Genistein-induced Anticancer Effects on Acute Leukemia Cells Involve the Regulation of Wnt Signaling Pathway Through H4K20me1 Rather Than DNA Demethylation*

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[Abstract] Objective: To investigate the effects and mechanisms of genistein on the gene expression in the Wnt pathway in acute leukemia (AL) cells. **Methods:** The expression of Wnt pathway genes and cell cycle-related genes were analyzed in two AL cell lines. Pyrophosphate sequencing was performed to determine the methylation degree. Then, the enrichment of H4K20me1 and H3K9ac was determined using ChIP-qPCR. Flow cytometry was used to analyze the cell cycle. **Results:** The IC_{50} of genistein in the two AL cell lines was lower than that for the bone marrow mesenchymal stem cell line. Genistein upregulated H4K20me1, KMT5A and Wnt suppressor genes, including *Wnt5a*, and downregulated the downstream target genes of Wnt, such as c-myc and β-catenin. The methylation degree and H3K9ac enrichment in the *Wnt5a* promoter region remained unchanged. However, the enrichment of H4K20me1 in the *Wnt5a* promoter and coding regions increased. In addition, genistein upregulated Phospho-cdc2, Myt1, Cyclin A, Cyclin E2, p21 and Phospho-histone H3, but downregulated Phospho-wee1. Cell cycle arrest was induced in the G2/M phase. **Conclusion:** Genistein inhibits the activation of the Wnt pathway by promoting the expression of *Wnt5a* through the activation of KMT5A and enrichment of H4K20me1 in the *Wnt5a* gene promoter and coding regions, rather than demethylation. Genistein also blocks the cell cycle in the G2/M phase. Therefore, genistein is a potential anti-leukemia drug.
Key words: genistein; acute leukemia, H4K20me1; Wnt pathway; G2/M cell cycle arrest

In China, acute leukemia (AL) is the main cause of death from malignancies in people <35 years old. In the past four decades, treatment options were mainly limited to cytotoxic chemotherapy, and the 5-year survival rate was only 25%. In recent years, studies have confirmed that epigenetic modifications, such as DNA methylation, genomic imprinting, histone modification, RNA editing and chromatin remodeling, as regulatory mechanisms, affect the gene expression and regulate the phenotype of leukemia[1–3]. Epigenetic modifications and the reversibility of epigenetic changes have allowed for new drug targets and candidates.

Drugs with epigenetic regulation activity are called epigenetic therapeutic drugs, and the main drugs used in clinical settings are histone deacetylase and DNA methyltransferase inhibitors. Recently, some phytochemicals have received widespread attention as anticancer drugs, such as genistein, green tea polyphenols, curcumin and resveratrol. These have also been proven to influence the appearance of various cancer types, including leukemia, by genetic modification. Genistein (fig. 1A) is a soybean isoflavone, which is considered to be an antitumor drug that affects the epigenetic status of tumor suppressor genes in leukemia, and breast, prostate and colorectal cancer[4]. Previous studies have also revealed that genistein inhibits DNA methyltransferase activity and demethylates tumor suppressor genes, thereby promoting their expression[5–7]. In addition, genistein promotes histone H3K9 acetylation in prostate cancer[8]. However, the anti-leukemia mechanism is not yet fully elucidated, and further exploration is needed

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to determine whether other histone modifications are involved. Therefore, the present study aims to evaluate the anticancer effects of natural flavonoid genistein on AL cells, and determine the potential anticancer mechanism.

1 MATERIALS AND METHODS

1.1 Chemicals and Reagents

Genistein (≥98.0%, HPLC), dimethyl sulfoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI) and 5-Azacytidine (≥98.0%, HPLC) were purchased from Sigma-Aldrich Corp. (USA). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from GIBCO (USA). The EpiTect Bisulfite Kit was obtained from QIAGEN (Germany). The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (USA). The NE-PERTM Nuclear and Cytoplasmic Extraction Reagents and Mem-PERTM Plus Membrane Protein Extraction Kit were procured from Thermo Fisher Scientific (USA). The FastStart Universal SYBR Green Master (ROX) was purchased from Roche (Switzerland). The RevertAid First Strand cDNA Synthesis Kit was purchased from Fermentas (USA). The Cell Counting Kit 8 (WST-8/CCK8) was purchased from Beyotime Technology (China). The anti-histone H3 (acetyl K9), rabbit anti-c-Myc, anti-KMT5A/SETD8/Pr-SET7, rabbit anti-GAPDH, and anti-sodium potassium ATPase antibodies were purchased from Abcam (UK). The Cell Cycle Regulation Antibody Sampler Kit II [including the Phospho-cdc2 (Tyr15) rabbit mAb, Cyclin A2 mouse mAb, Cyclin B1 rabbit mAb, Cyclin E2 antibody, Phospho-histone H3 (Ser10) rabbit mAb, Myt1 antibody, p21 Waf1/Cip1 rabbit mAb, Phospho-Wee1 (Ser642) rabbit mAb, anti-rabbit IgG, HRP-linked antibody, anti-mouse IgG, and HRP-linked antibody], β-Tubulin rabbit antibody, and Lamin B1 rabbit mAb were purchased from CST (USA). The purified mouse anti-β-Catenin, BD IntraSure TM Kit, PI/RNase staining buffer, and PE Annexin V Apoptosis Detection Kit I were purchased from BD (USA). The anti-Wnt-5a antibody, ChIPAb+ monomethyl-histone H4 (Lys20), and EZ-Magna ChIP™ A/G One-Day Chromatin Immunoprecipitation Kits were purchased from Millipore (USA). The Alexa Fluor® 594-conjugated AffiniPure goat anti-rabbit IgG (H+L), Fluor® 488-conjugated AffiniPure goat anti-mouse IgG (H+L), paraformaldehyde, and Triton X-100 were purchased from ZSGB-BIO (China).

