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ORIGINAL ARTICLE

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Genistein-induced apoptosis via Akt signaling pathway in anaplastic large-cell lymphoma

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Abstract More than half of anaplastic large-cell lymphoma (ALCL) are associated with chromosomal translocation t(2;5)(p23;q35) that leads to the expression of nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) oncoprotein. NPM-ALK activates the antiapoptotic phosphatidylinositol-3 kinase/Akt (PI3K/Akt) signaling pathway, which plays a critical role in cell survival and apoptosis. Inhibition of the PI3K/Akt pathway has been considered as a therapeutic target for cancer where PI3K/Akt activation is a causative factor. Genistein, a natural isoflavonoid found in soy products, has been shown to inhibit cell growth and induce apoptosis in a wide variety of cell lines. Here, we demonstrated that treatment of two t(2;5) ALCL cell lines, SUDHL-1 and Karpas299, with genistein induced apoptosis in a time- and dose-dependent manner. Concurrently, these cells exhibited a decrease in Akt protein levels and subsequent downregulation of Akt activity (Akt phosphorylation). Furthermore, genistein treatment induced mitochondrial membrane potential change, caspase-3 activation and PARP cleavage. From these results, we conclude that inhibition of the Akt signaling pathway and induction of apoptosis by genistein could be used as a new treatment modality for the prevention and/or treatment of t(2;5) ALCL and other hematopoietic malignancies.

Keywords Anaplastic large cell lymphoma · Genistein · Apoptosis · Akt · Caspase

Abbreviations ALCL: Anaplastic large-cell lymphoma · ALK: Anaplastic lymphoma kinase · NPM: Nucleophosmin · PARP: Poly(ADP-ribose) polymerase · PI3K: Phosphatidylinositol-3 kinase

Introduction

Anaplastic large-cell lymphoma (ALCL) is a distinct subgroup of non-Hodgkin's lymphoma usually composed of large pleomorphic tumor cells that express the membrane antigen CD30 [16]. Despite variations in histological features, ALCL is commonly associated with a t(2;5)(p23;q35) translocation that results in the fusion of the nucleophosmin (NPM) gene at 5q35 with the tyrosine kinase gene anaplastic lymphoma kinase (ALK) at 2p23 [25, 38]. Fusion of NPM to ALK results in the dimerization and constitutive activation of the NPM-ALK oncoprotein that is capable of transforming fibroblasts and inducing a lymphoma-like disease in mice [6, 19]. Also NPM-ALK recruits the C-terminal SH2 domain of the antiapoptotic phosphatidylinositol-3 kinase (PI3-kinase), which in turn activates the serine/threonine kinase Akt (also known as protein kinase B, PKB), which likely contributes to the molecular pathogenesis of ALCL [3, 29].

Genistein is a prominent isoflavonoid found in soy products that has been identified as an inhibitor of protein tyrosine kinases, which play a key role in cell growth and apoptosis [1, 28]. In vitro and in vivo experimental studies have shown that genistein can inhibit the growth of various cancer cell lines through the modulation of genes that are related to the homeostatic control of cell cycle and apoptosis [2, 10, 21, 22, 31]. It has also been reported that genistein inhibits the activation of the nuclear transcription factor NF-κB and the Akt signaling pathway, both of which are known to

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maintain the balance between cell survival and apoptosis [14, 20].

The apoptotic cascade in mammalian cells is a multistep process. In most cases, the apoptotic cascade is initiated by loss of integrity of the outer mitochondrial membrane accompanied by release of cytochrome *c* and its binding to apoptotic protease activating factor (Apaf-1). This Apaf-1 induces the cleavage and activation of caspase-9 which activates a caspase cascade that executes cell death [32]. Akt can prevent the activation and the processing of the caspases by maintaining mitochondrial membrane integrity and by preventing the critical early step of cytochrome c release [17].

In this study, we demonstrated that genistein inhibits cell growth, induces mitochondria-dependent apoptosis via specific inhibition of the Akt signaling pathway and caspase-3 activation in the t(2;5) ALCL cell lines SUDHL-1 and Karpas299.

