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Huai Qi Huang-induced Apoptosis via Down-regulating PRKCH and Inhibiting RAF/MEK/ERK Pathway in Ph+ Leukemia Cells*

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Summary: Imatinib mesylate (IM) is the first-line treatment for Philadelphia (Ph) chromosomal positive leukemia by inhibiting phosphorylation of substrates via binding to the ABL kinase domain. Because of the drug resistance, side effects and the high cost of IM, it is necessary to find anti-cancer drugs with relatively low toxicity and cost, and enhanced efficacy, such as traditional Chinese medicines (TCMs). As one of TCMs, Huai Qi Huang (HQH) was chosen to treat BV173 and K562 cells. Various concentrations of HQH were added to cells for 24–72 h. Co-treatment of HQH and trametinib, an MEK inhibitor, was used to verify the synergistic effects on cell viability and apoptosis. Knockdown and overexpression of mitogen-activated protein kinase kinase 4 (MEK4) were implemented to demonstrate the role of MEK in cell apoptosis. Cell viability and apoptosis were measured by cell counting kit-8 assay (CCK8) and flow cytometry, respectively. Western blotting and real-time quantitative PCR (RT-qPCR) were used to assess protein and mRNA expression levels, respectively. The results showed that HQH inhibited survival and promoted apoptosis of BV173 and K562 cells in a dose-dependent manner, accompanied with down-regulation of PRKCH mRNA as well as CRAF, MEK4, phospho-ERK (pERK) and BCL2 proteins, and up-regulation of cleaved caspase3 protein. Co-treatment of HQH and trametinib had a synergistic effect on inhibiting survival and promoting apoptosis. MEK4 knockdown increased apoptosis, and had a synergistic effect with HQH. In contrast, MEK4 overexpression decreased apoptosis, and had the opposite effect with HQH. Collectively, the results of this study may identify a therapeutic mechanism of HQH on promoting apoptosis, and provide a potential option for treatment of Ph+ leukemia.
Key words: Huai Qi Huang; leukemia; apoptosis; BCR-ABL; RAF/MEK/ERK

In the pathogenesis of Philadelphia (Ph) chromosomal positive leukemia, the BCR-ABL chimeric protein plays a central role, especially in chronic myeloid leukemia (CML). Protein tyrosine kinase (PTK) activity determines the malignant transformation by BCR-ABL[1]. As the first-line treatment for CML, imatinib mesylate (IM) inhibits phosphorylation of substrates by binding to the ABL kinase domain[2]. In the early-stage of the disease, IM can significantly increase the survival rate of patients, but not improve the curative effect. However, in advanced-stage of disease, resistance to IM and second-generation agents (niloditib, dasatinib) can lead to disease relapse and progression for patients with CML as well as Ph+ ALL[3–5]. Moreover, a long-time use of imatinib and second generation of tyrosine kinase inhibitors (TKIs) is hampered by the chronic mild toxicities and a significant burden in health care costs[6, 7]. Therefore, the urgent requirement is to find an effective and cheap drug for Ph+ leukemia.

Plants may be one of the choices of anticancer drugs[8]. The botanicals have different components which have synergistic therapeutic effects and, moreover, can buffer the toxic effects of single component[9]. Polysaccharides and amino acids are the effective ingredient of *Trametes robiniophila Murr* (Huaier), which has been used for about 1600 years as one of traditional Chinese medicines (TCMs)[10, 11]. Huaier extract has been used to treat a number of solid tumors and leukemia[12–20]. Huai Qi Huang (HQH), whose primary ingredient is Huaier, is composed of Huaier, Chinese wolfberry fruit and polygonatum and used to treat asthma, mycoplasma pneumoniae pneumonia and kidney disease[21–26], and also induces apoptosis in acute lymphoblastic leukemia cells[27].

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Trametinib is an MEK inhibitor, which is approved by U.S. Food and Drug Administration. Ma *et al* reported that the survival of mouse models with BCR-ABL-independent IM-resistant CML was prolonged by the combined treatment of IM and trametinib[28]. In the present study, we want to know if HQH has a synergistic effect with trametinib in treating Ph^+ Leukemia and the underlying mechanism.

1 MATERIALS AND METHODS

1.1 Reagents, Human Cell Lines and Cell Culture

The electuary HQH ointment was a kind gift from Qidong Gaitianli Medicine Co., Ltd. (China). Electuary ointmen was diluted to a solution of 1 g/mL by 0.9% sodium chloride solution and the prepared HQH solution was sterilized with a 0.22 μm filter. Trametinib (GSK1120212) (A3018) was obtained from APExBIO (APExBIO Technology LLC, USA) and dissolved in dimethyl sulfoxide (DMSO). Then they were all stored at –20°C. Human Ph^+ K562 and BV173 cells were obtained from American Type Culture Collection (ATCC) (Rockville, USA) and the Wuhan University Cell Preservation Center (China), respectively. They were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, which was supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (GNM15140, Hangzhou, China), in a 5% CO_2 humidified atmosphere at 37°C.

