Int J Clin Exp Med 2014;7(11):4031-4037
www.ijcem.com /ISSN:1940-5901/IJCEM0002151

Original Article

Effects of lentivirus mediated STAT3 silencing on human chronic myeloid leukemia cells and leukemia mice

Xinyan Jia[1*], Wenzhong Yang[1*], Jia Han[2], Hong Xiong[2]

[1]*Department of Hematology, East Hospital, Tongji University School of Medicine, Shanghai 200120, China;* [2]*Department of Hematology, Shanghai Xuhui Central Hospital & Shanghai Clinical Center, Chinese Academy of Sciences, Shanghai 200031, China.* [*]*Equal contributors.*

Received August 28, 2014; Accepted October 23, 2014; Epub November 15, 2014; Published November 30, 2014

Abstract: Objective: To investigate the effects of lentivirus mediated STAT3 silencing on human chronic myeloid leukemia cells (K562) and the growth of chronic myeloid leukemia mice as well as to explore the potential mechanisms. Methods: Unbtreated K562 cells (CON), blank lentivirus transfected K562 cells (NC) and K562 cells expressing STAT3 siRNA (STAT3 siRNA) were injected into SCID mice to establish the chronic myeloid leukemia model in mice. The growth, peripheral white blood cell count and spleen index in these mice were determined. Results: In vitro experiment showed, when compared with control group, the interference efficiency of STAT3 expression was as high as 97.5% in K562 cells. Western blot assay revealed that the expression of c-Myc, Bcl-xL and Cyclin D1 reduced by 17.01%, 7.3% and 6.82%, respectively, showing significant difference when compared with control group ($P < 0.01$). These findings were consistent with those from fluorescence quantitative PCR. In vivo experiment showed the body weight of mice reduced progressively and the peripheral white blood cell count increased gradually in control group, accompanied by dragging hind limbs and progressive enlargement of the spleen. The body weight remained unchanged, peripheral white blood cell count reduced gradually and the spleen did not enlarge in mice treated with STAT3 siRNA expressing cells. Conclusion: Lentivirus mediated STAT3 silencing may inhibit the expression of its downstream genes (c-Myc, Bcl-xL and Cyclin D1) related to cell proliferation, apoptosis and cycle to suppress the malignant biological behaviors, and STAT3 silencing also inhibit the leukemogenic potency of K562 cells in mice.

Keywords: Chronic myeloid leukemia, STAT3 siRNA, lentivirus, animal model

Introduction

Chronic myeloid leukemia (CML) is an acquired malignant myeloproliferative disorder of hematopoietic stem cells and has the annual incidence of 1/100000. CML is the most common chronic leukemia in China. A majority of CML patients have the characteristic BCR-ABL fusion gene [1] which encodes P210 protein with tyrosinase activity. P210 protein can act on several signaling pathways such as Janus kinase/signal transduction and signal transduction and activator of transcription (JAK/STATs) signaling pathway and phospholipids phthalocyanine 3 kinase (PI3K)/protein kinase B signaling pathway [2-5] to influence the cell proliferation and apoptosis. In addition, it may also induce the deficiency of cell adhesion which is required for the regulation of growth and differentiation of hematopoietic stem cells, resulting in excessive proliferation and reduced apoptosis of bone marrow progenitor cells as well as decreased adhesion of bone marrow stromal cells, and thus a large amount of myeloid cells are released into circulation causing CML.

Among the downstream signaling pathways of BCR-ABL, the sustained STAT3 activation is closely associated with the malignant behaviors of cells (such as survival, abnormal proliferation and anti-apoptosis), and has been an important molecular mechanism underlying the blocked differentiation of hematopoietic stem cells induced leukemia [6-8]. Bhardwaj et al found to inhibit the STAT3 activation in multiple myeloma cells could down-regulate the expression of Bcl-2, Bcl-xl and Cyclin D1 to promote cell apoptosis [9]. In our previous study, lentivirus expressing STAT3 siRNA was prepared and

