Original Research

Effects of Twist1 on drug resistance of chronic myeloid leukemia cells through the PI3K/AKT signaling pathway

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Abstract: This study aimed to investigate the effects of Twist1 on the drug resistance of chronic myeloid leukemia (CML) cells through the PI3K/AKT signaling pathway. K562 and KCL-22 cells were modeled for imatinib resistance, so as to analyze the effects of inhibiting Twist1 and the pathway on the therapeutic effect of imatinib on imatinib-resistant CML cells, and to find the mechanism of action of Twist1 on affecting the resistance. After the CML cells were successfully resistant to imatinib, Twist1 expression increased again in the cells and the PI3K/AKT signaling pathway was further activated. After the silence of the Twist1 expression, the imatinib-resistant CML cells were more sensitive to imatinib, and the PI3K/AKT signaling pathway was inhibited, and the expression level of p-AKT protein significantly reduced. According to further experiments, imatinib enhanced its inhibitory effect on the growth of the imatinib-resistant CML cells after the activation of the pathway was inhibited by an LY3023414 inhibitor. In conclusion, Twist1 and the PI3K/AKT signaling pathway are over-activated during the formation of the CML cells resistant to imatinib. The silence of Twist1 can reverse the resistance through the pathway.

Key words: Twist1; PI3K/AKT; Chronic myeloid leukemia; Drug resistance.

Introduction

Chronic myeloid leukemia (CML) is an unusual type of bone marrow cancer. The bone marrow is the spongy tissue inside the bones that makes up blood cells. CML increases the number of white blood cells. The term "chronic" leukemia suggests that cancer tends to progress gradually to acute types of leukemia. The term "myeloid" refers to the type of cells with this cancer. CML is a disease in which the bone marrow of many white blood cells develops. Symptoms and signs of CML include fever, night sweats, and fatigue. CML is a very rare disease caused by Ph translocation. Its global annual incidence is 0.6-2.8/100,000 people and its male-female ratio is 1.2-1.8:1 (1, 2). In addition, the incidence of the disease among people of different ages significantly varies. According to statistics in 20 EU countries, the incidence among people aged 20-29 years is 0.39/100,000 people, while that among people over 70 years old rapidly increases to 15.2/100,000 people (3). As the population ages, the incidence of CML will further increase (4).

Imatinib is a commonly used drug for CML chemotherapy, but as a result, imatinib resistance that is a major clinical challenge has become more widespread during the treatment (5). Therefore, to find out the mechanism of imatinib resistance is of great significance for complementing therapeutic strategies and improving the efficacy of imatinib. Twist1 is a protein-coding gene located on human chromosome 7p21.1, and proteins coded by the gene can promote the invasion and metastasis of various tumor cells (6, 7). Recent studies have shown that Twist1 plays an important role in the development of the drug resistance of tumor cells. According to Zhu et al., this gene mediates the cisplatin resistance of epithelial ovarian cancer cells (8). According to Liu et al., this gene promotes the vincristine resistance of colon cancer cells (9). According to Yochum et al., Twist1 inhibitors overcome the resistance of EGFR-mutant non-small cell lung cancer (NSCLC) mediated by the epithelial-mesenchymal transition to EGFR tyrosine kinase inhibitors (10). However, whether Twist1 plays an important role in the development of the imatinib resistance of CML cells remains unclear. According to Heidari et al., Twist1 expression further increases in the peripheral blood of patients that have no therapeutic effect of imatinib, but the researchers did not further analyze the reasons (11).

Therefore, in this study, the effects of Twist1 on the drug resistance of CML cells were explored, and the mechanism of action of the effects was investigated, so as to provide an experimental basis for the clinical improvement of imatinib resistance.

Materials and Methods

Cell sources

Human CML cell lines K562 (BNCC339825) and KCL-22 (BNCC341746) and human bone marrow cell line HS-27A (BNCC338456) were purchased from

BeNa Culture Collection. Culture mediums for K562 and HS-27A cells were 90% DMEM (high glucose) + 10% fetal bovine serum (FBS), while the culture medium for KCL-22 cells was 90% IMDM + 10% FBS. All cells were cultured at 37°C and with 95% air + 5% carbon dioxide (CO_2). The DMEM (high glucose), IMDM, and FBS were purchased from Gibco™, and their item numbers were 11995081, 12440061, and 10099141, respectively. The LY3023414 inhibitor (Item No.: HY-12513) was purchased from MCE, USA.

