Xiao-Ai-Ping inhibits proliferation and induces apoptosis of chronic myeloid leukemia cells through downregulating β-catenin signaling pathway

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ABSTRACT

The active ingredients of Xiao-Ai-Ping (XAP) injection are Marsdeniae tenacissimae extraction, which is a traditional Chinese herb has been used to treat multiple diseases for a long time. However, the possible roles and mechanisms of XAP in chronic myeloid leukemia (CML) still remain unknown. In this study, we examined the effects of XAP on proliferation and apoptosis of K562 cells (CML cell line) and peripheral blood mononuclear cell (PBMCs) from CML patients. Here, we found that XAP could inhibit the proliferation of K562 cells and induce the apoptosis of K562 cells via mitochondrial pathway. In addition, XAP inhibited the migration of K562 cells through downregulating the expression levels of chemokine SDF-1 and its receptor CXCR-4. In addition, XAP down-regulated β-catenin and CyclinD1 level, and up-regulated GSK3β level in K562 cells. Interestingly, over-expression of β-catenin restored the XAP-induced apoptosis of K562 cells. Furthermore, XAP significantly inhibited the proliferation and induced the apoptosis of PBMCs from CML patients, compared with healthy people. In conclusion, XAP could inhibit the proliferation and migration of CML cells, and induce the apoptosis of CML cells through down-regulation of β-catenin and SDF1/CXCR-4 signaling.

INTRODUCTION

Chronic myeloid leukemia (CML) is characterized by the expansion of morphologically abnormal and malignant uncontrolled accumulation of hematopoietic stem cells that arises from t (9:22) (q34:q11) reciprocal translocation, forming the Philadelphia (Ph) chromosome. *BCR-ABL* fusion protein, encoded by Ph chromosome, has constitutively tyrosine kinase activity, resulting in leukemogenesis [1–6]. Various signaling pathways were activated by the *BCR-ABL* oncoprotein, such as phosphoinositide 3-kinase (PI3K) pathway, the rat sarcoma (Ras) and extracellular signal-regulated kinase (ERK) pathway, which promote the proliferation of cells, inhibit the apoptosis and change cell adhesion [7–9]. *BCR-ABL* fusion protein plays a critical role in the pathogenesis of CML, certainly the therapy of CML is focusing on the specific protein inhibitors [10] such as imatinib mesylate (IM), which has become the standard therapy in CML, practically all patients received IM therapy when first

diagnosed CML [11]. Despite standard-dose imatinib achieved remarkable results, still one third of patients do not have an acceptable prognosis [11]. And a large number of patients were resistant to IM therapy and relapsed, due to the point mutations in the kinase domain of *BCR-ABL* enzyme [12, 13]. Also if patients are in the progressive stage of CML, it is even more likely to be IM resistant [14]. The second generation of tyrosine kinase inhibitors (TKIs), including nilotinib, dasatinib, bosutinib, and ponatinib, still did not achieve ideal therapeutic efficacy [15–17]. Additionally, only 15% CML patients got transplantation due to various reasons [18]. Therefore new antitumor drugs or compounds are deeply needed to improve the therapeutic efficacy of CML.

In the past years, dissimilar treatment protocols have been attempted to ameliorate the treatment status, suppressing the proliferation, promoting apoptosis, leading to cell cycle arrest, inducing differentiation by compounds or natural products [19, 20]. At present, more than half of the antitumor drugs come from natural plants directly or indirectly [21]. Chinese medicines have been widely used in the field of cancer treatment with less side effects and low costs [22]. Xiao-Ai-Ping (XAP) injection is extracted from Marsdenia tenacissima, which is a traditional Chinese medicine, the Asclepiadaceous plant Marsdenia tenacissima (Roxb.)'s dried stems. Wight et Arn. Pharmacology studies have revealed that Marsdenia tenacissima and its derivatives induced hematologic neoplasm cells apoptosis [23]. However the effective pharmacological ingredients and associated mechanisms have not been clarified.

In this study, we examined the efficacy and mechanism of XAP on CML cell line. The results showed that XAP inhibited proliferation, induced apoptosis and inhibited migration on K562 cells and peripheral blood mononuclear cell (PBMCs) from CML patients, compared with control groups. Further we found that Wnt/β-catenin signaling pathway played a key role in XAP's function on K562 cells. These findings indicate the potential therapeutic efficacy of XAP on CML through down-regulation of β-catenin and SDF1/CXCR-4 signaling.

RESULTS

XAP reduced the viability and promoted the apoptosis of K562 cells

To investigate the effects of XAP on growth of CML cells, we firstly performed CCK8 assays by using K562 cells (CML cell line). Cultured K562 cells were incubated for 24 h with increasing concentrations (from 0 to 256 μl/ml) of XAP. Cell viability (relative to control) and IC50 were measured by CCK-8 assays. As shown in Figure 1A, XAP significantly reduced the cell viability of K562 cells in a dose-dependent manner. IC50 value of XAP for 24 h of K562 cells was 31.77 μl/ml. XAP also inhibited the cell viability of K562 cells in a time-dependent manner (for 24 h, 48 h and 72 h) (Figure 1B).These results suggest that XAP could reduce the viability of K562 cells both in dose and time-dependent manners.

To further examine whether XAP affect the cell survival of K562 cells, we performed the flow cytometry assays. K562 cells were treated with 0–24 μl/ml XAP for 24 h, and then cells were stained with Annexin V and propidium iodide (PI) and analyzed by flow cytometry assays. As shown in Figure 1C–1G, different stages of cell death were distinguished: normal cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-), late apoptotic cells (Annexin V^-/PI^+), and cell debris (Annexin V^+/PI^+). XAP treatment significantly increased the number of apoptotic cells (early apoptotic cells plus late apoptotic cells) in a dose-dependent manner (Figure 1C–1G). The quantitative analysis revealed that the percentage of apoptotic cells was 4.29±1.48, 10.93 ± 1.95, 21.64 ± 2.05, 42.61±1.38, 54.69 ± 3.85, when treated with, 0 μl/ml, 6 μl/ml, 12 μl/ml, 24 μl/ml and 48 μl/ml of XAP, respectively (Figure 1H) .

To further confirm the increase of apoptosis of K562 cells induced by XAP, the expression level of apoptosis proteins was detected by western blot after XAP treatment. As shown in Figure 2A, 2C–2E, the pro-apoptotic protein Bax, c-PARP and c-Caspase-3 were significantly up-regulated in a dose-dependent manner, whereas, the expression levels of inhibiting apoptosis protein Bcl-2 was significantly down-regulated by XAP treatment, compared with control group (Figure 2B, 2F). To further study the mechanism of apoptosis induced by XAP, western blot was conducted to detected the expression of mitochondria - associated proteins. The results showed that the expression of Ndufs1, CLPP, COXⅣ were both down-regulated significantly (Figure 2C–2E, Supplementary Figure 1A), while the c-Caspase-9 expression level was up-regulated obviously (Supplementary Figure 1A, 1B). These results suggest that XAP could induce the apoptosis of K562 cells via the mitochondrial pathway.