Table 1. Primers for Real-time RT-PCR

	Forward	Reverse
Bcl-xL	CCCAGAAAGGATACAGCTGG	GCGATCCGACTCACCAATAC
Cyclin D1	CTGGCCATGAACTACCTGGA	GTCACACTTGATCACTCTGG
c-Myc	CTCTCAACGACAGCAGCCCG	CCAGTCTCAGACCTAGTGGA
STAT3	TGCGGAGAAGCATCGTGAGT	CCTCCAATGCAGGCAATCTGT
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

used to transfect K562 cells. Results showed the intracellular expression of STAT3 was significantly inhibited, the in vitro proliferation of these cells was also markedly suppressed, cell cycle was arrested and early apoptosis was present [10]. However, the molecular mechanisms of the inhibitory effects of STAT3 on the K562 cell growth and the influence of STAT3 on the leukemia cells (K562 cells) in vivo are still poorly understood.

In the present study, the protein and mRNA expressions of downstream factors of STAT3 were detected, aiming to explore the molecular mechanisms underlying the effects of STAT3 silencing on K562 cells. In addition, STAT3 siRNA lentivirus was prepared and used to transfect K562 cells which were then injected into mice to establish CML animal model. The effects of STAT3 silencing on the in vivo tumorigenicity of K562 cells were investigated in this model. Our findings may provide experimental evidence for the therapy of CML targeting STAT3.

Materials and methods

Cell line, lentivirus and animals

K562 cells were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences. K562 cells were maintained in RPMI I640 containing 10% fetal bovine serum (FBS) at 37°C in an environment with 5% CO_2. FBS, RPMI 1640 and α-MEM were purchased from Gibco Company. The primers for STAT3, Bcl-xL, Cyclin D1 and c-Myc were synthesized in Shanghai Sangon. Reverse transcription kit (RevertAidTM First Strand cDNA Synthesis Kit, K1622) was from Fermentas (MBI). Marker DL2000 (3427Q) and reagents used for PCR were from TaKaRa. Trizol for extraction of total RNA (15596-026; Invitrogen), RIPA cell lysis buffer (PP1901; Beijing BioTeke Corporation), Protease Inhibitor Cocktail (ab65621; Abcam, UK), BCA Protein Assay Kit (71285-3; merck-china), antibodies against STAT3 (9132), Bcl-xL (2762), Cyclin D1 (2922) and c-Myc (9402) (Cell Signaling Technology), Amersham ECL PlusTM Kit (GE Healthcare, USA), lentivirus expressing STAT3 siRNA and negative control lentivirus (Shanghai Invitrogen) were used in the present study. Healthy male NOD/SCID mice (6-8 weeks; 18-20 g) were purchased from the Experimental Animal Center of Nanjing Medical University.

Detection of mRNA expression of STAT3 and its downstream genes (Bcl-xL, Cyclin D1 and c-Myc) by RT-PCR

The concentration of K562 cells with good growth state was adjusted to 5×10^5/ml and these cells were seeded into a 6-well plate (1 ml/well). Lentivirus expressing STAT3 shRNA (STAT3 siRNA) and negative control lentivirus (NC) were used to transfect K562 cells at the multiplicity of infection (MOI) of 50. Cells without any treatment served as a blank control (CON). Cells in different groups were harvested and treated with Trizol reagent according to manufacturer's instructions for extraction of total RNA which was then used to be reverse-transcribed into cDNA. Subsequently, quantitative real time PCR was done with the cDNA. $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression of target genes. GAPDH served as an internal reference. PCR was done under following conditions: pre-denaturation at 95°C for 3 min, a total of 40 cycles of denaturation at 95°C for 25 s, annealing at 57°C for 25 s (Bcl-2: 60°C for 45 s) and extension at 72°C for 50 s, and a final extension at 72°C for 5 min. The primers for target genes and their sizes are shown in **Table 1**.