Material and Methods

Reagents

Genistein (Sigma, St. Louis, Mo.) was dissolved in DMSO to make a 100 m*M* stock solution that was added directly to the culture medium at various concentrations. The PI3K inhibitor, LY294002 (Calbiochem, San Diego, Calif.) was also dissolved in DMSO and added to the cultures at the desired concentrations. The broad caspase inhibitor (z-VAD-FMK) (Calbiochem) and caspase-3 inhibitor (z-DEVD-FMK) (Calbiochem) were used at a concentration of 100 μ*M*. A mouse monoclonal antibody against NPM-ALK was from DakoCytomation (Glostrup, Denmark) and phosphotyrosine was from Upstate Biotechnology (Lake Placid, N.Y.), rabbit polyclonal antibodies against Akt, pAkt, caspase-3, poly(ADP-ribose) polymerase (PARP) were from Cell Signaling (Beverly, Mass.). Rabbit polyclonal antibodies against Bax, Bcl-2, Bcl-xL were from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Cell lines

Karpas299 is a human cell line generated from a NPM-ALK-positive T-cell lymphoma of adult ALCL patients, and SUDHL-1 is a human NPM-ALK-positive T-cell lymphoma cell line derived from infant ALCL patients. Both cell lines were kindly provided by Dr. Ohno, Kyoto University. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% antibiotics in a 5% CO_2 atmosphere at 37°C.

MTS assay

Cells were seeded at a density of 1×10^4/well in a 96-well tissue plate in a total volume of 100 μl tissue culture medium. Cells were treated with genistein or DMSO for 24–48 h, and then incubated with MTS [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, Wis.) at 37°C for an additional hour. The spectrophotometric absorbance of the samples was determined using an Ultra Multifunctional microplate reader at 490 nm. Each condition was performed in triplicate and all experiments were performed at least three times.

Western blot analysis

To extract proteins, cells were washed twice with phosphate-buffered saline (PBS) and lysed with 2X sample buffer (20 m*M* Tris, pH 8.0, 2 m*M* EDTA, 2 m*M* dithiothreitol, 1 m*M* Na_3VO_4, 2% SDS and 20% glycerol). Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, Ill.). Total cellular proteins (30–50 μg) were then subjected to 10–15% SDS-PAGE and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfat dried milk in TTBS (0.1% Tween 20 in Tris-buffered saline) for 1 h at room temperature (RT). For Western blotting using anti-phosphotyrosine antibodies, the membranes were blocked with

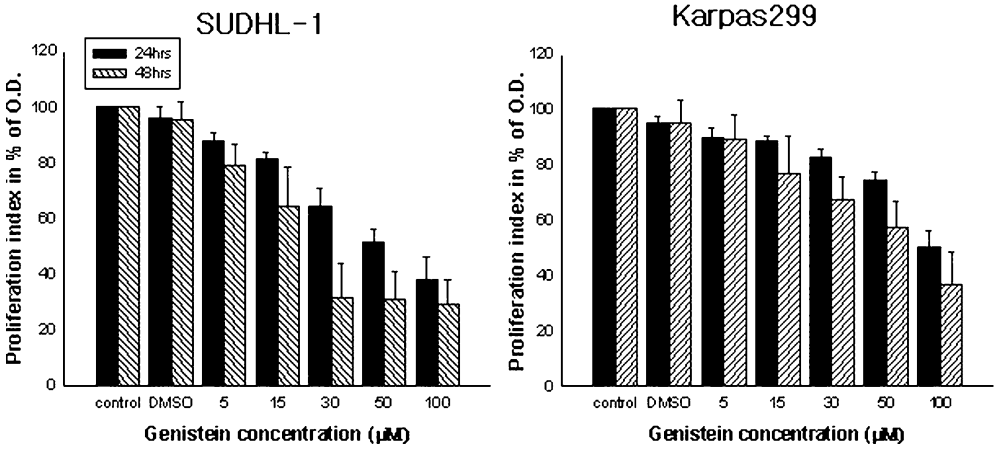


Fig. 1 Effect of genistein on viability of Karpas299 and SUDHL-1 cells. Karpas299 and SUDHL-1 cells were exposed to 0, 5, 15, 30, 50 and 100 μ*M* genistein or DMSO (vehicle control) for 24 and 48 h. Following these treatments, treated and untreated cells were subjected to the MTS assay as described in Material and methods. The data presented are the means ± SE of three independent experiments

0.25% gelatin in TTBS at 4°C overnight. The membranes were then incubated with anti-NPM-ALK (1:200), antiphosphotyrosine (1:2500), anti-Akt (1:1000), anti-pAkt (1:1000), anti-Bax (1:500), Bcl-2 (1:500), Bcl-xL (1:250), anti-caspase-3 (1:500), and anti-PARP (1:500) antibodies overnight at 4°C. Blots were then washed with TTBS three times, and incubated with a secondary antibody conjugated with horseradish peroxide at 1:5000 for 1 h. Blots were developed for visualization using an ECL (enhanced chemiluminescence) detection kit (Pierce).