1.2 Cell Culture

The human AL cell lines U937 and Jurkat, and the non-cancerous bone marrow mesenchymal stem cell line (hBMSCs) with STR authentication and no mycoplasma contamination was maintained at the Fujian Institute of Hematology (China). The U937 and Jurkat cells were grown in RPMI 1640 medium

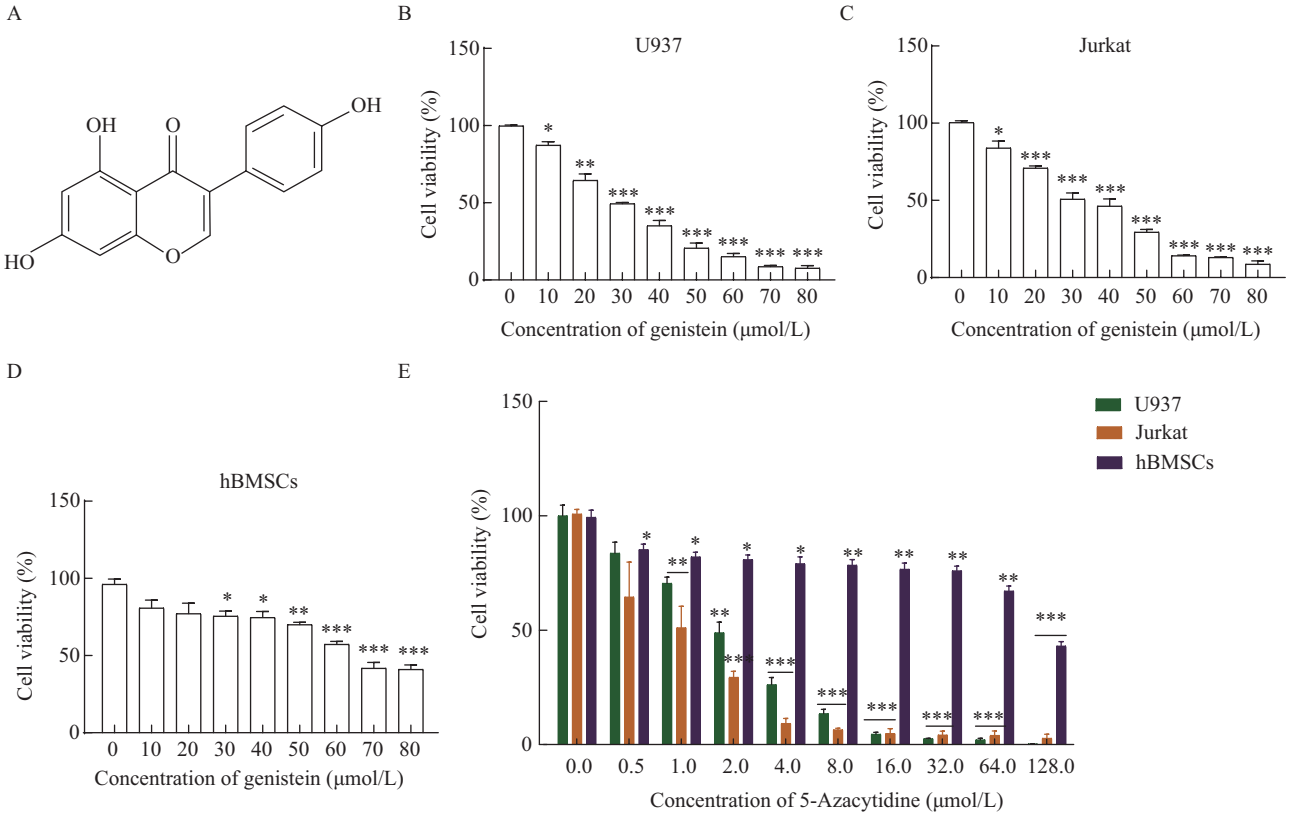

Fig. 1 A: chemical structure of genistein. Effects of different doses of genistein on cell viability of the (B) U937, (C) Jurkat, and (D) hBMSCs cell lines. (E) Effects of standard drug 5-Azacytidine on the activity of U937, Jurkat and hBMSC cells. The experiment was carried out in triplicate, and the results are expressed as mean±SD. The values were considered statistically significant at $^{*}P<0.01$, $^{**}P<0.001$ and $^{***}P<0.0001$.

(GIBCO), and the hBMSCs were maintained in Gibco Dulbecco's Modified Eagle Medium (DMEM) (GIBCO). All cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere (HealthForce, China), and supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL) and 10% heat-inactivated FBS (GIBCO).

1.3 CCK-8 Cell Viability Assay

The cytotoxic effect of genistein on U937, Jurkat and hBMSCs cells were determined using the CCK-8 assay (Beyotime Technology). The cells were seeded at a density of 1×10^4 cells/well in 96-well plates, and treated with genistein (0–80 μmol/L) for 72 h. The genistein was dissolved in DMSO, and the untreated cells received equal concentrations of DMSO. After the genistein treatment, 10 μL of CCK-8 was added to each well, and cells were incubated at 37°C for two h. The absorbance values at 450 nm and 630 nm were measured, in order to determine the number of living cells. The cytotoxicity of genistein on U937, Jurkat and hBMSCs cells was expressed as IC_{50}, and was calculated using GraphPad Prism 8.0.1 (GraphPad Software, USA). Since 5-Azacytidine can reverse DNA methylation, this was used as the standard drug, and the antiproliferative effect of 5-Azacytidine on all cell lines cultured *in vitro* was determined using the same procedure.

1.4 Reverse Transcription and Fluorescence Quantitative PCR (qRT-PCR)

The U937 and Jurkat cells were seeded at a density of 1×10^5 cells/mL in a 6-well plate. Genistein was added at concentrations of 0, 20, 40 and 50 μmol/L, and followed by 72 h of incubation. DMSO was used as the vehicle control. Thereafter, cells were collected and washed twice with phosphate buffered saline (PBS). qRT-PCR was performed to detect the relative mRNA expression of Wnt pathway genes (*Wnt5a*, *DACT1*, *WIF1*, *DKK1* and *c-myc*, *AXIN2*) in the genistein-treated U937 and Jurkat cells. The total RNA was isolated using TRIzol reagent, according to manufacturer's instructions. The same amount of total RNA (1 μg) was used to synthesize the cDNA using reverse transcription with the RevertAid First Strand cDNA Synthesis Kit. The qRT-PCR with primers specific for Wnt pathway genes was performed with the Faststart Universal SYBR Green Master Mix on the ABI 7500 Fluorescence quantitative PCR system. GAPDH was used as an internal control. qRT-PCR was performed at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method. The specific mRNA primers are presented in table 1, with GAPDH as the endogenous control.

1.5 Pyrophosphate Sequencing

Pyrophosphate sequencing was performed to determine the methylation rate of the CpG island of the *Wnt5a* promoter in genistein-treated U937 and Jurkat cells. U937 and Jurkat cells were treated with 0 and 40 μmol/L of genistein for 72 h. Thereafter, all cells were collected and washed twice with PBS. Then, the genomic DNA was extracted using phenol-chloroform. The EpiTect Bisulfite Kit was used for bisulfite modification and the purification of genomic DNA, in strict accordance with manufacturer's instructions. Then, the amplification of sulfated genomic DNA was carried out, and the methylation rate of the CpG island of the *Wnt5a* promoter was determined using pyrosequencing[9].