Detection of protein expression of STAT3 and its downstream genes (Bcl-xL, Cyclin D1 and c-Myc) by Western blot assay

The concentration of K562 cells with good growth state was adjusted to 5×10^5/ml and these cells were then transfected with lentivirus as abovementioned. At 5 days after transfection, cells were harvested and total protein was extracted. After determination of protein concentration with BCA method, 20 μg of total protein was subjected to 10% SDS-PAGE and electronically transferred onto PVDF membrane at 400 mA for 120 min. After blocking, the

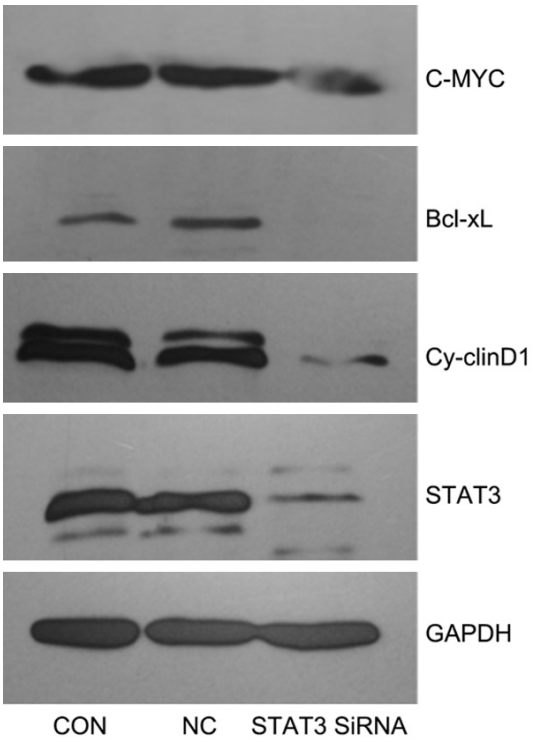

Figure 1. Detection of protein expression of c-Myc, Bcl-xL and Cyclin D1 in K562 cells after transfection with STAT3 siRNA expressing lentivirus by Western blot assay. When compared with cells without lentivirus transfection, the protein expression of c-Myc, Bcl-xL and Cyclin D1 reduced markedly in K562 cells after transfection with STAT3 siRNA expressing lentivirus.

membrane was treated with antibodies against Bcl-xL, Cyclin D1, c-Myc or GAPDH and then with HRP conjugated secondary antibody. Visualization was done with ECL, and protein bands were scanned into a computer, followed by detection of optical density by using Image J software. The protein expression of STAT3, Bcl-xL, Cyclin D1 and c-Myc was detected in cells of different groups.

Effects of STAT3 silencing on CML in vivo in a mouse model

NOD/SCID mice aged 8-10 weeks were housed in a specific pathogen free room. Before study, peripheral blood was collected from the tail vein, followed by detection of peripheral white blood cell (WBC) count. Mice with peripheral WBC count of 10-12 × 10^9/L were used for the establishment of CML mouse model. One day before experiment, mice were systemically irradiated with a linear accelerator (300 cGy/mouse) to increase the success rate. Then, cells with good growth state of different groups (K562, K562/NS and K562/STAT3) were injected into these mice via the tail vein to establish the CML mouse model. These mice were housed routinely and the activities, hair and appetite were observed every day and the body weight was measured once every 2 days. At 3, 5, 7 and 11 days and 15 d after preparation of animal model, mice in different groups were randomly selected, and blood was collected from the orbital venous plexus, followed by detection of peripheral WBC count. Then, these mice were sacrificed, and the spleen was collected and weighed, followed by calculation of spleen index: spleen index = (spleen weight/mouse body weight) × 10.

Statistical analysis

Statistical analysis was done with SPSS version 15.0, and data were expressed as means ± standard deviation. Comparisons between experiment group and control group were done with t test and those among groups with analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

STAT3 silencing inhibits the protein expression of c-Myc, Bcl-xL and cyclin D1 in K562 cells

Lentivirus expressing STAT2 siRNA was used to transfect K562 cells, and results showed the interference efficiency was as high as 97.5%. Western blot assay revealed the protein expression of STAT3 and its downstream genes (c-Myc, Bcl-xL and cyclin D1) was significantly inhibited in these cells (**Figure 1**).