IC50 determination

Cells in the logarithmic growth phase were taken out, digested with trypsin (Thermo Scientific™, Item No.: 89901), and then resuspended. After that, they were conventionally inoculated into a 96-well plate at about $3*10^3$ cells/well and cultured for 6 hours. Next, imatinib with different concentrations was added. The concentrations in parent cells were respectively set as 0, 4, 8, 12, 16, 20, and 24μmol/L, while the concentrations in drug-resistant cells were respectively set as 0, 10, 20, 30, 40, 50, and 60μmol/L, with 3 same wells set up at the same time. CCK-8 assay was used to detect changes in the optical density (OD) values of the cells, and to calculate the cells' growth inhibition rate and IC50. Cell growth inhibition rate = [OD (blank wells) -OD (intervention wells)] / OD (blank wells).

Construction of imatinib-resistant cell lines

Cells in the logarithmic growth phase were taken out, conventionally digested with trypsin, and then resuspended. They were induced to be resistant to imatinib through low concentration gradient increment in vitro combined with intermittent high-dose pulse therapy. The concentration of imatinib was 1% IC50 initially, but it was increased to 0.2μmol/L after the cells stably survived, to 0.5μmol/L after the cells stably survived at 10% IC50, to 1μmol/L after the cells stably survived at 1-fold IC50. The modeling was considered successful when the cells stably survived at 2-fold IC50.

Expression vector construction and transfection

All expression vectors were designed by Thermo Fisher Scientific (China), including low Twist1 expression vector (si-Twist1) and blank vector si-NC. Cells in the logarithmic growth phase were taken out, digested with trypsin, resuspended, and then conventionally inoculated into a 96-well plate. When the cells fused to about 80%, the expression vectors were transfected, with the specific steps carried out in accordance with the instructions of the kits. The cells were cultured in an incubator at 37°C and with 5% CO_2 for 48 hours, with the culture medium changed every 6 hours. Western blot was used to detect the results of the transfection. The LipofectamineTM2000 transfection kit was purchased from Invitrogen, USA, Item No.: 35050. Cells not intervened were considered as the blank group.

Western blot

Cells in the logarithmic growth phase were taken out for extracting total protein by using RIPA lysis method. BCA was used to detect the concentration of the protein. After the concentration was adjusted to 4μg/μL, the protein was separated with 12% polyacrylamide gel electrophoresis (PAGE), during which the voltage was 90V initially and then increased to 120V, so as to move the samples to the appropriate position of the separation gel. After that, the protein was transferred to the membrane (at 100V constant voltage for 100min) and sealed at 37°C for 60min. Next, the transferred membrane was sealed in 5% skim milk and subjected to an immune reaction. The membrane was incubated overnight with primary antibody (1: 1000) at 4°C, and then washed with PBS over 5min for 3 times the next day. Then, the membrane was incubated with secondary antibody (1: 1000) at room temperature for 1h. Finally, the membrane was developed with ECL luminescent reagents and fixed. The protein bands scanned by the film were statistically analyzed by Quantity One. The relative expression levels of proteins = the gray values of protein bands / the gray values of internal references. The RIPA kit, the BCA protein kit, and the ECL luminescent kit were purchased from Thermo Scientific™, and their item numbers were respectively 89901, 23250, and 35055. Rabbit anti-Twist1 monoclonal antibody, rabbit anti-PI3K monoclonal antibody, rabbit anti-AKT monoclonal antibody, rabbit anti-phosphorylated AKT (p-AKT) polyclonal antibody, and goat anti-rabbit IgG secondary antibody (monoclonal antibody) were purchased from Abcam, USA, and their item numbers were respectively ab187008, ab32089, ab179463, ab8805, and ab6721.

CCK-8 assay for cell proliferation

Cells in the logarithmic growth phase were taken out, digested with trypsin, and then resuspended. After the cell concentration was adjusted to $2*10^4$ cells/mL, the cells (100μL) were conventionally inoculated into a 96-well plate. CCK-8 mixed solutions (200μL, 10: 1) were respectively added at 12, 24, 48, 72, and 96 hours after culture, and then the cells were continuously cultured for 3 hours to measure the OD values at 450nm. The IC50 determination was conducted to measure the values at 48 hours after the culture. The CCK-8 detection kit (Item No.: C0037) was purchased from Beyotime, Beijing.