1.6 Determination of Protein Expression by Western Blotting

U937 and Jurkat cells were treated with 0 and 40 μmol/L of genistein for 72 h. The total protein was isolated using cell lysis buffer and a protease inhibitor cocktail. The nuclear and cytoplasmic proteins were isolated using NE-PERTM Nuclear and Cytoplasmic Extraction Reagents. The membrane proteins were isolated using the Mem-PERTM Plus Membrane Protein Extraction Kit. The BCA protein assay kit was used to determine the protein concentration. GAPDH was used as the loading control for the total protein.

Table 1 Sequences of the qRT-PCR and CHIP-qPCR primers

Gene	Designed RT-qPCR primer sequences (5' to 3')	
	Forward	Reverse
qPCR		
Wnt5a	GACCACATGCAGTACATCGGAGAAG	TCCACCTTCGATGTCGGAATTG
DACT1	AAGAGCACCTGGAGACAGACAG	GCTGGAATGACAACTGGATAAAC
WIF1	GAAGGTTGGCATGGAAGA	TGTAATTGGATTCAGGTGGA
DKK1	CTGCAAAAATGGAATATGTGT	CTTCTTGTCCTTTGGTGTGA
c-myc	GCTCCTGGCAAAAGGTCAGAGTCT	ACCAGTGGGCTGTGAGGAGGTT
AXIN2	CTGGCTCCAGAAGATCACAAAG	ATCTCCTCAAACACCGCTCCA
GAPDH	GGATGCAGGGATGATGTTCT	TGCCACTCAGAAGACTGTGG
CHIP-qPCR		
Wnt5a promoter region	GGTCTTTTGCACAATCACGCC	TTTCCAACGTCCATCAGCGAC
Wnt5a coding region	GATGGCTGGAAGTGCAATGTCT	ACCTGGGCGAAGGAGAAAAA
AXIN2 promoter region	CTGGAGCCGGCTGCGCTTTGATAA	CGGCCCCGAAATCCATCGCTCTGA
GAPDH	TACTAGCGGTTTTACGGGCG	TCGAACAGGAGGAGCAGAGAGCGA

Sodium potassium ATPase was used as the plasma membrane loading controller, β-tubulin was used as the cytoplasmic protein control, and lamin B1 was used as the nuclear protein control. Western blotting was performed[9]. Then, the primary antibodies used for the Western blotting analysis were as follows: mouse mono: *Wnt5a*, H4K20me1, Cyclin A2 and β-Catenin; rabbit mono: c-myc, GAPDH, sodium potassium ATPase, Phospho-cdc2, Cyclin B1, Phospho-histone H3, p21, Phospho-Wee1, β-Tubulin and Lamin B1; rabbit poly: KMT5A/SETD8/Pr-SET7, Cyclin E2 and Myt1. Anti-mouse and anti-rabbit secondary antibodies were used, in accordance to manufacturer's recommendations. The proteins were detected using the chemiluminescent substrate (ECL, Beyotime).

1.7 Immunofluorescence

U937 and Jurkat cells were collected and treated with 0 and 40 μmol/L of genistein for 72 h, and the cell concentration was adjusted to 1×10^6–2×10^6 cells/mL by adding PBS. Subsequently, 10 μL of the cell suspension was painted on the polylysine slide, and naturally dried for 10–15 min. Then, the cells were fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 15 min. Afterwards, these cells were incubated with the primary antibodies (anti-*Wnt5a* and anti-β-Catenin, 1:500), which were diluted with sheep serum blocking solution and blocked at 4°C overnight. Next, cells were incubated with Fluor 488-Conjugated AffiniPure goat anti-mouse IgG (H+L) (1:400) and Alexa Fluor 594-conjugated AffiniPure goat anti-rabbit IgG (H+L) (1:400) for two h at room temperature in the dark. Then, the nuclei were counterstained with DAPI. A Leica TCS SP8 laser scanning confocal microscope (Leica, Germany) was used for the co-localization analysis.

1.8 Chromatin Immunoprecipitation

U937 cells were seeded at a density of 1×10^5 cells/mL in 6-well plates, and treated with 0 and 50 μmol/L of genistein for 72 h. Thereafter, all cells were collected and washed twice with PBS. The EZ-Magna ChIP A/G One-Day Chromatin Immunoprecipitation kit was used for the chromatin immunoprecipitation (ChIP) of cells. Cell isolation, sonication, ChIP, eluting, de-crosslinking of protein complexes by immunoprecipitation, fluorescence qPCR, and data analysis were performed, as previously described[9]. The primers (table 1) were designed for different regions of the *Wnt5a* and *AXIN2* genes using *GAPDH* as the internal reference. The following antibodies were used: CHIPAb + monomethyl-histone H4 (Lys20) and anti-histone H3 (acetyl K9).

1.9 Estimation of Cell Cycle Distribution of Acute Leukemia Cells

The cells were plated in 6-well plates at a density of 1×10^5 cells/mL. Genistein was added to these cells at concentrations of 0, 20, 40, 50 and 70 μmol/L, and followed by 72 h of incubation. DMSO was used as the vehicle. Subsequently, these cells were washed with PBS, and fixed in 70% ethanol at 4°C overnight. This was followed by re-suspension in PBS containing 40 μg/mL of PI and RNase A (0.1 mg/mL) for 15 min in the dark at 37°C. Further analysis was carried out using flow cytometry[10].

1.10 Statistical Analysis

Experimental data are expressed as mean± standard deviation (SD). The experiments were repeated in triplicate, and the results were analyzed using SPSS 19.0 (IBM Corp., USA). The results were analyzed using one-way analysis of variance. *P*-values <0.05 were considered statistically significant.

2 RESULTS

2.1 Genistein Decreases the Viability of Acute Leukemia Cells

Genistein exhibited antitumor effects on AL cells (U937 and Jurkat, figs. 1B and 1C) in a concentration-dependent manner. The IC_{50} of genistein for U937 and Jurkat cells was 30 μmol/L. However, genistein had no obvious antiproliferative effect on non-cancerous hBMSCs (IC_{50}=70 μmol/L, fig. 1D). Next, 5-Azacytidine was used as a comparison, and to examine the anticancer effects of 5-aza-deoxycytidine on all cell lines used in the present study, as shown in fig. 1E.

2.2 Genistein Upregulates the Expression of Wnt Pathway Antagonist Genes and Inhibits the Wnt Signaling Pathway

After 72 h, as the genistein concentration increased (final concentrations: 0, 20, 40 and 50 μmol/L), the relative expression of the Wnt pathway antagonist genes (*Wnt5a*, *DACT1* and *WIF1*) exhibited a progressive increase, while the target genes of the Wnt signaling pathway (*AXIN2* and *c-myc*) demonstrated a progressive decrease. The genistein-induced gene expression changes in the Wnt pathway exhibited a dose-dependent pattern (figs. 2A and 2B).