STAT3 silencing inhibits the mRNA expression of c-Myc, Bcl-xL and cyclin D1 in K562 cells

QRT-PCR showed the mRNA expression of c-Myc, Bcl-xL and cyclin D1 reduced markedly in K562 cells after transfection with STAT3 siRNA expressing lentivirus. The mRNA expression of c-Myc, Bcl-xL and cyclin D1 in K562 cells transfected with STAT3 siRNA expressing lentivirus was 17.01%, 7.3% and 6.82% of that in control group, respectively ($P < 0.01$; **Figure 2**). These findings were consistent with those from Western blot assay.

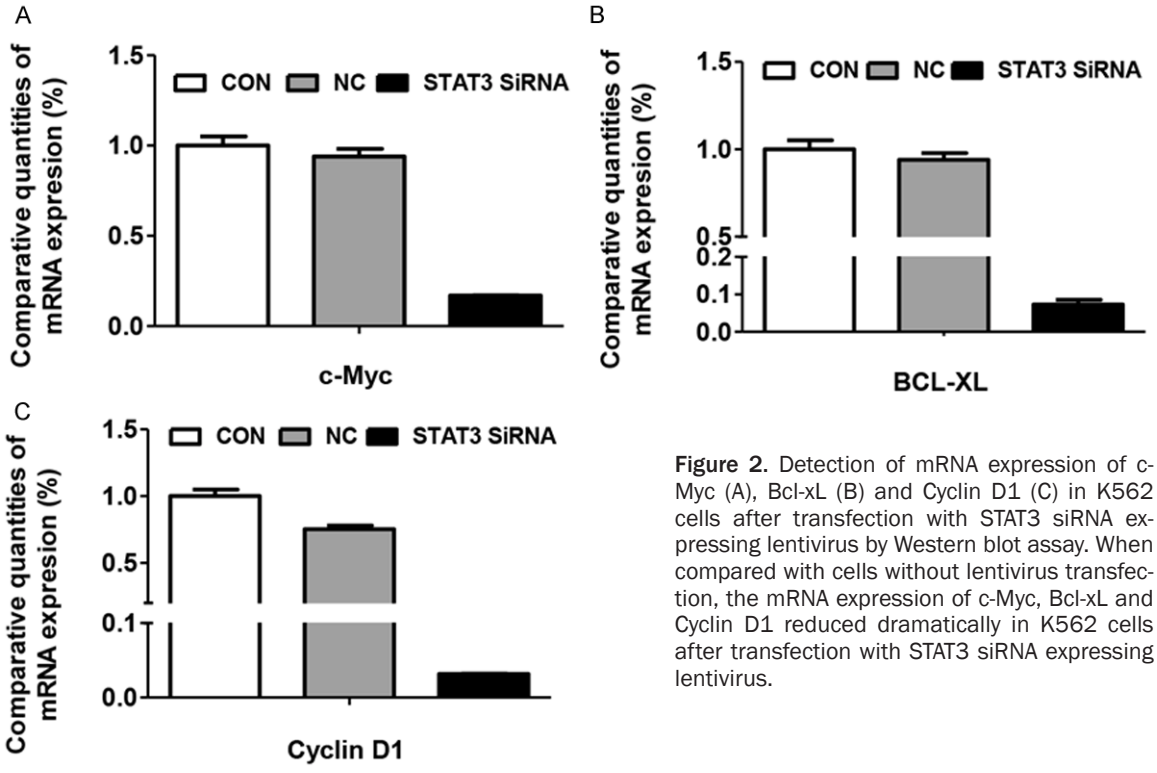

Figure 2. Detection of mRNA expression of c-Myc (A), Bcl-xL (B) and Cyclin D1 (C) in K562 cells after transfection with STAT3 siRNA expressing lentivirus by Western blot assay. When compared with cells without lentivirus transfection, the mRNA expression of c-Myc, Bcl-xL and Cyclin D1 reduced dramatically in K562 cells after transfection with STAT3 siRNA expressing lentivirus.

