

## Original Article

# Suppression of chronic lymphocytic leukemia progression by CXCR4 inhibitor WZ811

Shi Hui Li, Wen Chuan Dong, Li Fan, Guang Sheng Wang

Department of Oncology, Aviation General Hospital, No. 3 Beiyuan Road, Chaoyang District, Beijing 100012, China

Received May 28, 2016; Accepted July 30, 2016; Epub September 15, 2016; Published September 30, 2016

**Abstract:** CXCR4 is a chemokine and chemokine receptor pair playing critical roles in tumorigenesis. Overexpression of C-X-C chemokine receptor type 4 (CXCR4) is a hallmark of many hematological malignancies including acute myeloid leukemia, chronic lymphocytic leukemia and non-Hodgkin's lymphoma, and generally correlates with a poor prognosis. A highly potent competitive antagonist of CXCR4, WZ811, recently has been identified with suppression of cancer cells aggressive in a variety of cancers. However, the effects of WZ811 on chronic lymphocytic leukemia cells have not yet been defined. The effect of WZ811 on chronic lymphocytic leukemia cells TF-1 and UT-7 cells in proliferation, colony formation, and cell migration in vitro were measured respectively. Decreased in cell viability, colony formation, migration, and survival with cell cycle arrest and higher sensitivity to docetaxel in vitro was observed upon WZ811 treatment. In mouse xenograft models developed with human leukemia cells, WZ811 exhibited tumor growth inhibition. Collectively, we have demonstrated that CXCR4 inhibition by WZ811 has the potential for the treatment of human hematological malignancies. This study demonstrated that WZ811 may be a novel approach in the treatment of chronic lymphocytic leukemia.

**Keywords:** Chronic lymphocytic leukemia, CXCR4, WZ811, docetaxel

## Introduction

Chronic lymphocytic leukemia (CLL) is composed of cell cycle-arrested leukemic cells circulating in the blood and activated cells that are located in supportive zones in lymphoid organs, driven into proliferation by signals from the microenvironment [1]. The recirculating capacity of CLL cells does, therefore, contribute to clinical aggressiveness and key molecules involved in extravasation, such as chemokine receptors and integrins, may represent important prognostic markers and therapeutic targets. Controversial data have been reported on a prognostic value of the chemokine receptors C-X-C chemokine receptor type 3 (CXCR3) and CXCR4 in CLL [2]. Moreover, the complexity of the chemokine receptor network, with significant redundancy and cross-talk of receptors, e.g. via heterodimerization, represents a considerable hurdle in the development of chemokine-related drugs [3]. A better understanding of interactive chemokine receptor signals will help in more reliable prediction of responses to therapy.

Elevated CXCR4 expression was observed in in several different types of cancers including lung, kidney, brain, prostate, breast, ovarian, pancreas, and melanomas and supports tumor growth, metastasis, angiogenesis, and contributes to therapeutic resistance [4]. Besides in tumor cells, several studies have also identified increased expression of CXCR4 in cancer-associated fibroblasts (CAFs), which play an important role in tumorigenesis and have been implicated in neoplastic progression. Data from these studies suggest that soluble breast cancer factors initiate the trans-differentiation of normal human mammary fibroblasts to tumor-promoting CAFs through the induction of matrix metalloproteinase-1 (MMP-1) and CXCR4 expression [5]. In mouse models of human breast cancer and prostate cancer (PCa), high intratumoral C-X-C motif chemokine 12 (CXCL12) levels have been shown to attract CXCR4-positive inflammatory, vascular, and stromal cells into the tumors, where they eventually support tumor growth by secreting growth factors, chemokines, cytokines, and pro-angiogenic factors. In addition to contributing to the

tumor-stromal interactions, CXCR4 is also expressed on cancer stem-like cells and contributes to cancer recurrence [6]. Recent studies have shown the presence of a small subset of cancer cells, with very similar characteristics to stem cells, known as cancer stem cells (CSCs), which mediate tumor growth, metastasis, recurrence, as well as therapeutic resistance. CXCR4 expression in CSCs confers increased invasiveness and metastatic potential as well as improved self-renewal and survival capacity [7].

Similar observations were made in other hematological cancers such as chronic myeloid leukemia (CML), acute myelogenous leukemia (AML), and multiple myeloma (MM), where CXCR4 expression on cancer cells contributed to therapeutic resistance [8]. In CLL, CXCR4-expressing cancerous B-cells are attracted toward bone marrow stromal cells, which secrete high levels of CXCL12, resembling the homing of normal hematopoietic stem cells to bone marrow. Inhibition of CXCR4 was able to suppress cancer cells growth and metastasis, and studies on its mechanism mainly focused on protein kinase B (AKT) and mitogen-activated protein kinases (MAPK) signaling pathway [9]. Cancer cells are thought to hijack the chemokine CXCL12 and its specific receptor CXCR4 axis to establish distant organ metastasis. The CXCR4/CXCL12 axis plays a critical role in therapeutic resistance by (i) directly promoting cancer cell survival, invasion, and cancer stem (or tumor-initiating) cell phenotype; (ii) recruiting myeloid bone marrow-derived cells to indirectly facilitate tumor recurrence and metastasis; and (iii) promoting angiogenesis directly or in a paracrine manner [10]. CXCR4 contribute to the up-regulation of several factors involving in malignant tumor progression. Notably, the hypoxia-inducible factor (HIF)-1 $\alpha$ , growth factors such as basic fibroblast growth factor, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF), and transcription factors like nuclear respiratory factor-1 was positively up-regulate by CXCR4 activity [11].

Because CXCR4 is crucial for homing of tumor cells to the bone marrow microenvironment and drug resistance, CXCR4 antagonists have been explored as chemo-sensitizers in leukemia treatment [12]. In our study, our results showed that CXCR4 inhibitor WZ811 not only

inhibited cell proliferation, cell motility, cell survival, tumorigenic potential of chronic lymphocytic leukemia cells, and increased cell apoptosis and sensitivity to docetaxel, but also effectively block chronic lymphocytic leukemia cell growth in vivo. Taken together, WZ811 may be a novel approach to suppress chronic lymphocytic leukemia progression.