Annexin V staining

Approximately 2×10^5/ml cells were treated with different drugs for the indicated times. Cells were then harvested and washed twice with PBS and incubated for 20 min at RT with reagent comprising annexin V conjugated with fluorescein isothiocyanate (FITC) (2.5 μg/ml) (Pharmingen, San Diego, Calif.) and propidium iodide (5 μg/ml). Cells were analyzed on a FACScan flow cytometer (Epics XL, Coulter, Marseille, France). Annexin-V-positive propidium iodide-negative cells were considered as apoptotic.

Measurement of mitochondrial membrane potential (Ψ_m)

To measure $\Delta\Psi_m$ disruption, 5×10^5 cells were exposed to genistein for the indicated times and then incubated with 20 n *M* 3,3'-diethyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes, Eugene, Ore.) for 15 min at 37°C. $\Delta\Psi_m$ was determined by flow cytometric analysis using an Epics XL flow cytometer (Coulter).

Inhibition of caspases

Cells were pretreated with 100 μ*M* z-VAD-FMK (Calbiochem), a broad caspase inhibitor or 100 μ*M* z-DEVD-FMK (Calbiochem), a caspase-3 inhibitor for 1 h. Cells were then exposed to DMSO (control), or 50 or 100 μ*M* genistein for 24 h. A cell viability assay was performed as described above.

Results

Genistein-induced apoptosis in ALCL cell lines

To examine the effect of genistein on cell survival, Karpas299 and SUDHL-1 cells were treated with 5, 15, 30, 50 and 100 μ*M* genistein or DMSO (vehicle control) for 24 and 48 h, and cell viability was determined by MTS assay. As shown in Fig. 1, treatment with genistein resulted in a dose- and time-dependent inhibition of cell proliferation. Compared with Karpas299 cells, SUDHL-1 cells showed higher sensitivity to a lower dose of genistein. After 48 h of treatment with 30 μ*M* of genistein, growth inhibition of SUDHL-1 and Karpas299 cells was approximately 70% and 30%, respectively. However, treatment with 100 μ*M* genistein resulted in growth inhibition of more than 60% in both cell lines.

Growth inhibition could be secondary to cytostasis, apoptosis, or necrosis. To investigate these possibilities, we analyzed annexin V/PI-stained cells treated for 24 h with 5, 15, 30, 50 or 100 μ*M* of or DMSO (vehicle control) by flow cytometry. FACS analysis of annexin-V staining is a measure of externalization of phosphatidylserine to the outer plasma membrane leaflet representing a unique characteristic of apoptosis. Corresponding to the proliferation assay, both cell lines showed a marked apoptotic response to genistein, whereas DMSO did not affect cell viability. The degree of apoptosis was also genistein dose-dependent and directly correlated with the inhibition of cell growth (Fig. 2). Similar to the proliferation assay, over 50% of SUDHL-1 cells were apoptotic after treatment with genistein at concentrations of more than 30 μ*M*. However, even with 100 μ*M* genistein fewer than 50% of Karpas299 cells underwent apoptosis. Overall, genistein inhibited growth and induced apoptosis in both cell lines with a higher sensitivity in SUDHL-1 than in Karpas299 cells.

Inhibition of NPM-ALK and Akt/pAkt pathway by genistein

In order to further elucidate potential signaling pathways involved in genistein-induced apoptosis of ALCL cells, we first looked at changes in NPM-ALK, the major oncoprotein related to the pathogenesis of ALCL. Following exposure to 100 μ*M* genistein for 24 h, ALCL total cell lysates were collected and probed for 80 kDa NPM-ALK fusion protein by Western blotting (Fig. 3a). There was a decrease in NPM-ALK levels in both SUDHL-1 cells and Karpas299 cells. Since genistein is a broad-spectrum protein-tyrosine kinase inhibitor, changes in tyrosine phosphorylation after exposure to 100 μ*M* genistein for 24 h were also investigated by Western blotting (Fig. 3a). There was a significant decrease in phosphotyrosine content in both cell lines, with a more prominent response in SUDHL-1 cells. Interestingly, correlating with the reduction of NPM-ALK in genistein-treated cells, the tyrosine phosphorylation of 80 kDa proteins also considerably decreased, suggesting that 80 kDa phosphoprotein is NPM-ALK.