1.2 Cell Viability Assay

Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was used to measure cell proliferation rates of K562 and BV173 cells according to the manufacturer's instructions. Briefly, cells at a density of (3–5) × 10^4 cells/mL were seeded into 96-well plates at 100 μL/well (3 wells per group) at exponential growth phase. Wells containing no cells but medium were used as a blank control group in each plate. After 4-h incubation, HQH at different concentrations (0.1, 1, 5, 10 and 20 mg/mL) was added to the wells. 24, 48 or 72 h later, cell proliferation was tested, respectively. A co-culture was performed with different concentrations of trametinib (0.01, 0.1, 1, 10, 100, 500 and 1000 μmol/L) and 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562, respectively. Each well was added with 10 μL of CCK-8 solution and incubated for additional 4 h at 37°C. A microplate reader (Tecan Sunrise, Switzerland) was used to measure the absorbance (*A*) at 490 nm. The viability ratio was calculated using the following formula: the viability ratio = [(the *A* values of experimental group−the *A* values of blank group)/(the A values of untreated group−the *A* values of blank group)] × 100%[27]. The experiment was performed in triplicate.

1.3 Apoptosis Analysis

K562 and BV173 cells at a density of 1 × 10^5 cells/well were seeded in 6-well plates at exponential growth phase. Cells were incubated with 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562, 0.1 μmol/L trametinib or combination of HQH and trametinib, respectively, and cells incubated with HQH-free medium were used as a control. Cells were incubated with Annexin-V-FITC and propidium iodide (PI) (BD Biosciences, USA) according to the manufacturer's protocol. Then apoptosis of cells was analyzed using a FACScan™ flow cytometer (BD Biosciences, USA) and results were analyzed with FlowJo software (Tree Star, USA). The ratios of early and late apoptotic cells were calculated, and then compared between the experimental group and control group. The experiments were performed for 3 times.

1.4 Real-Time Quantitative PCR Analysis

Total RNA of K562 and BV173 cells was extracted by the RNAiso Plus (TaKaRa, China) and reversed into the first-strand cDNA using PrimeScript RT Master Mix (TaKaRa, China) according to the manufacturer's instructions. StepOne Plus Real-Time PCR System (Applied Biosystems AB, USA) was used to perform real-time quantitative PCR (RT-qPCR) analysis. StepOne Software v2.3 was used to analyze PCR products. Specific PCR primers were synthesized by HYcell biotechnology (China) and the sequences were listed in table 1. The relative gene expression differences were detected using $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH levels.

Table 1 Primer sequences

Genes	Sequence (5′-3′)
H-Caspase3	
Forward	GAACTGGACTGTGGCATTGAGAC
Reverse	GCACAAAGCGACTGGATGAAC
H-Bcl2	
Forward	TGGGGTCATGTGTGTGGAGAG
Reverse	AATCAAACAGAGGCCGCATG
H-GAPDH	
Forward	GAAGCTTGTCATCAATGGAAAT
Reverse	TGATGACCCTTTTGGCTCCC
H-PRKCH	
Forward	GATCAATGGACACAAGTTCATGG
Reverse	CGTTTATGGACGACACAGGTG
H-MEK4	
Forward	CATGCAGGGTAAGCGCAAAG
Reverse	CCAGTGTTGTTCAGGGGAGAT

1.5 Western Blotting

K562 and BV173 cells were lysed using RIPA lysis buffer (Beyotime Biotechnology, China) and protein concentration was detected using the BCA protein assay kit (Biosharp, China) following the manufacturer's instructions. After being boiled and cooled, 40 μg proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime Biotechnology, China) and transferred to polyvinylidene fluoride membranes

(PVDF, Millipore, USA), which were then blocked with 5% nonfat milk for 1 h at room temperature. Then the above membranes were incubated at 4°C overnight with primary antibody. Total ERK (4695T), phospho-ERK (pERK, 4370T), BCL2 (4223T), cleaved caspase3 (cleaved at Asp175) (9579T), β-actin (4970T) and horseradish peroxidase (HRP)-conjugated sheep anti-rabbit secondary antibodies (7074P2) were all purchased from Cell Signaling Technology (USA). CRAF (66592-1-Ig), MEK4 (17340-1-AP) and HRP-conjugated Affinipure goat anti-mouse IgG (H+L) (SA00001-1) were obtained from Proteintech (USA). The primary antibodies were diluted to 1:1000 and second antibodies were diluted to 1:5000. After being incubated with matched secondary antibodies for 1 h at room temperature, the protein signals were detected with the enhanced chemiluminescence (ECL) (Beyotime Biotechnology, China) by an ECL chemiluminescence detection system (UVP biospectrum 600 imaging system, USA). ImageJ software (National Institutes of Health, USA) was used to detect protein gray values, using the level of β-actin as an internal control.

1.6 Transient Transfection Assay

In order to analyze the role of MEK in apoptosis, small interfering RNA (siRNA) against MEK4 was used to knock down the expression of MEK4. The siRNAs targeting MEK4 and non-targeting negative control (NC) were purchased from JTS scientific (China). MEK4 knockdown was performed according to the manufacturer's instructions. K562 and BV173 cells were cultured with serum free medium for 12 h before transfection at a density of 0.5×10^6/mL cells and transfected with MEK4 gene-specific siRNA duplexes: sense, 5′-GUCGCAUGCUAUGUUUGUATT-3′ and antisense, 5′-UACAAACAUAGCAUGCGACTT-3′. Non-targeting control siRNAs were used as a control. Briefly, 100 pmol siRNA and 5 μL Lipofectamine 2000 (Invitrogen, USA) were separately diluted into 1:50 with 250 μL Opti-MEM® medium for 5 min at room temperature and then mixed. 30 min later, the mixtures were added to cells in a 6-well plate. 12 h later, the RPMI 1640 containing 10% fetal bovine serum was used to replace serum free media. Subsequent experiments were performed after 24–36 h for RT-qPCR or Western blotting.