## Materials and methods

### *Cell lines and drugs*

The chronic lymphocytic leukemia cell lines (TF-1 and UT-7) were supplied by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, California, USA), penicillin (100 Units/mL) and streptomycin (100 mg/mL) (Life Technologies, Carlsbad, California, USA). All of the cells were grown in a humidified incubator at 37°C with 5% CO<sub>2</sub>. The CXCR4 inhibitor WZ811 was purchased from Selleck (New Jersey, USA), while the docetaxel was obtained from Meilun (Dalian, China).

### *Cell proliferation assay and colony formation assay*

For cell proliferation assay, cells were seeded in 96-well plates at  $2.0 \times 10^3$  cells/well in a final volume of 100  $\mu$ l and incubated overnight. The viability of cells was determined with CellTiter 96 non-radioactive cell proliferation assay (MTS) (Promega BioSciences, Madison, Wisconsin, USA) following the manufacturer's protocol [13]. For colony formation assay, cell suspension was mixed with 0.3% soft agar in growth media containing 10% FBS and layered in triplicate onto 0.6% solidified agar in growth media containing 10% FBS ( $1 \times 10^3$  cells/well). After 14 days culture, colonies containing 50 cells or more were counted under a microscope at  $\times 100$  magnification as previously described. The colonies were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and counted [14].

### *Cell apoptosis assay by flow cytometry*

Cell apoptosis was detected using annexin V-APC apoptosis detection kit (eBioscience, 88-8007-72) and measured with flow cytometry. In brief, cells were treated with WZ811 at

37°C for 24 h. After collection and washing with phosphate-buffered saline (PBS) buffer, cells were re-suspended with staining buffer at a final density of  $1 \times 10^6$ /ml. Then, 5  $\mu$ l annexin V-APC was added to 100  $\mu$ l cell suspensions and incubated at room temperature in the dark for 10 min. Finally, cells were analyzed with FACS Calibur (Becton-Dickinson, USA) to determine cell apoptosis profiles [15].

## Cell migration assay

Migration assay was performed by suspending cells in serum-free medium and seeded them into the upper chambers of Transwell (Corning). The lower chamber of each well was added with 600  $\mu$ l growth media with 40% FBS. After incubated at 37°C for 6 h, cells were fixed and stained with the non-migratory cells on the upper chamber were removed. Stained cells were visualized by light microscopy and counted in 5 random high-power fields [16].

## Cell cycle analysis

Cells were seeded in 6-well cell culture plates and treated with various agents for indicated time period. For cell cycle analysis, cells were detached with trypsin and washed with cold PBS. Precipitated cells were fixed by 500  $\mu$ l cold 70% ethanol overnight at -20°C. After being washed in PBS, fixed cells were then incubated with RNase at 37°C for 30 min and stained with propidium iodide (PI) for 15 min at room temperature in dark and immediately analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA). For cell apoptosis analysis, cells were detached with trypsin and washed with cold PBS. Resuspended cells in 500  $\mu$ l binding buffer were double stained with FITC-conjugated Annexin V and PI. After 15 min of incubation at room temperature in dark, samples were immediately analyzed by flow cytometry.

## DAPI staining

Apoptotic nuclear morphology was observed using DAPI staining. Cells were seeded in 2-well slides and treated with the specified concentrations of WZ811 for 24 h, after which the 2-well slides were washed with phosphate-buffered saline (PBS). Next, cells were fixed with 4% paraformaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI) staining solution [17].

The 2-well slides were washed with PBS and mounted on microscope slides with mounting solution. Stained cells were observed using fluorescence microscopy (Olympus, Tokyo, Japan).

## Chemo-resistance analysis

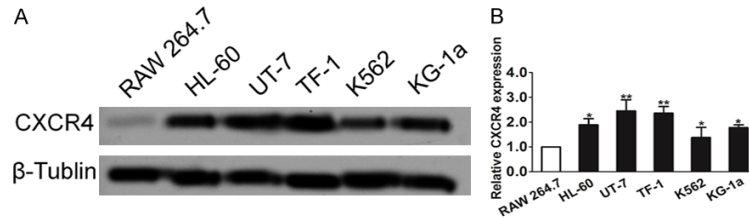
For chemo-sensitivity assay, cells were treated with a series of different concentrations of docetaxel or combination of WZ811 with docetaxel for 24 h. The cell viability was measured using the same method as MTS and the half inhibition concentration (IC50) were calculated by SPSS statistics software.

## Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated with TRIzol reagent (Invitrogen) and the cDNAs were synthesis with the reverse-transcription kit (Takara, Japan). The quantitative analysis was performed using the LightCycler® 480 SYBR Green I Master (Roche, Basel, Switzerland) on a LightCycler® 480 System (Roche) according to the manufacturer's instructions. The relative mRNA expression was calculated using the  $2^{-\Delta\Delta Ct}$  comparative CT method normalized to GAPDH and control. The primers used for PCR were as follows (sense and antisense, respectively): GAPDH: Forward primer: 5'-CGAGATCCCTCCAAATCAA-3' and Reverse primer: 5'-TTCACACCCATGACGAACAT-3'. CXCR4: Forward primer: 5'-AGGAAATGTTTTGGGAGGTTTG-3' and Reverse primer: 5'-TTTTGATTGAATGTGATTAGGG-3'. cDNAs amplification and relative expression values were obtained from three independent experiments.