Because the Akt signaling pathway is an important signal transduction pathway that plays a critical role in cell survival and the antiapoptotic process in ALK-positive ALCL, we investigated whether the status of Akt and phosphorylated Akt (pAkt) proteins were altered upon genistein treatment of ALCL cells. Genistein treatment caused a decrease in both Akt and active pAkt

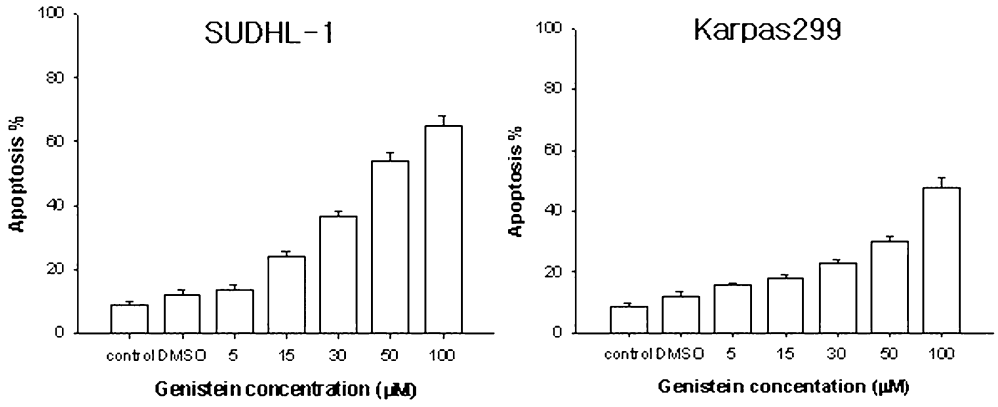


Fig. 2 Genistein-induced apoptosis in SUDHL-1 and Karpas299 cells. Cells were exposed to 0, 5, 15, 30, 50 or 100 μ*M* genistein or DMSO (vehicle control) for 24 h. Following this, untreated and treated cells were stained with annexin V/PI. Positively stained apoptotic cells were quantified by flow cytometry. The data are presented as the means ± SE of three independent experiments

levels. Interestingly, the maximum effect of genistein on downregulation of Akt/pAkt was different between the two cell lines. Neither Akt nor pAkt proteins were detectable after 24 h incubation with 100 μ*M* genistein in SUDHL-1 cells, while decreased levels of Akt and disappearance of phosphorylated active pAkt were observed after 48 h incubation with 100 μ*M* genistein in Karpas299 cells (Fig. 3b). This relatively rapid inhibition of Akt in SUDHL-1 cells correlated with the decrease in NPM-ALK protein levels (Fig. 3a) and with the higher sensitivity of SUDHL-1 cells to genistein-induced apoptosis (Figs. 1 and 2).

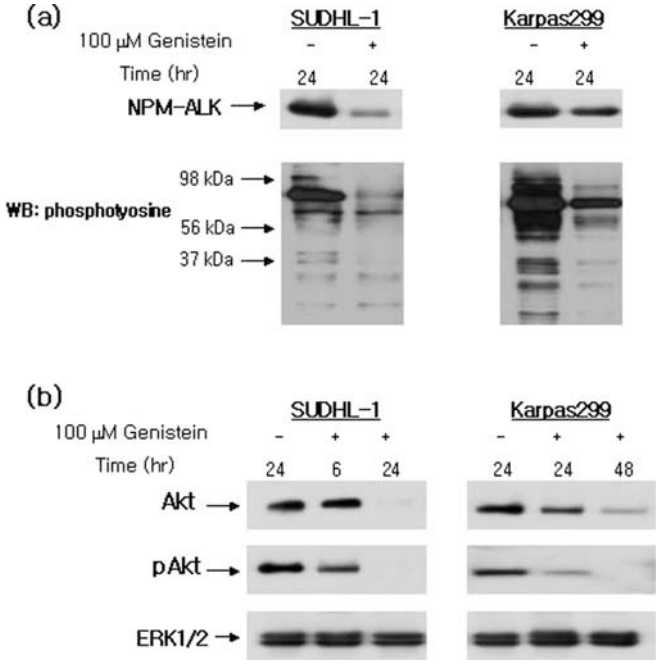


Fig. 3 **a** Effect of genistein on NPM-ALK expression and cellular phosphotyrosine kinase activity. SUDHL-1 and Karpas299 cells were treated without or with 100 μ*M* genistein for 24 h. Equal amounts of total cell lysates (30 μg protein) were loaded onto each lane and were probed for NPM-ALK and tyrosine-phosphorylated proteins by Western blotting. **b** Genistein-mediated reduction of Akt/pAkt proteins. SUDHL-1 cells were treated without or with 100 μ*M* genistein for 6 and 24 h and Karpas299 cells for 24 and 48 h. Cell lysates from each treatment (30 μg protein) were subject to Western blotting using anti-Akt and anti-pAkt antibodies