The MEK4-expressing vector pcDNA3.1 was obtained from JTS scientific (China). A total of 4×10^5 K562 or BV173 cells were seeded per well in six-well plates and grew for 24 h before transfection. We transfected K562 and BV173 cells using a mixture of 3 μg of pcDNA3.1plasmid DNA carrying an MEK4 and 15 μL of Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions and used null vector as a negative control (NC). 24 or 48 h later, 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562 were added to cells transfected with plasmid DNA carrying an MEK4, and after 24 h cells were used to test target mRNA by RT-qPCR or protein by Western blotting, respectively.

1.7 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, USA). All data were expressed as the means ± standard deviation (SD) and analyzed by the Student's *t* test and one-way analysis of variance (ANOVA). Differences between groups were considered statistically significant at *P*-values <0.05.

2 RESULTS

2.1 Inhibitory Effects of HQH on Proliferation of BV173 and K562 Cells

HQH at various concentrations of 0.1, 1, 5, 10 and 20 mg/mL was added to BV173 and K562 cells for 24–72 h and the cell viability was determined by CCK8 assay. Cell viability was inhibited by HQH in both BV173 (fig. 1A) and K562 (fig. 1B) cells, and compared with 48 h and 72 h, 24 h was the most suitable time to inhibit cell proliferation. Moreover, the inhibition of cell viability increased with the increase of HQH concentration, suggesting that HQH inhibited cell viability in a dose-dependent manner. The IC50 was 0.5 mg/mL for BV173 cells and 5.2 mg/mL for K562 cells. To demonstrate the effects of combined treatment of HQH and trametinib, cells were cultured with different concentrations of trametinib and 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562 for 24 h, respectively. The results showed that the combined use of HQH and trametinib had a synergistic effect on inhibiting cell proliferation in both BV173 (fig. 1C) and K562 (fig. 1D) cells ($P<0.05$, $P<0.01$ or $P<0.001$).

2.2 HQH-induced Apoptosis of BV173 and K562 Cells

BV173 and K562 cells were stained with Annexin V-FITC and PI, and apoptosis was detected by flow cytometry. Cells were cultured with 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562, 0.1 μmol/L trametinib alone or combination of HQH and trametinib for 24 h. The results showed that co-culture of HQH and trametinib had a higher apoptosis rate than HQH or trametinib alone on both BV173 (fig. 2A and 2C) and K562 (fig. 2B and 2D) cells ($P<0.01$ or $P<0.001$).

2.3 HQH Promotes Apoptosis through Inhibiting RAF/MEK/ERK Pathways and PRKCH

BV173 and K562 cells were co-cultured with different concentrations of HQH (1, 5 and 10 mg/mL) for 24 h and Western blotting was used to detect apoptosis-associated proteins (BCL2 and cleaved caspase3). The results showed that HQH induced apoptosis in a dose-dependent manner with an up-regulation of cleaved

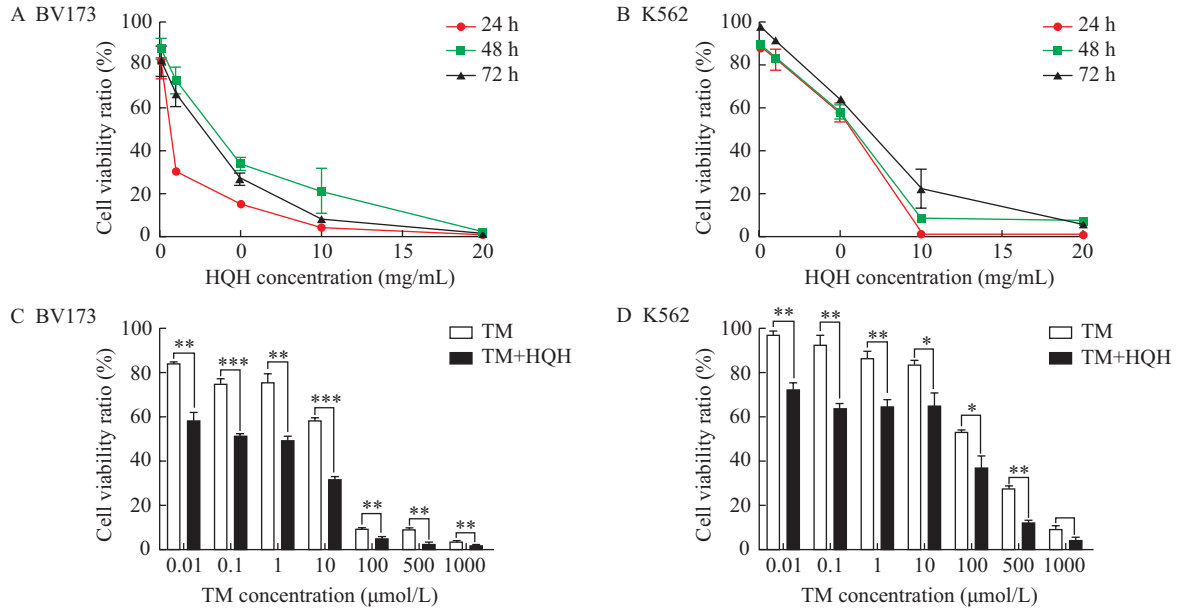

Fig. 1 Synergistic effect of Huai Qi Huang (HQH) and trametinib (TM) on inhibiting cell proliferation
HQH inhibited cell proliferation in a dose-dependent manner in BV173 (A) and K562 cells (B) cells. Cells were co-cultured with different concentrations of TM (0.01, 0.1, 1, 10, 100, 500 and 1000 μmol/L) and 0.5 mg/mL HQH for BV173 (C) and 5.2 mg/mL HQH for K562 (D) for 24 h, respectively. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