## Western blot

Whole-cell lysates were prepared with RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of cell lysates (30  $\mu$ g) were loaded on 10% SDS-PAGE and transferred onto PVDF membranes. After membranes were blocked, they were incubated with monoclonal antibody against CXCR4, Bax (1:500, Proteintech), Bcl-xL (1:500, Signalway Antibody), Caspase-3 (1:500, Signalway Antibody), PI3K p55 (1:500, Signalway Antibody) and phosphor-PI3K p55 Tyr199 (1:10000, Epitomics), AKT (1:5000, Epitomics) and phosphor-AKT Ser473 (1:1000, Cell Signaling Technology), mTOR and phosphor-mTOR Ser2448 (1:1000, Cell Signaling Technology), GSK-3 $\beta$



**Figure 1.** CXCR4 is up-expressed in chronic lymphocytic leukemia. A: The expression level of CXCR4 in chronic lymphocytic leukemia cells was examined using western blot assay. B: CXCR4 levels were further confirmed using RT-PCR. The data are presented as mean  $\pm$  SD. For indicated comparisons, \* $P < 0.05$ , \*\* $P < 0.01$ .

and phosphor-GSK-3 $\beta$  Ser9 (1:1000, Cell Signaling Technology), NF- $\kappa$ B p65 phosphor-NF- $\kappa$ B p65 Ser536 (1:5000, Bioworld Technology) and  $\beta$ -Tubulin (1:5000, Epitomics) followed by incubation with horseradish peroxidase-conjugated IgGs (1:10000, Bioworld Biotechnology). Target proteins were detected by the ECL system (Millipore, Braunschweig, Germany) and visualized with the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

#### *In vivo therapeutic studies*

A total of  $1 \times 10^6$  TF-1 cells in 100  $\mu$ l of PBS were injected subcutaneously into dorsal flanks of an immunodeficient nude mouse. The animals were treated with WZ811 (40 mg/kg), or WZ811 once daily by oral gavage once the tumors have reached 100 mm<sup>3</sup>. Tumor growth and body weight was measured every three days during the treatment. The tumor volume (TV) was calculated every 3 days according to the following standard formula: TV (mm<sup>3</sup>) = length  $\times$  width<sup>2</sup>  $\times$  0.5. All of the experiments on mice were approved by the local ethics committee of Aviation General Hospital [18].

#### *Statistical analysis*

SPSS 20.0 software (SPSS Inc, Chicago, USA.) was used to perform the statistical analysis. All results were given as the mean  $\pm$  standard deviation (SD) of three independent experiments, and the significance of the differences among three groups was calculated using one-way analysis of variance (ANOVA) while Student's t-test was used to ascertain the significance between two groups. A threshold of  $P < 0.05$  was considered to be statistical significant.

## **Results**

### *CXCR4 is over-expressed in chronic lymphocytic leukemia*

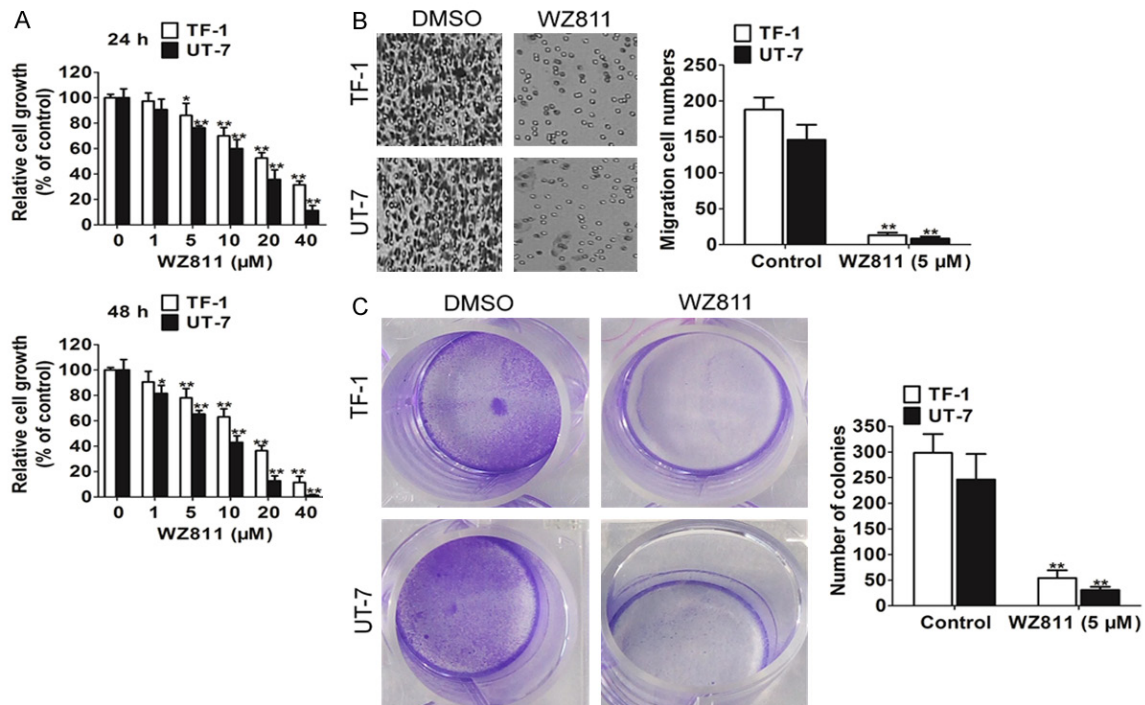
CXCR4 has been reported to be involved in the development and progression of in hematopoietic malignancies, and has previously been demonstrated to be association with the growth, survival and apoptosis of cancer cells [19].

To confirm, the role of CXCR4 in chronic lymphocytic leukemia. The expression level of CXCR4 in chronic lymphocytic leukemia cell lines HL-60, K562, KG-1a, UT-7, TF-1 and RAW 264.7 macrophages cell lines was examined using western blots. CXCR4 was found to be elevated in all gastric cancers, especially in UT-7 and TF-1 cells (**Figure 1A**). The elevated mRNA levels of CXCR4 in those cells were also observed, using real-time RT-PCR, which indicates the overexpression of CXCR4 in chronic lymphocytic leukemia cell lines at the transcriptional level (**Figure 1B**).

### *WZ811 inhibits CLL cell proliferation, cell motility and colony formation ability*

To access the anti-cancer activities of WZ811 in chronic lymphocytic leukemia, the anti-proliferative activity of WZ811 against the CLL cell lines TF-1 and UT-7 was examined. As shown in **Figure 2A**, we found WZ811 inhibited TF-1 and UT-7 cells proliferation in a dose responsive manner both after cells treatment with WZ811 for 24 h and 48 h. Cell motility ability is closely correlated with the process of tumor progression. The effect of WZ811 on CLL cell motility was determined by using a Transwell migration assay. As shown in **Figure 2B**, the average cell number that migrated into the lower chamber decreased by WZ811 treatment. This finding revealed that WZ811 might be an effective inhibitor of the migration of CLL cells. Next, we detected the effect of WZ811 on clone formation capability of cancer cells TF-1 and UT-7 in soft agar assay. As shown in **Figure 2C**, treatment with 5  $\mu$ M WZ811 markedly decreased the number of colonies compared to the untreated control cells; moreover, there were almost no clone forming in UT-7 cells under the WZ811 concentration of 5  $\mu$ M.