Induction of apoptosis by PI3-kinase inhibitors

Activation of PI3-kinase has been shown to elicit antiapoptotic signals including the activation of PKB/Akt [6, 12, 33]. As we observed the downregulation of Akt and a concurrent increase in apoptosis in genistein-treated ALCL cells, we examined the role of Akt activation in ALCL cell survival using the selective PI3-kinase inhibitor, LY294002, at concentrations of 20, 40, 60, and 100 μ*M* for 24 h. LY294002 has been used in a number of studies to demonstrate the importance of the PI3-kinase/Akt pathway in the transforming ability of oncogenic tyrosine kinases [11]. As shown in Fig. 4, LY294002 was able to induce apoptosis in both ALCL cell lines in a dose-dependent manner. Moreover, consistent with the genistein data (Figs. 1 and 2), SUDHL-1 cells were more sensitive to LY294002-induced cell death than Karpas299 cells. For example, 40 μ*M* LY294002 induced apoptosis in up to 48% of SUDHL-1 cells but in only 24% of Karpas299 cells. These similar phenomena were also observed with another PI3-kinase inhibitor, wortmannin (data not shown). Therefore, the effect of PI3-kinase inhibitors on cell viability supports the notion that antiapoptotic PI3-kinase activity is important in NPM/ALK-positive ALCL cell lines as an upstream regulator of the Akt/pAkt signaling pathway [3, 29].

Effect of genistein on mitochondrial membrane potential

While alterations in the mitochondrial membrane potential (Ψ_m) have been implicated in the execution of apoptosis [15], Akt is known to promote cell survival by maintaining mitochondrial integrity and by inhibiting both the release of cytochrome *c* and alteration in mitochondrial membrane potential induced by multiple apoptotic stimuli [17]. Pharmacological studies have suggested that the loss of membrane potential ($\Delta\Psi_m$) is due to alterations in permeability transition pores and in many cases it is preceded by cytochrome *c* release and caspase activation [35]. As we observed the downregulation of Akt and apoptosis following treatment with

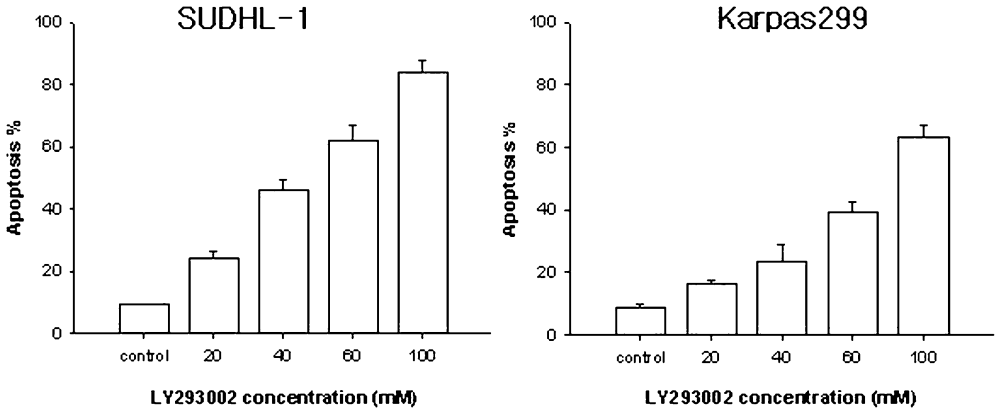


Fig. 4 LY294002-induced apoptosis in ALCL cell lines. Karpas299 and SUDHL-1 cells were incubated with 20, 40, 60 or 100 μ*M* of LY294002 or DMSO (control) for 24 h. The cells were stained with annexin V/PI and the results were evaluated using a flow cytometer

genistein, we investigated whether genistein-induced apoptosis is related to alterations in mitochondrial potential in ALCL cells. We examined the change in mitochondrial membrane potential by measuring the uptake of the mitochondrial specific dye $DiOC_6$ in ALCL cells undergoing apoptosis. As shown in Fig. 5, both cell lines showed alterations in the mitochondrial membrane potential after 24 h of treatment with genistein at 100 μ*M*. This change was more obvious in SUDHL-1 cells, which correlated with its sensitivity to genistein-induced apoptosis.

As Bcl-2 family members are well known critical regulators of the apoptosis cascade, we also investigated whether genistein affects the expression levels of Bcl-2 family members, including Bcl-2, Bcl-xL and Bax, by Western blot analysis. There was little or no detectable change in Bcl-2 and Bcl-xL protein levels, but there was an increase in Bax protein levels after genistein treatment in both cell lines (Fig. 6).