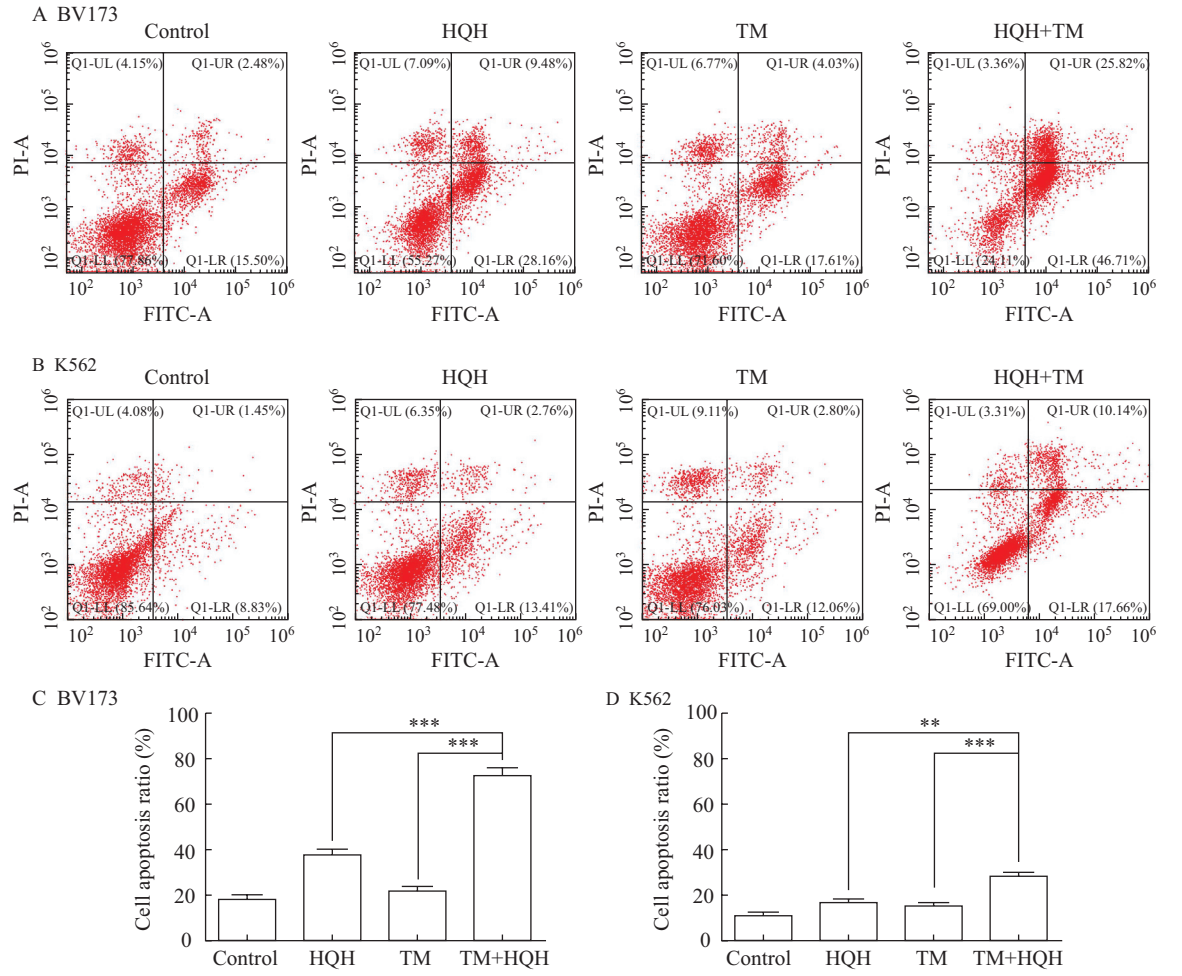

Fig. 2 Combination of Huai Qi Huang (HQH) and trametinib (TM) promotes cell apoptosis
Representative scatter plots of Annexin V- fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining were shown for BV173 (A) and K562 (B) cells treated with 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562, 0.1 μmol/L TM or combination of HQH and TM for 24 h, respectively. Apoptosis bar graphs were shown for percentage of apoptotic cells of BV173 (C) and K562 (D) cells. $^{**}P<0.01$, $^{***}P<0.001$

caspase3 and a down-regulation of BCL2 in BV173 and K562 cells (fig. 3A and 3B, respectively).