2.3 Genistein Promotes the Protein Expression of *Wnt5a* in the Acute Leukemia Cell Membrane, Reduces the Migration of β-catenin to the Nucleus, and Inhibits Wnt Pathway Activation

The treatment of U937 and Jurkat cells with genistein (40 μmol/L) caused significant alterations in Wnt pathway proteins, when compared to untreated cells. The Wnt pathway antagonist gene *Wnt5a* (figs. 3A and 3B) was upregulated in the cell membrane and cytoplasm, while the Wnt pathway target gene *c-myc* was downregulated in the nucleus and cytoplasm (figs. 3A and 3C). Compared to the control, the β-catenin expression in the cytoplasm and nucleus decreased after the genistein treatment (figs. 3A and 3C).

The control (drug concentration of 0 μmol/L) and

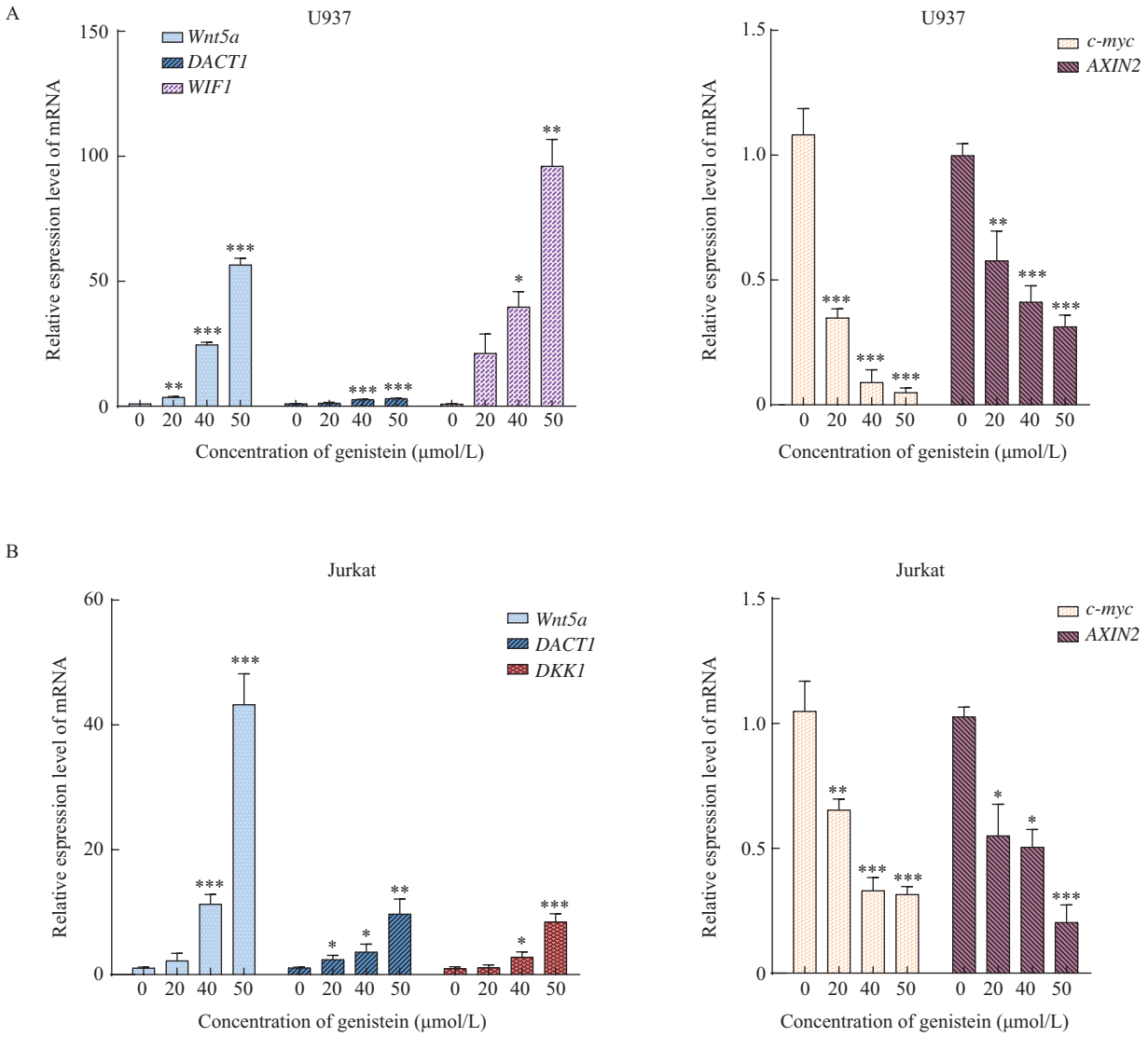

Fig. 2 Effects of genistein at the indicated doses on the mRNA expression of Wnt pathway genes in the U937 (A) and Jurkat (B) cell lines. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* control group

genistein-treated (drug concentration of 40 μmol/L) groups were observed using a laser confocal microscope. *Wnt5a* was labeled with green fluorescence, β-catenin was labeled with red fluorescence, and the nucleus was stained with DAPI with blue fluorescence (fig. 3D). In comparing the intensity of the red and green fluorescence between the control and genistein-treated groups, the cytoplasm and cell membranes of U937 and Jurkat cells in the control group were scattered with green fluorescence in the form of discontinuous dots on the cell membrane. The cell volume for the genistein-treated group significantly increased, and the green fluorescence on U937 and Jurkat cell membranes was significantly enhanced, exhibiting a continuous circular distribution. The cytoplasm and nucleus of U937 and Jurkat cells in the control group presented a scattered red fluorescence, and the red fluorescence intensity was significantly weaker in the genistein-treated group than in the control group. These results were consistent with the Western blotting results.

2.4 Genistein Cannot Reverse the Methylation Status of the *Wnt5a* Promoter Region

Compared to the control group, there was no significant difference in methylation for each of the six methylation sites in the genistein-treated group (drug concentration: 40 μmol/L; $P>0.05$, fig. 4A). Genistein did not reverse the methylation status of the *Wnt5a* promoter region.