Statistical analysis

SPSS 19.0 (Asia Analytics Formerly SPSS, China) was used for statistical analysis. Measurement data were expressed by mean ± standard deviation (mean±sd). The comparison between the two groups was analyzed by independent samples t-test, the comparison between multiple groups by one-way analysis of variance, post hoc test by LSD test. $P<0.05$ indicated a statistically significant difference.

Results

Expressions of Twist1 and PI3K/AKT signaling pathway-related proteins

The expressions of Twist1, PI3K, AKT, and p-AKT in human CML cell lines K562 and KCL-22 were higher than those in human bone marrow cell line HS-27A ($P<0.05$). (Table 1).

IC50 determination of K562 and KCL-22 cells

According to CCK-8 assay, the growth inhibition of

Table 1. Expressions of Twist1 and PI3K/AKT signaling pathway-related proteins

	K562	KCL-22	HS-27A	F	P
Twist1	1.472±0.121	1.287±0.102	0.455±0.039*	99.417	<0.001
PI3K	0.875±0.032	0.859±0.034	0.084±0.012*	791.671	<0.001
AKT	1.032±0.045	1.001±0.052	0.095±0.011*	525.701	<0.001
p-AKT	0.968±0.038	0.945±0.027	0.072±0.012*	1013.471	<0.001

Note: * indicated P<0.05 when compared with that in K562 and KCL-22 cells.
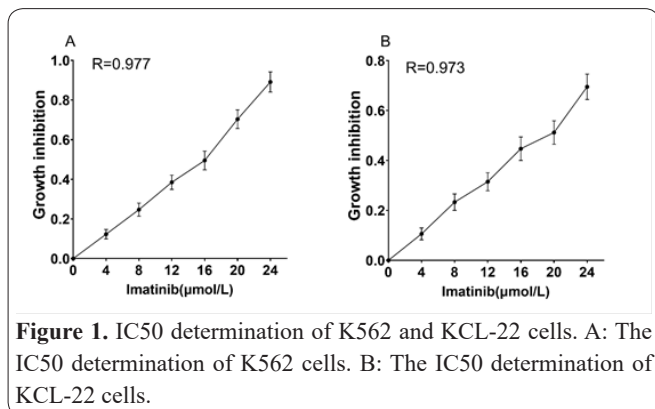

Figure 1. IC50 determination of K562 and KCL-22 cells. A: The IC50 determination of K562 cells. B: The IC50 determination of KCL-22 cells.
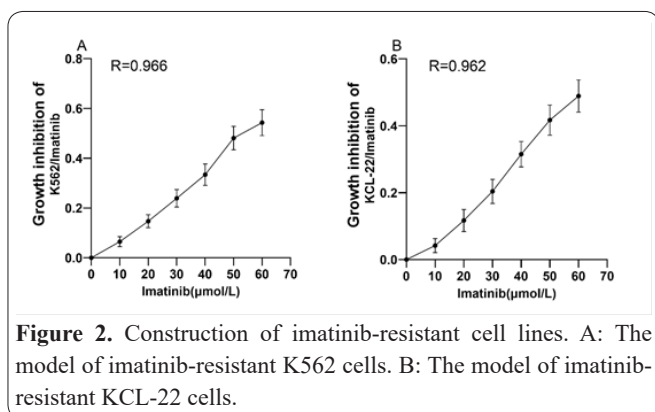

Figure 2. Construction of imatinib-resistant cell lines. A: The model of imatinib-resistant K562 cells. B: The model of imatinib-resistant KCL-22 cells.

K562 and KCL-22 cells by imatinib at various concentrations is shown in Figure 1. The linear regression equations $Y(K562) = 0.03644*X - 0.03096$ and $Y(KCL-22) = 0.02778*X - 0.003607$ were established. The IC50 values of the K562 and KCL-22 cells were calculated to be 14.57μmol/L and 18.13μmol/L, respectively.

Construction of imatinib-resistant cell lines

According to CCK-8 assay, the growth inhibition of K562/imatinib and KCL-22/imatinib cells is shown in Figure 2. The linear regression equations $Y(K562/imatinib) = 0.009457*X - 0.02529$ and $Y(KCl-22/imatinib) = 0.008625*X - 0.03246$ were established. The IC50 values of the K562/imatinib and KCL-22/imatinib cells were calculated to be 55.55μmol/L and 61.73μmol/L, respectively.