XAP inhibited the migration of K562 cells through downregulating the expression levels of chemokine SDF-1 and its receptor CXCR-4

To further test the effects of XAP on the migration of K562 cells, transwell chamber migration assays were performed. In this assay, K562 cells were allowed to migrate for 12 h in the presence or absence of XAP in the lower chamber. As shown in Figure 3A–3D, with the concentrations of XAP increasing, the number of K562 cells migrating through the transwell chamber was significantly decreased (Figure 3E). The chemokine SDF-1 and its receptor CXCR-4 contribute to stem cell homing and play a role in the migration of leukemic cells [24]. To examine whether XAP could inhibit the migration of K562 cells through regulating the expression levels of

SDF-1 and CXCR-4 in K562 cells, western blot assay was performed after XAP treatment. As shown in Figure 3F–3I, XAP treatment significantly reduced the expression level of SDF-1 and CXCR-4 in K562 cells in a dose-dependent manner. These results suggest that XAP may down-regulate the expression of SDF-1 and CXCR-4, then regulate the homing and migration of CML cells.

XAP down-regulated Wnt/β-catenin signaling pathway in K562 cells

How does XAP induce apoptosis of K562 cells? Since Wnt/β-catenin signaling pathway mediates the apoptosis of cells [25], we next examined whether XAP induced the apoptosis of K562 cells through this pathway. As shown in Figure 4A, 4B, β-catenin protein level was significantly down-regulated in K562 cells in a dose-dependent manner after XAP treatment, whereas the inhibitor of β-catenin upstream signaling, the expression levels of p-GSK-3β (phospho-glycogen synthase kinase-3β) and GSK-3β (glycogen synthasekinase-3β) protein were significantly increased in K562 cells in a dose-dependent manner (Figure 4A, 4C, 4D). We also detected the expression of downstream protein of β-catenin signaling, CyclinD1. Western blot results showed that after treated with XAP, CyclinD1 protein was also significantly down-regulated (Figure 4A, 4E). The message RNA (mRNA) expression levels of Wnt/β-catenin signaling pathway were also detected. The results showed mRNA levels of β-catenin and CyclinD1 were significantly decreased in K562 cells after XAP treatment (Figure 4F, 4H), while mRNA level of GSK-3β was

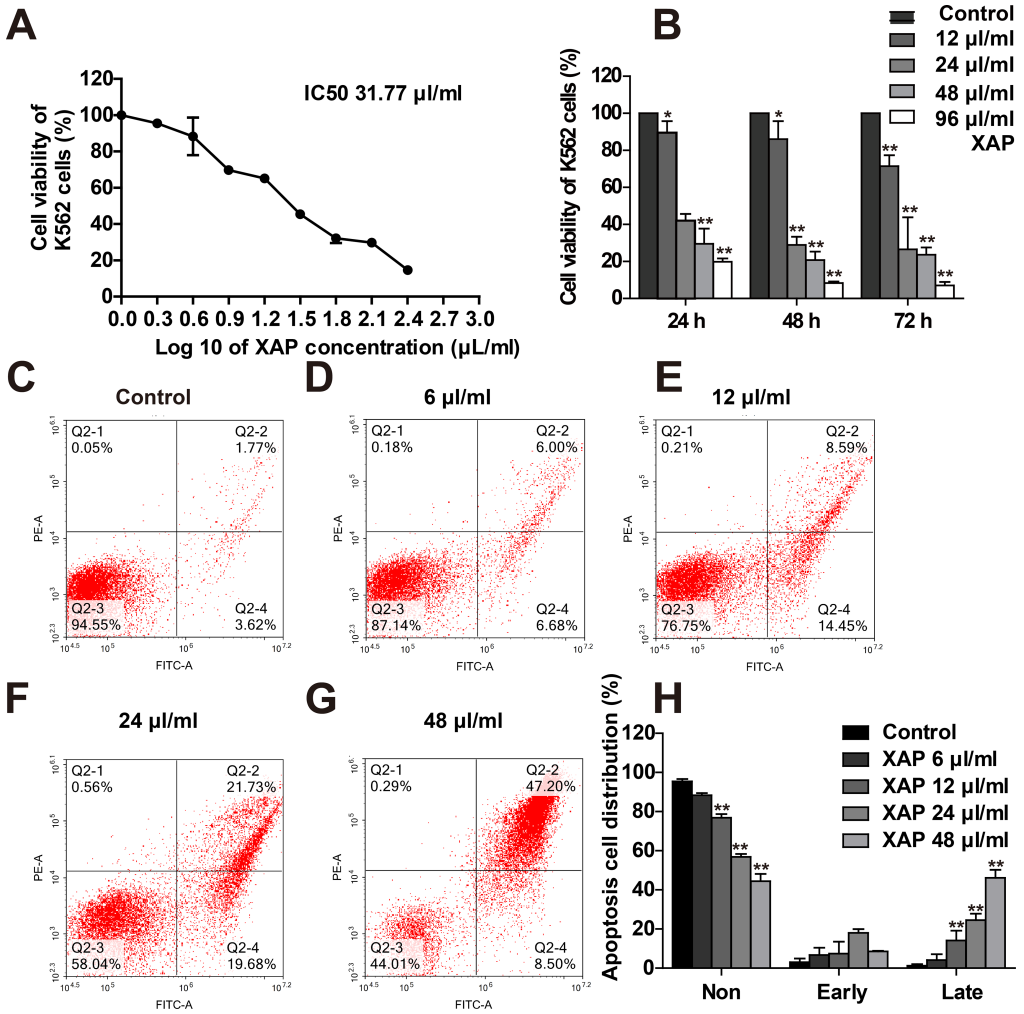

Figure 1: Xiao-Ai-Ping (XAP) treatment reduced the viability and promoted the apoptosis of K562 cells. (**A**) CCK8 assays detected the dose-dependent inhibition of K562 cell viability by XAP treatment after 24 h. The results were shown as a feature of μl drug concentrations (log scale). The IC50 value was counted by SPSS21 software. (**B**) CCK8 assays detected time-dependent inhibition of K562 cell viability. K562 cells were treated with XAP (the concentration of XAP from 0 to 96 μl/ml) after 24 h, 48 h and 72 h. **(C-G).** Flow cytometric analysis were performed on K562 cells after 24 h of XAP treatment at a growing concentrations range (from 0 to 48 μl/ml) with AnnexinV-FITC/PI double-staining. (**H**)**.** The apoptosis rates of K562 cells with ascending concentrations of XAP were analyzed quantitatively shown in C-G. Data were represented as mean ± SD of three independent trails. Student's *t*-test, compared with control, $^{*}P < 0.05$ and $^{**}P < 0.01$. The control was treated with RPMI 1640.