2.5 Genistein Affects the Enrichment of Histones H4K20me1 and H3K9ac in *Wnt5a* and *AXIN2* in Acute Leukemia Cells

The Western blotting results revealed that the histone H4K20me1 and histone methyltransferase KMT5A expression in U937 and Jurkat cells gradually increased when the genistein concentration was increased (fig. 4B). Next, the enrichment of H4K20me1 and H3K9ac was further detected in *Wnt5a* and *AXIN2* in U937 cells after genistein treatment. In comparing

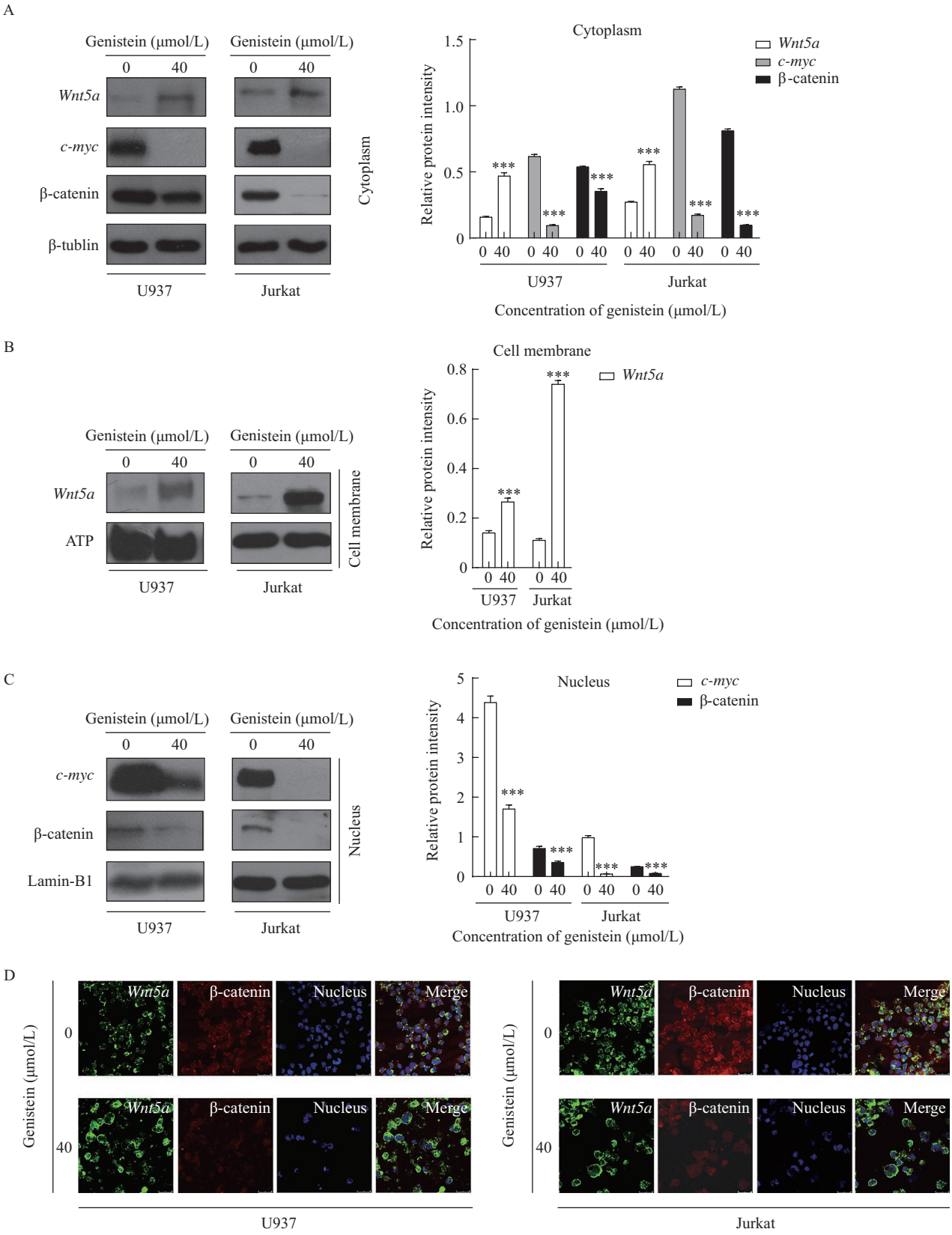

Fig. 3 Effects of the indicated doses of genistein on the Wnt pathway gene protein expression and intracellular localization in the U937 and Jurkat cell lines

Representative images of (A) Wnt5a, c-myc and β-catenin protein expression in the cytoplasm, (B) Wnt-5a protein expression in the cell membrane, and (C) c-myc and β-catenin protein expression in the nucleus, as detected by Western blotting. β-tubulin was used as a cytoplasmic control, ATP was the membrane control, and lamin-B1 was the nuclear control. D: estimation of the changes in intracellular localization for Wnt5a and β-catenin with the indicated doses of genistein by immunofluorescence staining, using a laser confocal microscope. The experiments were carried out in three biological replicates. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