Effects of silencing Twist1 on imatinib resistance of drug-resistant cell lines

The results of Western blot showed that the expression level of Twist1 in the K562/imatinib and KCL-22/imatinib cells was significantly higher than that in the K562 and KCL-22 cells (P<0.05). After silencing the Twist1 expression, the resistance of Twist1 to imatinib in the K562/imatinib and KCL-22/imatinib cells significantly reduced, and the growth inhibition of the cells by imatinib was enhanced at IC50 concentration (P<0.05).

Moreover, the PI3K/AKT signaling pathway was also inhibited, and the expression levels of PI3K, AKT, and p-AKT proteins significantly reduced (P<0.05). (Figure 3).

Effects of inhibiting PI3K/AKT signaling pathway on imatinib resistance of drug-resistant cell lines

After the CML cells were resistant to imatinib, the PI3K/AKT signaling pathway in the cells was further activated. The expression levels of PI3K, AKT, and p-AKT proteins in the K562/imatinib and KCL-22/imatinib cells were significantly higher than those in the K562 and KCL-22 cells. After the LY3023414 inhibitor was used to inhibit the activation, the growth inhibition of the K562/imatinib and KCL-22/imatinib cells by imatinib was enhanced at IC50 concentration (P<0.05). (Figure 4).

Discussion

The PI3K/AKT signaling pathway is a classic intracellular pathway that regulates cell cycles. Through phosphorylating downstream factors such as various enzymes, kinases, and transcription factors, it regulates the proliferation and dormancy of normal cells and the proliferation, survival, invasion, and metastasis of tumor cells (12, 13). This pathway promotes the epithelial-mesenchymal transformation of tumor cells (14), and the transformation is an important reason for the drug resistance of the tumor cells (15). Recent studies have found that the PI3K/AKT signaling pathway plays an important role during drug resistance (16). Twist1 is a basic helix-loop-helix transcription factor and a major regulatory factor for epithelial-mesenchymal transformation. According to Mikheev et al., this factor promotes the epithelial-mesenchymal transformation of glioma cells and enhances their invasion by activating this pathway (17). Accordingly, Twist1 activating the PI3K/AKT signaling pathway may be an important reason for Twist1 to affect the drug resistance of tumor cells.