Table 1: Primer sequences of β-catenin, cyclin D1, GSK-3β, GAPDH

Gene	Forward	Reverse
β-catenin	GCCAAGTGGGTGGTATAGAGG	GCGGGACAAAGGGCAAGA
GSK3β	AGACGCTCCCTGTGATTTATG	AACAAGAGGTTCTGCGGTTT
cyclinD1	AGGCGGAGGAGAACAAACAG	TGAGGCGGTAGTAGGACAGGA
GAPDH	CATCAATGGAAATCCCATCA	GACTCCACGACGTACTCAGC

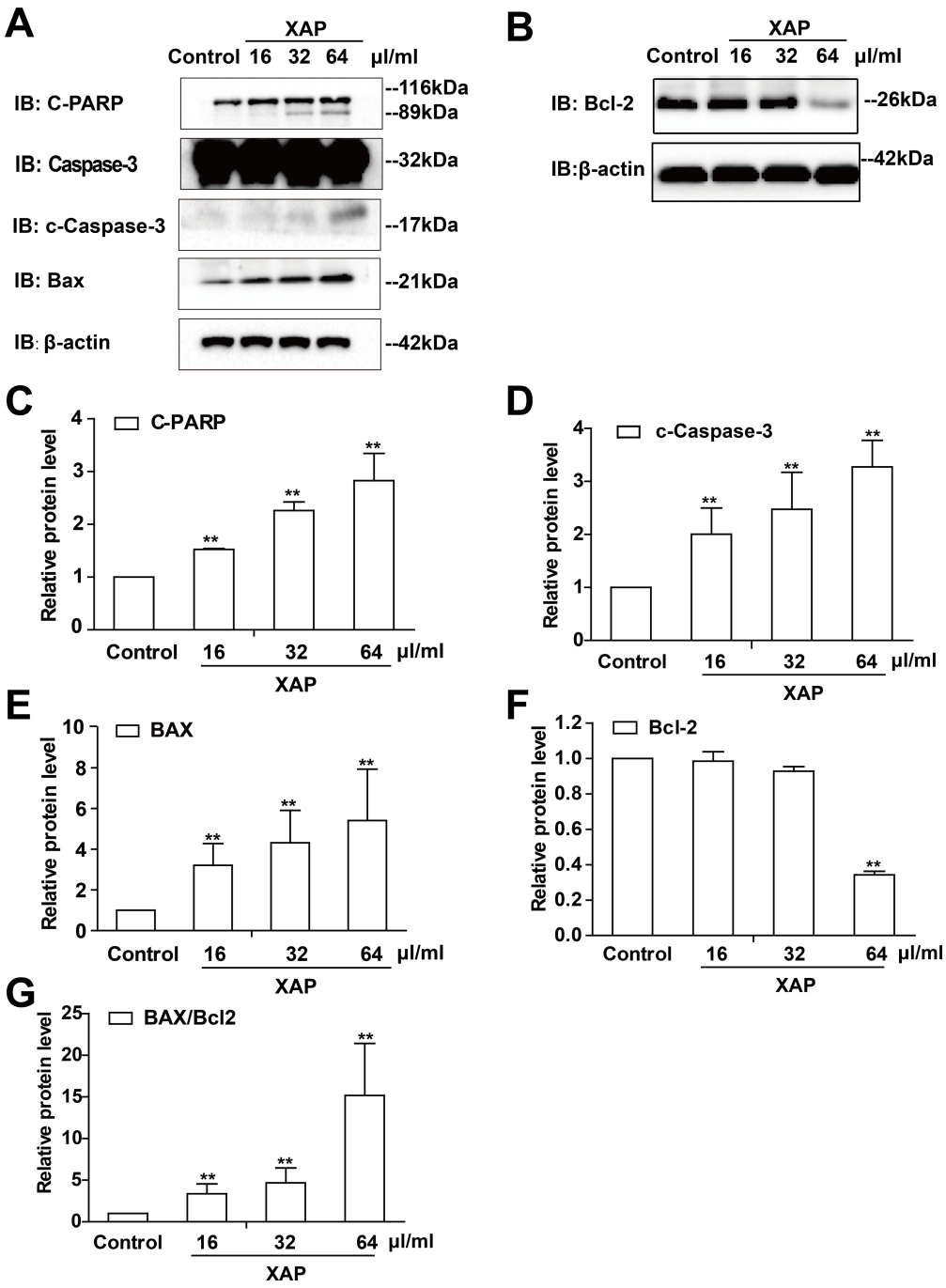

Figure 2: Effects of XAP treatment on the expression pattern of apoptosis-related downstream proteins in K562 cells. A–B. Western blot detected the expression of cleaved-PARP (c-PARP), cleaved caspase-3 (c-Caspase-3) and Bax (**A**), Bcl-2 (**B**) in K562 cells. Cells were treated with various concentrations of XAP (from 0 to 64 μl/ml) after 24 h. (**C-G):** The relative expression of the proteins described above (shown in A-B) and Bax/Bcl2 ratio were analyzed quantitatively. Data were represented as mean ± SD of three independent trails. Student's *t*-test, compared with control, $^{*}P < 0.05$ and $^{**}P < 0.01$. The control was treated with RPMI 1640.

significantly increased after XAP treatment (Figure 4G). Taken together, these results suggest that XAP treatment down-regulated Wnt/β-catenin signaling pathway in K562 cells in the mRNA levels, which might mediate the XAP-induced apoptosis of K562 cells.

Over-expression of β-catenin rescued the apoptosis of K562 cells induced by XAP treatment

To further examine whether down-regulating of Wnt/β-catenin signaling mediated the apoptosis of K562 cells induced by XAP, we constructed β-catenin plasmid (blank PV vector) and transfected these plasmids into K562 cells to rescue the down-regulation of β-catenin by XAP. There was no significant difference between blank plasmid group and β-catenin plasmid group in apoptosis of K562 cells (Figure 5A, 5B, 5E). However, after XAP (30 μl/ml) treatment, as shown in the Figure 5C, 5D, 5E, flow cytometric analysis revealed that overexpression of β-catenin in K562 cells, indeed significantly restored XAP-induced apoptosis of K562 cells, compared with control group (blank constructs). Furthermore, western blot further revealed that 24 h after XAP (30 μl/ml) treatment, overexpression of β-catenin in