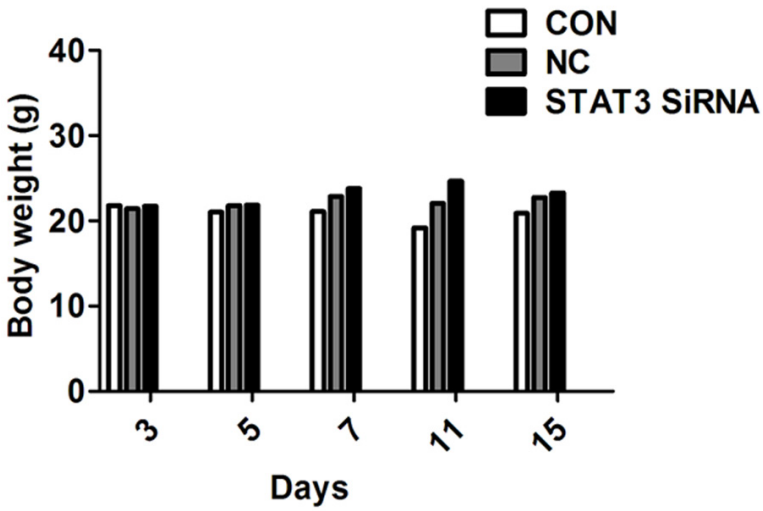

Figure 3. Change in body weight of mice in different groups.

Effects of STAT3 silencing on the growth of CML mice

In CON, mice showed gray hair, reduced appetite and dragging hind limbs at about 7 days after injection and then presented hair loss. After cell injection, cells in CON group showed progressive reduction in body weight (emaciation). In NC group, mice also showed weight loss, but the extent of weight loss was lower than that in CON group. In STAT siRNA group, the growth of mice was in a good state, the hair was bright, activities were free and there was no evident weight loss (**Figure 3**).

Effect of STAT3 silencing on the spleen index of CML mice

At the end of study, mice were sacrificed, and the major organs were observed macroscopically. The liver size in CON group remained unchanged, the spleen enlarged slightly and became dark, and hemorrhagic foci were observed in several mice. In NC group, the spleen enlarged significantly, but there were no obvious changes in other organs. When compared with Con group and NC group, the spleen in STAT3 siRNA group did not enlarge, but became a little bit smaller, and other organs also had no enlargement and were light-red.

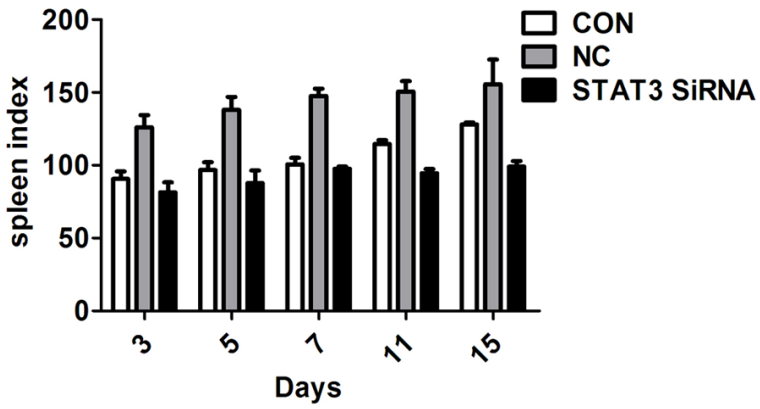

Figure 4. Change in the spleen index of different groups of mice.

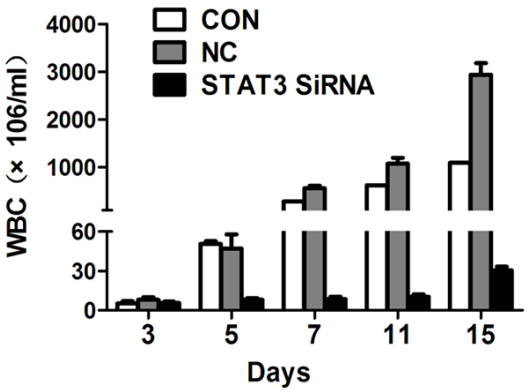

Figure 5. Peripheral WBC count of mice in different groups.