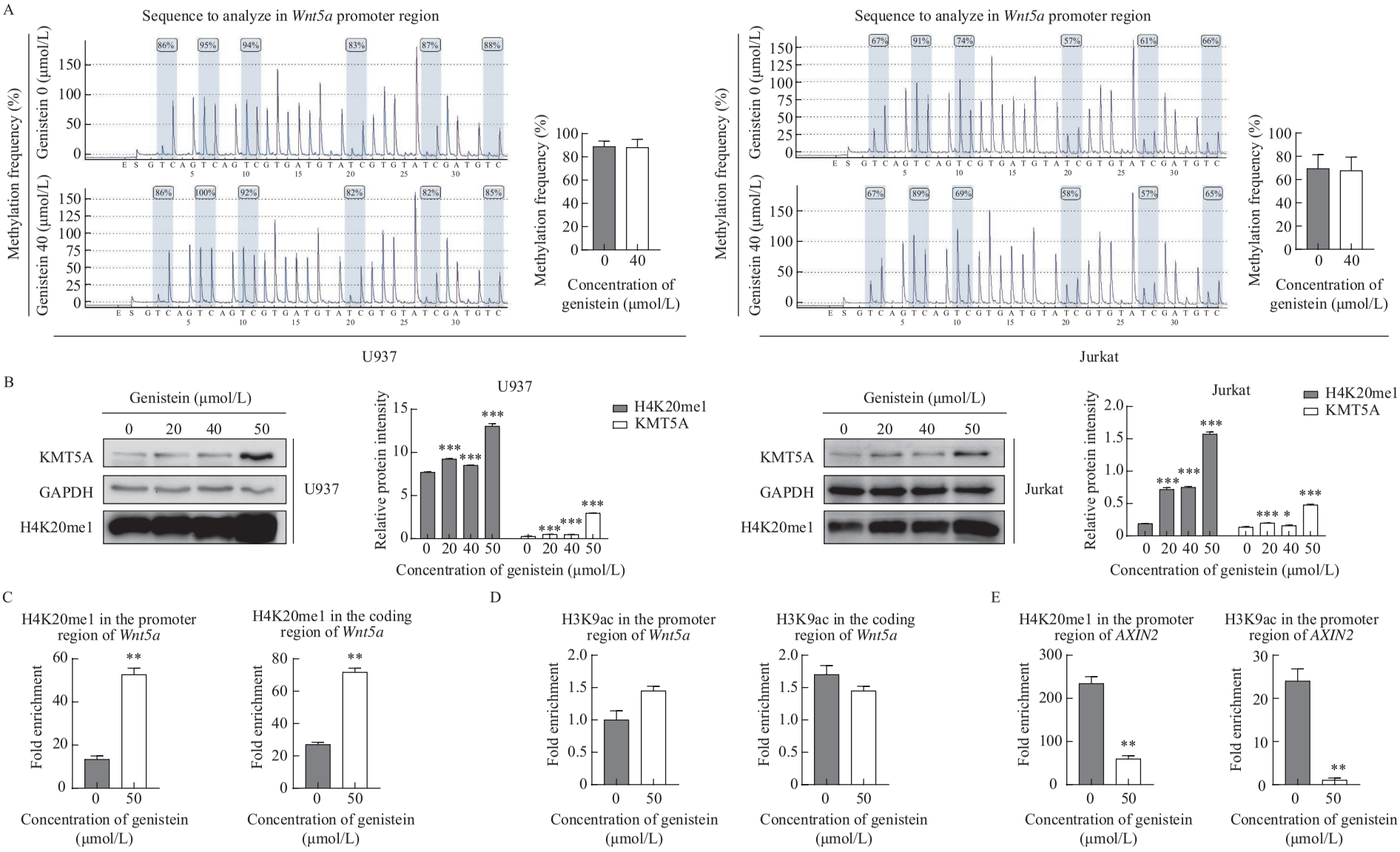

Fig. 4 A: Detection of changes in the methylation status of the *Wnt5a* promoter region with the indicated doses of genistein by pyrophosphate sequencing. Effects of the indicated concentrations of genistein on (B) protein expression of H4K20me1 and KMT5A in U937 and Jurkat cells, as detected by Western blotting, and the enrichment of (C) H4K20me1 and (D) H3K9ac in the promoter and coding regions of *Wnt5a*, and the enrichment of (E) H4K20me1 and H3K9ac in the promoter region of *AXIN2* in the U937 cell line, as detected by ChIP-qPCR. The experiments were carried out in three biological replicates. The values were considered statistically significant at $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* control group

the enrichment of H4K20me1 in the *Wnt5a* promoter and coding regions between the control (genistein concentration: 0 μmol/L) and genistein-treated (genistein concentration: 50 μmol/L) groups, the enrichment of H4K20me1 in the *Wnt5a* promoter and coding regions was significantly higher in the genistein-treated group than in the control group (P<0.01, fig. 4C). However, there were no significant differences in the enrichment of H3K9ac in the *Wnt5a* promoter and coding regions between the control and genistein-treated groups (P>0.05, fig. 4D). The enrichment of H4K20me1 and H3K9ac in the AXIN2 gene promoter region was significantly lower in the genistein-treated group than in the control group (P<0.01, fig. 4E).

2.6 Genistein Caused the G2/M Phase Arrest in Acute Leukemia Cells

The cell cycle analysis revealed that with the increase in genistein concentration, the ratio of U937 and Jurkat cells in the G0/G1 phase and S phase gradually decreased, while the ratio of cells in the G2 phase gradually increased. The difference between the control and genistein-treated groups (genistein concentrations of 20, 40, 50 and 70 μmol/L) was statistically significant (fig. 5). Furthermore, the genistein-induced G2/M phase arrest of U937 and Jurkat cells was dose-dependent.