**Figure 2.** WZ811 inhibits chronic lymphocytic leukemia cells proliferation and aggressive effectively. A: Cells were exposed to indicated concentrations of WZ811 (1, 5, 10, 20, 40 μM) for 24 h and 48 h, respectively. Cell viability was determined by MTS proliferation assay. The data are presented as mean ± SD. The values are expressed as percentage of viable cells normalized to percentage of viable cells in 0.5% DMSO-treated cells. \* $P < 0.05$  and \*\* $P < 0.05$  compared with the DMSO-treated group. B: TF-1 and UT-7 cells were treated with the indicated concentrations of WZ811 for 6 h. The migration activity of the cells in the WZ811n group was reduced obviously compared with the control cells. \* $P < 0.05$  and \*\* $P < 0.05$  compared with the control group. C: The effects of WZ811 on the colony forming ability of TF-1 and UT-7 cells. Images are representative of three independent experiments. \* $P < 0.05$  and \*\* $P < 0.05$  compared with the control group.

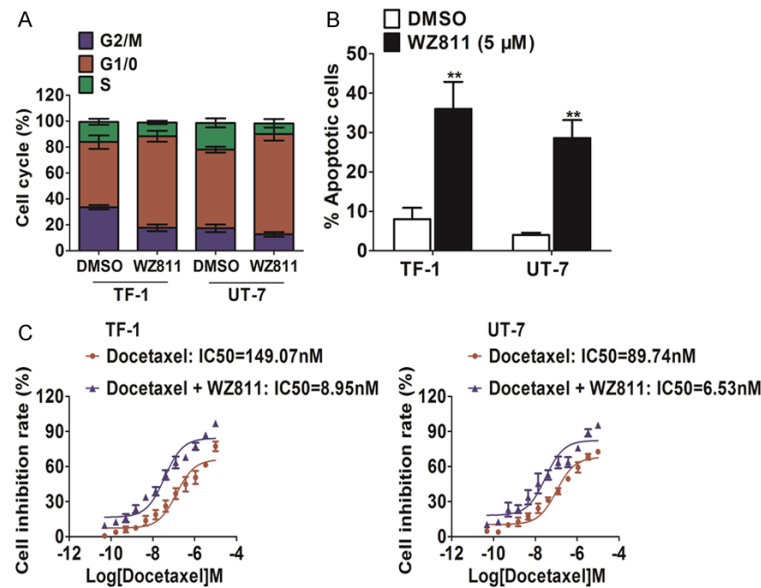
#### WZ811 induces cell apoptosis and increases the sensitivity of cells to docetaxel

The cell cycle is a critical characteristic that could accurately reflect the cell survival and apoptosis of cancer cells. To examine whether WZ811 have an effect on the cell cycle of CLL cells, flow cytometry analysis was used. As shown in **Figure 3A**, there was a decreased in the rate of S-phase and significantly increased in the rate of G1/O-phase after treatment with WZ811. Moreover, our results showed an increased rate of apoptosis in CLL cells 24 h after WZ811 treatment (**Figure 3B**). To determine whether WZ811 influenced the sensitivity of CLL cells to docetaxel, these cells were treated with different concentrations of docetaxel for 24 h and detected by MTS proliferation assay. As shown in **Figure 3C**, docetaxel resulted in more effective inhibition of pro-

liferation in TF-1 and UT-7 cells treated with WZ811 than in those with DMSO (control). The IC<sub>50</sub> for docetaxel in those cells treated with DMSO was high than 80 nM, but was under 10 nM in WZ811-treated cells.

#### WZ811 down-regulated the expressions of aggressiveness markers

Previous studies have established the CXCR4 as an upstream regulator of molecules involved in cancer development [20]. The serine/threonine kinase AKT, a downstream effector of phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K), is involved in cell survival, anti-apoptotic signaling, and chronic lymphocytic leukemia progression. To investigate the possible mechanisms of WZ811 on CLL aggressiveness, we assayed the expressions of several signaling involved in aggressiveness. In



**Figure 3.** WZ811 increases the sensitivity to docetaxel in TF-1 and UT-7 cells. A: The results of flow cytometry showed that CLL cells treated with WZ811 exhibited an increased rate of the G1-phase and a decrease in the S-phase. The data are presented as mean  $\pm$  SD. B: In accordance with the inhibition of the cells survival by WZ811, cells in the WZ811 group exhibited an increased proportion of apoptosis compared with the control group. The values are expressed as percentage of apoptotic cells normalized to percentage of viable cells in 0.5% DMSO-treated cells. For indicated comparisons, \*\* $P < 0.05$  compared with the DMSO-treated group. C: Chemo-sensitivity assays showed that WZ811 can obviously increase the sensitivity of TF-1 and UT-7 cells to docetaxel ( $P < 0.05$ ;  $n = 3$ ) with a lower IC<sub>50</sub> values in WZ811 group than control group ( $P < 0.05$ ;  $n = 3$ ).