Genistein induced cleavage of caspase-3 and PARP

The transduction and execution of apoptotic signals requires proper signal transduction and the coordinated action of caspases [9]. The caspases are present in cells as inactive procaspases, with the active tetramer being formed by removal of the prodomain and cleavage between large and small subunits [23]. Caspase activity is responsible, either directly or indirectly, for cleavage of cellular proteins which are characteristically proteolysed during apoptosis. For example, activation of caspase-2, -3, -7, and -9 leads to the cleavage of PARP, and caspase-6 activation results in cleavage of nuclear lamins [27].

To further explore the downstream effect of the altered mitochondrial membrane potential, we evaluated genistein-induced caspase-3 activation. As shown in Fig. 6a, treatment with genistein converted caspase-3 from its inactive form (32 kDa) to its active form (19 kDa) in both cell lines, as illustrated by a decrease in the inactive form and a concomitant increase in the active form as assessed by Western blotting using an anti-caspase-3 antibody that recognizes both forms. Subsequently, we further examined genistein-induced caspase-3 activation by PARP cleavage. Treatment of SUDHL-1 and Karpas299 cells with 100 μ*M* genistein

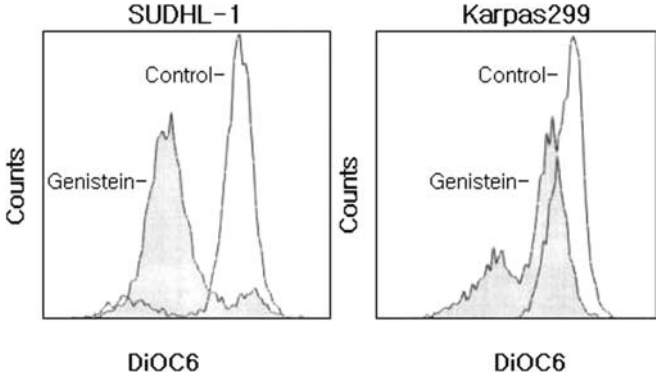


Fig. 5 Mitochondrial membrane depolarization induced by genistein. Cells were treated without or with 100 μ*M* genistein for 24 h, and then incubated with $DiOC_6$ to monitor $\Delta\Psi_m$ by FACS. The loss in $\Delta\Psi_m$ corresponded with decreased fluorescence and a shift to the left

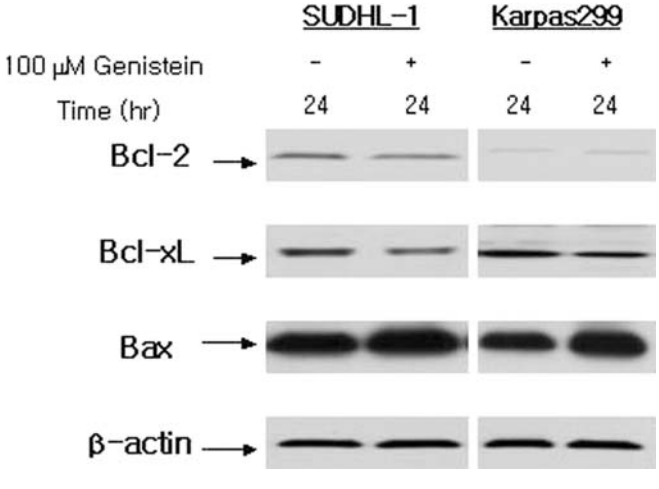


Fig. 6 Effects of genistein on the levels of Bcl-2 family members. Cells were incubated with 100 μ*M* genistein for 24 h. Cells grown in culture medium without genistein were used as a control. Cell lysates were labeled with polyclonal anti-Bcl-2, anti-Bcl-xL, and anti-Bax antibodies and analyzed by Western blotting

caused a time-dependent proteolytic cleavage of PARP, with accumulation of the 85-kDa fragments and a concomitant disappearance of the full length 116-kDa protein (Fig. 6b). Interestingly, consistent with the results in Figs. 1, 2 and 3, genistein-induced activation of caspase-3 and PARP cleavage were much slower in Karpas299 cells than in SUDHL-1 cells that are highly responsive to genistein-induced apoptosis and Akt downregulation.