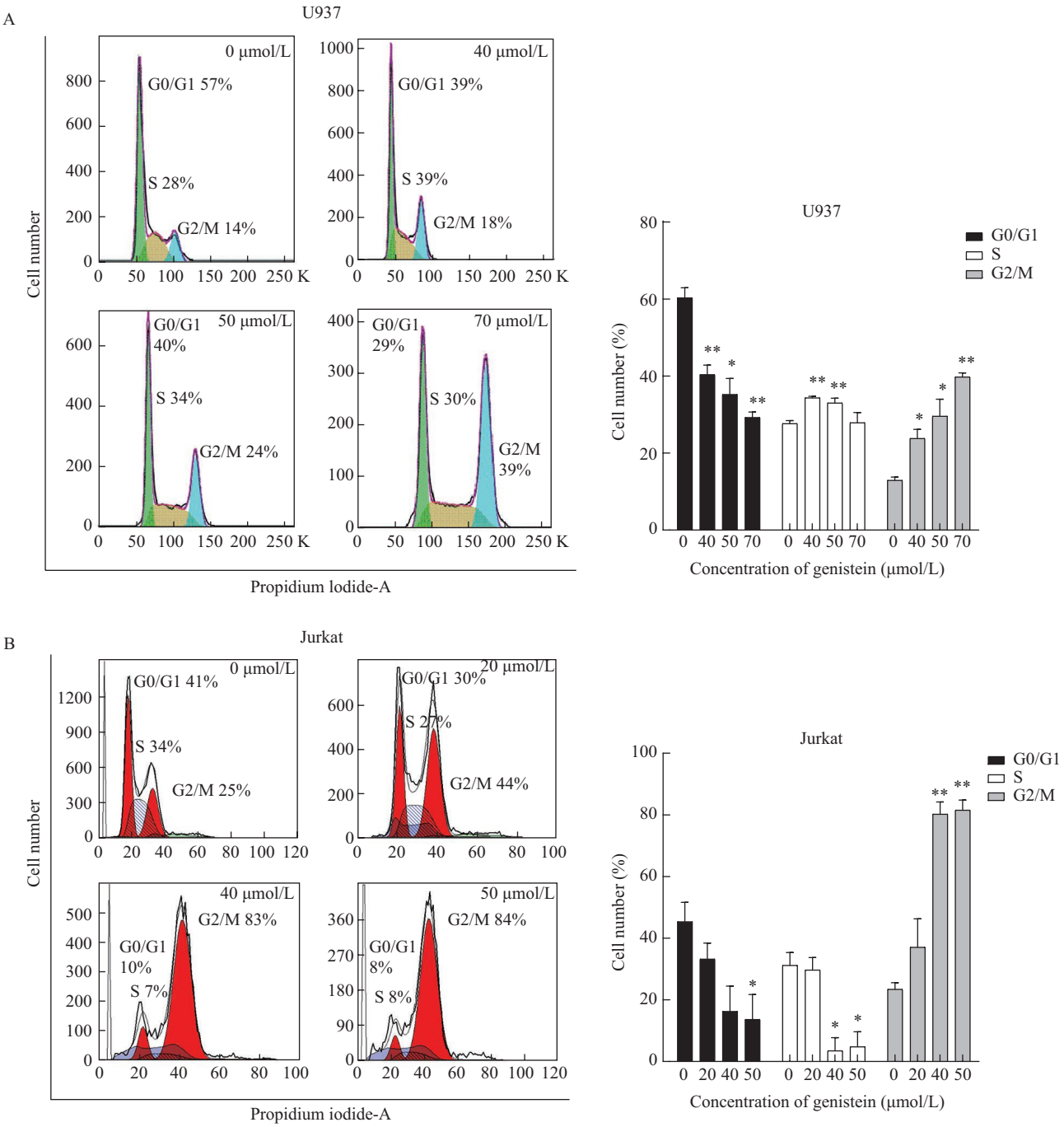

Fig. 5 Cell cycle distribution of U937 (A) and Jurkat (B) cells at the indicated doses of genistein, as determined by flow cytometry. The results are representative of three biological replicates. $^{*}P$<0.05, $^{**}P$<0.01 and $^{***}P$<0.001

2.7 Effect of Genistein on the Expression of G2/M Phase-related Genes in the Cell Cycle

In order to understand how genistein induces G2/M phase arrest in U937 and Jurkat cells, the expression of G2/M phase-related genes (*Cyclin A*, *Cyclin B1*, *Cyclin E2*, *Phospho-Wee1*, *Phospho-histone H3*, *p21*, *Phospho-cdc2* and *Myt1*) was tested. The Western blotting revealed that compared to the control group, the *Phospho-Wee1* gene expression was downregulated, while the expression of *Phospho-histone H3*, *p21*, *Phospho-cdc2, Myt1*, *Cyclin A*, *Cyclin B1* and *Cyclin E2* was upregulated in the genistein-treated group (40 μmol/L, fig. 6).

3 DISCUSSION

Changes in epigenetic information, such as transcriptional inhibition, genome imprinting, cell apoptosis and chromosome inactivation, can have extensive effects on mammalian genomes, including humans. Epigenetic information can be directly linked to drugs, diet, lifestyle and environmental factors. These factors can not only induce epigenetic changes that promote cancer, but can also improve the epigenetics to prevent cancer. Therefore, a full understanding of the epigenetic changes and mechanisms that affect gene expression would be helpful for the deeper understanding of the mechanisms of tumor occurrence, development and prevention.

Genistein is the main isoflavone extracted from soybeans, accounting for approximately 50% of soy isoflavones. *In vivo* and *in vitro* experiments have confirmed that genistein can protect against prostate, colorectal, oral, skin and bladder cancers, leukemia, and renal cell carcinoma. An early study reported that in colorectal tumor cells, this can reverse the methylation of the *Wnt5a* promoter region to promote the re-expression, and suppress the Wnt signal pathway[11]. However, the exact epigenetic mechanism by which genistein exerts anti-AL effect has not been thoroughly studied.

In the present study, genistein was evaluated for its potential anticancer activity against AL cell lines U937 and Jurkat, and non-cancer cell line hBMSCs. Genistein has significant anticancer activity against two AL cell lines (U937 and Jurkat), with a higher cytotoxic effect on U937 and Jurkat (IC_{50}=30 μmol/L, fig. 1). Furthermore, genistein has a relatively low inhibitory effect on the proliferation of hBMSCs (IC_{50}=70 μmol/L). Therefore, these results suggest that genistein may be an effective anti-AL drug.

The present results indicated that the mRNA expression was upregulated for *Wnt5a* and *DACT1*. Furthermore, the protein expression was upregulated for total H4K20me1, KMT5A, and cell membrane and cytoplasm *Wnt5a*, while the protein expression was downregulated for cytoplasmic and nuclear β-catenin, and cytoplasmic and nuclear Wnt pathway downstream

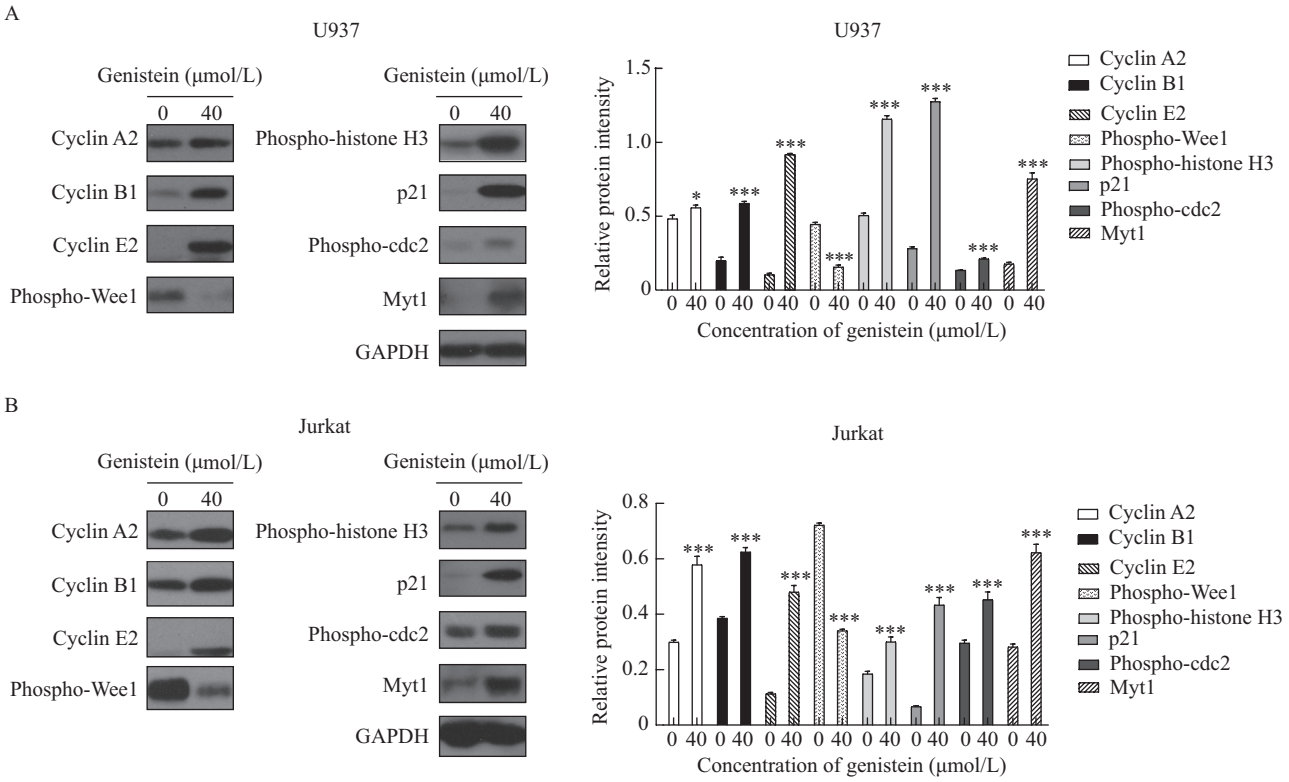

Fig. 6 Effects of the indicated doses of genistein on the G2/M phase-related protein expression in the U937(A) and Jurkat (B) cell lines. The representative images of Cyclin A, Cyclin B1, Cyclin E2, Phospho-Wee1, Phospho-histone H3, P21, Phospho-cdc2 and Myt1, as detected by Western blotting. The images are representatives of three biological replicates. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