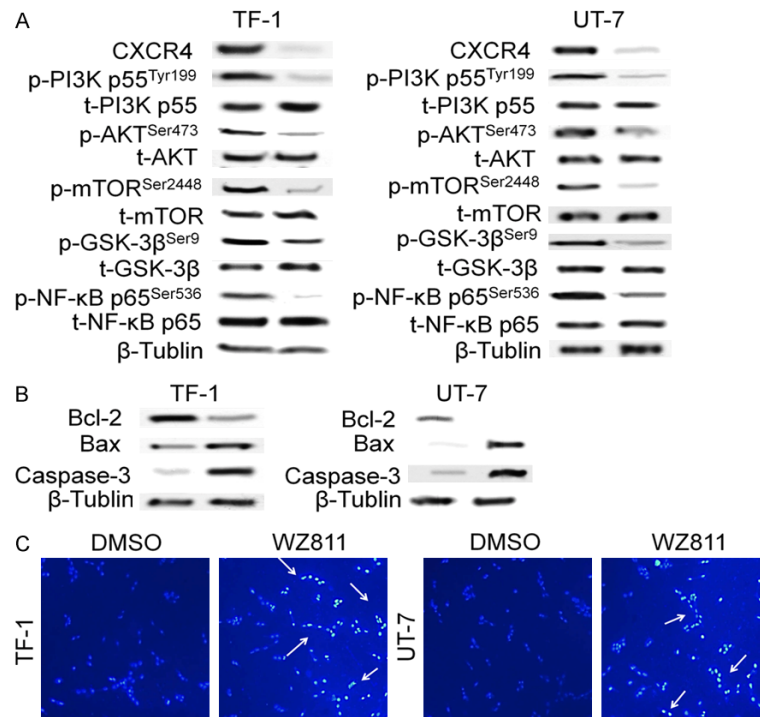
this study, we observed that phosphor-PI3K, phosphor-AKT, total-AKT, phosphor-mammalian target of rapamycin (mTOR), phosphor-glycogen synthase kinase 3 beta (GSK-3 $\beta$ ) and phosphor-nuclear factor kappa-light-chain-enhancer of activated B cells p65 (NF- $\kappa$ B p65) were strongly decreased with the addition of WZ811 in a dose-dependent manner (Figure 4A). In addition, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) significantly decreased as well as Caspase-3 occurred after WZ811 treatment (Figure 4B). DAPI staining was utilized to observe nucleus brightness, a marker of apoptosis. Nucleus brightness was significantly increased in the WZ811-treated cells in comparison with that of the control cells (Figure 4C).

#### WZ811 inhibited chronic lymphocytic leukemia growth in vivo

Based on the above in vitro findings, we next conducted in vivo experiment to confirm the effect of WZ811 on cancer cells. 100  $\mu$ l TF-1

single-cell suspensions ( $1 \times 10^6$  cells) were injected to the right limb subcutaneous of BALB/c nude mice. After one week, mice were randomly divided into two cohorts, one of which received vehicle and the other administrated with WZ811. The tumor volume was monitored, as shown in Figure 5A, the tumor growth in WZ811 group was dramatically retarded as compared with it in control group. At the end of the experiment, the average tumor volume and tumor weight of the group treated with WZ811 was significantly lower compared with that of the control group (Figure 5B). By 25 d, the tumor burden in mice treated with vehicle ( $n = 6$ ) had grown to an average of 1.8 g whereas (Figure 5C); those mice treated with WZ811 had a tumor burden of merely 0.23 g ( $n = 6$ ). Interestingly, no difference was detected in body weight between the WZ811 and vehicle treated

mice (Figure 5D). Consistent with in vitro studies, the immunohistochemistry results showed that the proliferation of chronic lymphocytic leukemia cells was inhibited by WZ811 administration as there was less and weak expression for Ki67 in WZ811 group compared with that in control group (Figure 5E). Meanwhile, numbers of TUNEL-positive cells (apoptotic cell death) were greater in the tumor tissues from WZ811 treated mice than in those of control mice (Figure 5E). To further decipher the underlying mechanism of WZ811 inhibited proliferation and progression in chronic lymphocytic leukemia growth in vivo; the CXCR4 down-regulation signaling pathway was then assayed by immunohistochemistry (Figure 6A) and western blot assay (Figure 6B). As shown in Figure 6A, the tumor tissues from mice in WZ811 group exhibited low level of CXCR4, PI3K, and AKT staining compared with that from mice in control group. As would be expected, the phosphorylation level of PI3K (p-PI3K), AKT (p-AKT), mTOR (p-mTOR), GSK-3 $\beta$  (p-GSK-3 $\beta$ ) and p-NF- $\kappa$ B p65



**Figure 4.** WZ811 inhibits aggressiveness markers and induces apoptosis in chronic lymphocytic leukemia cells. A. WZ811 suppressed the activation of CXCR4 induced by PI3K-AKT signaling pathway in TF-1 and UT-7 cells by western blot analysis. B. WZ811 down-regulated the expression of Bcl-2, Bax and caspase-3 in both TF-1 and UT-7 cells by western blot analysis.  $\beta$ -Tubulin was shown as loading control. C. Fluorescence microscopic images of TF-1 and UT-7 cells treated with WZ811 for 24 h. Nuclear brightness were observed.

(p-NF- $\kappa$ B p65) in the WZ811-treated group was significantly increased compared to the control group (Figure 6B). These data showed that WZ811 can suppress the in vivo tumor growth through inhibiting CXCR4/PI3K/AKT/mTOR signaling.

## Discussion

Chemoattractant cytokines are a large family of secreted cytokines by tumor cells and stromal cells whose main function is to mediate cancer cell directional migration and invasion [21]. Chemokines and their receptors play important roles in the pathogenesis of a wide range of human diseases, including chronic inflammatory diseases, multiple neurological disorders and cancer. Most cancers contain chemokines and express chemokine receptors as a consequence of the activity of deregulated transcription factors or cancer-promoting genes [22]. A large body of scientific literature has reported that CXCL12 and CXCR4, a chemokine and chemokine receptor pair play important roles