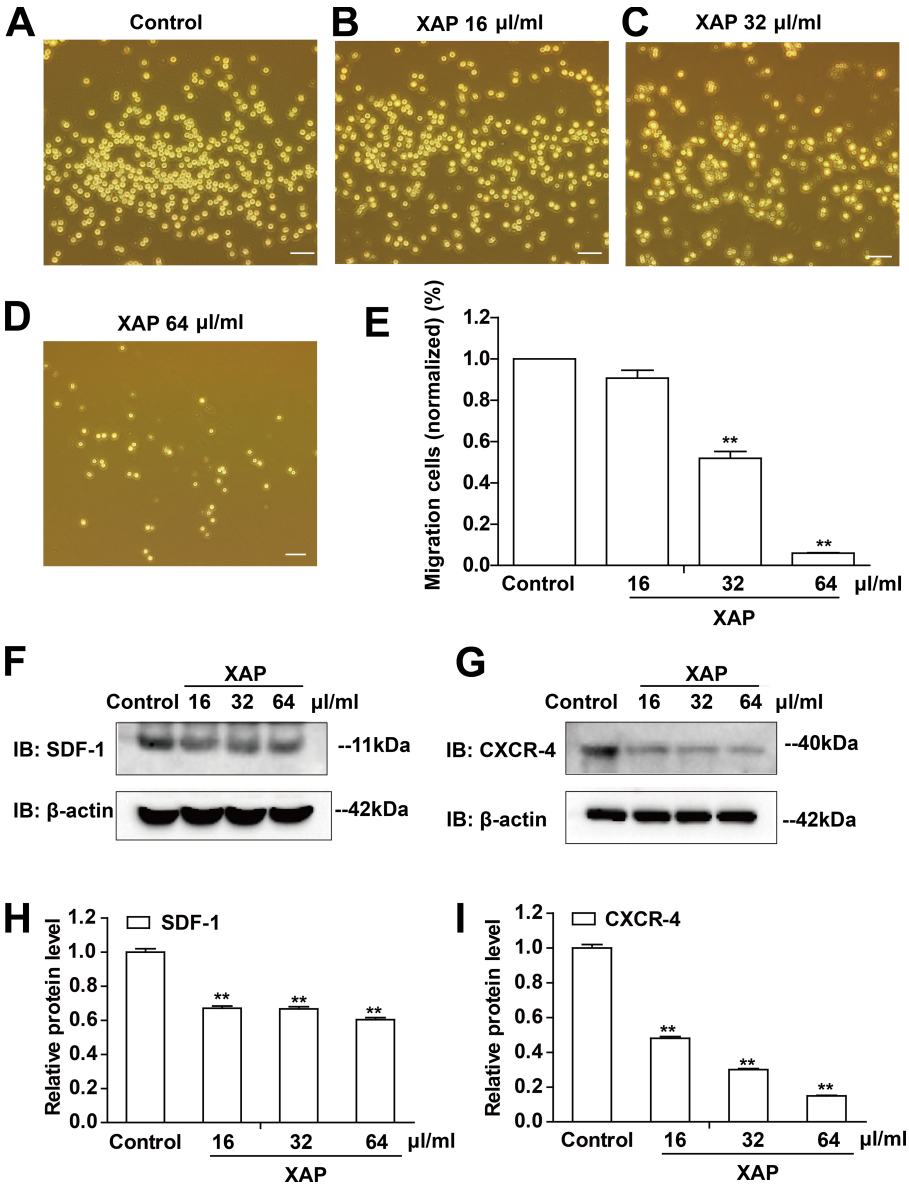

Figure 3: XAP treatment inhibited the migration of K562 cells. (**A-D**). Typical images of K562 cells treated by 0, 16, 32 and 64 μl/ml, respectively for 12 h in the transwell migration assay. (**E**) Quantitative analysis of the number of cells through the transwell membrane. K562 cells were treated with a growing concentration of XAP (from 0 to 64 μl/ml) after 12 h. (**F-G**). Western blot detected the expression of the chemokine SDF-1 and its receptor CXCR-4 proteins in K562 cells treated by different XAP concentrations. (**H-I**) Quantitative analysis of the relative SDF-1 and CXCR-4 proteins expression levels. Data were represented as mean ± SD of three independent trails. Student's *t*-test, compared with control, $^{*}P < 0.05$ and $^{**}P < 0.01$. Control was treated with RPMI 1640.

K562 cells, indeed significantly restored XAP-induced increase of Bax and decrease of Bcl2, compared with the blank plasmid transfection (Figure 6A, 6C–6E). Moreover, western blots also showed that the expression levels of other Wnt signaling pathway proteins, such as CyclinD1, p-GSK3β and GSK-3β, were significantly restored in XAP (30 μl/ml)-treated K562 cells by the overexpression of β-catenin, compared with blank construct (Figure 6B, 6F–6I). Taken together, these results suggest that over-expression of β-catenin rescued the apoptosis of K562 cells induced by XAP treatment.

XAP reduced the viability and promoted the apoptosis in PBMCs from CML patients

To examine whether XAP has the similar effects on CML cells, PBMCs from 4 different CML patients and 4 different healthy people were collected and treated with XAP (30 μl/ml). As shown in Figure 7B, 7E, 7F and 7H, XAP could significantly reduce the viability and increase the apoptosis of PBMCs from CML patients, compared with healthy people (Figure 7A, 7C, 7D and 7G) after XAP (30 μl/ml) treatment. These results suggest that