To further explore the mechanism of apoptosis induced by HQH, the proteins of CRAF, MEK4, pERK and ERK were detected by Western blotting and PRKCH mRNA was detected by RT-qPCR in BV173 and K562 cells, respectively. HQH reduced the expression levels of CRAF, MEK4, pERK proteins and PRKCH mRNA in a dose-dependent manner in both BV173 (fig. 3C and 3E) and K562 (fig. 3D and 3F) cells. There was no significant change in ERK protein level (P<0.05 or P<0.01).

2.4 HQH Promotes Apoptosis by Combining with the MEK Inhibitor Trametinib

BV173 and K562 cells were treated with 0.1 μmol/L trametinib alone or with a co-treatment of 0.1 μmol/L trametinib and 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562. 24 h later, pERK and ERK expression levels were determined by Western blotting. As shown in fig. 4, the co-treatment of trametinib and HQH significantly promoted apoptosis of BV173 (fig. 4A and 4C) and K562 (fig. 4B and 4D) cells, accompanied with up-regulation of cleaved caspase3 and down-regulation of pERK and BCL2. These results suggested that HQH and trametinib had synergistic effects on promoting apoptosis through downregulation of the MEK/ERK pathway in BV173 and K562 cells.

2.5 MEK4 Knockdown Promotes Apoptosis

To further evaluate the effects of MEK on apoptosis of BV173 and K562 cells, MEK4 knockdown was performed by MEK4 specific siRNA. The results showed that the mRNA expression levels of MEK4 and BCL2 decreased and that of caspase3 increased, accompanied with up-regulation of cleaved caspase3 and down-regulation of MEK4 and BCL2 on protein levels in BV173 (fig. 5A and 5C) and K562 (fig. 5B and 5D) cells. Moreover, co-treatment of MEK4 knockdown and 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562 had a synergistic effect in promoting apoptosis of both BV173 (fig. 5C) and K562 (fig. 5D) cells (P<0.05 or P<0.01).

2.6 MEK4 Overexpression Reduces Apoptosis

To further confirm the role of MEK4 in apoptosis of BV173 and K562 cells, MEK4 was overexpressed by plasmid DNA carrying an MEK4. As shown in fig. 6, the mRNA expression levels of MEK4 and BCL2 increased and that of caspase3 decreased in BV173 (fig. 6A) and K562 (fig. 6B) cells. Meanwhile, the protein expression levels of MEK4 and BCL2 increased and that of cleaved caspase3 decreased in BV173 (fig. 6C) and K562 (fig. 6D) cells. However, co-treatment of MEK4 overexpression and 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562 decreased MEK4, increased apoptosis, and had an opposite effect in promoting apoptosis compared with

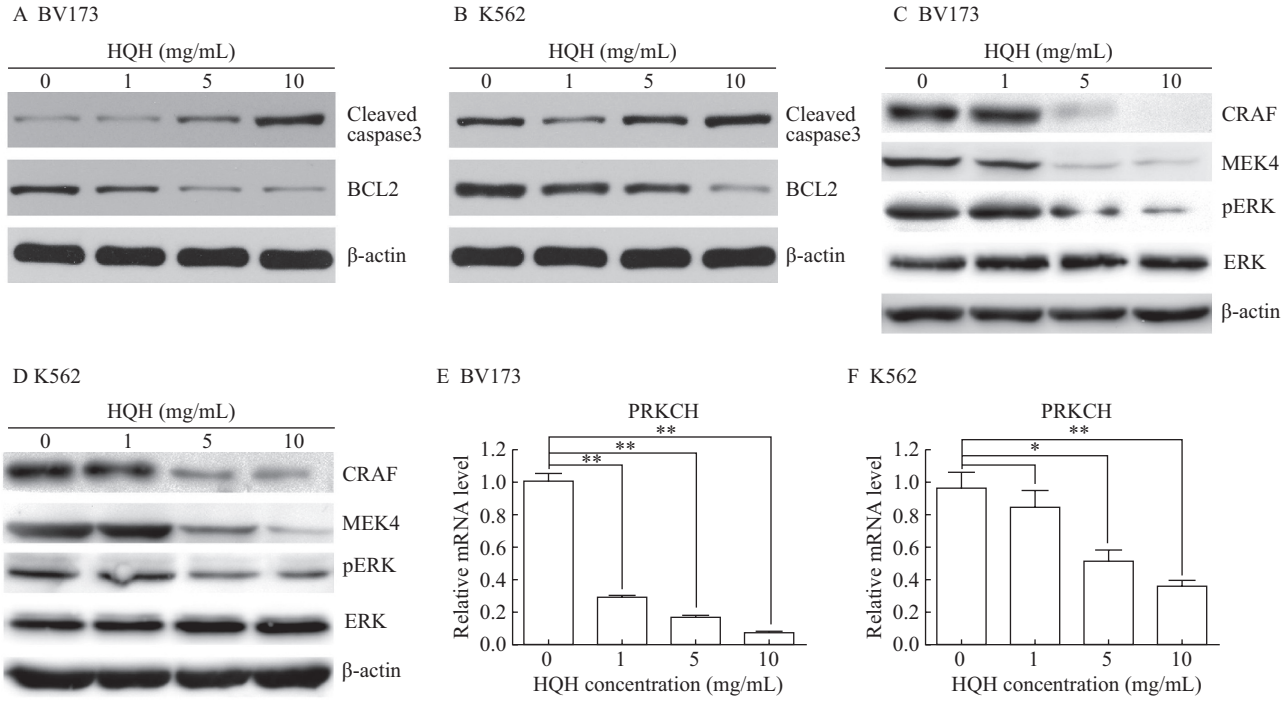

Fig. 3 Huai Qi Huang (HQH) promotes apoptosis of BV173 and K562 cells by downregulating expression of CRAF, MEK4, pERK and PRKCH

