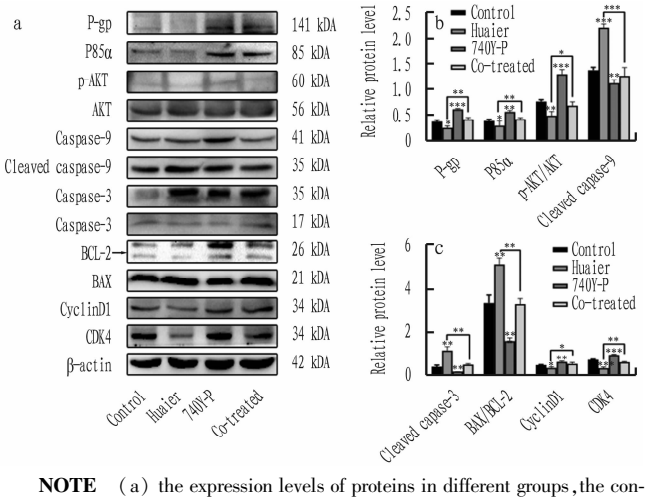
3.4 Regulating PI3K/AKT signaling pathway Western blot proved that Huaier can regulate PI3K/AKT signaling pathway. The expression levels of P85α and p-AKT/AKT in HCT8/L cells were significantly higher than HCT8, while the expression levels of P85α and p-AKT/AKT were significantly reduced in drug-treated groups, that is, the activative level of PI3K/AKT signal pathway in HCT8/L cells is higher than HCT8, but Huaier can inhibit the activation of PI3K/AKT signal pathway (Fig. 4).



NOTE (a) the expression levels of P85α, p-AKT and AKT on HCT8 and HCT8/L, and compared different concentrations groups with non-drug group; (b) column figure showed P85α and p-AKT/AKT ratio of different groups obtained from three independent assays.

Fig. 4 Effects of Huaier on the expression level of proteins related PI3K/AKT signaling pathway

3.5 Inducing apoptosis and arresting cell cycle through PI3K/AKT signaling pathway to reverse oxaliplatin resistance in CRC By comparing with the function of 740Y-P, we can know that Huaier can induce apoptosis and arrest cell cycle to reverse oxaliplatin-resistant of CRC through PI3K/AKT signaling pathway. As shown in Fig 5, Huaier could significantly inhibit the expression of P-gp, P85α, p-AKT/AKT, CyclinD1 and CDK4,



NOTE (a) the expression levels of proteins in different groups, the control group is the no-drug group, Huaier group is the concentration of Huaier (9 mg/mL), 740 Y-P group is the concentration of PI3K/AKT signal pathway agonist (5μmol/L), and co-treated group is Huaier (9 mg/mL) combined with 740 Y-P (5 μmol/L) on HCT8/L cells; (b) and (c) are the histogram is the expression levels of corresponding proteins in different groups obtained by three independent assays.

Fig. 5 Western blot of protein expression level

and increase the expression of BAX/BCL-2, Cleaved caspase-3/caspase-3, cleaved caspase-9/caspase-9; while 740Y-P increased the expression of P-gp, P85α, p-AKT/AKT, CyclinD1 and CDK4, and inhibited the expression of BAX/BCL-2, cleaved caspase-3/Caspase-3 and cleaved caspase-9/caspase-9. The expressions of P-gp, P85α, p-AKT/AKT, CyclinD1 and CDK4 in the co-treated group were higher than the Huaier group but lower than 740Y-P group; and the expressions of BAX/BCL-2, cleaved caspase-3/caspase-3 and cleaved caspase-9/Caspase-9 were lower than Huaier group but higher than the 740Y-P group. That is, the effect of Huaier is opposite to the 740Y-P, and it can partially counteract the effect of it, which further proves that Huaier inducing apoptosis and arresting cell cycle through PI3K/AKT signaling pathway to reverse oxaliplatin resistance of CRC.

4 Discussion

CRC is a malignant tumor that occurs in the colorectal tract, with the second highest mortality rate in the world[2]. In China, the morbidity and mortality rate are increasing year by year[18]. It is often found in the middle and late stage in clinic, so it is especially difficult to treat it, and the emergence of chemotherapy resistance makes treatment even more difficult. So far, many methods have been tried to improve the sensitivity of tumors to chemotherapy, including inhibiting the activity of drug metabolism enzymes[19], inhibiting the activity or expression of membrane transporters[20-21], increasing the activity or expression of topoisomerase[22], inhibiting the activation of signal pathways[23], inhibiting the activation of selective receptors[24], inducing programmed cell death and regulating tumor microenvironment[25]. Mechanism of drug resistance may be related to the classical drug resistance pathway of P-gp and the inhibition of apoptosis, which may be the two most studied mechanisms of tumor drug resistance. Our results also show that PI3K/AKT signal pathway in drug-resistant cells is abnormally activate, which leads to downstream target proteins abnormally express, such as proliferous proteins CyclinDl and CDK4, apoptotic proteins BAX/BCL-2, cleaved caspase-3/caspase-3, cleaved caspase-9/caspase-9 (Fig. 2). Abnormal expression of these effector proteins leads the abnormal proliferation and apoptosis of tumor cells, which eventually leads the increased expression of P-gp and drug resistance of tumors (Fig. 3). However, Huaier can inhibit the activation of PI3K/AKT signaling pathway and cell proliferation, inducing apoptosis to reverse drug resistance (Fig. 4-5). This provides a new idea for us to study the treatment of drug-resistant CRC. However, the current results are based on cell assays, which may deviate from the clinical treatment effect. We still need more clinical treatment results to further confirm the drug resistance effect. Traditional Chinese medicine is a traditional treasure of our country, which has aroused widespread concern of scholars in recent years. The good curative effect of Huaier including good curative effect, low toxicity and difficult to cause drug-resistance, therefore, Huaier has become an adjuvant treatment drug on clinic. However, there are many components in the extract of Huaier. We did not clearly study the correlation and interaction mechanism among the components, and it is even more likely that Huaier regulates the progress of tumor cells through various ways, which are still unclear. We haven't fully confirmed the specific effect of multi-drug combined with Huaier, and more in-depth research is still needed to clarify its effects.

5 Conclusions

The above data prove that Huaier can regulate PI3K/AKT signaling pathway to significantly reduce the cell viability of HCT8/L, arrest cell cycle in G1/G0 phase and promote cell apoptosis, inhibit the expression of P-gp and effectively reverse drug resistance. Generally speaking, these findings reveal the molecular mechanism of Huaier reversing the drug resistance of CRC. This study is expected to a foundation and guarantee for the research of reversing drug resistance and adjuvant chemotherapy.

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