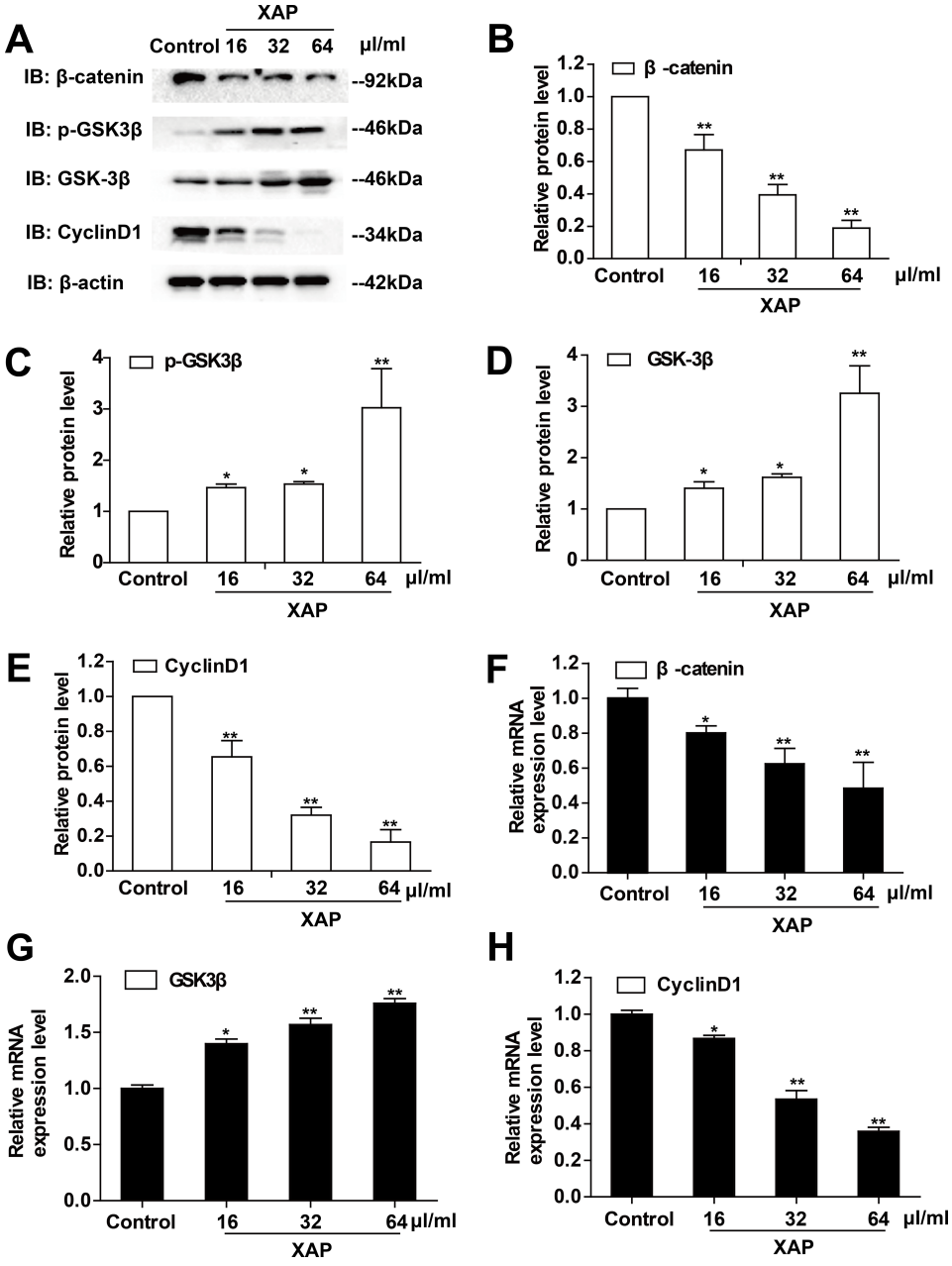

Figure 4: XAP treatment down-regulated Wnt/β-catenin signaling pathways in K562 cells. (**A**) Western blot detected the expression levels of Wnt signaling pathway proteins (β-catenin, p-GSK3β, GSK3β, CyclinD1) in K562 cells under XAP concentrations. (**B-E**) Quantitative analysis the above proteins relative expression levels as shown in (A). **(F-H)** RT-PCR detected β-catenin, GSK3β and CyclinD1 mRNA expression levels and quantitative analysis the relative expression. Data were represented as mean ± SD of three independent trails. Student's *t*-test, compared with control, $^{*}P < 0.05$ and $^{**}P < 0.01$. Control was treated with RPMI 1640.

effects of XAP on CML patients were similar to the effects of XAP on K562 cells. In summary, XAP might promote the apoptosis of CML patients PBMCs, suggesting the XAP might be a promising antitumor drug in curing CML.

DISCUSSION

In the present study we revealed that XAP suppressed the proliferation and promoted apoptosis of CML cells (K562 cell line) through inhibiting the Wnt/β-catenin signaling pathway. We presented a potential therapeutic efficacy of XAP on CML cells. This study not only defines the XAP's function in anti-CML cells being a valuable adjuvant therapy for CML therapy but also identifies a novel Wnt/β-catenin signaling pathway related to XAP for the effects of XAP on CML cells.

Wnt/β-catenin signaling pathway is well known to get involved in almost every aspects of tissues development and cellular homeostasis [26]. Wnt/β-catenin signaling pathway includes Wnt (WNT family secretory protein), frizzled (Curl family transmembrane receptor), GSK-3β (glycogen synthase kinase-3, negative regulatory kinases), β-catenin (a cytoplasmic protein) and other proteins. Wnt ligands combine with receptor complexes which are made up by frizzled and LRP5/6 (low density lipoprotein receptor related protein 5/6) proteins. Then the complexes phosphorylate Dsh (dishevelled protein locates in cell membranes) and combine with Axin (tumor suppressor protein), leading to β-catenin

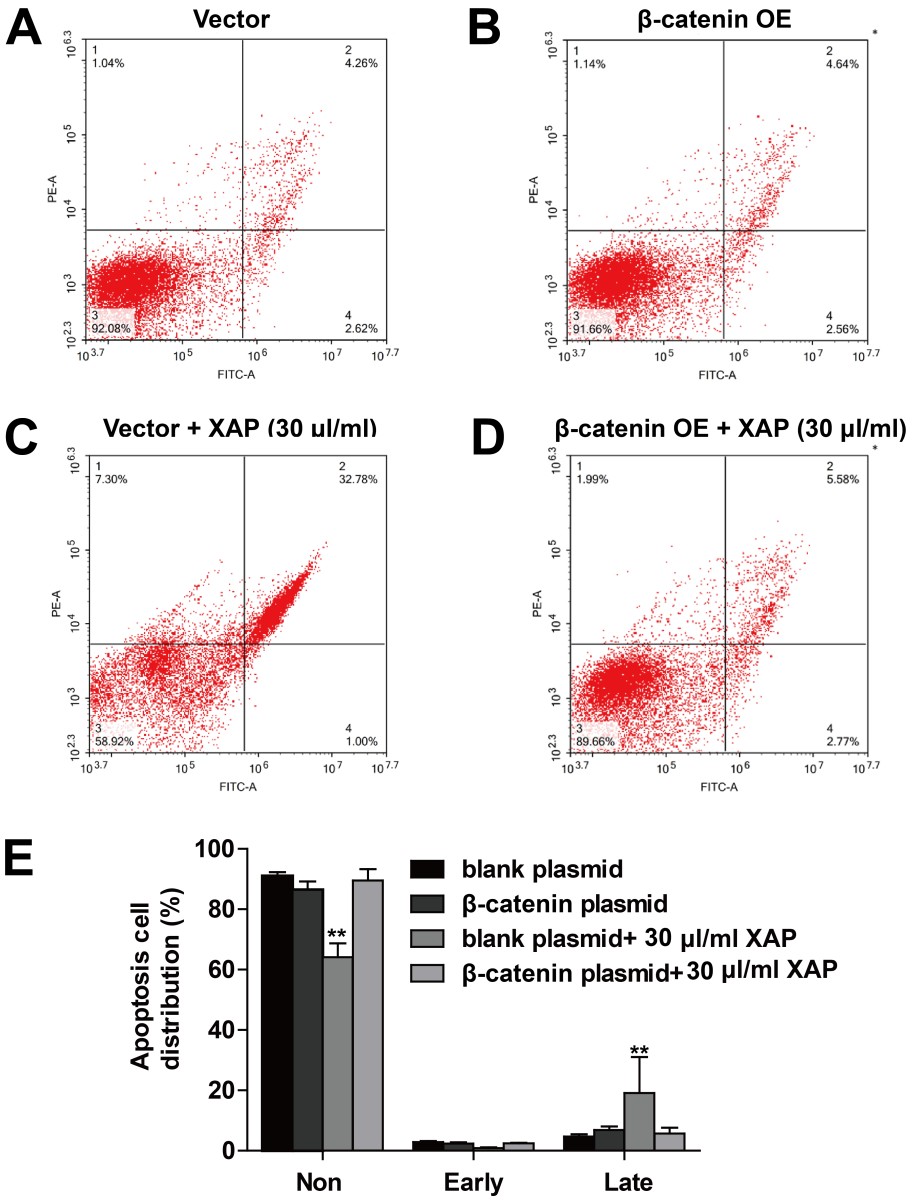

Figure 5: Over-expression of β-catenin rescued the apoptosis of K562 cells induced by XAP treatment. (**A-D**). Flow cytometric analysis by AnnexinV-FITC/PI double-staining were performed on K562 cells transfected with blank PV vector and the β-catenin plasmid, treated with XAP (30 μl/ml) for 48 h. (**E**) The quantitative analysis of the apoptosis rate of K562 cells as shown in E. Student's *t*-test, compared with control (blank PV vector), $^{*}P < 0.05$ and $^{**}P < 0.01$. OE: over-expression.

destruction complex building up by GSK-3β、Axin and APC (adenomatous polyposis coli) disintegration. β-catenin is fail to phosphorylate and degrade. The un-phosphorylated β-catenin accumulates in cytoplasm then translocates to the nucleus, integrateing with TCF/LEF (T cell factor/lymphoid enhance factor) and activating downstream events, such as CyclinD1, c-myc [27, 28].