HQH enhanced expression of cleaved caspase3 and reduced expression of BCL2 in a dose-dependent manner in both BV173 (A) and K562 (B) cells. HQH increased apoptosis by downregulating expression levels of CRAF, MEK4, pERK and PRKCH with no significant change in ERK level in BV173 (C and E) and K562 (D and F) cells. β-actin was used as a loading control for Western blotting. GAPDH was used as the control for RT-qPCR. $^{*}P$<0.05, $^{**}P$<0.01

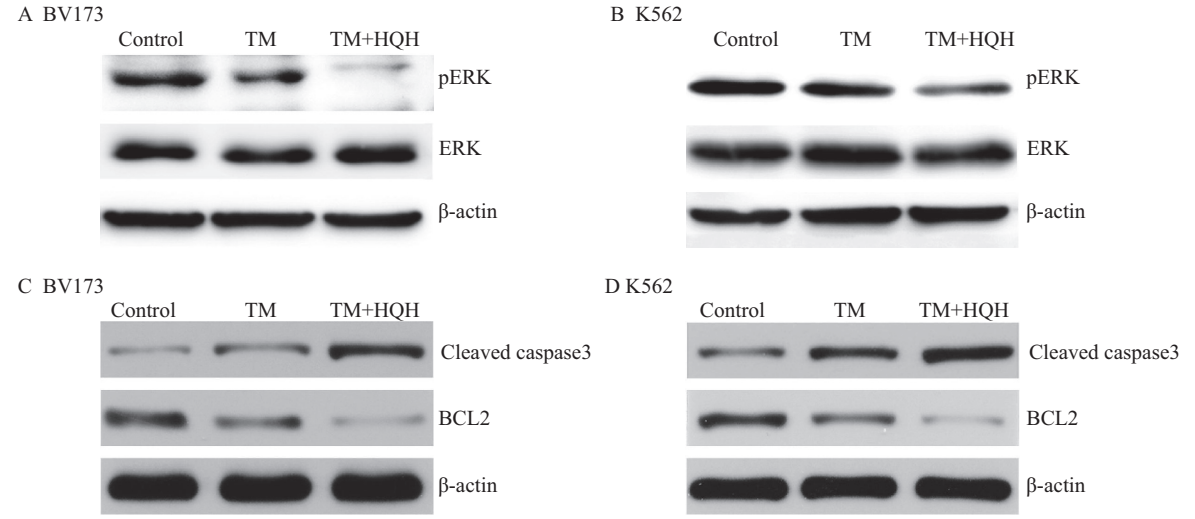

Fig. 4 Co-treatment of trametinib (TM) and Huai Qi Huang (HQH) increases expression of cleaved caspase3 and reduces expression of BCL2 by downregulating the expression of pERK

Co-treatment of 0.1 μmol/L TM and 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562 reduced the expression of pERK in BV173 (A) and K562 (B) cells. Meanwhile, the co-treatment of TM and HQH upregulated the expression of cleaved caspase3 and downregulated the expression of BCL2 in BV173 (C) and K562 (D) cells. β-actin was used as the loading control for Western blotting.