Statistical analysis showed the spleen index in STAT3 siRNA group was significantly different from that in control group ($P < 0.01$) (**Figure 4**).

Effect of STAT3 interfering on the peripheral WBC count in CML mice

In CON group, the peripheral WBC count increased markedly at 5 days after injection and reached 50-55 × 10^6/mL. The peripheral WBC count was 1000-1100 × 10^6/mL in CON group at 15 days. In NC group, the peripheral WBC count increased progressively. In STAT3 siRNA group, the peripheral WBC count remained unchanged and was significantly different from that in CON group and NC group ($P < 0.01$) (**Figure 5**).

Discussion

As an important member of downstream signaling pathway of BCR-ABL in CMl, STAT3 plays a crucial role in the malignant proliferation and differentiation of leukemia cells. STAT3 mainly promote the expression of pro-survival genes (such as Cyclin D1) or anti-apoptotic genes (such as Bcl-XL and survivin) to facilitate the malignant proliferation and inhibit the cell apoptosis [11]. Thus, to block the STAT3 and its downstream signal transduction has been a new strategy in the anti-tumor therapy.

In previous studies, lentivirus expressing human STAT3 siRNA was constructed and transfected into CML cells to knock-down STAT3 expression. Results showed it could significantly inhibit the cell growth and arrest these cells in G1-S phase, and these cells also showed early apoptosis [10]. Preliminary studies confirm that lentivirus mediated STAT3 silencing can inhibit the growth of leukemia cells. To further explore the mechanisms underlying the influence of STAT3 siRNA on CML cells, the expression of downstream molecules of STAT3 were detected in K562 cells undergoing STAT3 silencing. Results showed the mRNA and protein expressions of c-Myc, Bcl-xL and Cyclin D1 (3 downstream molecules of STAT3) were inhibited significantly after STAT3 silencing, suggesting that lentivirus expressing STAT3 siRNA may inhibit the expression of genes related to regulation of cell proliferation, apoptosis and cell cycle (such as c-Myc, Bcl-xL and Cyclin D1) in K562 cells, which may reverse the malignant phenotype of K562 cells. These findings were consistent with those reported by Zhang et al [7, 12, 13].

To further confirm the influence of lentivirus expressing STAT3 siRNA on K562 cells *in vivo*, K562 cells were employed to establish CML mouse model and the influence of STAT3 silencing on the growth of CML mice and the tumorigenicity of K562 cells were investigated. Our results showed CML mice in CON group had poor spirit, poor appetite and weight loss soon after injection and thereafter developed slow movement and hind limb paralysis. In NC group, there were also weight loss, enlargement of the spleen and increase in peripheral WBC count.

In STAT3 siRNA group, mice had good spirit and had no weight loss and spleen enlargement and the increase in peripheral WBC count was not obvious. These findings indicate that STAT3 silencing may inhibit the *in vivo* proliferation of K562 cells and delay the occurrence of leukemia, exerting an inhibitory effect on *in vivo* tumorigenicity of K562 cells to a certain extent.

In addition, although the peripheral WBC count in NC group did not increase significantly when compared with CON group, the spleen index in NC group was significantly higher than that in CON group, which seem to be conflicting. We speculate that this discrepancy might be ascribed to the use of lentivirus. The spleen is a major organ related to immune response and has a large amount of lymphocytes. After lentivirus infection, the virus may enter the cells and intergrade into cell genome. The encoded proteins may serve as antigens to induce immune response, resulting in spleen enlargement and hyperplasia. This also reminds us that, as in other gene therapy, the safety of lentivirus mediated anti-tumor therapy is required to be further studied.

In the present study, results confirm that STAT3 silencing may influence the expression of its downstream genes related to cell proliferation, growth and apoptosis, to reverse the malignant phenotype of leukemia cells. This strategy may be promising in the target therapy of CML.

Acknowledgements

The study was supported by Natural Science Foundation of Shanghai (13ZR1438800).

Disclosure of conflict of interest

None.