The continuous activation of Wnt/β-catenin signaling pathway is a key factor for tumorigenesis [29–31]. It also plays a key role in self-renewal of HSCs, accelerating CML progression and inducing *BCR-ABL* kinase-independent resistance [32–45]. The controversies about the important roles of Wnt/β-catenin signaling pathway in aspect of LSCs self-renewal have been thoroughly introduced [46]. Studies showed that when β-catenin was inhibited in mouse hematopoietic cells, the leukemic transformation mediated by *BCR-ABL* were significantly decreased [34, 47]. Combinatorial strategy (Combined inhibition of *BCR-ABL* and β-catenin) showed better effect in CML therapy, even in TKI-resistant blast crisis (BC)-CML [48]. In contrast, Wnt/β-catenin signaling pathway was activated once, progenitor cells resume self-renewal ability, further resulting in tumor development, recrudescence and drugs resistance [49–51]. The current academic points of reasons leading to *BCR-ABL* kinase-independent resistance are mainly focused on two aspects:

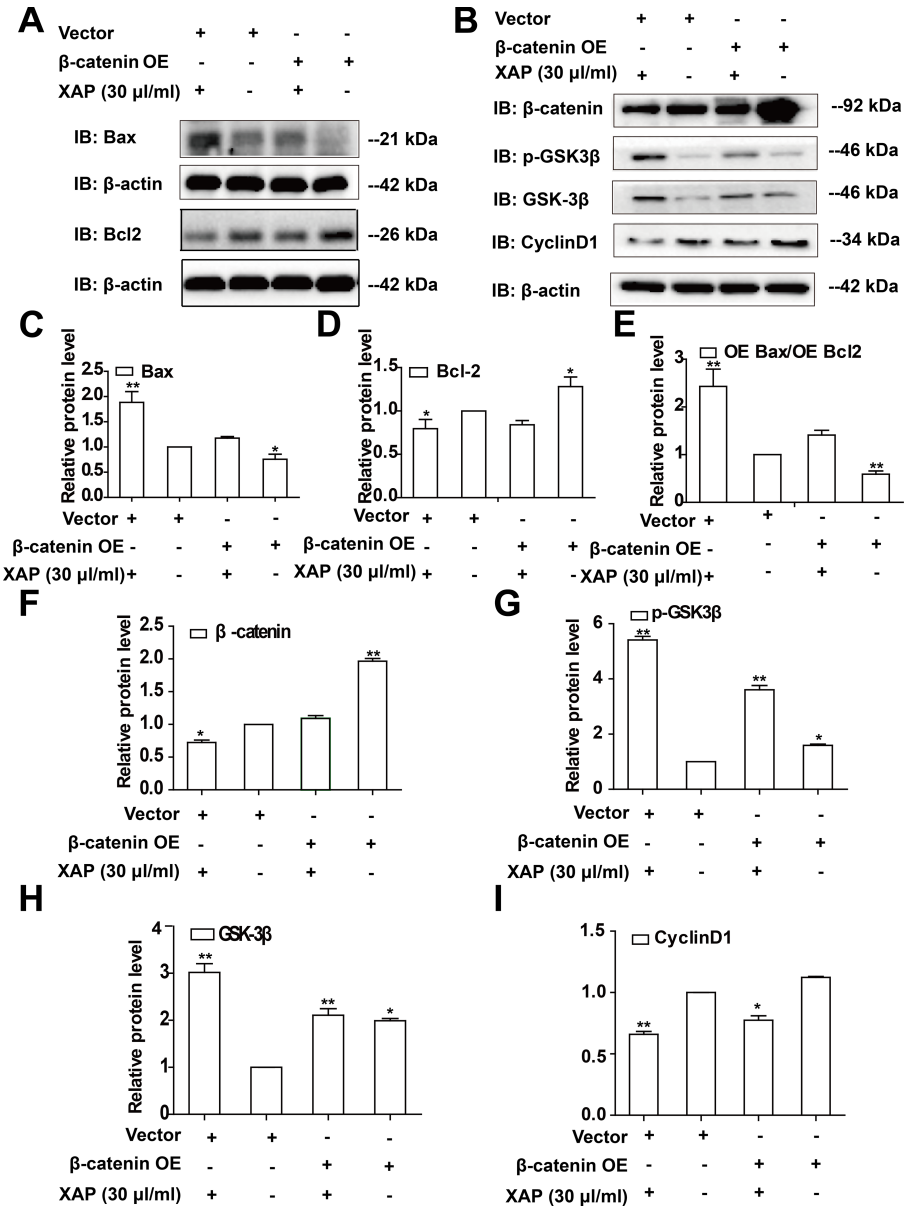

Figure 6: Over-expression of β-catenin rescued the apoptosis of K562 cells and inactivation of downstream of Wnt/β-catenin signaling in K562 cells induced by XAP treatment. (**A**) Western blot detected the expression level of Bax and Bcl2 proteins after blank PV vector and the β-catenin plasmid transfected. (**B**) Wnt signaling pathway proteins (β-actenin, p-GSK3β, GSK3β and CyclinD1) were detected. (**C–I**). Quantitative analysis of the relative protein expression levels. Data were represented as mean ± SD of three independent trails. Student's *t*-test, compared with control (the blank plasmid). $^{*}P < 0.05$ and $^{**}P < 0.01$. OE: over-expression.