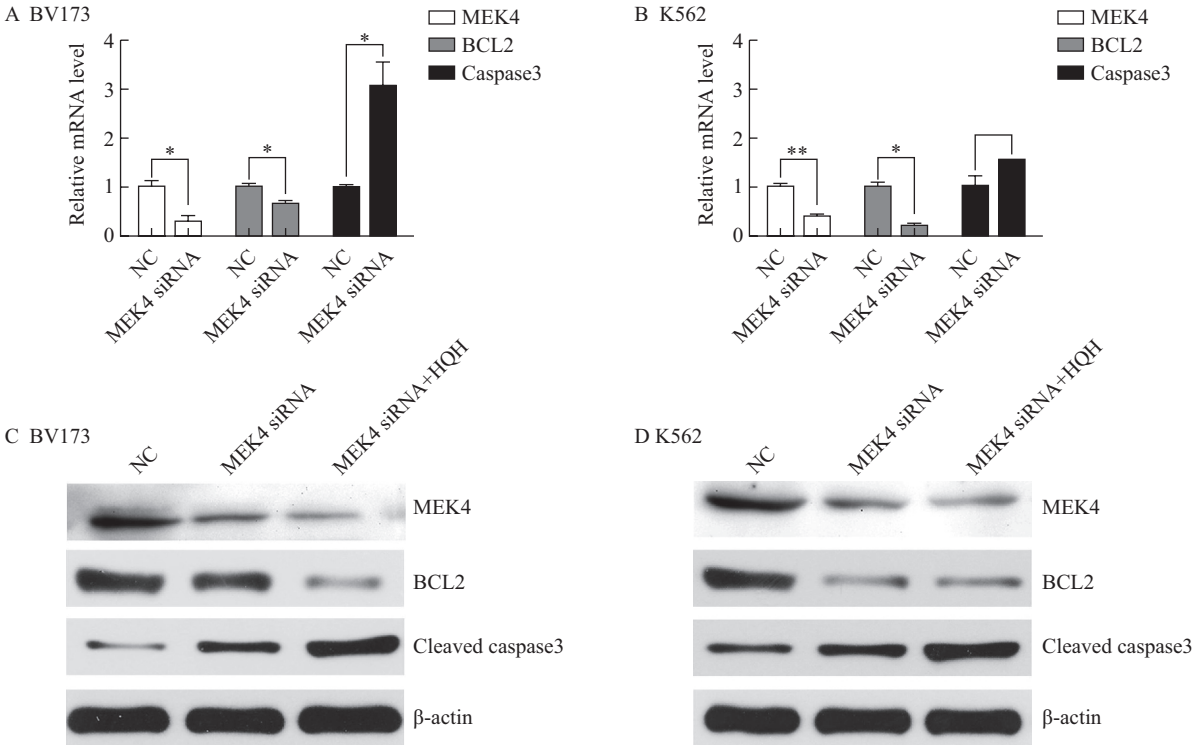

Fig. 5 MEK4 knockdown promotes cell apoptosis

MEK4 knockdown enhanced mRNA expression of caspase3 and reduced mRNA expression of BCL2 and MEK4 in BV173 (A) and K562 (B) cells. GAPDH was used as the control for RT-qPCR. MEK4 knockdown also upregulated the expression of cleaved caspase3 and downregulated the expression of MEK4 and BCL2 on protein levels, and there was a synergistic effect by combination of MEK4 knockdown and 0.5 mg/mL HQH for BV173 (C) and 5.2 mg/mL HQH for K562 (D) cells. β-actin was used as the loading control for Western blotting. $^{*}P<0.05$, $^{**}P<0.01$. HQH, Huai Qi Huang; NC, negative control

MEK4 overexpression alone ($P<0.05$ or $P<0.01$).

3 DISCUSSION

As a selective tyrosine kinase inhibitor, it is the major problem for IM that resistance quickly emerges and Ph^+ cells persist, although IM is effective in Ph^+ CML and ALL at early treatment stage[29]. Therefore, it is necessary to explore the resistance mechanism independent of BCR-ABL. BCR-ABL, produced by