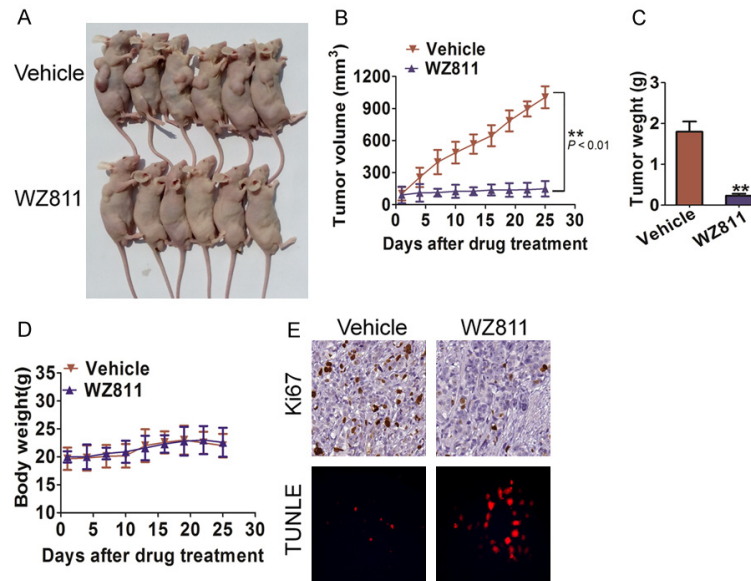
in multiple phases of tumorigenesis, including tumor cell proliferation, survival, invasion and metastasis, and angiogenesis. In clinical diagnosis, it has been demonstrated that CXCR4 is constitutively expressed in a variety of human cancers, such as breast, kidney, lung, colon, and brain cancer [23]. Several forms of hematologic malignancies including leukemia and lymphoma are also shown to express high levels of CXCR4. CXCR4 over-expression is especially noticeable in advanced and metastatic cancers and generally linked to cancer pathogenesis and outcome.

As a G-protein-coupled receptor (GPCR) the mechanism of CXCR4 receptor activation is mediated by coupling to an intracellular heterotrimeric G-protein associated with the inner surface of the plasma membrane. The heterotrimer is composed of  $G\alpha$ ,  $G\beta$  and  $G\gamma$

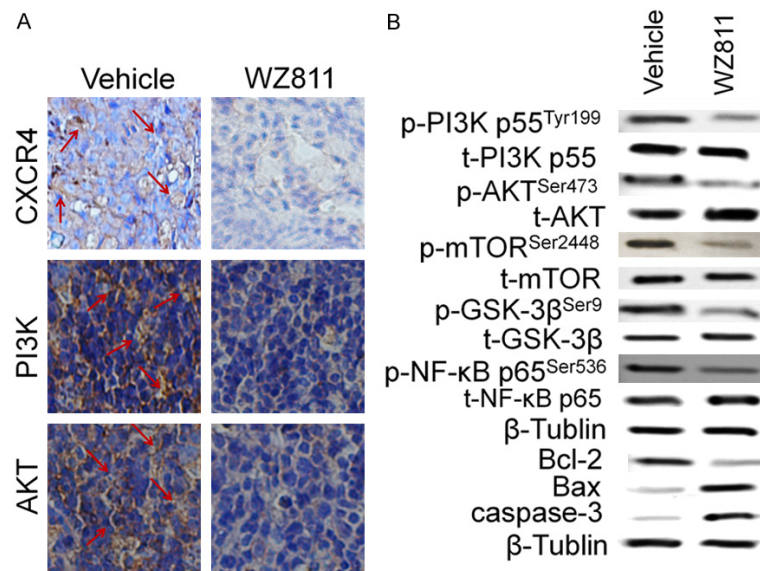
subunits, which in its basal state binds the guanine nucleotide GDP [24]. Upon activation by ligand binding, GDP is released and replaced by GTP, which leads to subunit dissociation into a  $\beta\gamma$  dimer and the  $\alpha$  monomer to which the GTP is bound. The GTP is rapidly hydrolyzed to GDP resulting in association of the receptor and the trimeric G-protein complex [25]. CXCR4-mediated chemotaxis is mediated by PI3K. PI3K activation can result in the phosphorylation of several focal adhesion components such as proline-rich kinase-2 (Pyk-2), Crk-associated substrate (p130Cas), focal adhesion kinase (FAK), Paxillin, Nck, Crk, and Crk-L. Crk, which belongs to the adaptor family of proteins composed of SH2 and SH3 domains, has a putative role in signaling. PI3K lead to the activation of the serine-threonine kinase AKT, which has been found to play a key role in tumor cell survival, and possibly proliferation [26]. Signaling through Gxi has been linked to transcription and expression through the PI3K-AKT-NF- $\kappa$ B axis, and also via MEK1/2 and ERK 1/2. ERK can phosphorylate and



## WZ811 suppress chronic lymphocytic leukemia progression



**Figure 5.** WZ811 suppresses the lymphocytic leukemia cells growth on mouse xenograft models. A: Immunosuppressed mice with established TF-1 cells were given WZ811 (40 mg/kg) by oral gavage or vehicle (control). The mice treated with WZ811 showed marked reduction in tumor growth compared with the mice treated with vehicle. B: The volume of the tumors was significantly lower in mice treated with WZ811 than in the control group mice.  $**P < 0.01$  compared with the control group. Each data point represents the Mean  $\pm$  SD of 6 mice. C: The weight of the tumors was significantly decreased in WZ811-treated mice than in vehicle-treated mice ( $**P < 0.01$ ). D: Body weight was measured and plotted as absolute value from 0-25 days post-treatment. E: Tumor sections were analyzed by immunohistochemistry for detection of Ki67 expression in each group of nude mice. Apoptotic cells were examined by TUNEL staining. Each image was representative of six independent mice.



**Figure 6.** WZ811 suppresses CXCR4/PI3K/AKT signaling pathway in mouse xenograft model of lymphocytic leukemia. A: Immunosuppressed mice with established TF-1 cells were given WZ811 (40 mg/kg) by oral gavage or vehicle (control). The mice treated with WZ811 showed marked reduction in tumor growth compared with the mice treated with vehicle. B: The volume of the tumors was significantly lower in mice treated with WZ811 than in the control group mice.  $**P < 0.01$  compared with the control group. Each data point represents the Mean  $\pm$  SD of 6 mice. C: The weight of the tumors was significantly decreased in WZ811-treated mice than in vehicle-treated mice ( $**P < 0.01$ ). D: Body weight was measured and plotted as absolute value from 0-25 days post-treatment. E: Tumor sections were analyzed by immunohistochemistry for detection of Ki67 expression in each group of nude mice. Apoptotic cells were examined by TUNEL staining. Each image was representative of six independent mice.

cle (control). Tumor sections were analyzed by immunohistochemistry for detection of CXCR4, PI3K, and AKT expression in each group of nude mice. Each image was representative of six independent mice. B: The proteins were extracted from tumor xenografts and were subjected to western blot for measuring protein levels of phosphor-PI3K, phosphor-AKT, phosphor-mTOR, phosphor-GSK-3 $\beta$ , phosphor-NF- $\kappa$ B p65, and Bcl-2, Bax as well as Caspase-3 expression respectively.

activate other cellular proteins (like p90RSK), as well as translocate into the nucleus and phosphorylate and/or activate transcription factors, leading to changes in gene expression and cell cycle progression [27].