In this study, we verified for the first time that Twist1 promoted the CML cells resistant to imatinib by activating the PI3K/AKT signaling pathway, which was consistent with the results of previous studies (18, 19). In addition, Twist1 was overexpressed in the K562 and KCL-22 cells, and the PI3K/AKT signaling pathway was also activated. The two cell lines were used as parent cells to construct a model of imatinib-resistant CML cells. After the CML cells were successfully resistant to imatinib, the expression levels of Twist1 and PI3K/AKT signaling pathway-related proteins in the cells were detected. The results showed that the Twist1 expression increased again and the pathway was further activated in the imatinib-resistant CML cells. These
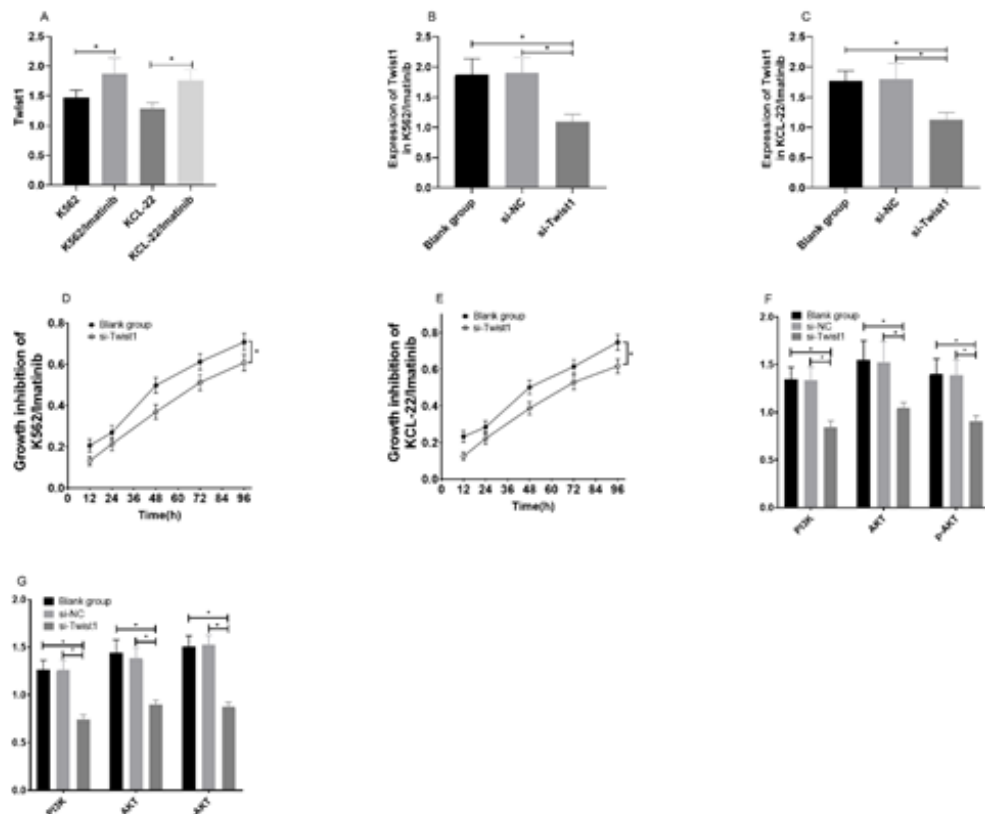
Figure 3. Effects of silencing Twist1 on imatinib resistance of drug-resistant cell lines. A: The expression levels of Twist1 in drug-resistant cells. B: The results of transfecting the K562/imatinib cells with si-Twist1. C: The results of transfecting the KCL-22/imatinib cells with si-Twist1. D: Effects of silencing Twist1 on the therapeutic effect of imatinib on the K562/imatinib cells. E: Effects of silencing Twist1 on the therapeutic effect of imatinib on the KCL-22/imatinib cells. F: Effects of silencing Twist1 on the PI3K/AKT signaling pathway in the K562/imatinib cells. G: Effects of silencing Twist1 on the PI3K/AKT signaling pathway in the KCL-22/imatinib cells. * indicates P<0.05.
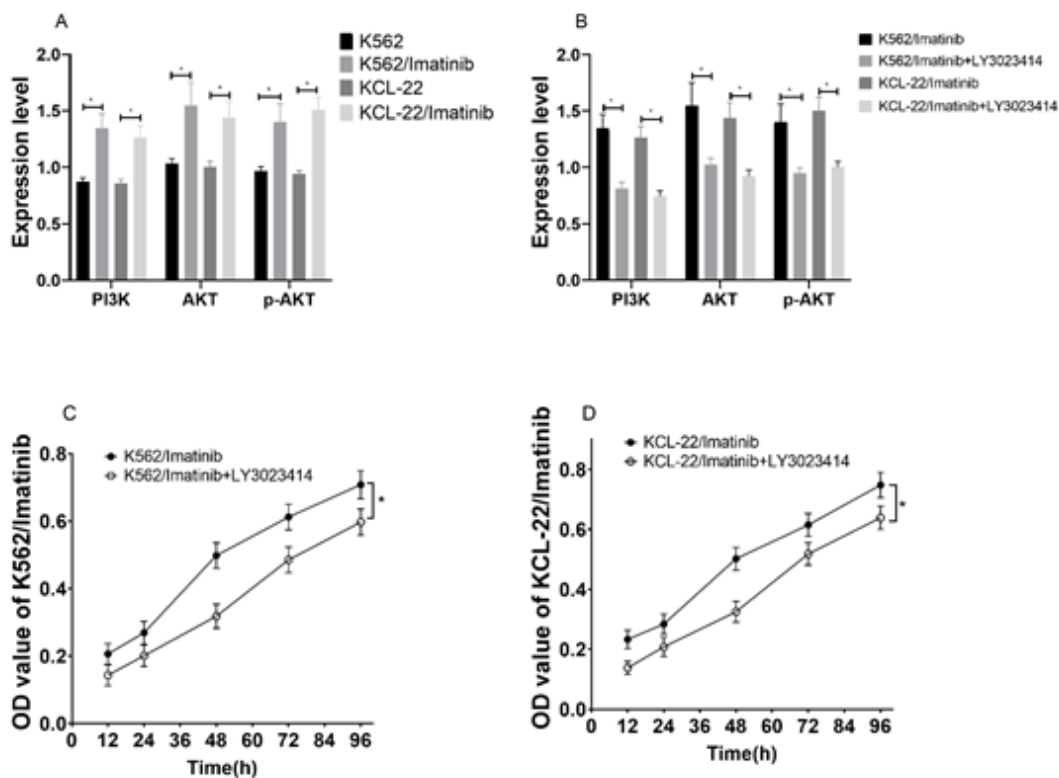