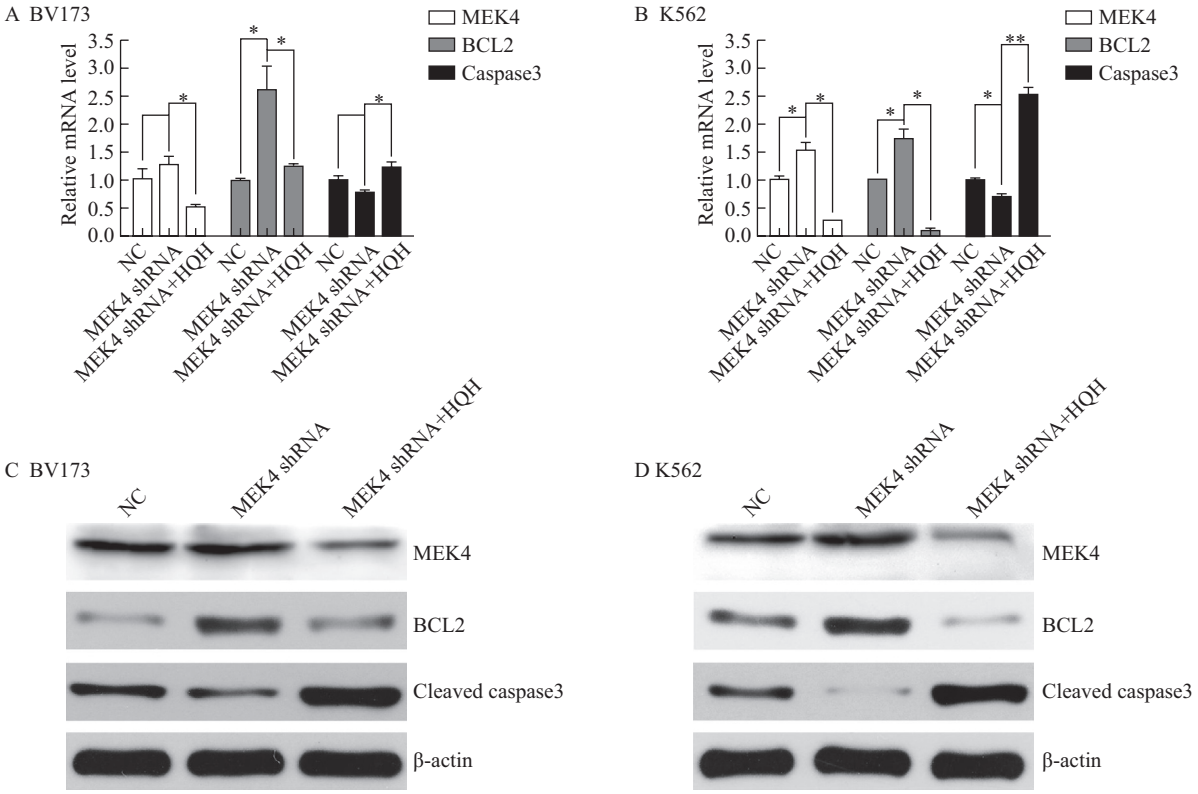

Fig. 6 MEK4 overexpression inhibits cell apoptosis

When MEK4 was overexpressed, the expression of MEK4 and BCL2 mRNA increased and that of caspase3 mRNA decreased in BV173 (A) and K562 (B) cells. GAPDH was used as the control for RT-qPCR. Meanwhile, the protein expression of cleaved caspase3 also decreased and that of MEK4 and BCL2 increased in MEK4 overexpressed BV173 (C) and K562 (D) cells. β-actin was used as the loading control for Western blotting. The combination of MEK4 overexpression and 0.5 mg/mL HQH for BV173 and 5.2 mg/mL HQH for K562 had an opposite effect on cell apoptosis. $^{*}P<0.05$, $^{**}P<0.01$. HQH, Huai Qi Huang; NC, negative control

chromosomal translocation, activates ABL tyrosine kinase, transforms myeloid progenitor cells developing 95% of CML cases and promotes leukemogenesis by activating downstream signaling proteins[30], such as the RAF/MEK/ERK[31]. Ma *et al* reported that due to upregulation of PRKCH, RAF/MEK/ERK signaling was sustained in IM-sensitizing genes (IMSG) knockdown cells. PRKCH encodes PKCη, a member of protein kinase C (PKC) family, which phosphorylates and activates CRAF to increase RAF/MEK/ERK signaling[28]. Savage *et al* and Vilimas *et al* reported that in the control of hematopoietic cell proliferation, the RAF1/MEK/ERK pathway interrupted cell apoptosis signals preceding pro-apoptotic signals[32, 33]. Therefore, we assume that inhibition of RAF/MEK/ERK pathway can promote apoptosis of Ph^+ cells, which are BCR-ABL independent or resistant to IM. As an MEK inhibitor, trametinib reduces the steady-state levels of RAF-MEK complexes[34]. In RAS-driven leukemias, the MAPK pathway is activated and sensitive to trametinib, which inhibits ERK *in vitro*[35]. Trametinib reduces peripheral blast counts and provides 6 months of disease control for a patient in spite of no complete remission (CR), and this response is consistent with pre-clinical models of RAS-activated AML[36, 37]. BCR-ABL^+ IMSG knockdown cells could be killed by a co-treatment of IM and trametinib (a U.S. Food and Drug Administration–approved MEK inhibitor)[28].

TCMs are widely used as anticancer drugs in recent years[38–40]. As a TCM, Huaier has anti-cancer properties on anti-proliferative effects, induction of apoptosis and anti-metastatic effects via pAKT/mTOR/S6 pathway, ER/NF-κB pathway and Wnt/β-catenin pathway, etc[41]. ERK1 and ERK2 (ERK1/2) are expressed in all human tissues[42]. Yan *et al* reported that Huaier suppressed motility of cervical cancer cells by downregulating p-ERK on cervical cancer SiHa and C33A cells[43]. Wang *et al* reported that Huaier extract suppressed the activation of ERK in a dose-dependent manner on human umbilical vein endothelial cell line[44]. This study showed that HQH inhibited proliferation by inhibiting the expression of CRAF, MEK4 and pERK in a dose-dependent manner in BV173 and K562 cells. Moreover, the combined treatment of HQH and trametinib synergistically promoted apoptosis by inhibiting MEK/ERK pathway. Andrews *et al* reported that >50% cellular proliferation of K562 cells could be inhibited by 100 nmol/L of trametinib[45].

In order to demonstrate the role of MEK in cell apoptosis, we knocked down MEK4 (MKK4/

MAP2K4) kinase, a member of MEK, and the results showed an increase in cell apoptosis and decrease in MEK4. Moreover, MEK4 knockdown combined with HQH had the synergistic effect on promoting apoptosis by reducing MEK4. Jones *et al* reported that MEK4 knockdown promoted cell apoptosis by increasing the levels of the glucocorticoid receptor, which increased sensitivity specifically to prednisolone[46]. MEK4 drove prostate cancer metastasis, which could be inhibited by inhibiting MEK4 in mice[47, 48]. Knocking down the expression of MEK4 inhibited metastasis of human pancreatic cancer cells in mice[49].

On the contrary, when we overexpressed MEK4, apoptosis of BV173 and K562 cells decreased. Xu *et al* reported that overexpression of MEK4 increased cell invasion in prostate cell lines PC3-M, PC3, 1532NPTX, 1542NPTX, 1532CPTX, and 1542CPTX, and the opposite effects were observed when MEK4 expression was decreased[50].

Ma *et al* reported that BCR-ABL-independent IM resistance was caused by high levels of PRKCH, which existed in CML stem cells, promoted RAF/MEK/ERK signaling and allowed CML stem cells to survive independent on BCR-ABL[28, 51, 52]. Our results showed that HQH also inhibited PRKCH mRNA expression in a dose-dependent manner, indicating HQH could inhibit stem cells of K562 and BV173 to induce apoptosis.

Collectively, the present study firstly provides a rationale that HQH efficiently kills BV173 and K562 cells by inhibiting PRKCH and RAF/MEK/ERK pathway *in vitro*. Combination of HQH and trametinib has a synergistic effect on promoting apoptosis of BV173 and K562 cells, which provides a new therapeutic strategy for Ph^+ leukemia.

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Conflict of Interest Statement

None.

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