In addition to the above typical signal pathway, CXCR4 signaling has been shown to involve the Ras-activated signaling pathway, several Src-related kinases such as Src, Lyn, Fyn, and Lck [28]. Moreover, CXCR4 may promote tumor cell survival by two mechanisms: post-translational inactivation of the cell death machinery and an increased transcription of cell survival-related genes. All the evidence suggests the CXCR4 offer a therapeutic opportunity for anti-cancer drug development [29]. So far at least, only few study focused on the effect of CXCR4 inhibitor in chronic lymphocytic leukemia. To determine the role of CXCR4 inhibitor WZ811 in chronic lymphocytic leukemia, we first examined the effect of WZ811 on chronic lymphocytic leukemia cell lines TF-1 and TU-7. Our results indicated that WZ811 could inhibit cell proliferation, cell motility, cell survival, colo-



ny formation ability, and induced cell apoptosis in vitro and tumorigenesis in vivo. Additionally, the protein expressions of chronic lymphocytic leukemia cell aggressive markers, including PI3K, AKT and NF- $\kappa$ B were decreased after coping with WZ811.

At present, docetaxel-based chemotherapy is widely administered for patients with cancer worldwide. Although docetaxel confers a significant survival benefit for many patients, all patients inevitably develop resistance to docetaxel and their disease will continue to progress over time. The treatment outcome may be improved by modulating the sensitivity of cancer cells to docetaxel [30]. In this study, our data showed that WZ811 could significantly increase the sensitivity of chronic lymphocytic leukemia cells to docetaxel, suggesting the combination of WZ811 and docetaxel may have complementary and additive anti-tumor effects on chronic lymphocytic leukemia. We also investigated the anti-tumor effect of WZ811 in chronic lymphocytic leukemia xenograft models in athymic mice. Immunohistochemistry and western blotting analysis showed that the expression of Ki67, CXCR4 and its downstream signal molecules in xenograft was remarkably decreased. In summary, our data demonstrate that WZ811 significantly inhibits chronic lymphocytic leukemia progression and tumorigenesis in vitro and in vivo via suppression of chronic lymphocytic leukemia cells aggressive. Future studies should focus on exploring the potential mechanisms of WZ811, for it could be vital in clinical use.

#### Disclosure of conflict of interest

None.

**Address correspondence to:** Shi Hui Li, Department of Oncology, Aviation General Hospital, No. 3 Beiyuan Road, Chaoyang District, Beijing 100012, China. E-mail: LSH20022005@126.com

#### References

- [1] Muggen AF, Singh SP, Hendriks RW and Langerak AW. Targeting signaling pathways in chronic lymphocytic leukemia. *Curr Cancer Drug Targets* 2016; [Epub ahead of print].
- [2] Kashyap MK, Kumar D, Jones H, Amaya-Chanaga CI, Choi MY, Melo-Cardenas J, Ale-Ali A, Kuhne MR, Sabbatini P, Cohen LJ, Shelat

- SG, Rassenti LZ, Kipps TJ, Cardarelli PM and Castro JE. Ulocuplumab (BMS-936564 / MDX1338): a fully human anti-CXCR4 antibody induces cell death in chronic lymphocytic leukemia mediated through a reactive oxygen species-dependent pathway. *Oncotarget* 2016; 7: 2809-2822.
- [3] Ganghammer S, Gutjahr J, Hutterer E, Krenn PW, Pucher S, Zelle-Rieser C, Johrer K, Wijnmans M, Leurs R, Smit MJ, Gattei V, Greil R and Hartmann TN. Combined CXCR3/CXCR4 measurements are of high prognostic value in chronic lymphocytic leukemia due to negative co-operativity of the receptors. *Haematologica* 2016; 101: e99-e102.
- [4] Chen SS, Chang BY, Chang S, Tong T, Ham S, Sherry B, Burger JA, Rai KR and Chiorazzi N. BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia. *Leukemia* 2016; 30: 833-843.
- [5] Zhang S and Kipps TJ. The pathogenesis of chronic lymphocytic leukemia. *Annu Rev Pathol* 2014; 9: 103-118.
- [6] ten Hacken E and Burger JA. Molecular pathways: targeting the microenvironment in chronic lymphocytic leukemia—focus on the B-cell receptor. *Clin Cancer Res* 2014; 20: 548-556.
- [7] Mittal AK, Chaturvedi NK, Rai KJ, Gilling-Cutucache CE, Nordgren TM, Moragues M, Lu R, Opavsky R, Bociek GR, Weisenburger DD, Iqbal J and Joshi SS. Chronic lymphocytic leukemia cells in a lymph node microenvironment depict molecular signature associated with an aggressive disease. *Mol Med* 2014; 20: 290-301.
- [8] Lopez-Guerra M, Xargay-Torrent S and Colomer D. CXCR5-mediated shaping of the lymphoid follicle in chronic lymphocytic leukemia. *Cancer Discov* 2014; 4: 1374-1376.
- [9] Han TT, Fan L, Li JY and Xu W. Role of chemokines and their receptors in chronic lymphocytic leukemia: function in microenvironment and targeted therapy. *Cancer Biol Ther* 2014; 15: 3-9.
- [10] Nannini PR, Borge M, Mikolaitis VC, Abreu C, Morande PE, Zanetti SR, Oppezzo P, Palacios F, Ledesma I, Bezares RF, Giordano M and Gamberale R. CCR4 expression in a case of cutaneous Richter's transformation of chronic lymphocytic leukemia (CLL) to diffuse large B-cell lymphoma (DLBCL) and in CLL patients with no skin manifestations. *Eur J Haematol* 2011; 87: 80-86.
- [11] Stamatopoulos B, Meuleman N, De Bruyn C, Delforge A, Bron D and Lagneaux L. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces apoptosis, down-regulates the CXCR4 chemokine receptor and im-