Figure 3. Effects of inhibiting PI3K/AKT signaling pathway on imatinib resistance of drug-resistant cell lines. A: Effects of the LY3023414 inhibitor on the PI3K/AKT signaling pathway in the K562/imatinib cells. B: Effects of the LY3023414 inhibitor on the PI3K/AKT signaling pathway in the KCL-22/imatinib cells. C: Effects of inhibiting the PI3K/AKT signaling pathway on the therapeutic effect of imatinib on the K562/imatinib cells. D: Effects of inhibiting the PI3K/AKT signaling pathway on the therapeutic effect of imatinib on the KCL-22/imatinib cells. * indicates P<0.05.

findings suggest that the activation of Twist1 and the PI3K/AKT signaling pathway is related to the imatinib resistance of CML cells. After silencing the Twist1 expression, the imatinib-resistant CML cells were more sensitive to imatinib, and the pathway was inhibited, and the expression level of p-AKT protein significantly reduced. After the LY3023414 inhibitor was used to inhibit the activation of the pathway, imatinib had an enhanced inhibitory effect on the growth of the imatinib-resistant CML cells. Therefore, Twist1 can promote CML cells resistant to imatinib by activating the PI3K/AKT signaling pathway, while the silence of Twist1 can reverse the resistance.

Roberts and others reported in Scientific Reports in 2016 that Twist1 could promote the cisplatin resistance of ovarian cancer cells by activating the AKT signaling pathway (20). Later Yang and others have also found that Twist1 could also be used as a downstream target of STAT3 that could promote the cisplatin resistance by up-regulating Twist1. Moreover, Twist1 could also be activated by COL11A1 to promote the cisplatin and paclitaxel resistance of ovarian cancer cells (21, 22). These studies indicate that Twist1 has a more complex role in the regulation of the drug resistance of tumor cells, which should be further explored and verified. In this regard, genome editing (23) may be helpful in the future.

In summary, Twist1 and the PI3K/AKT signaling pathway are over-activated during the formation of the CML cells resistant to imatinib. The silence of Twist1 can reverse the resistance through the pathway.

References

1.   Qin L, Deng HY, Chen SJ, Wei W. Relationship between cigarette smoking and risk of chronic myeloid leukaemia: a meta-analysis of epidemiological studies. Hematol 2017; 22(4):193-200.

2.   Rohrbacher M, Hasford J. Epidemiology and Etiology of Chronic Myeloid Leukemia. Neoplast Dis Blood 2018; (pp. 9-17). Springer, Cham.

3.   Hoffmann VS, Baccarani M, Hasford J, Lindörfer D, Burgstaller S, Sertic D, Costeas P, Mayer J, Indrak K, Everaus H, Koskenvesa P. The EUTOS population-based registry: incidence and clinical characteristics of 2904 CML patients in 20 European Countries. Leuk 2015; 29(6):1336-43.

4.   Höglund M, Sandin F, Simonsson B. Epidemiology of chronic myeloid leukaemia: an update. Ann Hematol 2015; 94(2): 241-247.

5.   Huang WK, Akçakaya P, Gangaev A, Lee L, Zeljic K, Hajeri P, Berglund E, Ghaderi M, Åhlén J, Bränström R, Larsson C. miR-125a-5p regulation increases phosphorylation of FAK that contributes to imatinib resistance in gastrointestinal stromal tumors. Exp Cell Res 2018; 371(1):287-96.

6.   Li CW, Xia W, Lim SO, Hsu JL, Huo L, Wu Y, Li LY, Lai CC, Chang SS, Hsu YH, Sun HL. AKT1 inhibits epithelial-to-mesenchymal transition in breast cancer through phosphorylation-dependent Twist1 degradation. Cancer Res 2016; 76(6):1451-62.

7.   Xu Y, Qin L, Sun T, Wu H, He T, Yang Z, Mo Q, Liao L, Xu J. Twist1 promotes breast cancer invasion and metastasis by silencing Foxa1 expression. Oncogene 2017; 36(8):1157-66.