To further evaluate the effect of genistein-induced caspase activation on apoptosis, ALCL cells were preincubated with z-VAD-FMK and z-DEVD-FMK, membrane-permeable irreversible pan-caspase and caspase-3 inhibitors, respectively, before treatment with genistein. Cell viability as measured by MTS assay revealed that caspase inhibitors could attenuate genistein-induced apoptosis, especially the pancaspase inhibitor z-VAD-FMK (Fig. 6c). Together, these data strongly suggest that genistein-induced apoptosis in ALCL is a mitochondrial-dependent process and that activation of caspases including caspase-3 plays a key role in the final pathway to apoptosis. Also correlating with genistein-induced apoptosis levels and Akt/pAkt downregulation, SUDHL-1 cells showed higher sensitivity to genistein in terms of mitochondrial membrane potential change and caspase activation.

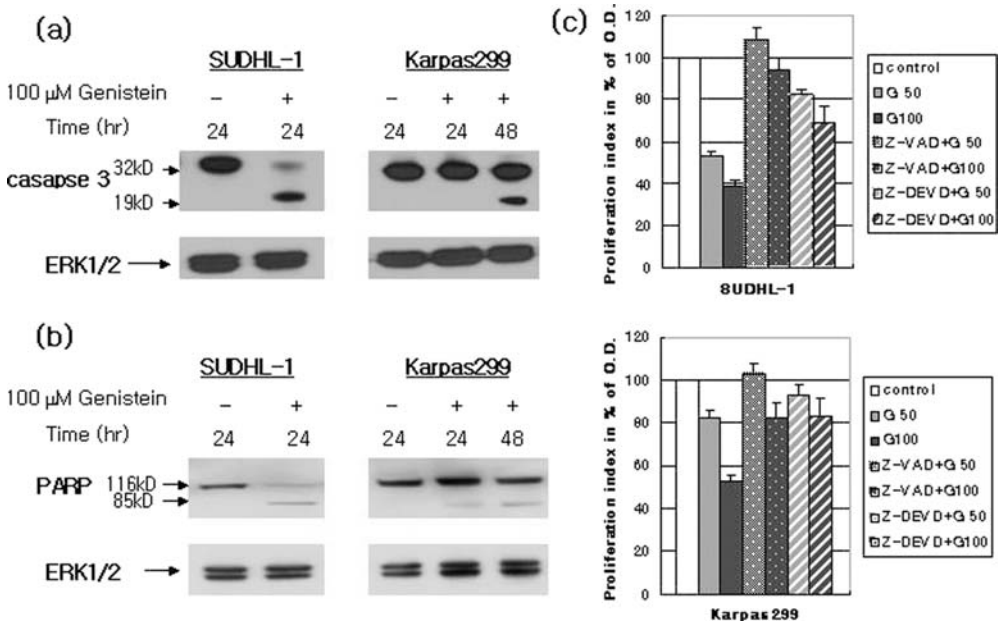
Fig. 7 Effects of genistein on caspase activation and apoptosis. Western blot analysis of caspase-3 (**a**) and PARP (**b**) cleavage. Cells were incubated with 100 μ*M* genistein for 24 h. Cells grown in culture medium without genistein were used as a control. Cell lysates were labeled with polyclonal anti-caspase-3 antibody (**a**) and anti-PARP antibody (**b**) and analyzed by Western blotting. **c** Effects of caspase inhibitors on genistein-induced apoptosis. The inhibitory effects of caspase inhibitors (z-VAD-FMK and z-DEVD-FMK) were analyzed by adding 100 μ*M* of each compound 1 h prior to genistein treatment. Cells were exposed to 50 and 100 μ*M* genistein or DMSO (control) for 24 h and incubated with MTS. Spectrophotometric absorbance was determined at 490 nm

Discussion

Chromosomal translocations are responsible for the expression of abnormal fusion proteins that possess constitutive tyrosine kinase activity, which play an essential role in cell proliferation, apoptosis, differentiation, and malignant transformation [18]. These translocations occur often in hematopoietic cells, leading to their transformation and the development of leukemias or lymphomas [30]. About 50–60% of ALCLs possess the reciprocal chromosomal translocation, t(2;5)(p23;q35), that fuses the NH2-terminal portion of ubiquitously expressed NPM to the entire cytoplasmic portion of the insulin-like receptor ALK tyrosine kinase, resulting in constitutive activation of the NPM-ALK oncoprotein [5, 25, 38]. The NPM-ALK oncoprotein recruits the C-terminal SH2 domain of the PI3-kinase p85 subunit, thus activating the antiapoptotic PI3-kinase/Akt pathway, which likely contributes to the molecular pathogenesis of ALCL [3, 29].