target proteins, such as c-myc. It was speculated that the anti-leukemia mechanism may be correlated to the activation of H4K20me1 methyltransferase (KMT5A) and the increased enrichment of H4K20me1 in the *Wnt5a* promoter and coding regions, which can loosen the chromatin and promote *Wnt5a* transcription. The synthesized *Wnt5a* protein is mainly concentrated in the cell membrane, and binds to the corresponding receptor. By activating the non-canonical Wnt pathway, the synthesis of β-catenin is reduced. Therefore, the amount of β-catenin entering the nucleus is reduced, thereby inhibiting the canonical Wnt/β-catenin signaling pathway and reducing the synthesis of proteins of downstream target genes (*c-myc* and *AXIN2*).

The present study demonstrated that there was no difference in *Wnt5a* methylation status between the genistein-treated and control groups. Consequently, it was considered that genistein's activation of *Wnt5a* gene expression is isolated from the methylation status of the promoter. Previous studies have shown that the antitumor effects involve various mechanisms, such as the inhibition of DNA methyltransferase activity and the demethylation of tumor suppressor genes to promote re-expression[12–15]. The present results are consistent with new international research results. That is, in the prostate and colon cancer cell lines, genistein was observed to promote methylated Wnt pathway antagonistic genes, but this did not change the methylation status of the gene promoter region[6, 8]. The relationship between the genistein-induced tumor suppressor gene expression and H4K20me1 has not been explored. It was speculated that H4K20me1 initiates the regulation of gene transcription without removing the gene methylation. Furthermore, *Wnt5a* had an obvious enrichment of H4K20me1 in both the promoter and coding regions, which is consistent with the theory that H4K20me1 can promote transcription initiation and extension[16]. The whole genome sequencing revealed that H4K20me1 and KMT5A are closely correlated to the activation of transcription[17]. A novel study revealed that KMT5A promotes the reactivation of neural stem cells by regulating the Wnt signaling and cell cycle[18]. In addition, the present results revealed that the expression of the downstream target gene *AXIN2* of the Wnt pathway in the genistein-treated group was downregulated. Moreover, the ChIP-qPCR results indicated that H4K20me1 and H3K9ac in the promoter regions of *AXIN2* exhibited a decreased enrichment. Considering that these two histone modifications can promote gene expression, the decrease of these histones in the promoter region may be the direct cause of the downregulation of *AXIN2*. The histone changes in the opposite direction in the same cell indicate that intracellular regulation is extremely delicate and complex. Different genes have different histone modifications at various times. Thus, each gene is maintained at an appropriate level. Although in prostate cancer, genistein can promote the histone H3K9 acetylation of genes whose promoter region is methylated[8]. The present study did not observe the enrichment of H3K9ac in the promoter region of the *Wnt5a* gene, which may be correlated to the selected gene and tumor type. Therefore, a new molecular mechanism of genistein in leukemia was proposed. Genistein can promote the expression of tumor suppressor gene *Wnt5a* by enriching H4K20me1 in the promoter and coding regions of *Wnt5a*, activate the non-canonical Wnt pathway, and inhibit the β-catenin-mediated classic Wnt pathway.

The present results revealed that in U937 and Jurkat cells, genistein can block the cell cycle progression in the G2/M phase. The DNA damage checkpoint in the G2/M phase is a very important cell cycle checkpoint in eukaryotes obtained from yeast to mammals. If a cell enters mitosis due to a defect in the G2/M phase checkpoint and cannot repair the DNA, the cell will die after mitosis. Cyclin B combines with cell cycle-dependent kinase CDC2 to form CDK1 kinase, which drives mitosis. The phosphorylation of the 14th and 15th tyrosine residues of CDC2 regulates the activity of CDC2. This regulation involves wee1 and Myt1, and the phosphorylation of the 14th and 15th tyrosine residues of CDC2 inhibits its activity. Consequently, the activity of CDK1 kinase cannot be expressed. In the M phase, phosphatase CDC25 can activate CDC2 with dephosphorylation. In the G2/M phase, Cyclin A and Cyclin B bind to CDK1, and CDK1 phosphorylates the substrate protein, such as histone H3, which leads to chromosome condensation[19].

Genistein has been reported to induce G2/M cell cycle arrest in ovarian cancer cells[20], and G0/G1 cell cycle arrest in pancreatic cancer cells[15]. However, the mechanism of the G2/M cycle arrest induced by genistein has not been studied. It was determined whether genistein could induce cell cycle arrest in AL cells, and it was found that genistein promotes cell cycle arrest in the G2/M phase in a dose-dependent manner. Through the detection of G2/M phase-related cyclins, it was found that phosphorylated CDC2, Myt1, Cyclin A, Cyclin B1, Cyclin E2, p21 and Phospho-histone H3 were upregulated to varying degrees, while phosphorylated wee1 was downregulated. It was speculated that the upregulation of Cyclin A promotes the cell cycle through the S phase, and that the upregulation of Cyclin E2 induced cells to pass through the G1/S checkpoint. Myt1 and wee1 promote the phosphorylation and inactivation of cdc2 Try15/Try14. Although the upregulation of Cyclin B1 can activate the cell cycle process, if the activity of CDK1 kinase (a complex formed by Phospho-cdc2 and Cyclin B1) is inhibited, the upregulation of p21 also inhibits the

Cyclin-CDK complex, the cell cycle will be blocked at the G2/M stage.

In summary, genistein, which is abundant in soybeans, has a strong antitumor effect on AL cells. It inhibits the Wnt pathway activation by inducing the expression of *Wnt5a* and enriching H4K20me1 in the *Wnt5a* promoter and coding regions, rather than demethylation. Furthermore, genistein blocks the cell cycle. Genistein may have good application prospects in AL therapy, as an epigenetic drug, in the future.

Conflict of Interest Statement

There are no conflicts of interest.

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