8.   Zhu X, Shen H, Yin X, Long L, Xie C, Liu Y, Hui L, Lin X, Fang Y, Cao Y, Xu Y. miR-186 regulation of Twist1 and ovarian cancer sensitivity to cisplatin. Oncogene 2016; 35(3):323-32.

9.   Liu YR, Liang L, Zhao JM, Zhang Y, Zhang M, Zhong WL, Zhang Q, Wei JJ, Li M, Yuan J, Chen S. Twist1 confers multidrug resistance in colon cancer through upregulation of ATP-binding cassette transporters. Oncotarget 2017; 8(32):52901.

10. Yochum ZA, Cades J, Wang H, Chatterjee S, Simons BW, O'Brien JP, Khetarpal SK, Lemtiri-Chlieh G, Myers KV, Huang EH, Rudin CM. Targeting the EMT transcription factor TWIST1 overcomes resistance to EGFR inhibitors in EGFR-mutant non-small-cell lung cancer. Oncogene 2019; 38(5):656-70.

11. Heidari N, Vosoughi T, Mohammadi Asl J, Saki Malehi A, Saki N. How is the relationship between TWIST-1 and BCR-ABL1 gene expressions in chronic myeloid leukaemia patients? Biomark 2018; 23(3):271-6.

12. Vara JÁ, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 2004; 30(2):193-204.

13. Mayer IA, Arteaga CL. The PI3K/AKT pathway as a target for cancer treatment. Annu Rev Med 2016; 67:11-28.

14. Baek SH, Ko JH, Lee JH, Kim C, Lee H, Nam D, Lee J, Lee SG, Yang WM, Um JY, Sethi G. Ginkgolic acid inhibits invasion and migration and tgf-β-induced emt of lung cancer cells through pi3k/akt/mtor inactivation. J Cell Physiol 2017; 232(2):346-54.

15. Shibue T, Weinberg RA. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nat Rev Clin Oncol 2017; 14(10):611.

16. Guerrero-Zotano A, Mayer IA, Arteaga CL. PI3K/AKT/mTOR: role in breast cancer progression, drug resistance, and treatment. Cancer Metastasis Rev 2016; 35(4):515-24.

17. Mikheev AM, Mikheeva SA, Severs LJ, Funk CC, Huang L, McFaline-Figueroa JL, Schwensen J, Trapnell C, Price ND, Wong S, Rostomily RC. Targeting TWIST 1 through loss of function inhibits tumorigenicity of human glioblastoma. Mol Oncol 2018; 12(7):1188-202.

18. Cosset E, Hamdan G, Jeanpierre S, Voeltzel T, Sagorny K, Hayette S, Mahon FX, Dumontet C, Puisieux A, Nicolini FE, Maguer-Satta V. Deregulation of TWIST-1 in the CD34+ compartment represents a novel prognostic factor in chronic myeloid leukemia. Blood, J Am Soc Hematol 2011; 117(5):1673-6.

19. Lu Y, Li Y, Chai X, Kang Q, Zhao P, Xiong J, Wang J. Long noncoding RNA HULC promotes cell proliferation by regulating PI3K/AKT signaling pathway in chronic myeloid leukemia. Gene 2017; 607:41-6.

20. Roberts CM, Tran MA, Pitruzzello MC, Wen W, Loeza J, Dellinger TH, Mor G, Glackin CA. TWIST1 drives cisplatin resistance and cell survival in an ovarian cancer model, via upregulation of GAS6, L1CAM, and Akt signalling. Sci Rep 2016; 6:37652.

21. Yang J, Xing H, Hong L, Lu D, Li B, Tang J, Min J. STAT3-mediated Twist1 upregulation contributes to epithelial-mesenchymal transition in cisplatin resistant ovarian cancer. Int J Clin Exp Med 2018; 11(7):6749-57.

22. Wu YH, Huang YF, Chang TH, Chou CY. Activation of TWIST1 by COL11A1 promotes chemoresistance and inhibits apoptosis in ovarian cancer cells by modulating NF-κB-mediated IKKβ expression. Int J Cancer 2017; 141(11):2305-17.

23. Bordbar M, Darvishzadeh R, Pazhouhandeh M, Kahrizi D. An overview of genome editing methods based on endonucleases. Mod Genet J 2020; 15(2): 75-92.