- pairs migration of chronic lymphocytic leukemia cells. *Haematologica* 2010; 95: 1136-1143.
- [12] O'Hayre M, Salanga CL, Kipps TJ, Messmer D, Dorrestein PC and Handel TM. Elucidating the CXCL12/CXCR4 signaling network in chronic lymphocytic leukemia through phosphoproteomics analysis. *PLoS One* 2010; 5: e11716.
- [13] Kurenova EV, Hunt DL, He D, Fu AD, Massoll NA, Golubovskaya VM, Garces CA and Cance WG. Vascular endothelial growth factor receptor-3 promotes breast cancer cell proliferation, motility and survival in vitro and tumor formation in vivo. *Cell Cycle* 2009; 8: 2266-2280.
- [14] Tian T, Li A, Lu H, Luo R, Zhang M and Li Z. Six1 promotes glioblastoma cell proliferation and invasion by upregulation of connective tissue growth factor. *Am J Cancer Res* 2015; 5: 1823-1830.
- [15] Ma H, Yang F, Lian M, Wang R, Wang H, Feng L, Shi Q and Fang J. Dysregulation of zinc finger protein, X-linked (ZFX) impairs cell proliferation and induces apoptosis in human oral squamous cell carcinoma. *Tumour Biol* 2015; 36: 6103-6112.
- [16] Zhan Q, Huang RF, Liang XH, Ge MX, Jiang JW, Lin H and Zhou XL. FRAS1 knockdown reduces A549 cells migration and invasion through downregulation of FAK signaling. *Int J Clin Exp Med* 2014; 7: 1692-1697.
- [17] Yuan CX, Chu T, Liu L, Li HW, Wang YJ, Guo AC and Fan YP. Catalpol induces oligodendrocyte precursor cell-mediated remyelination in vitro. *Am J Transl Res* 2015; 7: 2474-2481.
- [18] Lv ZD, Liu XP, Zhao WJ, Dong Q, Li FN, Wang HB and Kong B. Curcumin induces apoptosis in breast cancer cells and inhibits tumor growth in vitro and in vivo. *Int J Clin Exp Pathol* 2014; 7: 2818-2824.
- [19] Niedermeier M, Hennessy BT, Knight ZA, Henneberg M, Hu J, Kurtova AV, Wierda WG, Keating MJ, Shokat KM and Burger JA. Isoform-selective phosphoinositide 3'-kinase inhibitors inhibit CXCR4 signaling and overcome stromal cell-mediated drug resistance in chronic lymphocytic leukemia: a novel therapeutic approach. *Blood* 2009; 113: 5549-5557.
- [20] Burger M, Hartmann T, Krome M, Rawluk J, Tamamura H, Fujii N, Kipps TJ and Burger JA. Small peptide inhibitors of the CXCR4 chemokine receptor (CD184) antagonize the activation, migration, and antiapoptotic responses of CXCL12 in chronic lymphocytic leukemia B cells. *Blood* 2005; 106: 1824-1830.
- [21] Barretina J, Junca J, Llano A, Gutierrez A, Flores A, Blanco J, Clotet B and Este JA. CXCR4 and SDF-1 expression in B-cell chronic lymphocytic leukemia and stage of the disease. *Ann Hematol* 2003; 82: 500-505.
- [22] Cheng ZH, Shi YX, Yuan M, Xiong D, Zheng JH and Zhang ZY. Chemokines and their receptors in lung cancer progression and metastasis. *J Zhejiang Univ Sci B* 2016; 17: 342-351.
- [23] Kim B, Yoon J, Yoon SW and Park B. Onbaekwon Suppresses Colon Cancer Cell Invasion by Inhibiting Expression of the CXCR4 Chemokine Receptor 4. *Integr Cancer Ther* 2016; [Epub ahead of print].
- [24] Wang Z, Sun J, Feng Y, Tian X, Wang B and Zhou Y. Oncogenic roles and drug target of CXCR4/CXCL12 axis in lung cancer and cancer stem cell. *Tumour Biol* 2016; [Epub ahead of print].
- [25] Wang Y, Xie Y and Oupicky D. Potential of CXCR4/CXCL12 Chemokine Axis in Cancer Drug Delivery. *Curr Pharmacol Rep* 2016; 2: 1-10.
- [26] Morimoto M, Matsuo Y, Koide S, Tsuboi K, Shamoto T, Sato T, Saito K, Takahashi H and Takeyama H. Enhancement of the CXCL12/CXCR4 axis due to acquisition of gemcitabine resistance in pancreatic cancer: effect of CXCR4 antagonists. *BMC Cancer* 2016; 16: 305.
- [27] Mills SC, Goh PH, Kudatsih J, Ncube S, Gurung R, Maxwell W and Mueller A. Cell migration towards CXCL12 in leukemic cells compared to breast cancer cells. *Cell Signal* 2016; 28: 316-324.
- [28] Lee H, Ko JH, Baek SH, Nam D, Lee SG, Lee J, Yang WM, Um JY, Kim SH, Shim BS and Ahn KS. Embelin Inhibits Invasion and Migration of MDA-MB-231 Breast Cancer Cells by Suppression of CXCR4 Chemokine Receptor 4, Matrix Metalloproteinases-9/2, and Epithelial-Mesenchymal Transition. *Phytother Res* 2016; 30: 1021-32.
- [29] Billard MJ, Fitzhugh DJ, Parker JS, Brozowski JM, McGinnis MW, Timoshchenko RG, Serafin DS, Lininger R, Klauber-Demore N, Sahagian G, Truong YK, Sassano MF, Serody JS and Tarrant TK. G Protein Coupled Receptor Kinase 3 Regulates Breast Cancer Migration, Invasion, and Metastasis. *PLoS One* 2016; 11: e0152856.
- [30] Yang DL, Xin MM, Wang JS, Xu HY, Huo Q, Tang ZR and Wang HF. Chemokine receptor CXCR4 and its ligand CXCL12 expressions and clinical significance in bladder cancer. *Genet Mol Res* 2015; 14: 17699-17707.