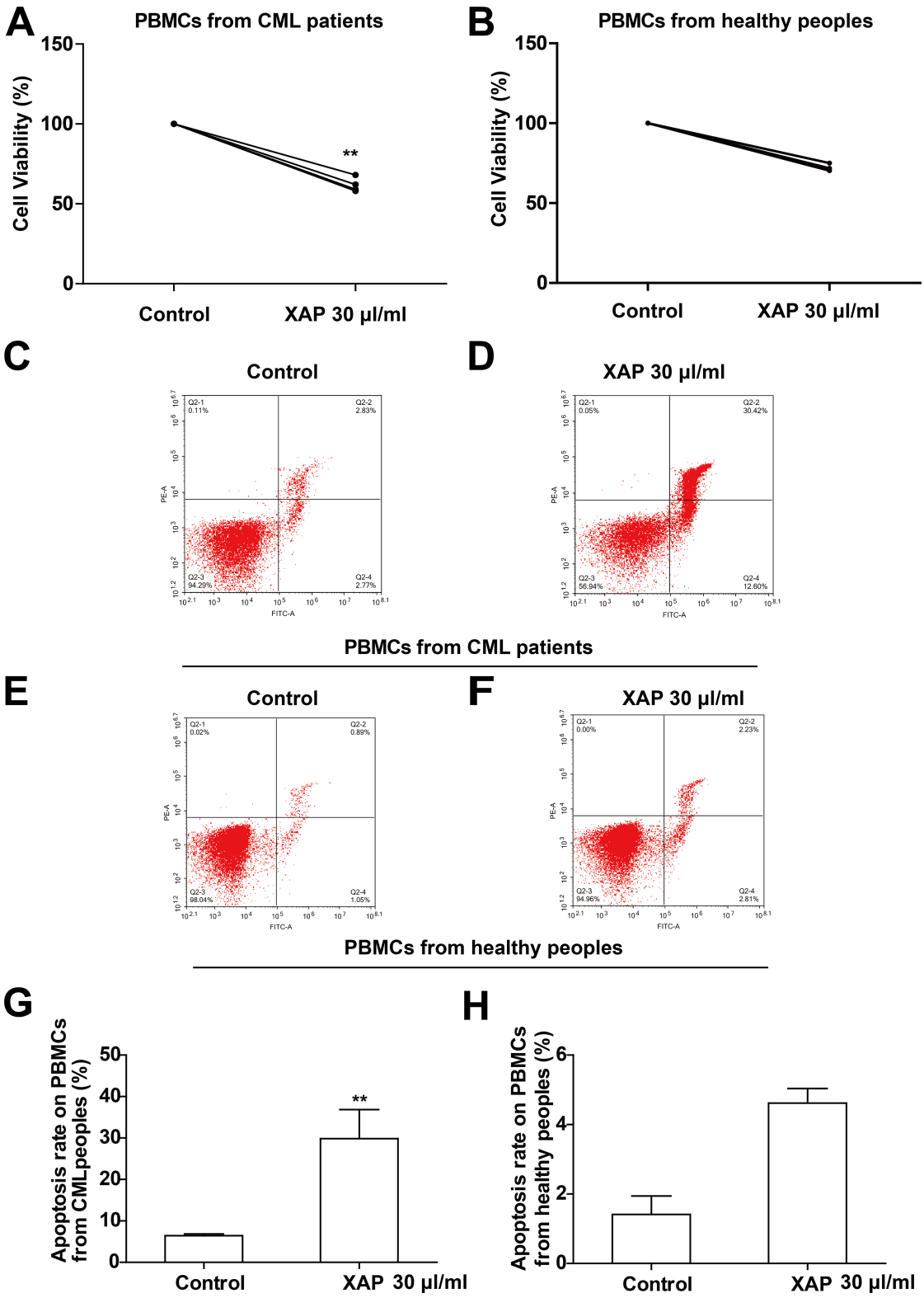

Figure 7: XAP reduced the viability and promoted the apoptosis of PBMCs from CML patients. (**A-B**) CCK8 assays were performed on PBMCs from healthy people (A) ($n = 4$) or CML patients (B) ($n = 4$) treated by XAP for 4 h. (**C–D**) Flow cytometric analysis were performed on PBMCs from healthy people after 4 h of control and XAP treatment at 30 μl/ml with AnnexinV-FITC/PI double-staining. (**E–F**) Flow cytometric analysis were performed on PBMCs from CML patients after 4 h of control and XAP treatment at 30 μl/ml with AnnexinV-FITC/PI double-staining. (**G–H**) Quantitative analysis of the apoptotic percentage of PBMCs from healthy people and CML patients, respectively. Data were represented as mean ± SD ($^{*}p < 0.05$ and $^{**}p < 0.01$). Student's *t*-test, compared with control, $*P < 0.05$ and $**P < 0.01$. Control was treated with RPMI 1640.

activation of signaling pathways or extrinsic bone marrow (BM) microenvironmental conditions [42, 43, 45, 52]. In addition, nuclear β-catenin leads to cells TKI resistance, but not BM-caused TKI resistance [45].

Based on above evidences, inactivated the Wnt/β-catenin signaling pathway and stabilization β-catenin in the cytoplasm are the key steps in CML therapy. In our study, we demonstrated that XAP promoted apoptosis and suppressed proliferation of K562 cells and PBMCs from CML patients, through antagonizing the Wnt/β-catenin signaling pathway. We found that XAP treatment could down-regulate the expression of β-catenin, and up-regulate the GSK-3β and p-GSK3β level, further inactivate CyclinD1. In addition, over-expression of β-catenin by transfection, restored XAP-induced apoptosis of K562 cells. These consequences intensely revealed that XAP could mediate the Wnt/β-catenin signaling pathway and induce K562 cells apoptosis.

Beyond this, literatures revealed that other signaling pathways have been found involved in CML. In our study, we demonstrated that PTEN/PI3K/AKT signaling pathway and SDF1/CXCR4 axis were involved in XAP-treated K562 cells (some of data not shown). Some studies showed the crosstalk between Wnt/β-catenin and PI3K/AKT signaling pathways in other tumors [53, 54]. And the key factor between two signaling is GSK-3β. PI3K/AKT activation leads to GSK-3β phosphorylation, suppressed GSK-3β activity [55–57], and inhibited β-catenin degradation. However, the detailed relationships between two signaling pathways are still need further examinations in future.

In addition, XAP could also inhibit the activity of SDF1/CXCR4 axis, then further affect K562 cells migration. Chemokines are a family of small proinflammatory chemoattractant proteins that take part in many aspects of homeostatic process. SDF1/CXCR4 mediates lymphocytes homing to secondary lymphoid organs, and is closely related to tumor process including metastasis, angiogenesis, and survival of tumor progression [58, 59]. SDF1, a homeostatic chemokine and the ligand of CXCR4, is produced by marrow-, lymph nodes-, muscle- and other types of cells [60]. CXCR4, is highly expressed on the membrane of normal hematopoietic cells and acute myeloid leukemic $CD34^+$ cells, $CD34^+38^-$ cells were also included [61], has been found to regulate the $CD34^+$ stem cells homing to marrow microenvironment [62]. At present, the IM resistance is a big challenge for CML therapy, even patients get complete responses after IM therapy, the quiescent original cell still remain and resist to IM [63]. At present, the small molecule antagonists and blocking antibodies targeting to CXCR4, are still in the clinical trial stages [64, 65]. The important role of SDF1/CXCR4 axis is well recognized in many kinds of hematologic malignancies [66–68], however the exact connection with CML is still need to be further explored. Our research provided a certain basis for the link between SDF1/CXCR4 axis and CML to a certain extent.

In Wnt signaling pathway, *GSK-3*β plays an important role in coordinating signal input, the key function is the phosphorylation and stability of β-catenin [69, 70]. Meanwhile, studies have revealed that when inhibited *GSK-3*β, the expression level of CXCR4 was also increased, then enhanced the mesenchymal stem cells (MSCs) migration [71, 72]. Simultaneously, when co-cultivate the CML cells and MSCs, IM could restore CXCR4 expression and further influence the CML cells migration to bone marrow stromal cells (BMSCs). This is closely related to the mechanism of CML relapse [73]. The crosstalk between Wnt pathway and SDF1/CXCR4 axis is focused on *GSK-3*β. So it is obvious that *GSK-3*β plays an important role in regulating SDF1/CXCR4 axis and Wnt pathway. However the definite connection between two pathways and the link with CML still need further explored. Our results showed that XAP reduced K562 cells migration significantly and restored SDF1/CXCR4 expression. According to the references and results of experiments, XAP might play an important role in reducing recurrence/metastasis of patients who received IM therapy.