Address correspondence to: Hong Xiong, Department of Hematology, Shanghai Xuhui Central Hospital & Shanghai Clinical Center, Chinese Academy of Sciences, Shanghai 200031, China. E-mail: xionghong_xh@163.com

References

[1] Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, Geurts van Kessel A, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, et al. Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature 1983; 306: 277-280.

[2] Meshkini A and Yazdanparast R. Involvement of ERK/MAPK pathway in megakaryocytic differentiation of K562 cells induced by 3-hydrogenkwadaphnin. Toxicol In Vitro 2008; 22: 1503-1510.

[3] Stella S, Tirro E, Conte E, Stagno F, Di Raimondo F, Manzella L and Vigneri P. Suppression of survivin induced by a BCR-ABL/JAK2/STAT3 pathway sensitizes imatinib-resistant CML cells to different cytotoxic drugs. Mol Cancer Ther 2013; 12: 1085-1098.

[4] Nakamura S, Yokota D, Tan L, Nagata Y, Takemura T, Hirano I, Shigeno K, Shibata K, Fujisawa S and Ohnishi K. Down-regulation of Thanatos-associated protein 11 by BCR-ABL promotes CML cell proliferation through c-Myc expression. Int J Cancer 2012; 130: 1046-1059.

[5] Pellicano F, Scott MT, Helgason GV, Hopcroft LE, Allan EK, Aspinall-O'Dea M, Copland M, Pierce A, Huntly BJ, Whetton AD and Holyoake TL. The Antiproliferative Activity of Kinase Inhibitors in Chronic Myeloid Leukemia Cells Is Mediated by FOXO Transcription Factors. Stem Cells 2014; 32: 2324-2337.

[6] Harb JG, Neviani P, Chyla BJ, Ellis JJ, Ferenchak GJ, Oaks JJ, Walker CJ, Hokland P, Roy DC, Caligiuri MA, Marcucci G, Huettner CS and Perrotti D. Bcl-xL anti-apoptotic network is dispensable for development and maintenance of CML but is required for disease progression where it represents a new therapeutic target. Leukemia 2013; 27: 1996-2005.

[7] Zhang G and Fu Y. Antiproliferative effect of indomethacin on CML cells is related to the suppression of STATs/Bcl-XL signal pathway. Ann Hematol 2006; 85: 443-449.

[8] Cha Y, Moon BH, Lee MO, Ahn HJ, Lee HJ, Lee KA, Fornace AJ Jr, Kim KS, Cha HJ and Park KS. Zap70 functions to maintain stemness of mouse embryonic stem cells by negatively regulating Jak1/Stat3/c-Myc signaling. Stem Cells 2010; 28: 1476-1486.

[9] Bhardwaj A, Sethi G, Vadhan-Raj S, Bueso-Ramos C, Takada Y, Gaur U, Nair AS, Shishodia S and Aggarwal BB. Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells. Blood 2007; 109: 2293-2302.

[10] Ma LD, Zhou M, Wen SH, Ni C, Jiang LJ, Fan J and Xia L. Effects of STAT3 silencing on fate of

chronic myelogenous leukemia K562 cells. Leuk Lymphoma 2010; 51: 1326-1336.

[11] Kim D, Lee IH, Kim S, Choi M, Kim H, Ahn S, Saw PE, Jeon H, Lee Y and Jon S. A specific STAT3-binding peptide exerts antiproliferative effects and antitumor activity by inhibiting STAT3 phosphorylation and signaling. Cancer Res 2014; 74: 2144-2151.

[12] Bewry NN, Nair RR, Emmons MF, Boulware D, Pinilla-Ibarz J and Hazlehurst LA. Stat3 contributes to resistance toward BCR-ABL inhibitors in a bone marrow microenvironment model of drug resistance. Mol Cancer Ther 2008; 7: 3169-3175.

[13] Chang G, Zhang H, Wang J, Zhang Y, Xu H, Wang C, Zhang H, Ma L, Li Q and Pang T. CD44 targets Wnt/beta-catenin pathway to mediate the proliferation of K562 cells. Cancer Cell Int 2013; 13: 117.