Akt, also known as PKB, consists of a family of highly conserved serine/threonine kinases [4]. After stimulation with growth factors and cytokines, PI3K is activated and phosphorylates phosphoinositides. The interaction of the generated phospholipid second messenger molecule with the pleckstrin homology (PH) domain of Akt recruits Akt to the plasma membrane, where it is phosphorylated. Phosphorylation of both threonine and serine residues fully activates Akt, which provides a survival signal that protects cells from apoptosis and mediates growth factor-induced cell proliferation [7, 13, 36, 37].

Genistein, a prominent isoflavonoid found in soy products, has been shown to inhibit cell growth and induce apoptosis in a wide variety of cultured cells, mostly solid tumor-derived cells of epithelial cell origin [2, 10, 14, 20, 21, 22, 26, 31]. In this study genistein



significantly inhibited growth and induced apoptosis of both SUDHL-1 cells and Karpas299 cells, two ALCL cell lines used as models of hematopoietic malignancy. This cell growth inhibition and apoptosis induction occurred in a time- and dose-dependent manner, with an increased sensitivity in SUDHL-1 cells.

Recent studies done by Li and Sarkar [20] and Gong et al. [14] emphasize the importance of the Akt signaling pathway in genistein-induced apoptosis in prostate and breast cancer cell lines. In our study, the expression of both total Akt and phosphorylated Akt was downregulated by genistein, and correlating with the apoptosis status, SUDHL-1 cells showed a marked decrease in Akt and pAkt protein levels compared to Karpas299 cells.

Turturro et al. [34] have shown that the Akt pathway is constitutively activated in both SUDHL-1 cells and Karpas299 cells, but higher levels of Akt/pAkt are present in SUDHL-1 cells. Their study also demonstrated greater NPM-ALK-associated autophosphorylation activity and phosphotyrosine-containing proteins in SUDHL-1 cells than in Karpas299 cells. This correlates with the findings seen in our study which show that SUDHL-1 cells are more sensitive to genistein. Treatment with genistein showed a more prominent reduction of NPM-ALK level and Akt/pAkt downregulation in SUDHL-1 cells, resulting in higher apoptosis levels and a better response to the PI3-kinase inhibitor LY294002. Taken together, these data demonstrate that SUDHL-1 cells are more dependent on the NPM-ALK-associated autophosphorylation activity and PI3K/Akt signaling pathway for cell survival than Karpas299 cells.

One of the major regulatory and effector pathways of apoptosis consists of a distinct class of aspartyl proteases referred to as caspases [9]. Caspases typically reside in the cytoplasm as inactive zymogens, which upon receipt of "death stimuli" are proteolysed at Asp residues to their active forms [23]. In our investigation, treatment with genistein caused a change in the mitochondrial membrane potential, caspase-3 activation and degradation of PARP. Also, pretreatment of cells with caspase inhibitors, Z-VAD-FMK and Z-DEVD-FMK, inhibited genistein-induced apoptosis. Blockade of apoptosis with both caspase inhibitors strongly suggests an active role of caspases in the genistein-induced apoptosis of ALCL cell lines. Correlating with genistein-induced apoptosis levels and Akt/pAkt downregulation, SUDHL-1 cells showed a greater sensitivity to genistein than Karpas299 cells in terms of mitochondrial membrane potential change and caspase activation.

The Bcl-2 family members are important modulators of mitochondria-initiated apoptosis. We investigated the levels of Bcl-2, Bcl-xL and Bax protein by Western blot analysis. There was little or no detectable change in Bcl-2 and Bcl-xL protein levels, but there was an increase in Bax protein levels after genistein treatment. This correlates with the findings of a previous study by Li et al. [21] that emphasized the role of Bax in genistein-induced apoptosis in breast cancer. But a recent study by Coluccia et al. [8] demonstrates the importance of Bcl-xL in ALK-positive ALCL as a downstream activator of NPM-ALK-mediated oncogenicity. In our study although the upregulation of Bax by genistein was not prominent, this slight increase in Bax might be more critical than a decrease in bcl-2 and Bcl-xL for genistein-induced apoptosis of hematopoietic malignancies.

In conclusion, we demonstrated for the first time that genistein, a natural tyrosine kinase inhibitor, significantly inhibited the growth and induced apoptosis in t(2;5) ALCL through its effect on the Akt/pAkt pathway. This finding is of the utmost importance considering the fact that children and young adults are the main victims of ALK-positive ALCL, and that the use of genistein as a new treatment modality in combination with other chemotherapeutic agents might enhance its activities and lower the side effects caused by high-dose multimodality treatment. A recent study has shown that genistein enhances the antitumor effect of CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) in diffuse large-cell lymphoma [24]. The results of our study support the further investigation of the use of genistein in additional preventive or therapeutic strategies in ALCL and other hematopoietic malignancies.

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