In this study, we mainly aimed at canonical Wnt pathway. Although previous studies have found that besides the canonical Wnt pathway, the non-canonical Wnt-pathway might also involve in the pathogenesis of CML [74]. Here we found that XAP could suppress the proliferation and promote the apoptosis of K562 cells, through inhibiting Wnt/β-catenin signaling pathway. Our studies represented a novel strategy for CML therapy, and the underlying mechanism.

MATERIALS AND METHODS

XAP injection preparation

XAP injection (Crude drug concentration 5g/ml) was purchased from Sanhome Pharmaceutical Co., Ltd (Nanjing, China) and stored at 4°C. *In vitro* experiments, XAP was diluted in RPMI 1640 (from Gibco, Carlsbad, CA, USA).

Cell lines and culture conditions

Human chronic myelogenous leukemia cell line K562 were purchased from cell bank of the Chinese Academy of Science (Shanghai, China) and cultured in RPMI 1640 containing with 10% fetal bovine serum (FBS) (from Gibco, Carlsbad, CA, USA) and 100 U/ml of penicillin-streptomycin at 37°C saturated humidity containing 5% CO_2. Logarithmically growing cells (4–6 × 10^5 cells/ml) were used to perform following experiments.

PBMCs were collected from CML patients, on the basis of obtaining patients' Informed Consent Form. Then

apply Ficoll (TBD, HaoYang, Tianjin, China) to extract PBMCs. 4 diagnosed CML patients whose original cells in peripheral blood accounted for more than 70% from Zhejiang Provincial People's Hospital (Oct, 2016-Feb, 2017) and 4 healthy people were accepted with Informed Consent. Collect 4 ml anti-coagulated blood from every people. Take anti-coagulated blood and mix well with the same amount of PBS solution, then was layered onto 4 ml Ficoll-Paque. After centrifuge 2000 rpm × 20 min, liquid was divided into three layers and middle layer was collected and diluted with 20 ml RPMI 1640, then centrifuge 2000 rpm for another 10 min. Discard the supernatant and re-suspended PBMCs and cultured in RPMI 1640 containing with 10% fetal bovine serum (FBS). Exposed cells to XAP (30 μl/ml) treatment. The control group cells were cultured with RPMI 1640.

Proliferation assay (CCK-8 assay)

The effects of XAP on viability and proliferation of K562 cells were assessed by CCK8 colorimetric assays according to the previous described [75]. Briefly, seed exponentially growing K562 cells in 96-well plates at a density of 2.0×10^4 cells/well in 100 μl RPMI 1640 medium then exposed to XAP with concentrations of 0 (control group cells were treated with RPMI 1640), 12, 24, 48 and 96 μl/ml for 24, 48 and 72 h, respectively. Each group included 3 parallel wells, then added CCK-8 to each well. After 2 h incubation, the absorbance was measured at 450 nm using a micro-plate reader (BIOTEK, Vermont, USA). Cell viability was calculated by this: Cell viability (%) = (OD treatment-OD blank)/(OD control-OD blank).

Cell apoptosis assay

AnnexinV/PI assay K562 cells were exposed for 24 h to various concentrations of XAP, the control cells were also treated with RPMI 1640. After treatment, cells were washed by PBS twice, then cells underwent AnnexinV/PI double staining for cell death assays (Beyotime, Haimen, China) according to manufacturer's protocol. The percentages of early and late stages of apoptosis cells were measured by flow cytometry (ACEA NovoCyte, USA).

Migration analysis

K562 cells were cultivated with XAP at 16, 32, 64 μl/ml, respectively for 12 h, the control cells were treated with RPMI 1640 as usual. Treated cells were washed by PBS twice, then 5×10^4 cells were resuspended with 200 μl serum-free medium and seeded in transwells (purchased from Corning, NY, USA). Each transwell was put in 24-well plate contained with 400 μl RPMI 1640 medium supplement with 10% FBS. After 12 h, the migrated cells were observed by light microscope.

Western blot analysis

K562 cells were treated with XAP at 16, 32 and 64 μl/ml for 24 h, respectively, the control cells were also treated with RPMI 1640, after treatment, then tumor cells were washed by PBS for twice. Remove supernatant and cells were lysed with RIPA added PMSF (Beyotime, Haimen, China) for 30 min at 4°C. After this, the concentration of harvested proteins were measured by protein assay kit (Thermo). Next, the same amount of proteins were collected and separated by 12% SDS-polyacrylamide gel electrophoresis. Then proteins were transferred to polyvinylidene fluoride (PVDF) membranes (ImmunobilinP; Millipore) then used 10% skim milk to block proteins for 1 h. The blots were detected with primary antibodies against β-actin (1:1000, abcam, USA), Bax (1:1000, Cell Signaling Technology, USA), Bcl2 (1:1000, Cell Signaling Technology, USA), c-Caspase-3 (1:1000, abcam, USA), cleaved-PARP (1:1000, Cell Signaling Technology, USA), β-catenin (1:1000, Cell Signaling Technology, USA), GSK-3β (1:1000, Cell Signaling Technology, USA), p-GSK3β (1:1000, Cell Signaling Technology, USA), CyclinD1 (1:1000, abcam, USA), PTEN (1:1000, Cell Signaling Technology, USA), AKT (1:1000, Cell Signaling Technology, USA), p-AKT (1:1000, Cell Signaling Technology, USA), PI3K (1:1000, Cell Signaling Technology, USA), SDF-1 (1:1000, abcam, USA), CXCR-4 (1:1000, abcam, USA), CD44 (1:1000, abcam, USA) at 4°C incubation overnight. Then, wash away the primary antibodies, the corresponding secondary antibodies were added (1:5000, Beyotime) for 1 h at room temperature, at last, the enhanced chemiluminescence (ECL) solution (Amersham Biosciences) was used to detect the target bands. The gray values of membrane signals were measured by Gel-Pro-Analyzer software (Bethesda, MD, USA) and the results were calculated. β-actin served as loading control.

Reverse transcription-PCR (RT-PCR) assay

K562 cells were cultured with XAP at 0 16 32 and 64 μl/ml for 24 h, respectively, the control cells were also treated with RPMI 1640, after XAP incubation, the mRNA from K562 cells was extracted (MagnaPure LC RNA Isolation Kit; Roche Applied Science) and underwent reverse transcription into cDNA according to Transcription High Fidelity cDNA Synthesis kit (Roche Applied Science) respectively following the corresponding manufacturer's instructions. With template DNA prepared, the parameter of amplification reaction was as follows: 3 min at 95˚C followed by 40 cycles for 10 sec at 95˚C, 30 sec at 60˚C. And primer pairs were as Table 1:

Cell transfection

β-catenin was constructed into PV vectors and was transfected into K562 cells with lipofectamine

3000 (Invitrogen, USA) following the manufacturer's instructions. 24 h after transfection, cells (1×10^6/ml) were treated with XAP (30 μl/ml) for 48 h. The apoptosis of cells count was determined by flow cytometry and some related proteins were detected by western blot.

Statistics

All data were presented as standard deviation (SD), results expressed from at least 3 independent experiments. Effects of difference between control and XAP treatments were analyzed by Student's *t*-test, performed by the SPSS statistics 21.0. $p < 0.05$ was defined as significant statistically.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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